Chapter 19
Inflammation and White Matter Injury in Animal Models of Ischemic Stroke

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Abbreviations

AchAo	Anterior choroidal artery occlusion
AMPA	2-Amino-3-(3-hydroxy-5-methyl-isoxazol-4-yl)propanoic acid
APC	Adenomatous polyposis coli
APP	Amyloid precursor protein
ATP	Adenosine triphosphate
BBB	Blood–brain barrier
BCCAo	Bilateral common carotid artery occlusion
CBF	Cerebral blood flow
CNS	Central nervous system
COX-2	Cyclooxygenase-2
CR3A	Complement component receptor 3 alpha
dMBP	Degraded myelin basic protein
eNOS	Endothelial nitric oxide synthase
ET-1	Endothelin-1
FACS	Fluorescence-activated cell sorting
GABA	Gamma-aminobutyric acid
GFP	Green fluorescent protein

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H&E	Hematoxylin and eosin
HI	Hypoxia–ischemia
IB_4	Isolectin B4
Iba1	Ionized calcium binding adapter molecule 1
ICA	Internal carotid artery
ICH	Intracerebral hemorrhage
IL	Interleukin
ITGAM	Integrin alpha M
LCA	Leukocyte common antigen
LFB	Luxol fast blue
Mac-1	Macrophage-1 alpha antigen
MBP	Myelin basic protein
MCAo	Middle cerebral artery occlusion
MHC	Major histocompatibility complex
MMP	Matrix metallopeptidase
MPO	Myeloperoxidase
MRI	Magnetic resonance imaging
NKT	Natural killer T cells
NO	Nitric oxide
NOS	Nitric oxide synthase
OGD	Oxygen-glucose deprivation
RAG1	Recombination activating gene 1
TGFβ	Transforming growth factor beta
TIMP	Tissue inhibitor of metallopeptidase
TNF-α	Tumor necrosis factor alpha
tPA	Tissue plasminogen activator

19.1 Introduction

19.1.1 Statement of the Problem

This entire book focuses on white matter; ranging from structure and distribution in the central nervous system (CNS), to methods for monitoring injury and repair, and mechanisms underlying white matter injury. Many types of CNS insult in patients and in animal models are considered, and several chapters deal specifically with stroke in patients, and with ischemia in animal models. Ischemic stroke is much more prevalent than hemorrhagic stroke (Adams et al. 1993) and is the leading cause of long-term disability in the USA and the second leading cause of death worldwide (Kelly-Hayes et al. 1998; Lopez and Mathers 2006). Primary ischemic stroke can be global (usually the result of cardiac arrest or an aortic occlusion) (Traystman 2003) or focal, if caused by a transient or permanent occlusion of a major cerebral artery or one or more deep blood vessels (so-called lacunar stroke) (Durukan and Tatlisumak 2007). Intracerebral hemorrhage (ICH) results from rupture of small brain arteries or arterioles, and accounts for the remaining 10–15 % of strokes in

Western populations (Anderson et al. 1994). Up to 30–40 % of ischemic strokes spontaneously undergo hemorrhagic transformation (Wang and Lo 2003), and ICH patients are at a high risk of developing global ischemia (Qureshi et al. 2009). In addition, ischemic stroke is a risk factor when treating hemorrhage with the blood-clot promoter; factor VIIa, the only drug used for treating ICH. While factor VIIa can decrease hematoma expansion (Diringer et al. 2008; Mayer et al. 2005, 2008), the outcome is often unimproved (Mayer et al. 2008; Lin et al. 2012).

The only effective drug treatment for ischemic stroke patients is the thrombolytic, tissue plasminogen activator (tPA). While tPA can, in limited cases, produce a remarkable reperfusion of damaged tissue, its use is limited by the need to inject it within the first 3–4.5 h of stroke onset (Wang et al. 2004). Because most patients are not in hospital and assessed in that time window, only about 5 % of patients can currently benefit from tPA therapy (del Zoppo 1998). This has spawned a huge and ongoing effort to identify new therapeutic targets and drugs. Hundreds of chemicals that target early neurotoxicity have been identified, and dozens have proven promising in preclinical studies in rodents but have been ineffective in improving survival or functional outcomes in clinical trials (Brott and Bogousslavsky 2000; Ginsberg 2009; Lakhan et al. 2009). This failure has led to deeper consideration of potential shortcomings of experimental approaches and models used in preclinical stroke research (Braeuninger and Kleinschnitz 2009; Dirnagl 2006; O'Collins et al. 2006). Several reports from European and North American Stroke Consortia (Stroke Therapy Academic Industry Roundtable) (Endres et al. 2008; Meairs et al. 2006; Fisher 1999) have emphasized that most experimental studies target events that are too early to be "drug-able," are restricted to healthy, young male rodents, lack relevant comorbidities (e.g., hypertension, diabetes), monitor infarct size as their primary outcome measure, and have been highly neurocentric. Most stroke research has explored neural cell death and neural cell repair (Petty and Wettstein 1999). Many studies have relied on showing treatment-related changes in neuron apoptosis (e.g., TUNEL, caspase 3 activation), damaged (e.g., Fluorojade) or remaining neurons (e.g., NeuN staining). Recommendations from stroke consortia for improving preclinical studies include: (1) focus more on white matter injury; (2) identify strategies to protect vascular and glial cells, not just neurons; (3) focus more on aged animals; (4) develop small animal models of lacunar stroke and compare mechanisms of damage with large vessel occlusion; and (5) identify and understand neuroimmune interactions and develop therapeutic strategies to target inflammation. This chapter will address several of these recommendations for preclinical studies.

19.1.2 Why Study Inflammation and White Matter Injury After Ischemic Stroke?

Historically, white matter was thought to be less susceptible to stroke than gray matter (Marcoux et al. 1982). More recent advances in imaging technology have led to a paradigm shift. Development of neuroimaging techniques, such as magnetic resonance imaging (MRI) and Diffuse Tensor Imaging, has provided the means

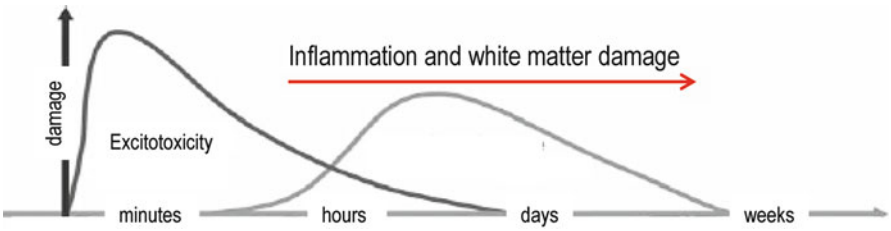


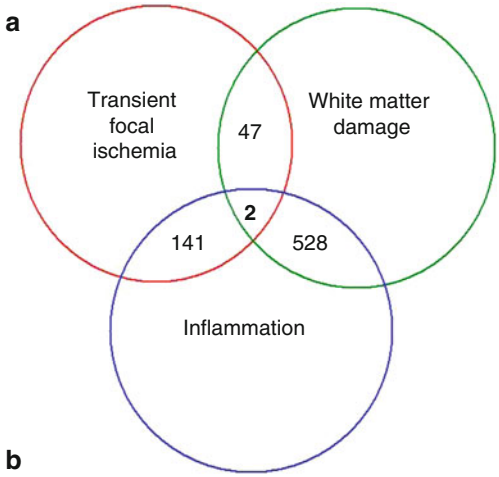
Fig. 19.1 The generally accepted time course of damaging events after stroke in humans (Sect. 19.1.2). Until recently, most experimental studies in animals focused on neurotoxicity, which is initiated very rapidly after stroke. This chapter addresses the inflammation phase which is delayed and prolonged

to map networks of white matter in the human brain at the macroscopic level. Resulting clinical observations have shown that ischemic stroke is rarely confined to gray matter (Goldberg and Ransom 2003), and primary white matter injury occurs in about 25 % of human strokes (Matute et al. 2013). At the microscopic level, more recent research has demonstrated the sensitivity of the major cellular components of white matter to ischemia. Several animal models have shown the susceptibility to ischemic damage of oligodendrocytes, the myelin forming cells of the CNS (reviewed by (Arai and Lo 2009)), myelin and axons (Petty and Wettstein 1999; Hughes et al. 2003; Irving et al. 2001; Lively and Schlichter 2012; Medana and Esiri 2003; Moxon-Emre and Schlichter 2010, 2011; Pantoni et al. 1996). Thus, white matter is now considered an important contributor to stroke outcome, and a tractable therapeutic target.

A second paradigm shift has been the change in focus of preclinical investigations searching for new post-stroke therapies. The emphasis is moving away from the rapidly occurring primary neurotoxicity to the secondary injury phase, which occurs in a time window that is amenable to treatment in hospital. This secondary phase is characterized by a prominent inflammatory response within and surrounding the core infarct that can last for many hours to days (Emsley and Tyrrell 2002; Jin et al. 2010; Zhang and Stanimirovic 2002). However, inflammation is complex. Understanding its temporal-spatial development and which components are harmful versus beneficial will be essential for developing better therapeutic strategies. Figure 19.1 illustrates the commonly proposed time course of events after stroke in humans.

19.1.3 Scope of This Chapter

Much of this book is devoted to white matter damage after acute injury, including ischemic stroke and intracerebral and subarachnoid hemorrhage. Thus, we will not review findings that focus solely on white matter damage. Instead, the chapter will focus on the intersection of inflammation and white matter injury in the context of experimental models of ischemia. In focusing on ischemic stroke (the vast majority), the chapter begins by describing the main models used to monitor white matter injury



b

Stroke-related	White matter-related	Inflammation-related
Transient focal ischemia	White matter	Inflammation
Hypoxia ischemia	Axon damage	Microglia
	Myelin	Macrophage
	Corpus callosum	Neutrophil
	Oligodendrocytes	Lymphocyte
	Internal capsule	Dendritic cell

Fig. 19.2 As discussed in Sect. 19.1.3, very few studies consider all three elements. (**a**) Venn diagram showing the approximate number of articles found when each pair of terms or the combined term "transient focal ischemia AND white matter AND inflammation" was entered into PubMed. (Human studies were excluded.) (**b**) To broaden the scope of the search, additional search terms and combinations were included. There was a slight variation in the number of articles found, depending on which search terms were combined. However, in every case, the intersection of elements from all three columns retrieved very few papers

in experimental stroke studies (mainly rodents). We describe methods used to monitor white matter damage in these models, and provide some figures to illustrate why we prefer some approaches over others. Because this is the only chapter in this book that explicitly deals with inflammation, we then provide a brief primer on inflammation. This section describes the main immune cells involved in animal models of ischemia, how to monitor them, and key findings. Then, we summarize the limited literature concerning the intersection of all three elements (ischemic stroke, white matter injury, inflammation) in both adult and neonatal rodent ischemia models. The wrap-up section comments on needs for further research.

In selecting literature related to this chapter, we searched the PubMed database up to March 2013. As shown in Fig. 19.2, the search began with original and review articles that contained all three topics: transient focal ischemia + white matter + inflammation. Remarkably, only two publications were found. We then broadened the search using combinations of multiple search terms, and included studies that examined inflammation and white matter damage in neonatal hypoxia–ischemia and adult chronic ischemia models.

19.2 Animal Models Used to Study White Matter Injury After Ischemia

19.2.1 White Matter Locations in the Brain

It is often noted that the ratio of white matter to gray matter in the human brain is 60:40, but much lower in the rat (14:86) and mouse (10:90) (Krafft et al. 2012). While this might be considered an impediment to studying white matter injury, rodents have several brain regions with high densities of white matter (Johnson et al. 2012). Several of these regions are commonly used to study white matter damage in rodents. The corpus callosum is comprised of white matter tracts (nerve fibers) that connect the two cerebral hemispheres, transferring and integrating information from the left and right hemispheres (Bruni and Montemurro 2009). The internal capsule is a compact band of fibers that lies deep in the brain, separating the caudate nucleus from the putamen. It consists of projection fibers that relay information from the cortex and spinal cord, brain stem, and subcortical structures (Bruni and Montemurro 2009). The optic nerve, formed by axons of retinal ganglion cells, carries information from the retina to the thalamus and other subcortical nuclei (Perry and Cowey 1984; Perry et al. 1984). Other white matter-dense brain regions include, but are not limited to, the anterior commissure, cingulum, extreme capsule, and external capsule (Bruni and Montemurro 2009). Some brain regions such as the striatum provide an excellent template for studying white and gray matter injury side-by-side. As Fig. 19.3 illustrates, the striatum contains a large number of myelinated axon bundles that are surrounded by nerve cell bodies and dendrites, and astroglial and microglial cells. We have exploited this structure in rats to simultaneously analyze inflammation, neuronal death, and damage to myelin and axons after both ischemic and hemorrhagic stroke (Lively and Schlichter 2012; Moxon-Emre and Schlichter 2010, 2011; Lively et al. 2011; Wasserman and Schlichter 2007, 2008; Wasserman et al. 2008).

19.2.2 Ischemia Models Used to Study White Matter Damage

It is clear that no animal model can fully recapitulate all components of human stroke (Durukan and Tatlisumak 2007; Howells et al. 2010). Most preclinical studies are conducted in small animals, especially rodents (Durukan and Tatlisumak 2007), and almost all results relevant to this chapter come from rats and mice. While mouse models facilitate using transgenic animals, which are of limited availability in rats (Durukan and Tatlisumak 2007), we mainly exploit rats for several reasons. The cerebrovascular anatomy and physiology is reasonably similar between rats and humans (Macrae 1992). In the most commonly used mouse model of ischemia (middle cerebral artery occlusion (MCAo), discussed below), the infarct size

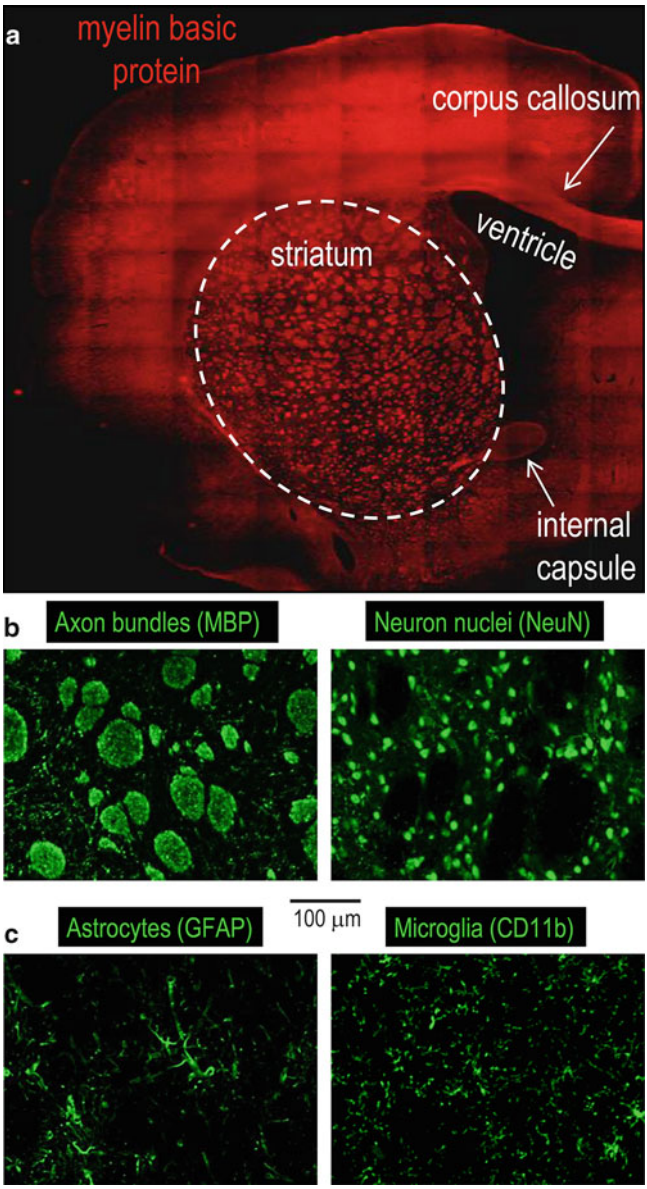


Fig. 19.3 As discussed in Sect. 19.2.1, the architecture of the rat striatum provides advantages for studying white matter and gray matter together with glial responses and inflammation. (**a**) Coronal section of healthy adult rat brain labeled with an antibody against myelin basic protein (MBP). Note the structure of the striatum (*circled*), which contains numerous white matter bundles (axon tracts) cut in cross section. Also shown are two regions of relatively pure white matter: the corpus callosum and internal capsule. Low magnification images were digitally stitched to show an entire hemisphere. (Modified from Moxon-Emre I and Schlichter LC. 2010. Evolution of inflammation and white matter injury in a model of transient focal ischemic stroke. J. Neuropathol. Expt'l Neurol. 69:1–15.) (**b**) Higher magnification images show bundles of myelinated axons (*left*: MBP labeled), surrounded by gray matter and neuropil (*right*) in which neuronal nuclei are labeled with NeuN antibody. (**c**) Outside the axon bundles, there are astrocytes (immunolabeled for glial fibrillary acidic protein, GFAP) and ramified, resting microglia (labeled with OX-42 antibody, which recognizes CD11b)

is highly variable and shows strain-dependence that is not seen in rats (Carmichael 2005). The positive correlation between brain size and amount of white matter (Zhang and Sejnowski 2000) and larger brain size of rats yields more material for analysis. The larger size is especially useful when one seeks to analyze several parameters or stains, and to quantify differences across brain regions.

19.2.2.1 Vessel Occlusion

Middle cerebral artery occlusion. Most human ischemic strokes occur in the area surrounding the middle cerebral artery (MCA). A rodent model of MCAo was developed early and has since been refined (Koizumi et al. 1986; Robinson et al. 1975). Robinson et al. (1975) used craniotomy to expose the MCA and distally ligated the artery, which produced an ischemic lesion that extended into the cortex. Now, a more commonly used method for occluding blood flow is to advance an intraluminal suture through the internal carotid artery (ICA) until it reaches the origin of the MCA (del Zoppo et al. 1992). With this method, transient ischemia is produced by removing the suture to allow reperfusion. The duration of MCAo needed to produce a significant nonlethal lesion is variable. The common duration for rat is 60, 90, or 120 min (Carmichael 2005), and usually results in neuron death throughout the striatum and into the dorsolateral cortex, but not in contralateral brain regions (Garcia et al. 1995). Based on the distribution of injury, the MCAo model is useful for simultaneously analyzing damage that is mainly isolated to gray matter (in the cortex), white matter (in the corpus callosum), or occurs in both (in the striatum). Despite its popularity, an important limitation of the MCAo model is the variable size and location of the infarct (Liu and McCullough 2011). Some of the variability seen in the literature can be attributed to differences in size and quality of the suture/filament used (Kuge et al. 1995); silicone-coated thread induces a larger lesion than uncoated thread (Laing et al. 1993). When using mice, some strains demonstrate substantially larger infarcts after MCAo than others (Connolly et al. 1996a). Other limitations of the MCAo model are the risk of subarachnoid hemorrhage (Carmichael 2005), and the added difficulty in aged animals, which have less flexible blood vessels and higher mortality (Liu and McCullough 2011).

Fig. 19.4 (continued) putamen+caudate of the striatum. Staining with 2 % TTC (2,3,5,-triphenyl-2H-tetrazolium chloride) appears pale in the metabolically compromised infarcted regions. (**c**) Example of sampling sites for quantification in a TTC-stained section at Day 7 after ET-1 injection: infarct core (*red*), edge (*white*), surrounding striatum (*blue*), uninjured contralateral striatum (*green*). We sample multiple regions to quantify parameters that include staining area, staining intensity, density of specific cell types. For statistical comparisons, the average of four "boxes" from each of several sections is then averaged for multiple animals. Comparisons include: naïve animals, saline-injected control animals, and the contralateral hemisphere of ET-1 injected animals. (Modified from Moxon-Emre I and Schlichter LC. 2010. Evolution of inflammation and white matter injury in a model of transient focal ischemic stroke. J. Neuropathol. Expt'l Neurol. 69: 1–15)

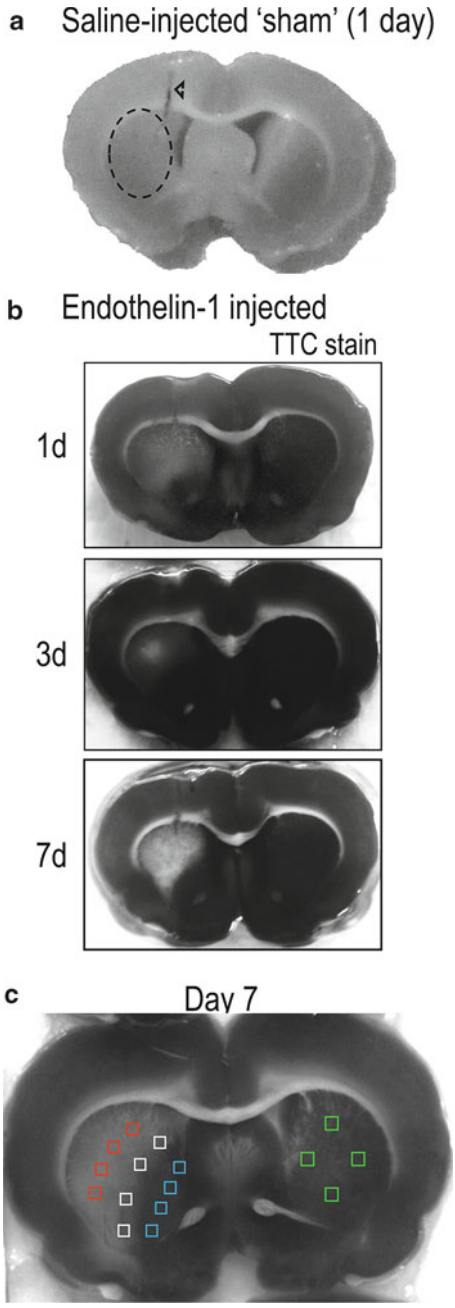


Fig. 19.4 Focal ischemia induced by endothelin-1 (ET-1) injection in the rat (Sect. 19.2.2.2). (**a**) There was no infarct in saline-injected control rats. The location of the needle penetration track is indicated by a small amount of bleeding seen 1 day after saline injection (*arrowhead*). (**b**) Stereotaxic injection of ET-1 into the anterior striatum produced a lesion that was restricted to the

Anterior choroidal artery occlusion. Occlusion of the anterior choroidal artery (AchAo) can be obtained by advancing an intraluminal suture through the ICA to a region *proximal* to the MCA (He et al. 1999). As illustrated in detail in their follow-up paper, the infarct location is similar in MCAo and AchAo (He et al. 2000). The main differences are that after AchAo, the infarct is generally smaller but nearly always encompasses the internal capsule. Thus, AchAo reliably produces ischemia in a white matter-dense region, and facilitates studies of deep lacunar strokes with a less-distributed lesion than MCAo.

19.2.2.2 Vasoconstriction

An increasingly popular model uses injection of the potent vasoconstrictor, endothelin-1 (ET-1). ET-1 is a naturally occurring, 21 amino acid peptide produced by endothelial cells, which binds to the endothelial receptors, ET_A and ET_B (Verhaar et al. 1998). There are several advantages of this model over vessel occlusion models. Provided the potency of each ET-1 batch is tested, the injected dose can be titrated to obtain different degrees of transient ischemia, and lesion sizes that are relatively reproducible. For instance, with moderate amounts of ET-1 injected directly into the brain parenchyma, a reduction of cerebral blood flow (CBF) of ~60 % for up to 3 h has been reported (Hughes et al. 2003).

ET-1 is sometimes applied to the MCA to cause constriction, instead of ligating the artery in rats (Gresle et al. 2006; Robinson et al. 1990). Importantly, stereotaxic ET-1 injection can be used to produce a focal infarct in brain regions that are selected to focus on white matter (e.g., corpus callosum, internal capsule), gray matter (e.g., cortex), or both (e.g., striatum) (Sozmen et al. 2012). When injected into white matter-rich regions, ET-1 produces hallmarks of white matter injury seen in humans, including axonal damage, demyelination, inflammation, and glial scar formation (Hughes et al. 2003; Lively and Schlichter 2012; Moxon-Emre and Schlichter 2010). Figure 19.4 shows the development of a typical infarct after ET-1 injection into the anterior striatum (putamen + caudate) of the rat, and illustrates sample sites for quantitative spatial analysis.

There are few mouse studies using ET-1 injection to evoke transient ischemia, and the results have been inconsistent. Injecting ET-1 into the striatum failed to induce ischemia in multiple strains of mice, and increasing the dose increased mortality without inducing a lesion (Horie et al. 2008). Another study showed a dose-dependent increase in infarct size in mouse brain subjected to multiple focal injections of ET-1 (Sozmen et al. 2009). Differences in expression of endothelin receptor subtypes might be a confounding factor. Mouse brain expresses a lower proportion of ET_A receptors, which evoke the vasoconstriction needed to produce ischemia, than ET_B receptors, which generally cause vasodilation (but see below) (Sozmen et al. 2012; Wiley and Davenport 2004). The possibility was raised that the larger proportion of ET_B receptors interferes with induction of ischemia in the mouse brain (Sozmen et al. 2012). That differences in ET-1 affinities are responsible

is unlikely: the K_d for ET-1 binding is 0.12 nM for ET_B and 0.6 nM for ET_A receptors (Haynes et al. 1995). A further complication is that in humans, ET_B receptors can mediate both vasodilation and vasoconstriction (Haynes et al. 1995; Clozel et al. 1992; Seo et al. 1994). This might explain the finding that although the rat striatum contains mainly ET_B receptors (Tayag et al. 1996), a focal ET-1 injection reliably produces an ischemic infarct (described above). Further research is needed to clarify the regional and cell-specific expression, and roles of the ET-1 receptor subtypes in rodents. Nevertheless, focal ET-1 injection into the rat CNS is a widely accepted model of transient ischemia that can create reproducible lesions restricted to a desired region of the brain (Sozmen et al. 2012).

19.2.2.3 Neonatal Hypoxia–Ischemia Models

The most commonly used model of ischemic injury in immature rodents is the hypoxia–ischemia (HI) model (Vannucci and Hagberg 2004). To induce ischemia, the common carotid artery is permanently ligated, and the whole body is temporarily exposed to hypoxia (often 8 % oxygen for up to 3 h). This procedure reduces CBF to the ipsilateral brain region by 40–60 %, and then CBF returns to normal immediately after re-exposure to normoxic conditions (Vannucci et al. 1988). Unlike adult rodents, ligation or hypoxia alone does not induce ischemia in neonates: both are required (Vannucci and Hagberg 2004). The resulting infarct is large but restricted to the ipsilateral hemisphere; damage to the contralateral hemisphere is rare (Towfighi et al. 1995; Vannucci and Vannucci 1997). On the ipsilateral side, the infarct extends through the cerebral cortex, striatum, thalamus, hippocampus, and most importantly for the topic of this chapter, the subcortical white matter. HI is exploited as an animal model of cerebral palsy (Johnston et al. 2005), a disorder seen in about 1/300 8-year-old children in the USA (Kirby et al. 2011). Several modifications of the HI model have also been used, including bilateral carotid ligation or variations in the duration of hypoxia. In this chapter, we will restrict discussion to rodent models using unilateral ligation followed by 6–8 % oxygen.

19.2.2.4 Optic Nerve Ischemia

The myelinated axon tracts that make up the optic nerve have proven extremely useful for studying mechanisms underlying white matter injury. Much of the stroke-related mechanistic work has used isolated optic nerves subjected to varying periods of oxygen-glucose deprivation (OGD) to simulate ischemia (Arai and Lo 2009). However, the optic nerve is one of the most vulnerable white matter regions following experimental chronic ischemia (Wakita et al. 1994). ET-1 has been injected into the microvasculature of the optic nerve in vivo (Cioffi et al. 1995), and some consider the resulting ischemia and white matter injury to be a good model of human glaucoma (Cioffi 2005).

19.2.3 Methods Used to Monitor White Matter Damage After Ischemia

As mentioned above, neuronal axons and the myelin sheath are both susceptible to ischemic injury. Despite their intimate connections, the timing and mechanisms underlying damage to these structures differs. The vulnerability of oligodendrocytes to ischemic injury can lead to myelin loss and prevent its repair. Therefore, to comprehensively examine white matter injury after ischemia, it is important to distinguish myelin from axons (both healthy and damaged) and to monitor oligodendrocytes.

19.2.3.1 Staining Healthy and Damaged Myelin

Histological stains for myelin have been in use for many years, with loss of staining indicating white matter pathology. Healthy myelin can be labeled with colorimetric stains, such as the lipid soluble dyes, Luxol Fast Blue (LFB) (Kluver and Barrera 1953; Salthouse 1962) and Sudan Black B (Stilwell 1957) but these dyes provide low contrast and resolution (Schmued et al. 2008). There are several recent improvements in myelin labeling that are less expensive than antibody-based methods. Gold-chloride and, especially, the improved gold-phosphate complex ("Black-Gold") provide excellent contrast under bright-field and dark-field illumination (Wasserman and Schlichter 2008; Schmued et al. 2008). If fluorescence is required, FluoroMyelin™ stain is attractive because it can be combined with immunohistochemistry in fixed tissue. For live imaging, FluoroMyelin™ Red is a vital dye that labels myelinated axons (Monsma and Brown 2012).

The use of antibody-based immunohistochemistry for detecting myelin damage has become popular because it can provide excellent contrast and allows use of multiple stains. The most commonly used protein target is myelin basic protein (MBP), which constitutes up to 30 % of myelin in the CNS (Sternberger et al. 1978). A reduction in antibody staining for MBP has been used to monitor myelin damage or loss in animal models of transient ischemia (Irving et al. 2001; Lively and Schlichter 2012; Moxon-Emre and Schlichter 2010; Souza-Rodrigues et al. 2008) and HI (Carty et al. 2008; Villapol et al. 2011). A cautionary note in relying solely on MBP labeling is that the staining can appear faint if healthy myelinated axon bundles are very tightly packed (Sternberger et al. 1978). If so, then the swelling of damaged axon bundles might actually increase the MBP labeling. An important advance that overcomes this limitation is the immunological detection of degraded MBP (dMBP) (Matsuo et al. 1997). We prefer this approach and, as shown in Fig. 19.5, have found that the increase in dMBP labeling coincides temporally and spatially with loss of MBP after transient ischemia evoked by injecting the vasoconstrictor, ET-1, into the rat striatum.

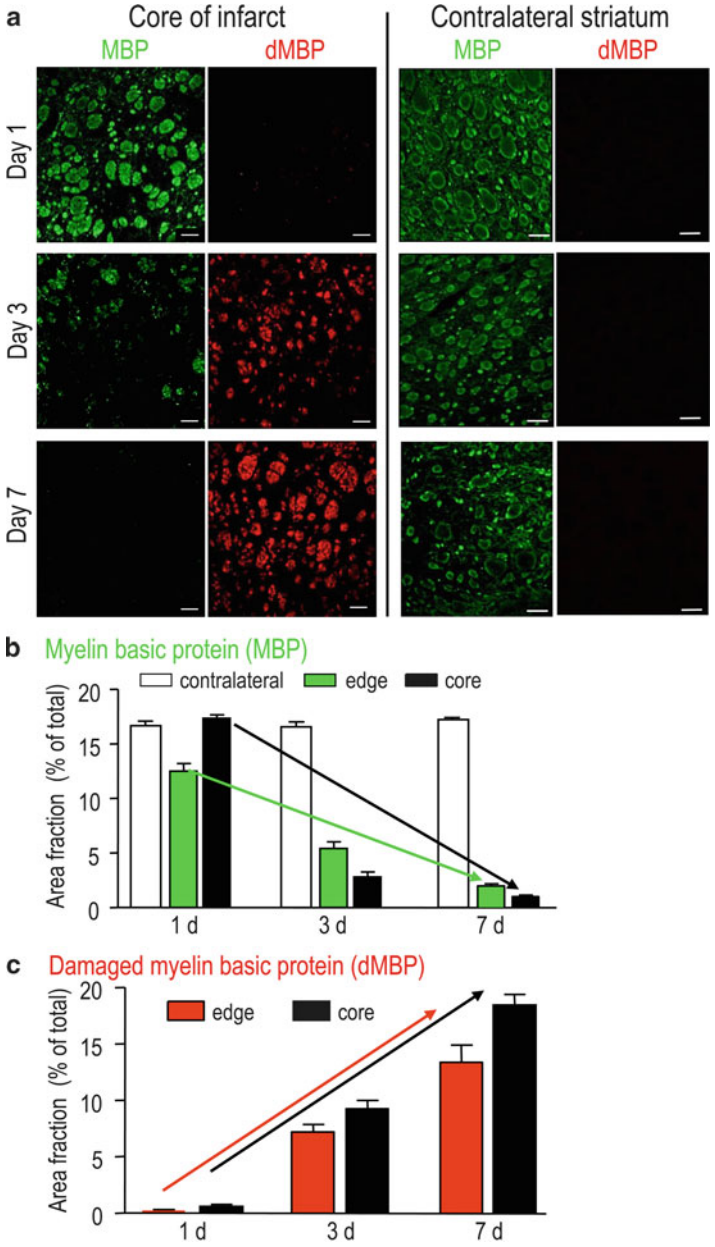


Fig. 19.5 Temporal evolution of myelin damage in the infarct core is shown during the first week after ET-1 injection into the rat striatum (Sects. 19.2.3.1 and 19.4.1). (**a**) The *left-hand panels* show sections immunolabeled for normal myelin (*green*; mouse monoclonal anti-MBP antibody) and damaged myelin (*red*; rabbit polyclonal anti-dMBP antibody). The *right-hand panels* show the normal myelin staining in uninjured contralateral striatum. (**b**) Quantification shows the loss of normal myelin basic protein staining in the infarct core and at the infarct edge. In the contralateral striatum (*white bars*), there was no loss of staining. The area of staining (expressed as percent of total area examined) was determined with ImageJ software, averaged for four rats at each time point, and shown as mean ± SEM. (**c**) A time-dependent increase in staining for damaged MBP is seen, using the same quantification procedure as in (**b**). Only the core and edge were analyzed because there was no dMBP staining on the undamaged contralateral side (see (**a**), *right*). (Modified from Moxon-Emre I and Schlichter LC. 2010. Evolution of inflammation and white matter injury in a model of transient focal ischemic stroke. J. Neuropathol. Expt'l Neurol. 69: 1–15)

19.2.3.2 Identifying Damaged Axons

For histological assessment of axon pathology, the Bielschowski silver impregnation method was developed many years ago (Nauta 1952). This stain labels degenerating axon terminals, and although it is still in use, it is capricious and cannot be used for co-labeling (Castellani et al. 2007). With increased understanding of axon pathology, new markers of damaged axons continue to be identified. For instance, when fast axonal transport is disrupted in injured axons, there is swelling and accumulation of proteins and organelles. Accumulation of amyloid precursor protein (APP) can be readily detected by immunohistochemistry (Fig. 19.6). APP is considered a more reliable indicator of early axon injury than traditional silver stains (Ferguson et al. 1997; Gentleman et al. 1995; Smith et al. 2003) but accumulation does not indicate whether axons are terminally damaged or can recover. As shown in Fig. 19.6, we recently found that the matricellular molecule, SC1/hevin, identifies early axon damage after transient ischemia (and other forms of acute damage) much like APP (Lively and Schlichter 2012). Another indication of damage is loss of integrity of the axon cytoskeleton, which can be monitored using antibodies raised against neurofilaments (e.g., NF200) (Lively and Schlichter 2012; Moxon-Emre and Schlichter 2011; McCracken et al. 2002). In the brain, the heavy neurofilament chain is considered the most highly phosphorylated protein, and changes in its phosphorylation state have been used to detect axon damage (reviewed in (Petzold 2005)). Nonphosphorylated heavy neurofilaments can be detected using the SMI-32 antibody (Sternberger and Sternberger 1983), which labels damaged axons in humans following cerebral ischemia (Leifer and Kowall 1993). The interpretation of changes in SMI-32 staining intensity might be complicated. After transient forebrain ischemia in rats, an increase in SMI-32 intensity was seen in swollen, degenerating axons in the core of the infarct, and the staining gradually decreased as white

Fig. 19.6 (continued) axon bundles (not showing MBP). Scale bar: 100 μm. Panel C shows time-dependent increases in the fractional area of SC1 and APP staining (percent of total area examined). Values are the mean ± SEM of three animals, with each measurement being the mean of four sampling regions per striatum. (**d**) APP and SC1/hevin distribution in damaged white matter tracts; examples are 1 day after a hemorrhagic stroke in the striatum of adult rats. The labels are: NF200 for axon neurofilaments (*green*), DAPI (*blue*) for cell nuclei, and SC1 or APP (*red*). *Left*: The image from a normal white matter tract in the contralateral striatum shows NF200 distribution, and lack of APP. In the surrounding striatum, further from the lesion, an apparently normal white matter tract is labeled for NF200, but not SC1. *Right*: At the edge of the lesion, adjacent serial sections were labeled for NF200 to align the images, and either APP or SC1. The distributions of APP and SC1 were similar. (**e**) High-magnification, deconvolved images of cross-sectioned axons (color-separated and merged) near the inner edge of the lesion. The immunostains are for: SC1 + NF200, SC1 + pan-axonal neurofilament, SC1 + MBP. Scale bar = 5 μm. The axons in the *top* and *bottom* images are cut in cross-section, while axons in the center image were selected to be at a more oblique angle. In damaged white matter tracts, SC1 staining was inside swollen axons (compare with NF200). ((**b**–**e**) are modified from Lively S and Schlichter LC. 2012. SC1/hevin identifies early white matter injury after ischemia and intracerebral hemorrhage in young and aged rats. J. Neuropathol. Expt'l Neurol. 71:480–493)

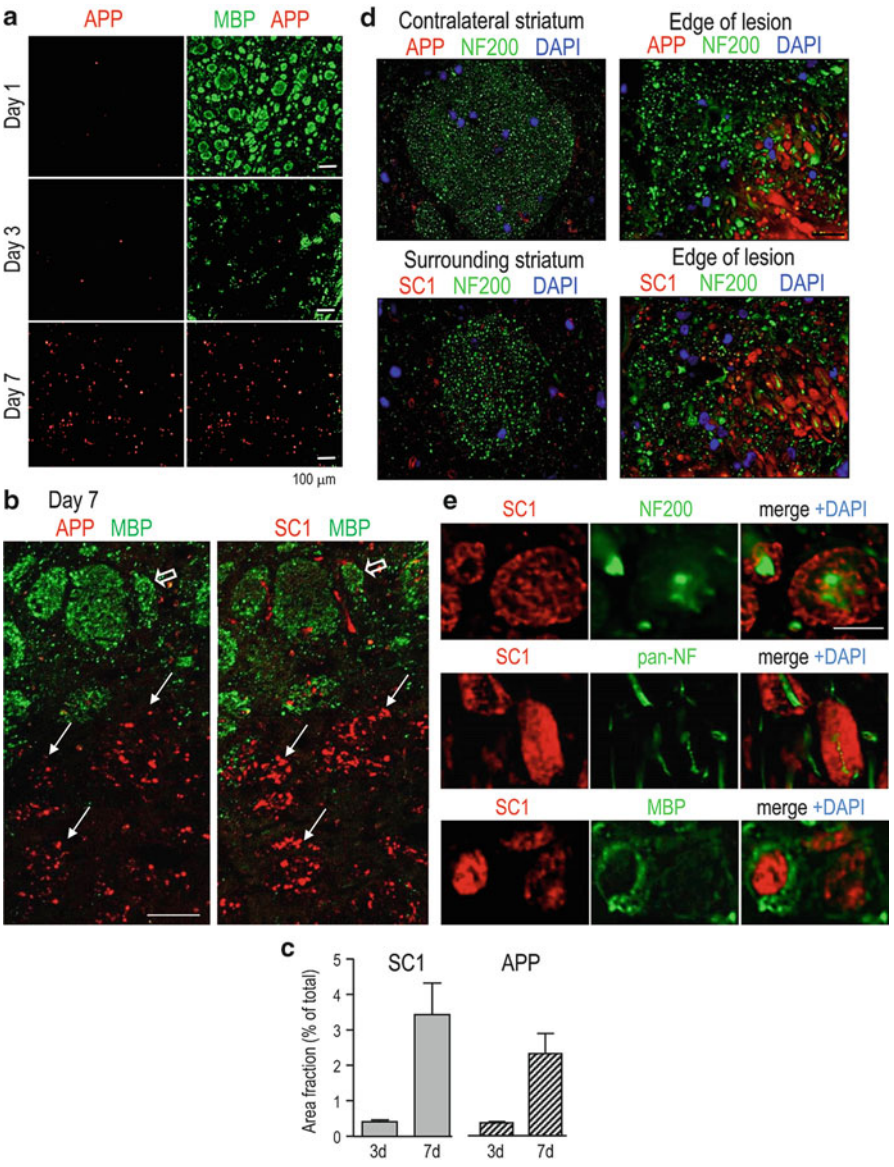


Fig. 19.6 To monitor axon damage after ET-1 induced ischemia, we quantified accumulation of amyloid precursor protein (APP) and the matricellular molecule, SC1 (also known as hevin) (Sects. 19.2.3.2 and 19.4.1). (**a**) In the infarct core, APP (rabbit polyclonal anti-APP antibody, *red*) accumulated in injured axons as staining for normal myelin decreased (mouse monoclonal anti-MBP antibody, *green*). (Modified from Moxon-Emre I and Schlichter LC. 2010. Evolution of inflammation and white matter injury in a model of transient focal ischemic stroke. J. Neuropathol. Expt'l Neurol. 69:1–15.) (**b**, **c**) Confocal images taken at the edge of the infarct 7 days after ischemia (**b**). Adjacent serial sections were immunolabeled for MBP, and individual axon bundles were identified for alignment purposes (*open arrows*). SC1 and APP were prominent in damaged

matter damage progressed (Gresle et al. 2006). However, at the inside edge of the lesion (delineated by ballistic light analysis), increased staining was also seen in morphologically normal-appearing axons. The authors suggest that SMI-32 immunohistochemistry might distinguish the edge of the peri-infarct region.

19.2.3.3 Monitoring Oligodendrocyte Damage

The microtubule-associated protein, Tau-1, might be a good early indicator of oligodendrocyte pathology. After transient (Valeriani et al. 2000) and permanent ischemia (Valeriani et al. 2000; Irving et al. 1997), Tau-1 immunoreactivity increases rapidly (<1 h) and transiently (1–3 days) in oligodendrocytes within the infarct. However, the fate of the Tau-1-labeled cells is unclear and they are not necessarily lost (Gresle et al. 2006). Tau-1 is normally present in healthy axons so it is necessary to either show lack of co-labeling with a neuron marker, or the presence of co-labeling with an oligodendrocyte marker. Loss of oligodendrocytes can be assessed using markers of mature oligodendrocytes, including antibodies directed against 2′, 3′cyclic nucleotide 3′ phosphodiesterase (CNPase) (Sprinkle et al. 1983) and adenomatous polyposis coli (APC) protein (Bhat et al. 1996). For a comprehensive review of oligodendrocyte markers used during development, please see (Baumann and Pham-Dinh 2001).

19.3 A Primer on CNS Inflammation After Ischemia

19.3.1 Overview

The brain is highly vascularized, and when healthy, is protected by the blood–brain barrier (BBB) from cells and molecules of the circulatory system (reviewed in (Engelhardt and Sorokin 2009)). This isolation from blood and lymphatic systems contributed to the long-standing notion that the CNS is immune-privileged (Streilein 1993). However, tissues that are at high risk of infection generally possess resident macrophages (e.g., Kupffer cells in the liver, “dust cells” in the lung) that are positioned to rapidly respond, and to help recruit blood-borne immune cells into the injured tissue. The CNS is no exception: it possesses microglia, resident macrophage-like cells that rapidly respond to a wide variety of insults, and with a myriad of outcomes (discussed below). After a stroke, the BBB integrity is compromised and CNS inflammation then involves “activation” and extravasation of blood-borne innate immune cells (neutrophils, monocytes). The innate immune system can respond rapidly because, unlike the adaptive immune system mediated by T and B lymphocytes, it does not require antigen recognition, processing, cell priming, and proliferation. Thus, it is expected that within minutes of arriving at the damage site, resident microglia and infiltrating innate immune cells (neutrophils, macrophages) can begin to

secrete inflammatory mediators. From in vitro work, we (Kaushal et al. 2007; Kaushal and Schlichter 2008; Sivagnanam et al. 2010) and many others (Colton and Gilbert 1987; Mao et al. 2007; Piani et al. 1991; Smith et al. 1998) have shown that activated microglia and macrophages can produce pro-inflammatory molecules, excitatory amino acids, lipases, proteases, and reactive oxygen and nitrogen species.

When considering acute inflammation after stroke, the damage-induced ("danger") response is generally more relevant than a pathogen-induced ("stranger") response. However, stroke often occurs in an inflammatory environment (Jin et al. 2010; Chapman et al. 2009; Denes et al. 2010; Drake et al. 2011; McColl et al. 2009; Whiteley et al. 2009), with comorbidity arising from bacterial infection, atherosclerosis, hypertension, diabetes, or obesity (Drake et al. 2011; Fischer et al. 2006). Inflammation in peripheral tissue or within the brain is thought to be an important determinant of stroke outcome (Kleinig and Vink 2009). In rodent models of transient ischemia, a wave of inflammation occurs within the brain during the first week: microglial activation is seen early, followed by infiltration of blood-borne immune cells (macrophages, neutrophils) (Moxon-Emre and Schlichter 2010; Stevens et al. 2002). Infiltration of blood-borne immune cells is mediated by their interactions with endothelial cells through receptors that include the lymphocyte adhesion receptor α4 integrin; and significantly, blocking this interaction reduced the infarct volume and improved the neurological outcome after transient MCAo (Becker et al. 2001; Relton et al. 2001).

Animal models are widely used to study inflammation after transient ischemia; for detailed reviews, see (Jin et al. 2010; Ceulemans et al. 2010; Jordan et al. 2008; Wang et al. 2007). Often called a "double-edged sword," there is strong evidence that inflammation can both contribute to secondary damage and to post-stroke recovery. A simplistic view has been that early inflammatory responses are directed toward destroying damaged cells, and later responses help resolve the pro-inflammatory state, promoting tissue recovery and repair. There are many recent reviews on microglia (Kettenmann et al. 2011; Luo and Chen 2010; Saijo and Glass 2011) and macrophages (Van Dyken and Locksley 2013; Wynn et al. 2013) summarizing that these cells can function as antigen-presenting cells, phagocytose damaged cells and debris, and produce a plethora of cytokines, chemokines, proteases, and growth factors that affect themselves and other cells. Here, we will not catalogue individual inflammatory molecules and cell functions. Instead, we will briefly present methods for monitoring the most relevant inflammatory cells, and then present an overview of findings on inflammation in rodent models of transient focal ischemia.

19.3.2 Methods to Assess Inflammation in Ischemia Models

19.3.2.1 Monitoring Neutrophils

After transient ischemia, neutrophils have been identified using routine hematoxylin and eosin (H&E) staining, by recognizing cells with a unique multi-lobed

nucleus (Phillips et al. 2000; Williams et al. 2003; Zhao et al. 2009). Limitations are that the staining resolution is relatively low, especially in densely packed tissues, and it is not conducive to double- and triple labeling. The extent of neutrophil infiltration after ischemia (and other types of injury) is most commonly monitored by biochemical detection of myeloperoxidase (MPO) activity, which is high in activated neutrophils (Barone et al. 1991; Batteur-Parmentier et al. 2000; Beray-Berthat et al. 2003; Bradley et al. 1982; Lerouet et al. 2002). A major limitation of this indirect detection is its inability to reveal spatial and cell-specific MPO expression. This problem can be overcome by exploiting anti-MPO antibodies, as has been done by us and numerous others in animal models of transient ischemia (Moxon-Emre and Schlichter 2010; Justicia et al. 2003; Matsuo et al. 1994). Immunodetection is more sensitive than the biochemical assay (Zhang and Chopp 1997) and can be paired with other cell-specific antibodies to identify the cellular source (Weston et al. 2007). This is important because MPO can also be expressed by activated macrophages and microglia, as shown after transient MCAo (Zhang and Chopp 1997). MPO levels increase when microglia and macrophages become phagocytic, and they can also phagocytose neutrophils that contain MPO (Weston et al. 2007). In mice, neutrophils have also been labeled in situ by an antibody against the Ly6G antigen, which is commonly used in fluorescence-activated cell sorting (FACS) analysis (Gelderblom et al. 2009). Two papers used antibodies against unknown neutrophil antigens: HB199 (Hughes et al. 2003) and MBS-1, with antigen retrieval (Souza-Rodrigues et al. 2008).

19.3.2.2 Monitoring Microglia and Macrophages

In the healthy adult brain, resting microglia can be readily distinguished from other cell types by their tiny cell bodies and highly ramified processes, and by immunohistochemical staining (reviewed in (Kettenmann et al. 2011)). There are several markers that are widely used to detect microglia: the "integrin alpha M" subunit (ITGAM), which is also known as CD11b, complement component receptor 3 alpha (CR3A), and macrophage-1 alpha antigen (Mac-1A) (Akiyama

Fig. 19.7 (continued) indistinguishable from macrophages. After transient ischemia induced by ET-1 injection, ramified microglial cells are still present in the striatum surrounding the infarct, and are found throughout the uninjured contralateral striatum. Cells in the core of the infarct rapidly adopt a macrophage-like appearance. (**c**) By 3 days after ischemia, the infarct is defined by large numbers of activated microglia/macrophages that can be labeled with Iba1 or the OX-42 antibody, which labels CD11b (or several other markers, see Sect. 19.3.2.2). (**d**) Spatial correlation of activated microglia/macrophages with damaged axons. *Left*: Activated microglia/macrophages were immunolabeled with Iba1 (*red*) and ED1 (*green*). *Middle*: the adjacent serial section was immunolabeled with APP to indicate damaged axons, and ED1 to label activated microglia/macrophages. *Right*: Higher magnification images of the boxed regions show activated microglia inside damaged axon bundles closer to the infarct. Scale bars: 100 μm (*left*, *middle*), 10 μm (*right*). ((**d**) is from Moxon-Emre I and Schlichter LC. 2011. Neutrophil depletion reduces BBB breakdown, axon injury inflammation after intracerebral hemorrhage. J. Neuropathol. Expt'l Neurol. 70:218–235)

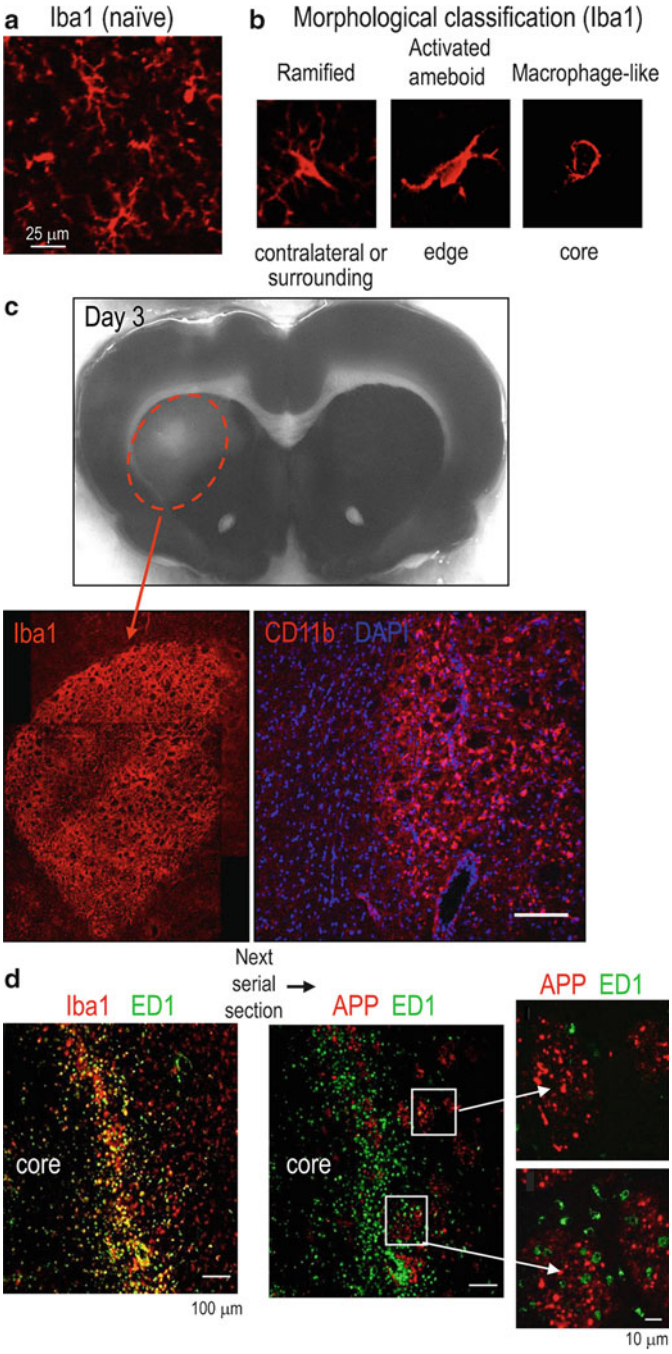


Fig. 19.7 Immunolabeling for microglia and macrophages (Sects. 19.3.2.2 and 19.4.1). (**a**) Iba1 labels ramified, resting microglia in the normal striatum of a healthy young adult rat. (**b**) Iba1 labeling shows that as microglia activate, their processes are retracted until they are morphologically

and McGeer 1990; Robinson et al. 1986); "ionized calcium binding adapter molecule 1" (Iba1) (Imai et al. 1996; Ito et al. 1998); and the surface glycoprotein, F4/80 (Perry et al. 1985). ITGAM and F4/80 also label dendritic cells (Whiteland et al. 1995), endogenous macrophage-like cells that are mainly in myelinated fiber tracts (e.g., corpus callosum, striatal axon bundles, fimbria) and at sites of interstitial fluid drainage (i.e., perivascular space, interface between choroid plexus and cerebral spinal fluid) (reviewed in (Colton 2013)). Microglia (and infiltrating macrophages) can also be identified by lectin staining, notably *Griffonia simplicifolia* B_4 isolectin (IB_4) and tomato lectin; however, these stains are less specific and also label blood vessels (Acarin et al. 1994; Streit and Kreutzberg 1987). Figure 19.7 illustrates some markers we exploit for labeling resting microglia in the healthy rat brain, and both activated microglia and macrophages after transient ischemia.

After acute brain damage, some of the markers are up-regulated (Isaksson et al. 1999; Ito et al. 2001) but the continuing challenge is to discriminate microglia from blood-derived macrophages in damaged tissue. Guillemin and Brew (Guillemin and Brew 2004) have reviewed biochemical and morphological similarities and differences between activated microglia and macrophages. The key issues are that microglia respond to injury by retracting their processes and can adopt a rounded morphology that closely resembles perivascular macrophages and infiltrating macrophages, and all three cell types share many markers. Because of the difficulty in distinguishing between these cells in the damaged brain, we prefer using the combined term, "activated microglia/macrophages." Matters are further complicated because the commonly used marker, CD11b, is also present on neutrophils. After infiltrating the brain following transient ischemia, neutrophils can also label with IB_4 (Matsumoto et al. 2007), although staining might also reflect phagocytosis by neutrophils of microglia and/or macrophages. CD11b is part of an integrin complex on leukocytes, whose interaction with adhesion molecules on endothelial cells allows cell extravasation. Consequently, CD11b inhibition has been tested as a means of reducing stroke damage. Intravenous injection of a function-blocking anti-CD11b antibody at the time of reperfusion after MCAo, reduced neutrophil infiltration, infarct volume, and neurological deficits (Chen et al. 1994). This was presumed to result from reduced neutrophil infiltration but the possibility that macrophage infiltration and microglial activation were affected was not addressed. Another antibody often used as a pan-macrophage marker is CD68 (also known as ED1), which was initially thought to label an intracellular antigen (Dijkstra et al. 1985). Later, ED1 was shown to be a lysosomal marker whose increased expression correlates with the amount of phagocytic activity (Damoiseaux et al. 1994). In the acutely damaged rat brain, ED1 labels phagocytic microglia and macrophages (Moxon-Emre and Schlichter 2010, 2011; Wasserman et al. 2008; Hansen et al. 2001) (and see Fig. 19.7). In an attempt to differentiate between microglia and macrophages, an alternative approach has been to use FACS analysis. It is most common to co-label with CD11b and the antigen, CD45 (also known as leukocyte common antigen [LCA]), which is a protein tyrosine phosphatase. Resting microglia are $CD11b^{+}CD45^{low}$; whereas, infiltrating macrophages are $CD11b^{+}CD45^{high}$

(Ford et al. 1995). One limitation is that CD45 can be up-regulated in activated microglial cells, which then closely resemble invading macrophages (Kettenmann et al. 2011; Sedgwick et al. 1991). Moreover, because all information about spatial localization of the cells is lost, FACS analysis is not useful for addressing the relationship between specific immune cells and white matter damage.

19.3.2.3 Monitoring Lymphocytes and Other Immune Cells

When examining lymphocytes, it can be useful to first monitor the presence of both activated T and B cells by staining with an antibody directed against CD25 (the IL-2 receptor alpha chain) (Whiteland et al. 1995). More specific markers can be used to distinguish between T and B cells. B cells can be labeled for CD45R/B220 (Whiteland et al. 1995), Ki-B1R antigen (Koch et al. 2008), CD19 or Pax5 (Ward et al. 2006); and the pan-T cell marker, CD3, is very useful for showing their presence in rodent tissues (Ward et al. 2006). All of these markers can be used both for immunohistochemistry on brain sections and for FACS analysis. Immunohistochemistry is of limited use for identifying T cell subtypes and double labeling is usually required. For example, CD4 labels helper T cells, and CD8 labels cytotoxic T cells and NK cells (Whiteland et al. 1995), but in rats, both have been reported to label macrophages and dendritic cells (Gibbings and Befus 2009). To further discriminate T cell subtypes, it is better to use FACS analysis (Liesz et al. 2011).

19.3.3 Findings on Brain Inflammation in Rodent Models of Ischemia

Here, we will address the intersection of inflammation and ischemia, with emphasis on the major immune cells involved, and animal models using vessel occlusion or vasoconstriction, which are more closely aligned with human ischemic stroke seen in the clinical setting. Microglia, macrophages, and neutrophils are the most abundant types of immune cells found in the ischemic infarct (Gelderblom et al. 2009), and consequently, have been the focus of most experimental stroke studies.

19.3.3.1 Results on Neutrophils

Circulating blood leukocytes are thought to have a more prominent and damaging role in transient ischemia, which is followed by reperfusion, than in permanent ischemia, in which blood flow is arrested. Reperfusion after transient ischemia increases endothelial cell expression of adhesion molecules that attract circulating neutrophils, and this recruitment can further clog blood vessels (Connolly et al. 1996b; del Zoppo et al. 1991; Hartl et al. 1996; Prestigiacomo et al. 1999). The prevailing view

is that neutrophils are the first hematogenous cells to infiltrate the ischemic infarct. For instance, using the ET-1 model of transient ischemia, we (Moxon-Emre and Schlichter 2010) and others (Souza-Rodrigues et al. 2008) observed peak neutrophil levels at 1 day, and they were undetectable by 7 days. However, another study reported a complete absence of neutrophils (Hughes et al. 2003). The reasons for this discrepancy are not clear but might reflect the labeling methods; i.e., different antibodies, occasional use of antigen retrieval (Sect. 19.3.2.1). The idea that neutrophils are harmful after ischemia is long-standing. The extent of ischemic injury correlates with the increase in neutrophil numbers (Weston et al. 2007). Neutrophils express several pro-inflammatory mediators, and although activated neutrophils are short-lived (1–3 days) (Moxon-Emre and Schlichter 2010; Lerouet et al. 2002; Matsuo et al. 1994; Weston et al. 2007), they can contribute to the early rise in cytokines, reactive oxygen species, and matrix metallopeptidases (MMPs) that can exacerbate BBB breakdown and tissue damage (Justicia et al. 2003; Hartl et al. 1996; Nguyen et al. 2007).

The most compelling evidence comes from studies that have depleted the pool of circulating neutrophils or inhibited their extravasation (reviewed in (Hartl et al. 1996)). Antibody-mediated neutrophil depletion has been exploited in several studies of cerebral ischemia (Matsuo et al. 1994; Gautier et al. 2009; Harris et al. 2005; Petrault et al. 2005). We used this method in a study of ICH in the rat striatum, and showed how blood neutrophil depletion affects their density in the lesion (Moxon-Emre and Schlichter 2011). Several studies implicate neutrophils in BBB breakdown, edema, and infarct volume (Matsuo et al. 1994; Gautier et al. 2009) but others reported that neutrophil depletion did not affect the infarct size (Beray-Berthat et al. 2003; Yilmaz et al. 2006). One possible explanation is that the contribution of neutrophils to ischemic damage might be region dependent. After transient MCAo, MPO activity was elevated for up to 3 days in the cortex and striatum of adult rats (Weston et al. 2007). Depleting neutrophils with vinblastine reduced MPO activity in the cortex and striatum, but oxidative stress and infarct volume decreased only in the cortex (Beray-Berthat et al. 2003). This is consistent with neutrophils being more abundant and long-lasting (up to 15 days post-ischemia) in the cortex than in the striatum (Weston et al. 2007). However, while increased MPO activity reflected accumulation of neutrophils, the sustained elevation at later times reflected neutrophil phagocytosis by microglia and macrophages (Weston et al. 2007).

In considering neutrophils as a potential target for reducing stroke damage, it is important to note their interactions with capillary endothelial cells, which regulate the BBB and control neutrophil infiltration (reviewed in (Tonnesen 1989)). Endothelial cells are more resistant to ischemic insults than neurons but are still vulnerable, and their damage can contribute to BBB breakdown and secondary injury (Won et al. 2011). A salient in vitro study that exposed bovine endothelial cells to OGD showed that they express inducible nitric oxide synthase (NOS-2) and produce nitric oxide (NO), which contributes to their OGD-induced apoptosis (Xu et al. 2000). However, not all sources of NO are detrimental. There is evidence that toxicity depends on the cellular source, type of NO synthase used (e.g., inducible versus constitutive), and timing. Some studies suggest that NO

production very early after transient ischemia is beneficial. That is, infarct volume and neutrophil infiltration increased if the NOS inhibitor, L-NAME (N^G-nitro-L-arginine methyl ester), was injected intraperitoneally immediately (but not 1 h) after the onset of ischemia (Batteur-Parmentier et al. 2000). That study suggested that high NO might limit neutrophil entry. Transgenic mice lacking constitutive endothelial NOS had larger infarcts after focal ischemia (Huang et al. 1996). A recent study has challenged the accepted view that neutrophils infiltrate the brain parenchyma after transient MCAo. Neutrophils did accumulate but remained in the capillary lumen or perivascular space in mice and in samples from human stroke patients (Enzmann et al. 2013). These studies have contributed to an ongoing debate concerning whether the contribution of neutrophils to stroke pathology has been overestimated (Kalimo et al. 2013).

19.3.3.2 Results on Microglia and Macrophages

A burgeoning area of experimental stroke research (reviewed in (Weinstein et al. 2010)) is the roles of macrophage-related innate immune cells: microglia, macrophages, and dendritic cells. Their roles are widely debated. A serious confounding factor is that most studies have failed to discriminate between activated microglia and macrophages (Sect. 19.3.2.2). Both cell types have the capacity to produce molecules and perform functions that can potentially either exacerbate damage or aid in repair. In vitro studies show that purified populations of microglia can produce pro-inflammatory cytokines (e.g., tumor necrosis factor (TNF)-α, interleukin (IL)-1β), and reactive oxygen and nitrogen species that can be cytotoxic (Wang et al. 2007; Hanisch and Kettenmann 2007). Conversely, microglia can produce anti-inflammatory cytokines (e.g., IL-10, TGFβ) that dampen the inflammatory response, and growth factors and neuroprotective molecules that can promote neuron survival and repair (Turrin and Rivest 2006; Wang and Dore 2007). In vivo studies are just beginning to discriminate their roles using selective monocyte depletion, genetic labels, and chimeric or parabiotic animals.

As CNS-resident cells, microglia have the potential to respond rapidly to ischemic events, including the initial insult to neurons, and the timing of events has sometimes been used to implicate microglia. After transient MCAo, one study concluded that microglia progress to the phagocytic state before macrophages enter (Schilling et al. 2005). As noted (Sect. 19.3.2.2), ED1 is up-regulated in phagocytic microglia and macrophages. Using the ET-1 injection model in rats, one study saw accumulation of ED1-labeled cells at 1 day, with maximal accumulation at 3 and 7 days (Souza-Rodrigues et al. 2008), while we (Moxon-Emre and Schlichter 2010) and others (Hughes et al. 2003) did not see them at 1 day. The timing of macrophage entry after stroke is uncertain, possibly reflecting different stroke models, but certainly because of identification issues. After transient MCAo in rats, round, $Iba1^+$ cells were present in the infarct at 12–24 h and interpreted as "may be blood-borne macrophages" (Ito et al. 2001). However, there was no proof of their identity. A similar study claiming monocyte infiltration as early as 1 day used H&E staining

and showed cells accumulating in the lumen of blood vessels (Clark et al. 1994). The timing of extravasation was not determined and the authors stated that macrophages were abundant in the infarct by 3 days. A better approach is to systemically inject super-paramagnetic iron oxide nanoparticles, which are endocytosed by blood-borne monocytes and can be detected by MRI in the brain. Using this method after cortical photo-thrombotic stroke in rats, blood-derived macrophages were detected in the ischemic infarct only after 5–8 days (Kleinschnitz et al. 2003). Based on their late entry, the authors postulate that after stroke, macrophages are involved in remodeling functions rather than acute responses.

Another major issue in considering potential roles of activated microglia and macrophages is defining what is meant by "activation." For microglia it is essential to move beyond conventional descriptions of morphological changes, and instead, assess their biological functions. Recent attempts to better define microglial activation have used macrophage activation as a starting point (Luo and Chen 2010; Boche et al. 2013; Colton 2009; Noda and Suzumura 2012). Over the past decade, up to five modes of macrophage activation have been proposed to describe their responses, with each mode depending on the stimulus and affecting different inflammatory mediators and cellular functions (reviewed in (Van Dyken and Locksley 2013; Gordon 2003; Varin and Gordon 2009)). In the absence of microbe infiltration, classical and alternative activation are especially relevant to stroke. While classical activation is evoked by bacterial lipopolysaccharide, it is postulated to occur after exposure to "danger" signals during early stroke events. Classical activation results in secretion of reactive oxygen and nitrogen species and pro-inflammatory mediators. Alternative activation induces anti-inflammatory molecules that antagonize the actions of the pro-inflammatory mediators produced during classical activation. Thus, it is thought to be involved in tissue remodeling and repair. Experimentally, alternative activation can be evoked by IL-4 and IL-13. The analogy between activation of macrophages and microglia is probably too simplistic. Monocytes are blood-borne cells that mature into macrophages in multiple tissues, while microglial cells normally reside behind the BBB. These very different chemical and structural environments will undoubtedly influence the specific responses and contributions of these two cell types to brain pathology.

Because blood-derived macrophages are absent from the healthy brain, it is much easier to analyze functions of "resting" microglia. Several studies have addressed motility, and we now know that the ramified processes of resting microglia are actually very active in sensing the environment. The processes make contact with various neural tissue elements, including neurons, astrocytes, and blood vessels, and engulf tissue particles that are then retrograde-transported to their somata (Nimmerjahn et al. 2005). The ramified processes of microglia monitor synaptic conditions, and their motility is modulated by neuron activity. Live imaging using two-photon microscopy shows that cortical microglia make brief (~5 min) contacts with synapses as often as once an hour, and the frequency decreases during reduced neuron activity (Wake et al. 2009). There is evidence that multiple neurotransmitters affect microglial motility. In an ex vivo retinal explant model, glutamatergic neurotransmission through AMPA and kainate receptors maintained microglial branching complexity and increased process motility indirectly through purinergic signaling

(Fontainhas et al. 2011). Ionotropic GABAergic neurotransmission had the opposite effects; it decreased branching complexity and reduced process motility. Changes in motility of microglial processes occur after ischemia. One study addressed this in the peri-infarct region after photo-thrombotic focal ischemia in the mouse somatosensory cortex (Wake et al. 2009). As early as 30 min after ischemia, the duration of contact between microglia and synapses increased, and was often followed by loss of the presynaptic terminal. A recent study found that after 60 min of focal ischemia, there was an increase in microglial branching complexity (hypertrophy) in the ischemic striatum but a decrease in number of contacts per microglia in the cortex (Morrison and Filosa 2013). However, following reperfusion for 8 or 24 h, progressive retraction of microglial processes (de-ramification) occurred in both regions.

Phagocytosis is an important function of microglia and macrophages after stroke; indeed, microglia were once considered the brain's "garbage men." The bulk of the evidence is that microglial cells can rapidly transform into active phagocytes, in some cases before macrophages have even begun to enter the brain. One such study depleted peripheral monocytes using clodronate-loaded liposomes, and then subjected the rats to photo-thrombotic focal ischemia (Schroeter et al. 1997). Accumulation of phagocytic cells in the peri-infarct area was mainly microglia at 3 days but both microglia and macrophages at 6 days. A study of transient MCAo in mice used a transgenic, bone-marrow chimera in which blood-borne monocytes were labeled with green fluorescent protein (GFP) (Schilling et al. 2005). Microglial activation, as judged by morphological changes of unlabeled cells, was seen as early as 1 day, was coincident with phagocytosis of neuronal debris, and preceded macrophage infiltration, which began on day 2. By 7 days, massive accumulation of these cells was observed in the infarct, but macrophages accounted for only 20 % of the cells. Using the ET-1 injection model in rats, our laboratory also observed huge numbers of activated microglia/macrophages in the infarct by 7 days, but we could not distinguish between the two cell types (Moxon-Emre and Schlichter 2010). It has been postulated that a protective role of microglia is to phagocytose infiltrating neutrophils, thereby reducing toxic molecules in the extracellular space (Denes et al. 2007; Neumann et al. 2008). However, it is also possible that by releasing inflammatory molecules during phagocytosis, microglia might damage bystander neurons. Given that most acute neuron death (gray matter injury) has already occurred by the time the majority of macrophages infiltrate, the question is: What are macrophages doing? Do they perpetrate delayed white matter damage? Are they mainly involved in repair mechanisms? These questions and others highlight the need to determine, in time and space, the distribution of the two cell types, what molecules they produce, and what specific cellular functions they perform.

19.3.3.3 Results on Lymphocytes and Other Immune Cells

The peripheral immune system is suppressed after stroke, perhaps as an attempt to offset the inflammatory reactions occurring in the brain, and T lymphocytes are thought to be involved in this process (Planas and Chamorro 2009). However, there is a continuing debate concerning the degree to which lymphocytes enter the

brain after stroke and whether they contribute to brain pathology. An early study reported T cell infiltration in the rat cortex as early as 1 day following photothrombotic stroke (Jander et al. 1995). FACS analysis and immunohistochemistry have identified both T and B cells in the ischemic hemispheres of mice 3 days after transient MCAo (Stevens et al. 2002; Gelderblom et al. 2009). Some findings suggest that T cells play a more prominent role in the evolving stroke damage, and they have been receiving more attention in experimental stroke research (reviewed in (Brait et al. 2012)). Transgenic mice in which "recombination activating gene 1" (RAG1) is deleted are deficient in both T and B cells, and RAG1-null mice had 70 % smaller ischemic infarcts at 24 h after reperfusion than their wild-type counterparts. If RAG1-null mice were reconstituted with $CD3^+$ T cells, the ischemic infarcts were larger and comparable to wild-type mice; whereas, reconstitution with B cells did not affect the infarct size (Kleinschnitz et al. 2010).

Differences in stroke models, subclasses of T cells examined and gender contribute to controversies about the role of T cells. A transient increase in natural killer T cells (NKT) cells (but not NK cells) has been identified by FACS analysis in the ipsilateral mouse hemisphere 3 days after transient MCAo (Gelderblom et al. 2009) but their role is unknown. As expected, T cells infiltrate better after reperfusion in a transient ischemia model, but surprisingly, the brains of male mice contained more T cells (Brait et al. 2010). On the one hand, there is evidence for a beneficial role of regulatory T cells (T_{reg}) in a permanent MCAo model in mice. Depletion of T_{reg} cells using a CD25 function-blocking antibody increased the infarct size, microglial "activation," neutrophil invasion, levels of pro-inflammatory cytokines in the brain and blood, and neurological deficits (Liesz et al. 2009). Conversely, a detrimental role was seen in mice after transient MCAo. Selective genetic depletion of T_{reg} cells in DEpletion of REGulatory T cells (DEREG) mice reduced the infarct size and improved neurological outcome scores (Kleinschnitz et al. 2012). Intriguingly, that study found that T_{reg} cells can exacerbate ischemic brain damage by causing dysfunction of the microvasculature, rather than through their typical immunosuppressive functions. Roles of other T cell subsets are beginning to be investigated. After transient MCAo, mice lacking $CD4^+$ or $CD8^+$ T cells had smaller infarct volumes, which correlated with less leukocyte adhesion and recruitment as early as 4 h after reperfusion (Yilmaz et al. 2006). Given that an adaptive immune response generally takes days to develop, this rapid effect is surprising and the mechanism is unknown. Related studies of T cell roles after ischemia in other organs, such as liver (Zwacka et al. 1997) and kidney (Yokota et al. 2002) further demonstrate their complexity, and highlight the need to clarify their roles in cerebral ischemia. We lack studies of the contributions of T cells to white matter injury after stroke; however, future investigations can be informed by studies that associate specific T cell subsets with white matter damage in multiple sclerosis (Scheikl et al. 2010; Walker et al. 2011).

19.4 Intersection of Inflammation and White Matter Damage After Transient Ischemia

19.4.1 Adult Rodents

The preceding section addressed the intersection of inflammation and transient ischemia (see Venn diagram in Fig. 19.2), focusing on the immune cells involved. Here, we add the third component: white matter damage. Much of the limited information about effects of inflammation on white matter injury after ischemia is based on the correlation of microglia and macrophage accumulation in regions of white matter damage.

As one of surprisingly few groups that address this three-way intersection, we have exploited the transient focal ischemia model induced by a single 400 pmol ET-1 injection into the anterior striatum (putamen + caudate) of adult Sprague–Dawley rats. In our first study, having determined that saline injection alone caused minimal damage at each time point, we quantitatively compared changes in the core and edge of the infarct with the undamaged contralateral striatum (Moxon-Emre and Schlichter 2010). We showed that neutrophils (strongly immunoreactive for MPO) were present in the ischemic infarct at 1 day following the insult, and were no longer detected by 7 days. When present, neutrophils were inside and around MBP-labeled myelinated white matter tracts. As previously discussed (Sect. 19.3.2.2), after microglia had rounded-up we could not readily distinguish them from macrophages, and therefore used the collective term: activated microglia/macrophages. [See Fig. 19.7 for examples of staining for CD11b, Iba1, ED1.] As shown in Fig. 19.8, by 3 days after ischemia, Iba1-labeled activated microglia/macrophages began to infiltrate MBP-labeled white matter tracts in the infarct. By 7 days, there was a massive infiltration of Iba1-labeled cells into white matter tracts that were damaged, as judged by loss of MBP signal. By co-immunolabeling adjacent serial sections for MBP and dMBP, we were further able to show that the infiltrated bundles were damaged. A similar study of transient ischemia injected a much smaller dose of ET-1 (10 pmol) into the striatum of adult Wistar rats (Souza-Rodrigues et al. 2008), and did not include quantitative analysis. Qualitative similarities include progressive loss of MBP staining in striatal axon bundles that was most prominent by 7 days; infiltration by 1 day of neutrophils (labeled with MBS-1); and later accumulation, at 3 and 7 days, of ED1-positive cells in the ischemic infarct. However, we must be cautious in comparing the results because that study did not show the infarct, report its volume, or show the position of the sample site. It also did not compare the locations of neutrophils or microglia/macrophages in the infarct, or their spatial relation to the damaged white matter.

We know very little about the specific activation state of the microglia/macrophages or their contribution to the evolving white matter injury. However, in the two studies just described, their increased expression of the lysosomal marker, ED1 (see Fig. 19.7), suggests that they are actively phagocytic; perhaps removing myelin

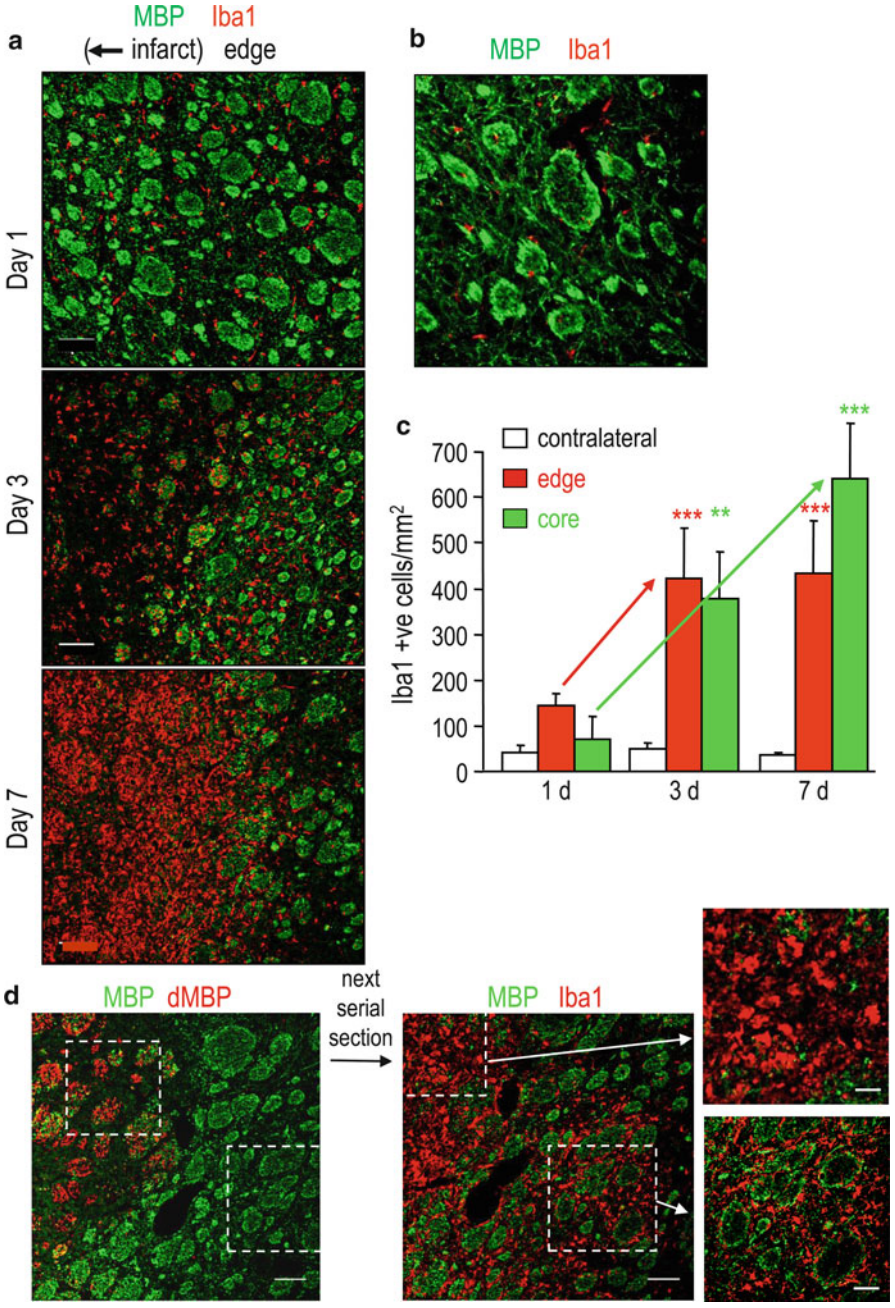


Fig. 19.8 Relationship between activated microglia/macrophages and myelin damage after ET-1-induced ischemia (Sect. 19.4.1). (**a**, **b**) At the edge of the infarct (**a**), there is a spatial-temporal correlation of the accumulation of activated microglia/macrophages (immunolabeled with rabbit polyclonal anti-Iba1 antibody, *red*) with loss of normal MBP-labeled myelin (labeled

debris. Both microglia and macrophages can phagocytose myelin in vitro (Smith 1993; Trotter et al. 1986). Myelin degradation products have been found in vivo within lesion-associated cells in the corpus callosum of cuprizone-fed mice (Olah et al. 2012). The authors concluded that these were microglia because this model of demyelination and re-myelination apparently has minimal involvement of peripheral immune cells.

More information comes from models of neonatal hypoxia–ischemia (Sect. 19.2.2.3) and adult chronic ischemia. In humans, chronic hypo-perfusion resulting from blocked vessels or impaired vascular function is thought to underlie vascular dementia (Roman et al. 2002), and is associated with neurodegeneration, impaired cognitive processes and psychiatric disorders (reviewed in (Farkas et al. 2007)). The main animal model of chronic hypo-perfusion uses bilateral common carotid artery occlusion (BCCAo) in adult rats, which results in memory impairment (Ohta et al. 1997). Two earlier studies used this model to correlate inflammation with white matter damage, as judged by LFB (Wakita et al. 1994) or by ultra-structural changes, including large vacuoles in swollen axons, and myelin sheaths that were irregular and loosely wrapped (Farkas et al. 2004). In the latter study, there was a prolonged (13 weeks) accumulation of $CD11b^+$ cells in the corpus callosum, internal capsule and, most notably, in the optic tract (Farkas et al. 2004). The white matter damage correlated with accumulation of $CD11b^+$ microglia (macrophages?), often in close proximity to damaged fibers (Farkas et al. 2004), and with infiltration of $CD4^+$ and $CD8^+$ lymphocytes (Wakita et al. 1994).

Accumulation of major histocompatibility complex (MHC) class I-labeled cells began as early as 1 day, was much more prominent in white matter than gray matter, and preceded the appearance of white matter lesions (Wakita et al. 1994). These cells were called "microglia" at the earlier time points (1–3 days). At 1 day, the white matter infiltrating cells had shortened processes, hypertrophic somata, and increased staining for MHC class I (Wakita et al. 1994). By 3 days, the bundle-infiltrating cells began to express MHC class II (Ia) antigen and LCA (CD45). Their numbers increased with time to a peak at 7–14 days, at which time they were round, looked like foamy macrophages, and from this time on, the authors called them "microglia/macrophages." In a recent study that did not directly correlate

Fig. 19.8 (continued) with mouse monoclonal anti-MBP, *green*). Scale bars=100 μm. For comparison, staining in the uninjured contralateral striatum is shown ((**b**), same scale). (**c**) There was a time-dependent increase in density of Iba1-labeled activated microglia/macrophages in the core and at the edge of the infarct. Values are shown as mean ± SD for four animals at each time point, and differences from day 1 are indicated (**$p<0.01$, ***$p<0.001$). (**d**) Activated microglia/macrophages infiltrate axon bundles displaying damaged myelin. *Left* and *middle*: Sections were double-stained with antibodies that recognize normal myelin (MBP) and damaged myelin (dMBP). The *boxes* outline the same white matter bundles in the adjacent serial sections. Scale bars = 100 μm. *Right*: Higher magnification images of the boxed regions from the *middle panels*. Scale bars = 50 μm. (Modified from Moxon-Emre I and Schlichter LC. 2010. Evolution of inflammation and white matter injury in a model of transient focal ischemic stroke. J. Neuropathol. Expt'l Neural. 69: 1–15)

inflammation with white matter damage, increased numbers of MHC class II-labeled microglia (macrophages?) were detected at 13 weeks after BCCAo in the hippocampal fimbria of rats exhibiting spatial memory impairments (Choi et al. 2011).

Several studies have used drugs to target inflammation following BCCAo. Rats that received intraperitoneal injections of the immunosuppressant, cyclosporin A, had less white matter damage, as judged by LFB and Bielschowsky silver stain (Wakita et al. 1995). The white matter tracts also contained fewer "microglia/macrophages" that were immunostained for LCA, MHC class I or II antigens. A very similar study by the same group used the immunosuppressant, FK506, and found similar results; i.e., less white matter damage and fewer bundle-infiltrating microglia/macrophages (Wakita et al. 1998). A third BCCAo study tested a 2 week treatment with the anti-inflammatory compound, minocycline (Cho et al. 2006). They found reduced white matter injury (LFB, Bielschowsky silver, MBP staining) in the corpus callosum and optic tract, and reduced microglia (and macrophage?) activation, as measured by the $CD11b^+$ area in the corpus callosum. Similarly, after MCAo in adult mice, axon damage and oligodendrocyte death were reduced by melatonin, which is considered to act an anti-inflammatory agent (Lee et al. 2005).

19.4.2 Neonatal Rodents

Although there are few investigations of the specific intersection between ischemia, inflammation, and white matter damage in neonatal animals, there are more publications than for adult animals. Neonatal stroke studies almost entirely exploit the HI model in rodents, which causes damage to both white and gray matter (Calvert and Zhang 2005). White matter damage is often assessed as the degree of myelin loss, axonal damage, and oligodendrocyte death (Carty et al. 2008; Villapol et al. 2011; Biran et al. 2006; Deng et al. 2008; Uehara et al. 1999; Wang et al. 2013). Subcortical white matter damage correlates with decreased numbers of immature (Carty et al. 2008; Wang et al. 2013) and mature oligodendrocytes (Carty et al. 2008; Villapol et al. 2011). Several HI studies have monitored inflammation concomitantly with white matter injury and have shown an increase in numbers of microglia/macrophages in white matter tracts. For instance, after HI in neonatal rats, white matter injury was accompanied by a 2–3 fold increase in the number of microglia (macrophages?) (IB_4 or CD11b labeled), which were assumed to be "activated" (Biran et al. 2006). However, their specific activation states have not been defined and, for the reasons addressed in Sect. 19.3.3.2, it is not known whether these two immune cells play similar or different roles.

It is well known from in vitro studies from our lab (Kaushal et al. 2007; Kaushal and Schlichter 2008; Sivagnanam et al. 2010; Fordyce et al. 2005; Khanna et al. 2001; Schlichter et al. 2010) and others (Colton and Gilbert 1987; Mao et al. 2007; Piani et al. 1991; Smith et al. 1998; Lai and Todd 2008; Rock et al. 2004) that activated neonatal microglia can produce neurotoxic molecules. Less is known about their products and roles in white matter injury in vivo. Several studies of neonatal HI have directly addressed correlations between inflammatory cell numbers and white matter

damage. Evidence that these cells directly contribute to white matter damage is the phagocytosis of immature oligodendrocytes in damaged white matter, as judged by colocalization of the microglia/macrophage marker, Iba1, and the immature oligodendrocyte marker, O1 (Biran et al. 2006). This is expected to hamper recovery and remyelination. In the corpus callosum of rat pups, a decrease in the density and activation of microglia (macrophages?) (Iba1 staining) accompanied a decrease in myelin loss (MBP staining intensity) and increase in premyelinating (O4+ cells) and immature oligodendrocytes (O1+ cells) (Carty et al. 2008). More recently, when the density of microglia (macrophages?) (tomato lectin staining) was reduced by melatonin administration, there was a decrease in HI-evoked white matter damage (MBP loss) and an increase in mature oligodendrocytes (APC-labeled cells) (Villapol et al. 2011).

Several studies support the view that inflammation, microglia/macrophage activation, and specific pro-inflammatory molecules contribute to white matter damage after neonatal HI. Some studies have begun to target functions of specific inflammatory mediators. Increased expression of the potentially toxic cytokines, IL-1β, and TNF-α, was seen in the rat corpus callosum after HI (Deng et al. 2008; Wang et al. 2013; Brochu et al. 2011; Carty et al. 2011). Administering the IL-1 receptor antagonist reduced white matter injury in the internal capsule (Girard et al. 2012). IL-1β can be processed to its active form by MMP9 (Schonbeck et al. 1998) and, after HI in MMP9-null mice, there were fewer activated microglia (macrophages?) (IB_4 staining) in the damaged white matter, which was judged by loss of MBP (Svedin et al. 2007). MMPs are regulated by several "tissue inhibitors of metallopeptidases" (TIMPs), and there is evidence from knockout mice that TIMP-3 contributes to TNF-α-dependent death of immature oligodendrocytes after MCAo in the adult (Yang et al. 2011). Cyclooxygenase-2 (COX-2) is an interesting target because it is inhibited by nonsteroidal anti-inflammatory drugs (such as aspirin and ibuprofen), and it increases after ischemic stroke in humans, rodents, and nonhuman primates (reviewed in (Candelario-Jalil and Fiebich 2008)). In rats, COX-2 was found in activated microglia (macrophages?) (Bauer et al. 1997), and the nonsteroidal anti-inflammatory drug, ibuprofen, inhibited COX-2 induction after HI (Carty et al. 2011). In mice, the pro-inflammatory cytokine, IL-18, was markedly increased in microglia and astrocytes after HI, and IL-18-null mice had less subcortical white matter damage (Hedtjarn et al. 2002, 2005). Several anti-inflammatory drugs reduced white matter damage in rodent HI models. Ibuprofen reduced IL-1β and TNF-α, and increased the amount of intact myelin (MBP stained) and immature (O1+) and mature oligodendrocytes (O4+) (Carty et al. 2011). White matter damage (loss of MBP) was also reduced by minocycline (Carty et al. 2008) and melatonin (Villapol et al. 2011).

19.5 Future Studies Needed

In 2010, we published our first paper on inflammation and white matter damage after focal ischemia in rat (Moxon-Emre and Schlichter 2010). At that time we stated that: "Despite substantial progress in understanding the pathogenesis of neuronal injury after stroke, most preclinical studies have failed to consider damage to

the white matter. Only recently have stroke studies begun to focus on the secondary injury phase and prominent inflammatory response, which is delayed, prolonged, and more amenable to treatment than acute neurotoxicity." White matter is increasingly recognized as a vulnerable region following ischemia (reviewed in (Petty and Wettstein 1999)); thus, we were surprised how little was known about the spatial and temporal progression of white matter damage. As described in Sect. 19.4.1, only two studies had addressed inflammation and white matter damage after transient ischemia in adult rodents (Hughes et al. 2003; Souza-Rodrigues et al. 2008), and they came to different conclusions about the infiltration of neutrophils and microglia/macrophages into the ischemic infarct. Thus, we set out to quantitatively analyze the temporal and spatial coevolution of white matter damage and inflammation using ET-1 injection to produce a transient, focal ischemic stroke in the rat striatum (Moxon-Emre and Schlichter 2010). ED1-labeled microglia/macrophages selectively infiltrated damaged white matter tracts in the rat striatum as early as 3 days after focal ischemia, but remained outside undamaged white matter tracts. As the Venn diagram in Fig. 19.2 illustrates, we found no additional publications addressing the intersection of inflammation and white matter damage after transient ischemia in adult rodents. This dearth of experimental animal research means there are many important aspects to address in future. Here, we will briefly discuss a few that are especially relevant to the material in this chapter.

1. While the ET-1 model of ischemia has shown that microglia/macrophages infiltrate damaged white matter tracts, we do not know their specific activation state or what functions they are carrying out (Sect. 19.3.2.2). We do not know if, or how, this infiltration affects neurotransmission, neuron survival, repair, or ultimate behavioral outcomes. We know little, if anything, about whether other inflammatory cells (e.g., lymphocytes, dendritic cells) contribute to white matter damage after transient ischemia (Sect. 19.3.2.3). We know almost nothing about how the inflammatory response affects white matter damage in aged or hypertensive animals, which are better models of most human strokes than using young, healthy animals.
2. Very few studies have *quantified* specific responses in transient ischemic stroke models. We need more detailed information on position- and time-dependent changes in the staining intensity or area of damaged white matter, whether it is myelin or axon damage, the density or numbers of specific immune cells, inflammatory molecules they are producing, and how these responses are changed by treatments.
3. As addressed throughout this chapter (e.g., Sect. 19.3.2), a major challenge is distinguishing activated microglia from infiltrating macrophages. Over the years, several experimental manipulations have been used in an attempt to distinguish their contributions. Several studies have ablated circulating monocytes using clodronate-loaded liposomes (Schroeter et al. 1997; Kanematsu et al. 2011; Mawhinney et al. 2012) or by bone marrow irradiation (Schilling et al. 2005). The recent development of knock-in fluorescent protein reporter mice for the fractalkine receptor (CX_3CR_1+/GFP) and chemokine receptor 2 (CCR2+/RFP) promises to help differentiate endogenous microglia from invading macrophages (Mizutani et al. 2012).

4. Recent insights into the ontogeny of microglia show that parenchymal CNS microglial cells develop earlier than previously thought, and from a surprising location: the embryonic yolk sac (Ginhoux et al. 2010). This site of origin differs from bone-marrow derived macrophages. A better understanding of their development and possible differential gene expression might provide insight into how to distinguish between activated microglia and infiltrating macrophages. There is some controversy about the potential of peripheral macrophages to replenish microglia after CNS injury. In transgenic mice engineered to allow ganciclovir-mediated depletion of proliferating microglia (CD11b-HSVTK transgenic mice), circulating Iba1$^+$ macrophages entered the brain, responded to ATP gradients and migrated towards sites of kainate-induced neuron death (Varvel et al. 2012). Unlike native resting microglia, which are highly ramified and have small somata, the infiltrating macrophages had large somata and short, irregular processes. This suggests that infiltrating macrophages remain as a distinct population. In future, identifying molecular expression profiles underlying the unique morphological and developmental differences might be useful in distinguishing microglia from macrophages.
5. It has been postulated that white matter is particularly vulnerable to inflammatory mediators (Coleman and Perry 2002). This means that inflammation after stroke might damage white matter at times and in locations where frank loss of neurons is not seen. Our discovery (Sect. 19.2.3.2) that SC1/hevin is a new early marker of axon damage might prove very useful. Its spatiotemporal expression correlated with microglia/macrophage infiltration of white matter bundles, and it showed aging-related differences in the progression of white matter damage (Lively and Schlichter 2012).
6. In neonatal HI models, attempts have been made to more closely evaluate the intersection of ischemia, white matter damage, and inflammation (Sect. 19.4.2). Most studies have been correlational, showing that white matter damage in a lesioned area was accompanied by an increase in microglia/macrophage numbers. While several studies used broad-spectrum anti-inflammatory drugs (minocycline, ibuprofen, melatonin), further research is needed to address the mechanisms. As for adult animals, an over-arching need is to parse out specific aspects of the inflammatory response, and determine whether they exacerbate the damage, aid in repair, or both.

References

Stroke therapy Academic Industry Roundtable, Fisher MC (1999) Recommendations for standards regarding preclinical neuroprotective and restorative drug development. Stroke 30:2752–2758

Acarin L, Vela JM, Gonzalez B et al (1994) Demonstration of poly-N-acetyl lactosamine residues in ameboid and ramified microglial cells in rat brain by tomato lectin binding. J Histochem Cytochem 42:1033–1041

Adams HP Jr, Bendixen BH, Kappelle LJ et al (1993) Classification of subtype of acute ischemic stroke. Definitions for use in a multicenter clinical trial. TOAST. Trial of Org 10172 in Acute Stroke Treatment. Stroke 24:35–41

Akiyama H, McGeer PL (1990) Brain microglia constitutively express beta-2 integrins. J Neuroimmunol 30:81–93

Anderson CS, Chakera TM, Stewart-Wynne EG et al (1994) Spectrum of primary intracerebral haemorrhage in Perth, Western Australia, 1989–90: incidence and outcome. J Neurol Neurosurg Psychiatry 57:936–940

Arai K, Lo EH (2009) Experimental models for analysis of oligodendrocyte pathophysiology in stroke. Exp Transl Stroke Med 1:6

Barone FC, Hillegass LM, Price WJ et al (1991) Polymorphonuclear leukocyte infiltration into cerebral focal ischemic tissue: myeloperoxidase activity assay and histologic verification. J Neurosci Res 29:336–345

Batteur-Parmentier S, Margaill I, Plotkine M (2000) Modulation by nitric oxide of cerebral neutrophil accumulation after transient focal ischemia in rats. J Cereb Blood Flow Metab 20:812–819

Bauer MK, Lieb K, Schulze-Osthoff K et al (1997) Expression and regulation of cyclooxygenase-2 in rat microglia. Eur J Biochem 243:726–731

Baumann N, Pham-Dinh D (2001) Biology of oligodendrocyte and myelin in the mammalian central nervous system. Physiol Rev 81:871–927

Becker K, Kindrick D, Relton J et al (2001) Antibody to the alpha4 integrin decreases infarct size in transient focal cerebral ischemia in rats. Stroke 32:206–211

Beray-Berthat V, Croci N, Plotkine M et al (2003) Polymorphonuclear neutrophils contribute to infarction and oxidative stress in the cortex but not in the striatum after ischemia-reperfusion in rats. Brain Res 987:32–38

Bhat RV, Axt KJ, Fosnaugh JS et al (1996) Expression of the APC tumor suppressor protein in oligodendroglia. Glia 17:169–174

Biran V, Joly LM, Heron A et al (2006) Glial activation in white matter following ischemia in the neonatal P7 rat brain. Exp Neurol 199:103–112

Boche D, Perry VH, Nicoll JA (2013) Review: activation patterns of microglia and their identification in the human brain. Neuropathol Appl Neurobiol 39:3–18

Bradley PP, Priebat DA, Christensen RD et al (1982) Measurement of cutaneous inflammation: estimation of neutrophil content with an enzyme marker. J Invest Dermatol 78:206–209

Braeuninger S, Kleinschnitz C (2009) Rodent models of focal cerebral ischemia: procedural pitfalls and translational problems. Exp Transl Stroke Med 1:8

Brait VH, Jackman KA, Walduck AK et al (2010) Mechanisms contributing to cerebral infarct size after stroke: gender, reperfusion, T lymphocytes, and Nox2-derived superoxide. J Cereb Blood Flow Metab 30:1306–1317

Brait VH, Arumugam TV, Drummond GR et al (2012) Importance of T lymphocytes in brain injury, immunodeficiency, and recovery after cerebral ischemia. J Cereb Blood Flow Metab 32:598–611

Brochu ME, Girard S, Lavoie K et al (2011) Developmental regulation of the neuroinflammatory responses to LPS and/or hypoxia-ischemia between preterm and term neonates: an experimental study. J Neuroinflammation 8:55

Brott T, Bogousslavsky J (2000) Treatment of acute ischemic stroke. N Engl J Med 343:710–722

Bruni EJ, Montemurro DG (2009) Human neuroanatomy: a text, brain atlas, and laboratory dissection guide, 3rd edn. Oxford University Press, New York

Calvert JW, Zhang JH (2005) Pathophysiology of an hypoxic-ischemic insult during the perinatal period. Neurol Res 27:246–260

Candelario-Jalil E, Fiebich BL (2008) Cyclooxygenase inhibition in ischemic brain injury. Curr Pharm Des 14:1401–1418

Carmichael ST (2005) Rodent models of focal stroke: size, mechanism, and purpose. NeuroRx 2:396–409

Carty ML, Wixey JA, Colditz PB et al (2008) Post-insult minocycline treatment attenuates hypoxia-ischemia-induced neuroinflammation and white matter injury in the neonatal rat: a comparison of two different dose regimens. Int J Dev Neurosci 26:477–485

Carty ML, Wixey JA, Reinebrant HE et al (2011) Ibuprofen inhibits neuroinflammation and attenuates white matter damage following hypoxia-ischemia in the immature rodent brain. Brain Res 1402:9–19

Castellani RJ, Alexiev BA, Phillips D et al (2007) Microscopic investigations in neurodegenerative diseases. In: Mendez-Vilas A, Diaz J (eds) Modern research and educational topics in microscopy, vol 1. Formatex, Badajoz, Spain, pp 171–182

Ceulemans AG, Zgavc T, Kooijman R et al (2010) The dual role of the neuroinflammatory response after ischemic stroke: modulatory effects of hypothermia. J Neuroinflammation 7:74

Chapman KZ, Dale VQ, Denes A et al (2009) A rapid and transient peripheral inflammatory response precedes brain inflammation after experimental stroke. J Cereb Blood Flow Metab 29:1764–1768

Chen H, Chopp M, Zhang RL et al (1994) Anti-CD11b monoclonal antibody reduces ischemic cell damage after transient focal cerebral ischemia in rat. Ann Neurol 35:458–463

Cho KO, La HO, Cho YJ et al (2006) Minocycline attenuates white matter damage in a rat model of chronic cerebral hypoperfusion. J Neurosci Res 83:285–291

Choi BR, Kwon KJ, Park SH et al (2011) Alternations of septal-hippocampal system in the adult wistar rat with spatial memory impairments induced by chronic cerebral hypoperfusion. Exp Neurobiol 20:92–99

Cioffi GA (2005) Ischemic model of optic nerve injury. Trans Am Ophthalmol Soc 103:592–613

Cioffi GA, Orgul S, Onda E et al (1995) An in vivo model of chronic optic nerve ischemia: the dose-dependent effects of endothelin-1 on the optic nerve microvasculature. Curr Eye Res 14:1147–1153

Clark RK, Lee EV, White RF et al (1994) Reperfusion following focal stroke hastens inflammation and resolution of ischemic injured tissue. Brain Res Bull 35:387–392

Clozel M, Gray GA, Breu V et al (1992) The endothelin ETB receptor mediates both vasodilation and vasoconstriction in vivo. Biochem Biophys Res Commun 186:867–873

Coleman MP, Perry VH (2002) Axon pathology in neurological disease: a neglected therapeutic target. Trends Neurosci 25:532–537

Colton CA (2009) Heterogeneity of microglial activation in the innate immune response in the brain. J Neuroimmune Pharmacol 4:399–418

Colton CA (2013) Immune heterogeneity in neuroinflammation: dendritic cells in the brain. J Neuroimmune Pharmacol 8:145–162

Colton CA, Gilbert DL (1987) Production of superoxide anions by a CNS macrophage, the microglia. FEBS Lett 223:284–288

Connolly ES Jr, Winfree CJ, Stern DM et al (1996a) Procedural and strain-related variables significantly affect outcome in a murine model of focal cerebral ischemia. Neurosurgery 38:523–531, discussion 32

Connolly ES Jr, Winfree CJ, Springer TA et al (1996b) Cerebral protection in homozygous null ICAM-1 mice after middle cerebral artery occlusion. Role of neutrophil adhesion in the pathogenesis of stroke. J Clin Invest 97:209–216

Damoiseaux JG, Dopp EA, Calame W et al (1994) Rat macrophage lysosomal membrane antigen recognized by monoclonal antibody ED1. Immunology 83:140–147

del Zoppo GJ (1998) Clinical trials in acute stroke: why have they not been successful? Neurology 51:S59–S61

del Zoppo GJ, Schmid-Schonbein GW, Mori E et al (1991) Polymorphonuclear leukocytes occlude capillaries following middle cerebral artery occlusion and reperfusion in baboons. Stroke 22:1276–1283

del Zoppo GJ, Poeck K, Pessin MS et al (1992) Recombinant tissue plasminogen activator in acute thrombotic and embolic stroke. Ann Neurol 32:78–86

Denes A, Vidyasagar R, Feng J et al (2007) Proliferating resident microglia after focal cerebral ischaemia in mice. J Cereb Blood Flow Metab 27:1941–1953

Denes A, Thornton P, Rothwell NJ et al (2010) Inflammation and brain injury: acute cerebral ischaemia, peripheral and central inflammation. Brain Behav Immun 24:708–723

Deng Y, Lu J, Sivakumar V et al (2008) Amoeboid microglia in the periventricular white matter induce oligodendrocyte damage through expression of proinflammatory cytokines via MAP kinase signaling pathway in hypoxic neonatal rats. Brain Pathol 18:387–400

Dijkstra CD, Dopp EA, Joling P et al (1985) The heterogeneity of mononuclear phagocytes in lymphoid organs: distinct macrophage subpopulations in the rat recognized by monoclonal antibodies ED1, ED2 and ED3. Immunology 54:589–599
Diringer MN, Skolnick BE, Mayer SA et al (2008) Risk of thromboembolic events in controlled trials of rFVIIa in spontaneous intracerebral hemorrhage. Stroke 39:850–856
Dirnagl U (2006) Bench to bedside: the quest for quality in experimental stroke research. J Cereb Blood Flow Metab 26:1465–1478
Drake C, Boutin H, Jones MS et al (2011) Brain inflammation is induced by co-morbidities and risk factors for stroke. Brain Behav Immun 25:1113–1122
Durukan A, Tatlisumak T (2007) Acute ischemic stroke: overview of major experimental rodent models, pathophysiology, and therapy of focal cerebral ischemia. Pharmacol Biochem Behav 87:179–197
Emsley HC, Tyrrell PJ (2002) Inflammation and infection in clinical stroke. J Cereb Blood Flow Metab 22:1399–1419
Endres M, Engelhardt B, Koistinaho J et al (2008) Improving outcome after stroke: overcoming the translational roadblock. Cerebrovasc Dis 25:268–278
Engelhardt B, Sorokin L (2009) The blood–brain and the blood-cerebrospinal fluid barriers: function and dysfunction. Semin Immunopathol 31:497–511
Enzmann G, Mysiorek C, Gorina R et al (2013) The neurovascular unit as a selective barrier to polymorphonuclear granulocyte (PMN) infiltration into the brain after ischemic injury. Acta Neuropathol 125:395–412
Farkas E, Donka G, de Vos RA et al (2004) Experimental cerebral hypoperfusion induces white matter injury and microglial activation in the rat brain. Acta Neuropathol 108:57–64
Farkas E, Luiten PG, Bari F (2007) Permanent, bilateral common carotid artery occlusion in the rat: a model for chronic cerebral hypoperfusion-related neurodegenerative diseases. Brain Res Rev 54:162–180
Ferguson B, Matyszak MK, Esiri MM et al (1997) Axonal damage in acute multiple sclerosis lesions. Brain 120(pt 3):393–399
Fischer U, Arnold M, Nedeltchev K et al (2006) Impact of comorbidity on ischemic stroke outcome. Acta Neurol Scand 113:108–113
Fontainhas AM, Wang M, Liang KJ et al (2011) Microglial morphology and dynamic behavior is regulated by ionotropic glutamatergic and GABAergic neurotransmission. PLoS One 6:e15973
Ford AL, Goodsall AL, Hickey WF et al (1995) Normal adult ramified microglia separated from other central nervous system macrophages by flow cytometric sorting. Phenotypic differences defined and direct ex vivo antigen presentation to myelin basic protein-reactive CD4+ T cells compared. J Immunol 154:4309–4321
Fordyce CB, Jagasia R, Zhu X et al (2005) Microglia Kv1.3 channels contribute to their ability to kill neurons. J Neurosci 25:7139–7149
Garcia JH, Liu KF, Ho KL (1995) Neuronal necrosis after middle cerebral artery occlusion in Wistar rats progresses at different time intervals in the caudoputamen and the cortex. Stroke 26:636–642, discussion 43
Gautier S, Ouk T, Petrault O et al (2009) Neutrophils contribute to intracerebral haemorrhages after treatment with recombinant tissue plasminogen activator following cerebral ischaemia. Br J Pharmacol 156:673–679
Gelderblom M, Leypoldt F, Steinbach K et al (2009) Temporal and spatial dynamics of cerebral immune cell accumulation in stroke. Stroke 40:1849–1857
Gentleman SM, Roberts GW, Gennarelli TA et al (1995) Axonal injury: a universal consequence of fatal closed head injury? Acta Neuropathol 89:537–543
Gibbings D, Befus AD (2009) CD4 and CD8: an inside-out coreceptor model for innate immune cells. J Leukoc Biol 86:251–259
Ginhoux F, Greter M, Leboeuf M et al (2010) Fate mapping analysis reveals that adult microglia derive from primitive macrophages. Science 330:841–845

Ginsberg MD (2009) Current status of neuroprotection for cerebral ischemia: synoptic overview. Stroke 40:S111–S114
Girard S, Sebire H, Brochu ME et al (2012) Postnatal administration of IL-1Ra exerts neuroprotective effects following perinatal inflammation and/or hypoxic-ischemic injuries. Brain Behav Immun 26:1331–1339
Goldberg MP, Ransom BR (2003) New light on white matter. Stroke 34:330–332
Gordon S (2003) Alternative activation of macrophages. Nat Rev Immunol 3:23–35
Gresle MM, Jarrott B, Jones NM et al (2006) Injury to axons and oligodendrocytes following endothelin-1-induced middle cerebral artery occlusion in conscious rats. Brain Res 1110:13–22
Guillemin GJ, Brew BJ (2004) Microglia, macrophages, perivascular macrophages, and pericytes: a review of function and identification. J Leukoc Biol 75:388–397
Hanisch UK, Kettenmann H (2007) Microglia: active sensor and versatile effector cells in the normal and pathologic brain. Nat Neurosci 10:1387–1394
Hansen R, Sauder C, Czub S et al (2001) Activation of microglia cells is dispensable for the induction of rat retroviral spongiform encephalopathy. J Neurovirol 7:501–510
Harris AK, Ergul A, Kozak A et al (2005) Effect of neutrophil depletion on gelatinase expression, edema formation and hemorrhagic transformation after focal ischemic stroke. BMC Neurosci 6:49
Hartl R, Schurer L, Schmid-Schonbein GW et al (1996) Experimental antileukocyte interventions in cerebral ischemia. J Cereb Blood Flow Metab 16:1108–1119
Haynes WG, Strachan FE, Webb DJ (1995) Endothelin ETA and ETB receptors cause vasoconstriction of human resistance and capacitance vessels in vivo. Circulation 92:357–363
He Z, Yamawaki T, Yang S et al (1999) Experimental model of small deep infarcts involving the hypothalamus in rats: changes in body temperature and postural reflex. Stroke 30:2743–2751, discussion 51
He Z, Yang SH, Naritomi H et al (2000) Definition of the anterior choroidal artery territory in rats using intraluminal occluding technique. J Neurol Sci 182:16–28
Hedtjarn M, Leverin AL, Eriksson K et al (2002) Interleukin-18 involvement in hypoxic-ischemic brain injury. J Neurosci 22:5910–5919
Hedtjarn M, Mallard C, Arvidsson P et al (2005) White matter injury in the immature brain: role of interleukin-18. Neurosci Lett 373:16–20
Horie N, Maag AL, Hamilton SA et al (2008) Mouse model of focal cerebral ischemia using endothelin-1. J Neurosci Methods 173:286–290
Howells DW, Porritt MJ, Rewell SS et al (2010) Different strokes for different folks: the rich diversity of animal models of focal cerebral ischemia. J Cereb Blood Flow Metab 30:1412–1431
Huang Z, Huang PL, Ma J et al (1996) Enlarged infarcts in endothelial nitric oxide synthase knockout mice are attenuated by nitro-L-arginine. J Cereb Blood Flow Metab 16:981–987
Hughes PM, Anthony DC, Ruddin M et al (2003) Focal lesions in the rat central nervous system induced by endothelin-1. J Neuropathol Exp Neurol 62:1276–1286
Imai Y, Ibata I, Ito D et al (1996) A novel gene iba1 in the major histocompatibility complex class III region encoding an EF hand protein expressed in a monocytic lineage. Biochem Biophys Res Commun 224:855–862
Irving EA, Yatsushiro K, McCulloch J et al (1997) Rapid alteration of tau in oligodendrocytes after focal ischemic injury in the rat: involvement of free radicals. J Cereb Blood Flow Metab 17:612–622
Irving EA, Bentley DL, Parsons AA (2001) Assessment of white matter injury following prolonged focal cerebral ischaemia in the rat. Acta Neuropathol 102:627–635
Isaksson J, Farooque M, Holtz A et al (1999) Expression of ICAM-1 and CD11b after experimental spinal cord injury in rats. J Neurotrauma 16:165–173
Ito D, Imai Y, Ohsawa K et al (1998) Microglia-specific localisation of a novel calcium binding protein, Iba1. Brain Res Mol Brain Res 57:1–9

Ito D, Tanaka K, Suzuki S et al (2001) Enhanced expression of Iba1, ionized calcium-binding adapter molecule 1, after transient focal cerebral ischemia in rat brain. Stroke 32:1208–1215
Jander S, Kraemer M, Schroeter M et al (1995) Lymphocytic infiltration and expression of intercellular adhesion molecule-1 in photochemically induced ischemia of the rat cortex. J Cereb Blood Flow Metab 15:42–51
Jin R, Yang G, Li G (2010) Inflammatory mechanisms in ischemic stroke: role of inflammatory cells. J Leukoc Biol 87:779–789
Johnson GA, Calabrese E, Badea A et al (2012) A multidimensional magnetic resonance histology atlas of the Wistar rat brain. Neuroimage 62:1848–1856
Johnston MV, Ferriero DM, Vannucci SJ et al (2005) Models of cerebral palsy: which ones are best? J Child Neurol 20:984–987
Jordan J, Segura T, Brea D et al (2008) Inflammation as therapeutic objective in stroke. Curr Pharm Des 14:3549–3564
Justicia C, Panes J, Sole S et al (2003) Neutrophil infiltration increases matrix metalloproteinase-9 in the ischemic brain after occlusion/reperfusion of the middle cerebral artery in rats. J Cereb Blood Flow Metab 23:1430–1440
Kalimo H, del Zoppo GJ, Paetau A et al (2013) Polymorphonuclear neutrophil infiltration into ischemic infarctions: myth or truth? Acta Neuropathol 125:313–316
Kanematsu Y, Kanematsu M, Kurihara C et al (2011) Critical roles of macrophages in the formation of intracranial aneurysm. Stroke 42:173–178
Kaushal V, Schlichter LC (2008) Mechanisms of microglia-mediated neurotoxicity in a new model of the stroke penumbra. J Neurosci 28:2221–2230
Kaushal V, Koeberle PD, Wang Y et al (2007) The Ca2+-activated K+ channel KCNN4/KCa3.1 contributes to microglia activation and nitric oxide-dependent neurodegeneration. J Neurosci 27:234–244
Kelly-Hayes M, Robertson JT, Broderick JP et al (1998) The American Heart Association stroke outcome classification. Stroke 29:1274–1280
Kettenmann H, Hanisch UK, Noda M et al (2011) Physiology of microglia. Physiol Rev 91:461–553
Khanna R, Roy L, Zhu X et al (2001) K+ channels and the microglial respiratory burst. Am J Physiol Cell Physiol 280:C796–C806
Kirby RS, Wingate MS, Van Naarden BK et al (2011) Prevalence and functioning of children with cerebral palsy in four areas of the United States in 2006: a report from the Autism and Developmental Disabilities Monitoring Network. Res Dev Disabil 32:462–469
Kleinig TJ, Vink R (2009) Suppression of inflammation in ischemic and hemorrhagic stroke: therapeutic options. Curr Opin Neurol 22:294–301
Kleinschnitz C, Bendszus M, Frank M et al (2003) In vivo monitoring of macrophage infiltration in experimental ischemic brain lesions by magnetic resonance imaging. J Cereb Blood Flow Metab 23:1356–1361
Kleinschnitz C, Schwab N, Kraft P et al (2010) Early detrimental T-cell effects in experimental cerebral ischemia are neither related to adaptive immunity nor thrombus formation. Blood 115:3835–3842
Kleinschnitz C, Kraft P, Dreykluft A et al (2012) Regulatory T cells are strong promoters of acute ischemic stroke in mice by inducing dysfunction of the cerebral microvasculature. Blood 121:679–691
Kluver H, Barrera E (1953) A method for the combined staining of cells and fibers in the nervous system. J Neuropathol Exp Neurol 12:400–403
Koch M, Broecker V, Heratizadeh A et al (2008) Induction of chronic renal allograft injury by injection of a monoclonal antibody against a donor MHC Ib molecule in a nude rat model. Transpl Immunol 19:187–191
Koizumi J, Yoshida Y, Nakazawa T et al (1986) Experimental studies of ischemic brain edema, I: a new experimental model of cerebral embolism in rats in which recirculation can be introduced in the ischemic area. Jpn J Stroke 8:1–8

Krafft PR, Bailey EL, Lekic T et al (2012) Etiology of stroke and choice of models. Int J Stroke 7:398–406
Kuge Y, Minematsu K, Yamaguchi T et al (1995) Nylon monofilament for intraluminal middle cerebral artery occlusion in rats. Stroke 26:1655–1657, discussion 8
Lai AY, Todd KG (2008) Differential regulation of trophic and proinflammatory microglial effectors is dependent on severity of neuronal injury. Glia 56:259–270
Laing RJ, Jakubowski J, Laing RW (1993) Middle cerebral artery occlusion without craniectomy in rats. Which method works best? Stroke 24:294–297
Lakhan SE, Kirchgessner A, Hofer M (2009) Inflammatory mechanisms in ischemic stroke: therapeutic approaches. J Transl Med 7:97
Lee EJ, Lee MY, Chen HY et al (2005) Melatonin attenuates gray and white matter damage in a mouse model of transient focal cerebral ischemia. J Pineal Res 38:42–52
Leifer D, Kowall NW (1993) Immunohistochemical patterns of selective cellular vulnerability in human cerebral ischemia. J Neurol Sci 119:217–228
Lerouet D, Beray-Berthat V, Palmier B et al (2002) Changes in oxidative stress, iNOS activity and neutrophil infiltration in severe transient focal cerebral ischemia in rats. Brain Res 958:166–175
Liesz A, Suri-Payer E, Veltkamp C et al (2009) Regulatory T cells are key cerebroprotective immunomodulators in acute experimental stroke. Nat Med 15:192–199
Liesz A, Zhou W, Mracsko E et al (2011) Inhibition of lymphocyte trafficking shields the brain against deleterious neuroinflammation after stroke. Brain 134:704–720
Lin Y, Stanworth S, Birchall J et al (2012) Recombinant factor VIIa for the prevention and treatment of bleeding in patients without haemophilia. Cochrane Database Syst Rev (3):CD005011
Liu F, McCullough LD (2011) Middle cerebral artery occlusion model in rodents: methods and potential pitfalls. J Biomed Biotechnol 2011:464701
Lively S, Schlichter LC (2012) SC1/hevin identifies early white matter injury after ischemia and intracerebral hemorrhage in young and aged rats. J Neuropathol Exp Neurol 71:480–493
Lively S, Moxon-Emre I, Schlichter LC (2011) SC1/hevin and reactive gliosis after transient ischemic stroke in young and aged rats. J Neuropathol Exp Neurol 70:913–929
Lopez AD, Mathers CD (2006) Measuring the global burden of disease and epidemiological transitions: 2002–2030. Ann Trop Med Parasitol 100:481–499
Luo XG, Chen SD (2010) The changing phenotype of microglia from homeostasis to disease. Transl Neurodegener 1:9
Macrae IM (1992) New models of focal cerebral ischaemia. Br J clin Pharmacol 34:302–308
Mao H, Fang X, Floyd KM et al (2007) Induction of microglial reactive oxygen species production by the organochlorinated pesticide dieldrin. Brain Res 1186:267–274
Marcoux FW, Morawetz RB, Crowell RM et al (1982) Differential regional vulnerability in transient focal cerebral ischemia. Stroke 13:339–346
Matsumoto H, Kumon Y, Watanabe H et al (2007) Antibodies to CD11b, CD68, and lectin label neutrophils rather than microglia in traumatic and ischemic brain lesions. J Neurosci Res 85:994–1009
Matsuo Y, Onodera H, Shiga Y et al (1994) Correlation between myeloperoxidase-quantified neutrophil accumulation and ischemic brain injury in the rat. Effects of neutrophil depletion. Stroke 25:1469–1475
Matsuo A, Lee GC, Terai K et al (1997) Unmasking of an unusual myelin basic protein epitope during the process of myelin degeneration in humans: a potential mechanism for the generation of autoantigens. Am J Pathol 150:1253–1266
Matute C, Domercq M, Perez-Samartin A et al (2013) Protecting white matter from stroke injury. Stroke 44:1204–1211
Mawhinney LA, Thawer SG, Lu WY et al (2012) Differential detection and distribution of microglial and hematogenous macrophage populations in the injured spinal cord of lys-EGFP-ki transgenic mice. J Neuropathol Exp Neurol 71:180–197
Mayer SA, Brun NC, Broderick J et al (2005) Safety and feasibility of recombinant factor VIIa for acute intracerebral hemorrhage. Stroke 36:74–79

Mayer SA, Brun NC, Begtrup K et al (2008) Efficacy and safety of recombinant activated factor VII for acute intracerebral hemorrhage. N Engl J Med 358:2127–2137
McColl BW, Allan SM, Rothwell NJ (2009) Systemic infection, inflammation and acute ischemic stroke. Neuroscience 158:1049–1061
McCracken E, Fowler JH, Dewar D et al (2002) Grey matter and white matter ischemic damage is reduced by the competitive AMPA receptor antagonist, SPD 502. J Cereb Blood Flow Metab 22:1090–1097
Meairs S, Wahlgren N, Dirnagl U et al (2006) Stroke research priorities for the next decade–a representative view of the European scientific community. Cerebrovasc Dis 22:75–82
Medana IM, Esiri MM (2003) Axonal damage: a key predictor of outcome in human CNS diseases. Brain 126:515–530
Mizutani M, Pino PA, Saederup N et al (2012) The fractalkine receptor but not CCR2 is present on microglia from embryonic development throughout adulthood. J Immunol 188:29–36
Monsma PC, Brown A (2012) FluoroMyelin Red is a bright, photostable and non-toxic fluorescent stain for live imaging of myelin. J Neurosci Methods 209:344–350
Morrison HW, Filosa JA (2013) A quantitative spatiotemporal analysis of microglia morphology during ischemic stroke and reperfusion. J Neuroinflammation 10:4
Moxon-Emre I, Schlichter LC (2010) Evolution of inflammation and white matter injury in a model of transient focal ischemia. J Neuropathol Exp Neurol 69:1–15
Moxon-Emre I, Schlichter LC (2011) Neutrophil depletion reduces blood–brain barrier breakdown, axon injury, and inflammation after intracerebral hemorrhage. J Neuropathol Exp Neurol 70:218–235
Nauta WJ (1952) Selective silver impregnation of degenerating axons in the central nervous system. Stain Technol 27:175–179
Neumann J, Sauerzweig S, Ronicke R et al (2008) Microglia cells protect neurons by direct engulfment of invading neutrophil granulocytes: a new mechanism of CNS immune privilege. J Neurosci 28:5965–5975
Nguyen HX, O'Barr TJ, Anderson AJ (2007) Polymorphonuclear leukocytes promote neurotoxicity through release of matrix metalloproteinases, reactive oxygen species, and TNF-alpha. J Neurochem 102:900–912
Nimmerjahn A, Kirchhoff F, Helmchen F (2005) Resting microglial cells are highly dynamic surveillants of brain parenchyma in vivo. Science 308:1314–1318
Noda M, Suzumura A (2012) Sweepers in the CNS: microglial migration and phagocytosis in the Alzheimer disease pathogenesis. Int J Alzheimers Dis 2012:891087
O'Collins VE, Macleod MR, Donnan GA et al (2006) 1,026 experimental treatments in acute stroke. Ann Neurol 59:467–477
Ohta H, Nishikawa H, Kimura H et al (1997) Chronic cerebral hypoperfusion by permanent internal carotid ligation produces learning impairment without brain damage in rats. Neuroscience 79:1039–1050
Olah M, Amor S, Brouwer N et al (2012) Identification of a microglia phenotype supportive of remyelination. Glia 60:306–321
Pantoni L, Garcia JH, Gutierrez JA (1996) Cerebral white matter is highly vulnerable to ischemia. Stroke 27:1641–1646, discussion 7
Perry VH, Cowey A (1984) Retinal ganglion cells that project to the superior colliculus and pretectum in the macaque monkey. Neuroscience 12:1125–1137
Perry VH, Oehler R, Cowey A (1984) Retinal ganglion cells that project to the dorsal lateral geniculate nucleus in the macaque monkey. Neuroscience 12:1101–1123
Perry VH, Hume DA, Gordon S (1985) Immunohistochemical localization of macrophages and microglia in the adult and developing mouse brain. Neuroscience 15:313–326
Petrault O, Ouk T, Gautier S et al (2005) Pharmacological neutropenia prevents endothelial dysfunction but not smooth muscle functions impairment induced by middle cerebral artery occlusion. Br J Pharmacol 144:1051–1058
Petty MA, Wettstein JG (1999) White matter ischaemia. Brain Res Brain Res Rev 31:58–64

Petzold A (2005) Neurofilament phosphoforms: surrogate markers for axonal injury, degeneration and loss. J Neurol Sci 233:183–198
Phillips JB, Williams AJ, Adams J et al (2000) Proteasome inhibitor PS519 reduces infarction and attenuates leukocyte infiltration in a rat model of focal cerebral ischemia. Stroke 31:1686–1693
Piani D, Frei K, Do KQ et al (1991) Murine brain macrophages induced NMDA receptor mediated neurotoxicity in vitro by secreting glutamate. Neurosci Lett 133:159–162
Planas AM, Chamorro A (2009) Regulatory T cells protect the brain after stroke. Nat Med 15:138–139
Prestigiacomo CJ, Kim SC, Connolly ES Jr et al (1999) CD18-mediated neutrophil recruitment contributes to the pathogenesis of reperfused but not nonreperfused stroke. Stroke 30:1110–1117
Qureshi AI, Mendelow AD, Hanley DF (2009) Intracerebral haemorrhage. Lancet 373: 1632–1644
Relton JK, Sloan KE, Frew EM et al (2001) Inhibition of alpha4 integrin protects against transient focal cerebral ischemia in normotensive and hypertensive rats. Stroke 32:199–205
Robinson RG, Shoemaker WJ, Schlumpf M et al (1975) Effect of experimental cerebral infarction in rat brain on catecholamines and behaviour. Nature 255:332–334
Robinson AP, White TM, Mason DW (1986) Macrophage heterogeneity in the rat as delineated by two monoclonal antibodies MRC OX-41 and MRC OX-42, the latter recognizing complement receptor type 3. Immunology 57:239–247
Robinson MJ, Macrae IM, Todd M et al (1990) Reduction of local cerebral blood flow to pathological levels by endothelin-1 applied to the middle cerebral artery in the rat. Neurosci Lett 118:269–272
Rock RB, Gekker G, Hu S et al (2004) Role of microglia in central nervous system infections. Clin Microbiol Rev 17:942–964, table of contents
Roman GC, Erkinjuntti T, Wallin A et al (2002) Subcortical ischaemic vascular dementia. Lancet Neurol 1:426–436
Saijo K, Glass CK (2011) Microglial cell origin and phenotypes in health and disease. Nat Rev Immunol 11:775–787
Salthouse TN (1962) Luxol fast blue ARN: a new solvent azo dye with improved staining qualities for myelin and phospholipids. Stain Technol 37:313–316
Scheikl T, Pignolet B, Mars LT et al (2010) Transgenic mouse models of multiple sclerosis. Cell Mol Life Sci 67:4011–4034
Schilling M, Besselmann M, Muller M et al (2005) Predominant phagocytic activity of resident microglia over hematogenous macrophages following transient focal cerebral ischemia: an investigation using green fluorescent protein transgenic bone marrow chimeric mice. Exp Neurol 196:290–297
Schlichter LC, Kaushal V, Moxon-Emre I et al (2010) The Ca2+ activated SK3 channel is expressed in microglia in the rat striatum and contributes to microglia-mediated neurotoxicity in vitro. J Neuroinflammation 7:4
Schmued L, Bowyer J, Cozart M et al (2008) Introducing Black-Gold II, a highly soluble gold phosphate complex with several unique advantages for the histochemical localization of myelin. Brain Res 1229:210–217
Schonbeck U, Mach F, Libby P (1998) Generation of biologically active IL-1 beta by matrix metalloproteinases: a novel caspase-1-independent pathway of IL-1 beta processing. J Immunol 161:3340–3346
Schroeter M, Jander S, Huitinga I et al (1997) Phagocytic response in photochemically induced infarction of rat cerebral cortex. The role of resident microglia. Stroke 28:382–386
Sedgwick JD, Schwender S, Imrich H et al (1991) Isolation and direct characterization of resident microglial cells from the normal and inflamed central nervous system. Proc Natl Acad Sci U S A 88:7438–7442
Seo B, Oemar BS, Siebenmann R et al (1994) Both ETA and ETB receptors mediate contraction to endothelin-1 in human blood vessels. Circulation 89:1203–1208

Sivagnanam V, Zhu X, Schlichter LC (2010) Dominance of E. coli phagocytosis over LPS in the inflammatory response of microglia. J Neuroimmunol 227:111–119
Smith ME (1993) Phagocytosis of myelin by microglia in vitro. J Neurosci Res 35:480–487
Smith ME, van der Maesen K, Somera FP (1998) Macrophage and microglial responses to cytokines in vitro: phagocytic activity, proteolytic enzyme release, and free radical production. J Neurosci Res 54:68–78
Smith DH, Meaney DF, Shull WH (2003) Diffuse axonal injury in head trauma. J Head Trauma Rehabil 18:307–316
Souza-Rodrigues RD, Costa AM, Lima RR et al (2008) Inflammatory response and white matter damage after microinjections of endothelin-1 into the rat striatum. Brain Res 1200:78–88
Sozmen EG, Kolekar A, Havton LA et al (2009) A white matter stroke model in the mouse: axonal damage, progenitor responses and MRI correlates. J Neurosci Methods 180:261–272
Sozmen EG, Hinman JD, Carmichael ST (2012) Models that matter: white matter stroke models. Neurotherapeutics 9:349–358
Sprinkle TJ, Sheedlo HJ, Buxton TB et al (1983) Immunochemical identification of 2′, 3′-cyclic nucleotide 3′-phosphodiesterase in central and peripheral nervous system myelin, the Wolfgram protein fraction, and bovine oligodendrocytes. J Neurochem 41:1664–1671
Sternberger LA, Sternberger NH (1983) Monoclonal antibodies distinguish phosphorylated and nonphosphorylated forms of neurofilaments in situ. Proc Natl Acad Sci U S A 80:6126–6130
Sternberger NH, Itoyama Y, Kies MW et al (1978) Myelin basic protein demonstrated immunocytochemically in oligodendroglia prior to myelin sheath formation. Proc Natl Acad Sci U S A 75:2521–2524
Stevens SL, Bao J, Hollis J et al (2002) The use of flow cytometry to evaluate temporal changes in inflammatory cells following focal cerebral ischemia in mice. Brain Res 932:110–119
Stilwell DL (1957) A sudan black B myelin stain for peripheral nerves. Stain Technol 32:19–23
Streilein JW (1993) Immune privilege as the result of local tissue barriers and immunosuppressive microenvironments. Curr Opin Immunol 5:428–432
Streit WJ, Kreutzberg GW (1987) Lectin binding by resting and reactive microglia. J Neurocytol 16:249–260
Svedin P, Hagberg H, Savman K et al (2007) Matrix metalloproteinase-9 gene knock-out protects the immature brain after cerebral hypoxia-ischemia. J Neurosci 27:1511–1518
Tayag EC, Jeng AY, Savage P et al (1996) Rat striatum contains pure population of ETB receptors. Eur J Pharmacol 300:261–265
Tonnesen MG (1989) Neutrophil-endothelial cell interactions: mechanisms of neutrophil adherence to vascular endothelium. J Invest Dermatol 93:53S–58S
Towfighi J, Zec N, Yager J et al (1995) Temporal evolution of neuropathologic changes in an immature rat model of cerebral hypoxia: a light microscopic study. Acta Neuropathol 90:375–386
Traystman RJ (2003) Animal models of focal and global cerebral ischemia. ILAR J 44:85–95
Trotter J, DeJong LJ, Smith ME (1986) Opsonization with antimyelin antibody increases the uptake and intracellular metabolism of myelin in inflammatory macrophages. J Neurochem 47:779–789
Turrin NP, Rivest S (2006) Molecular and cellular immune mediators of neuroprotection. Mol Neurobiol 34:221–242
Uehara H, Yoshioka H, Kawase S et al (1999) A new model of white matter injury in neonatal rats with bilateral carotid artery occlusion. Brain Res 837:213–220
Valeriani V, Dewar D, McCulloch J (2000) Quantitative assessment of ischemic pathology in axons, oligodendrocytes, and neurons: attenuation of damage after transient ischemia. J Cereb Blood Flow Metab 20:765–771
Van Dyken SJ, Locksley RM (2013) Interleukin-4- and interleukin-13-mediated alternatively activated macrophages: roles in homeostasis and disease. Annu Rev Immunol 31:317–343
Vannucci SJ, Hagberg H (2004) Hypoxia-ischemia in the immature brain. J Exp Biol 207:3149–3154

Vannucci RC, Vannucci SJ (1997) A model of perinatal hypoxic-ischemic brain damage. Ann N Y Acad Sci 835:234–249
Vannucci RC, Lyons DT, Vasta F (1988) Regional cerebral blood flow during hypoxia-ischemia in immature rats. Stroke 19:245–250
Varin A, Gordon S (2009) Alternative activation of macrophages: immune function and cellular biology. Immunobiology 214:630–641
Varvel NH, Grathwohl SA, Baumann F et al (2012) Microglial repopulation model reveals a robust homeostatic process for replacing CNS myeloid cells. Proc Natl Acad Sci U S A 109:18150–18155
Verhaar MC, Strachan FE, Newby DE et al (1998) Endothelin-A receptor antagonist-mediated vasodilatation is attenuated by inhibition of nitric oxide synthesis and by endothelin-B receptor blockade. Circulation 97:752–756
Villapol S, Fau S, Renolleau S et al (2011) Melatonin promotes myelination by decreasing white matter inflammation after neonatal stroke. Pediatr Res 69:51–55
Wake H, Moorhouse AJ, Jinno S et al (2009) Resting microglia directly monitor the functional state of synapses in vivo and determine the fate of ischemic terminals. J Neurosci 29:3974–3980
Wakita H, Tomimoto H, Akiguchi I et al (1994) Glial activation and white matter changes in the rat brain induced by chronic cerebral hypoperfusion: an immunohistochemical study. Acta Neuropathol 87:484–492
Wakita H, Tomimoto H, Akiguchi I et al (1995) Protective effect of cyclosporin A on white matter changes in the rat brain after chronic cerebral hypoperfusion. Stroke 26:1415–1422
Wakita H, Tomimoto H, Akiguchi I et al (1998) Dose-dependent, protective effect of FK506 against white matter changes in the rat brain after chronic cerebral ischemia. Brain Res 792:105–113
Walker CA, Huttner AJ, O'Connor KC (2011) Cortical injury in multiple sclerosis; the role of the immune system. BMC Neurol 11:152
Wang J, Dore S (2007) Inflammation after intracerebral hemorrhage. J Cereb Blood Flow Metab 27:894–908
Wang X, Lo EH (2003) Triggers and mediators of hemorrhagic transformation in cerebral ischemia. Mol Neurobiol 28:229–244
Wang X, Tsuji K, Lee SR et al (2004) Mechanisms of hemorrhagic transformation after tissue plasminogen activator reperfusion therapy for ischemic stroke. Stroke 35:2726–2730
Wang Q, Tang XN, Yenari MA (2007) The inflammatory response in stroke. J Neuroimmunol 184:53–68
Wang Y, Li B, Li Z et al (2013) Improvement of hypoxia-ischemia-induced white matter injury in immature rat brain by ethyl pyruvate. Neurochem Res 38:742–752
Ward JM, Erexson CR, Faucette LJ et al (2006) Immunohistochemical markers for the rodent immune system. Toxicol Pathol 34:616–630
Wasserman JK, Schlichter LC (2007) Neuron death and inflammation in a rat model of intracerebral hemorrhage: effects of delayed minocycline treatment. Brain Res 1136:208–218
Wasserman JK, Schlichter LC (2008) White matter injury in young and aged rats after intracerebral hemorrhage. Exp Neurol 214:266–275
Wasserman JK, Yang H, Schlichter LC (2008) Glial responses, neuron death and lesion resolution after intracerebral hemorrhage in young vs. aged rats. Eur J Neurosci 28:1316–1328
Weinstein JR, Koerner IP, Moller T (2010) Microglia in ischemic brain injury. Future Neurol 5:227–246
Weston RM, Jones NM, Jarrott B et al (2007) Inflammatory cell infiltration after endothelin-1-induced cerebral ischemia: histochemical and myeloperoxidase correlation with temporal changes in brain injury. J Cereb Blood Flow Metab 27:100–114
Whiteland JL, Nicholls SM, Shimeld C et al (1995) Immunohistochemical detection of T-cell subsets and other leukocytes in paraffin-embedded rat and mouse tissues with monoclonal antibodies. J Histochem Cytochem 43:313–320

Whiteley W, Jackson C, Lewis S et al (2009) Inflammatory markers and poor outcome after stroke: a prospective cohort study and systematic review of interleukin-6. PLoS Med 6:e1000145
Wiley KE, Davenport AP (2004) Endothelin receptor pharmacology and function in the mouse: comparison with rat and man. J Cardiovasc Pharmacol 44(suppl 1):S4–S6
Williams AJ, Hale SL, Moffett JR et al (2003) Delayed treatment with MLN519 reduces infarction and associated neurologic deficit caused by focal ischemic brain injury in rats via antiinflammatory mechanisms involving nuclear factor-kappaB activation, gliosis, and leukocyte infiltration. J Cereb Blood Flow Metab 23:75–87
Won SM, Lee JH, Park UJ et al (2011) Iron mediates endothelial cell damage and blood–brain barrier opening in the hippocampus after transient forebrain ischemia in rats. Exp Mol Med 43:121–128
Wynn TA, Chawla A, Pollard JW (2013) Macrophage biology in development, homeostasis and disease. Nature 496:445–455
Xu J, He L, Ahmed SH et al (2000) Oxygen-glucose deprivation induces inducible nitric oxide synthase and nitrotyrosine expression in cerebral endothelial cells. Stroke 31:1744–1751
Yang Y, Jalal FY, Thompson JF et al (2011) Tissue inhibitor of metalloproteinases-3 mediates the death of immature oligodendrocytes via TNF-alpha/TACE in focal cerebral ischemia in mice. J Neuroinflammation 8:108
Yilmaz G, Arumugam TV, Stokes KY et al (2006) Role of T lymphocytes and interferon-gamma in ischemic stroke. Circulation 113:2105–2112
Yokota N, Daniels F, Crosson J et al (2002) Protective effect of T cell depletion in murine renal ischemia-reperfusion injury. Transplantation 74:759–763
Zhang ZG, Chopp M (1997) Measurement of myeloperoxidase immunoreactive cells in ischemic brain after transient middle cerebral artery occlusion in the rat. Neurosci Res Commun 20:85–91
Zhang K, Sejnowski TJ (2000) A universal scaling law between gray matter and white matter of cerebral cortex. Proc Natl Acad Sci U S A 97:5621–5626
Zhang W, Stanimirovic D (2002) Current and future therapeutic strategies to target inflammation in stroke. Curr Drug Targets Inflamm Allergy 1:151–166
Zhao BQ, Chauhan AK, Canault M et al (2009) von Willebrand factor-cleaving protease ADAMTS13 reduces ischemic brain injury in experimental stroke. Blood 114:3329–3334
Zwacka RM, Zhang Y, Halldorson J et al (1997) CD4(+) T-lymphocytes mediate ischemia/reperfusion-induced inflammatory responses in mouse liver. J Clin Invest 100:279–289