Neuroprotective efficacy of minocycline after intracerebral hemorrhage and possible Kv1.3, Kv1.5, HERG and α7 nAChR channels as targets for regulating secondary brain injury in hemorrhagic and ischemic stroke

By

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A Thesis

Submitted to the Faculty of Graduate Studies

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Of

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II. LIST OF ABBREVIATIONS

AVM	arterio venous malformation
b FGF	basic fibroblast growth factor
Ca2+	calcium
CNS	central nervous system
COX-2	cyclooxygenase-2
EM	electron microscopy
ET-1	endothelin-1
FITC	fluorescein-5-isothiocyanate
HERG	human ether-a-go-go related gene
ICE	interleukin-1β-converting enzyme
ICH	intracerebral hemorrhage
IFN-γ	interferon-γ
iNOS	inducible nitric oxide synthase
IL-1β	interleukin-1β
K+	potassium ion
Kv	voltage-gated potassium ion channel
LM	light microscopy

M	molar
mM	millimolar
MMP	matrix metalloproteinases
NGS	normal goat serum
NMDA	N-methl-D-aspartate
PB	phosphate buffer
PBS	phosphate-buffered saline
PBST	PBS with Triton X-100
PMN	polymorphonuclear
PNS	peripheral nervous system
TBS	Tris-HCL buffered saline
TBS-T	TBS containing 0.2% Tween-20
TPA	tissue plasminogen activator

III. ABSTRACT

Ischemic stroke and intracerebral hemorrhage are among the most common life threatening neurological diseases and have limited therapeutic options. Previous studies demonstrated that there is an inflammatory response in the brain after intracerebral hemorrhage. Infiltrating leukocytes and activated microglia were found to release cytotoxic mediators contributing to secondary brain injury. Because the inflammatory response is a delayed process, the molecules participating in this secondary response, which may not be fundamental for physiologic brain functions, are potential targets for human therapy with a sufficiently wide therapeutic window. Minocycline is a semi-synthetic second-generation tetracycline that exerts anti-inflammatory effects completely separate from its antimicrobial action. Because tetracycline treatment is clinically well tolerated, we tested the hypothesis that minocycline would have neuroprotective effects in a rat model of intracerebral hemorrhage. In addition, previous studies have indicated that the voltage gated potassium channels Kv1.3, Kv1.5, HERG and the nicotinic acetylcholine receptors α7 unit are important regulators of T cells, microglia and macrophages, and these channels have become potential therapeutic targets for immunosuppression and for controlling brain inflammation. Thus, we tested the hypothesis that Kv1.3, Kv1.5, HERG and α7 channels are expressed in neutrophils, using immunohistochemical methods following intracerebral hemorrhage induced by injecting bacterial collagenase into rat striatum. Rats were treated with low dose minocycline (22.5 mg/kg ip at 1 and 12 hr, 11 mg/kg at 24 and 36 hr); high dose minocycline (45 mg/kg ip at 1 and 12 hr, 22.5mg/kg at 24 and 36 hr) and with high dose minocycline 45 mg/kg ip at

6 and 18 hr, 22.5 mg/kg at 30 and 42 hr. Immunohistochemistry for polymorphonuclear leukocyte marker (myeloperoxidase, MPO), microglial marker (OX42) and macrophage marker (ED1) was performed in minocycline injected and control animals to quantitatively determine the inflammatory cell count around the vicinity of the lesion. Results indicate a reduction in neutrophil and macrophage cell counts at the lesion site with a high dose of minocycline injected 1hr after ICH induction. However, the results appear to indicate no significant difference in neutrophil and macrophage cell counts when animals were treated with a high dose of minocycline 6 hrs after the induction of intracerebral hemorrhage. We conclude that minocycline might serve as a new therapeutic strategy for the treatment of stroke, but its potential to provide neuroprotection with a clinically relevant therapeutic window has to be still explored in other animal models of stroke. In separate studies using ICH and endothelin-induced ischemic models, double immunohistochemical staining for Kv1.3, Kv1.5, HERG and α7 in combination with neutrophil cell specific markers (HIS48 and RP3) revealed the expression of these channels in neutrophils, which is a novel observation. We suggest that Kv1.3, Kv1.5; HERG and α7 channels might serve as therapeutic targets to reduce the cytotoxic effects caused by neutrophils in the inflammatory process of secondary brain injury in hemorrhagic stroke.

IV. INTRODUCTION

IV. 1. Stroke

A stroke is a sudden interruption in the blood supply to an area of the brain. Because stroke occurs rapidly and requires immediate treatment, stroke is also called a brain attack. Stroke is one of the most common life threatening neurological diseases. Stroke was found to be the third leading cause of death after heart disease and cancer in western countries (Wolf et al., 1997). There are several types of stroke, and each type has different causes. Ischemic stroke, intracerebral hemorrhage (ICH) and subarachnoid hemorrhage represent the 3 main types of stroke. Unlike ischemic stroke and intracerebral hemorrhage which occur in the brain parenchyma, subarachnoid hemorrhage, most often caused by cerebral aneurysms, occur when a blood vessel just outside the brain ruptures and fills the subarachnoid space (area of the skull surrounding the brain). Ischemic stroke is the most common type of stroke accounting for almost 80% of all strokes. It is caused by a clot or a blockage within an artery leading to the brain. Intracerebral hemorrhage accounts for approximately 10% of strokes in western population (Brown et al., 1989) and up to 30% in oriental population (Lo et al, 1994). And hemorrhagic transformation occurs in 15 to 43% of patients presenting with ischemic stroke (Ly Den et al., 1993). Some of the etiological factors causing bleeding inside the skull leading to intracerebral hemorrhage are hypertension, trauma, aneurysms, arteriovenous malformation (AVM) and amyloid angiopathy. Approximately half of the clinical cases of ICH are associated with hypertension. Basal ganglia (putamen),

thalamus, cerebellum and pons are the most commonly affected brain areas with ICH (Garcia et al., 1991). The neurological deficits following ICH are due to direct tissue destruction leading to infarction, space-occupying effect of the hematoma with potential ischemic damage to the adjacent tissue, and cerebral edema and inflammation. Mortality during the first 30-day period after primary ICH was found to be 32% to 55% (Brown, 1989). Most ICH stroke survivors were left with some sort of neurological disability (Broderick et al., 1994; Fogelholm et al., 1985-89; Giroud et al., 1991).

IV.2. Inflammation in stroke

Inflammation is the response of living tissue to injury. It involves a cascade of complex events such as enzyme activation, mediator release, extravasation of fluid, cell migration, and tissue breakdown and repair. Inflammatory reactions are observed in both ischemic (Del Zoppo et al., 1991; Kochenek et al 1992, Schroetar et al 1994) and hemorrhagic (Del Bigio, et al 1996; Rosenberg et al., 1990) strokes, and were found to contribute to brain injury in several ways (Del Zoppo et al 1991, Garcia, 1997). Animal models of ischemic brain damage indicate that infiltration of leukocytes alone or in conjunction with activated glial cells contribute to damage by a variety of postulated mechanisms (Giulian et al., 1989, 1993; Giualian and Robertson., 1990; Hallenbeck and Dutka., 1990; Birdsall, 1991; Sloan et al., 1992; Mucke and Eddleston, 1993; Lees, 1993; Perry et al., 1993; Hara et al., 1993; Matsuo et al., 1995). While complete ischemia results in neuronal cell death, adjacent CNS areas with less compromised blood flow (the penumbra) may exhibit delayed neuronal death. Macrophages together with polymorphonuclear leukocytes (PMNs) (mostly neutrophils) infiltrate the brain from the circulation after an ischemic

episode and may contribute to neuronal death (Lassmann et al., 1991; Lees, 1993; Garcia et al., 1994; Jander et al., 1995; Siesjo and Siesjo, 1996; Feuerstein et al., 1997). It has been observed that neutrophilic inflammation is considerable in the vicinity of cerebral hematoma (Del Bigio et al., 1996). Studies by Akopov et al (1996) in humans indicate that neutrophil accumulation begins at 6 to 12 hr, and is maximum 12 to 24 hr after stroke, and poor neurological outcome and large infarct volumes correlated well with severity of neutrophil accumulation. That neutrophils enter brain after ischemia and contribute to injury is demonstrated by reduced infarct volume in animals depleted of these cells (Shiga et al., 1991; Chen et al., 1992; Heinel et al., 1994). Neutrophils release a variety of cytokines, such as tumor necrosis factor-α, interleukin-6 and interferon-γ (IFN-γ), which might play an important role in ischemic and traumatic brain damage (Arvin et al 1996; Ghirnikar et al 1998; Munoz-Fernandez et al 1998). Also, oxygen free radicals released by neutrophils may directly injure the endothelium, neurons and glia, and may promote edema in adjacent brain tissue. Several other studies have suggested that inflammation, involving infiltrating leukocytes and parenchymal microglia may contribute in the delayed progression of ischemic brain injury (Dirnage et al., 1999; Lee et al., 1999; Giulian, 1993). Glial cells play a vital role in the inflammatory mechanism. Astrocytes and microglia are found to be activated in the ischemic and hemorrhagic brains in response to glutamate excitotoxicity (Chao et al., 1993; Wu et al., 1998; Hu et al., 1996). Activated microglia can release a large variety of neurotoxins which include free radicals, hydrogen peroxide, super oxide. (Iadelula, 1997; Koistinato, 1997) glutamate and quinolinic acid, extra cellular proteases, eicosanoids, and cytokines, such as TNF-γ and interleukin-1β(IL-1β), which can cause further increase in microglial

proliferation and activation, resulting in release of more microglial toxins (Gulian et al 1993; Chao et al 1992; Hu et al 1996; Bhat et al 1998).

IV.3. Animal models used for studies of stroke and neuroprotection

A good in vivo animal model of stroke must reproduce the etiology, anatomical, functional and metabolic consequences of human pathology and must also permit the study of neuroprotective drugs in conditions pertinent to the clinical therapeutics.

Animal stroke models have been extensively applied to understand the mechanisms of ischemic and hemorrhagic brain injury and to develop novel therapeutic strategies for reducing brain damage after a stroke. Stroke models have been useful in characterizing the molecular cascades of injury processes. These injury pathways are also the targets of therapeutic interventions. The major achievements made in the past two decades applying animal stroke models include 1) the identification of the mediator role of excitotoxin and oxygen free radicals in ischemic brain injury (Matsuo et al., 1995); 2) the confirmation of apoptosis as a major mechanism of ischemic and hemorrhagic cell death (Quereshi et al., 2003); 3) the characterization of post ischemic gene expression (Dirnagl et al., 1999); 4) the delineation of post- ischemic inflammatory reaction; 5) the application of transgenic mice to confirm the roles of purported mediators in ischemic brain injury (Hara et al., 1996; George et al., 2002); 6) development of novel magnetic resonance imaging sequences for early noninvasive detection of ischemic brain lesions (Davis et al., 2004; Domingo et al., 2000; Liu et al., 2000); and 7) the development of novel therapeutic strategies based on preclinical findings derived from animal stroke models (Ahmed et al., 2000). With the failure of so many neuroprotective agents in clinical trials it is becoming

evident that existing animal models of stroke probably are an imperfect representation of human stroke and may be relevant only to a minority of human stroke types (Small et al., 2000). By understanding how animal models can be designed more relevant to human stroke and how the design of clinical trials may be improved, translational research can move forward for the development of stroke therapies.

IV.4. Neuroprotection in stroke

Neuroprotection is a term used to describe the putative effect of interventions protecting the brain from pathological damage. A neuroprotectant can extend the period for salvage of potentially reversible ischemic brain tissue and extend the time window for start of thrombolytic therapy. Neuroprotective strategies include targeting modulators of ion channels (Narahashi et al., 1997; Bowersox et al., 1997), scavengers of oxygen radicals and antagonists of excitotoxic neurotransmitters (Aarts et al., 2003; Bruno et al., 2001). Over the past two decades, neuroprotective agents have shown great promise in preclinical testing in animals, but very disappointing in clinical trials when tested in humans. Of more than 49 neuroprotective agents studied in more than 114 stroke trials, none has proven successful clinically (Kidwell et al., 2001). Currently, 2 thrombolytic therapy trials have shown efficacy for improving outcomes after ischemic stroke, the NINDS t-PA trial, with intravenous tissue plasminogen activator (TPA) given within 3 hours of stroke onset, and the PROACT-2 trial, with intra-arterial pro-urokinase given within 6 hrs (NINDS group 1995; Furlan et al., 1999). In a clinical trial the defibrinogenating agent ancrod demonstrated benefit when initiated within 3 hrs of stroke onset (Sherman et al., 1999).

IV.5. Criteria for effectiveness of neuroprotective therapy

Selection of appropriate time window for drug administration

Most neuroprotective studies have relied on drug administration either before the ischemic insult or very soon after the onset of ischemia (Jonas et al., 1997; Grotta et al., 1999). In contrast, time windows for entry in acute stroke neuroprotective trials have been longer and variable; in studies published between 1995 and 1999, the median time to entry was 12 hours (range, 4 hours to 12 days), with a median time to treatment of 14 hours (Kidwell et al., 2001). Various investigators (Grotto et al., 1999; Zivin et al., 1998; Marler et al., 1998) have indicated that treatment started within 3 hrs or 3-6 hrs after onset of stroke have a greater chance of efficacy because of the presence of potentially reversible ischemic tissue changes. Other studies (Fisher, 1997; Hakim, 1998) have shown that if reversible ischemic tissue is not present at the time of treatment, then neuroprotective therapy is least likely to work. So selection of appropriate time window (3-6 hours) for salvaging potentially reversible ischemic tissue is essential for a neuroprotective agent to be effective.

Extended neuroprotection and assessment at extended time points.

Studies done by Dyker (1998) and Lees (1998) recommend extended neuroprotective therapy. The concept of extended neuroprotective treatment is supported by magnetic resonance spectroscopy evidence which monitors ongoing neuronal loss over many days after stroke (Saunders et al., 1995). Studies of neuroprotective drugs have rarely shown

that early therapeutic benefit, when it is achieved, has a lasting impact. Most therapeutic attempts delayed but did not arrest cell death (Corbett et al., 1998; Drummond et al., 2000; Valtysson et al., 1994). For example, the NMDA antagonist MK-801 appeared neuroprotective at day 3, but at 4 weeks, there was no significant difference in infarct size (Valtysson et al., 1994). Similarly, the cyclin-dependant kinase inhibitor flavopiridol, AMPA antagonist NBQX, and N-type calcium channel antagonist SNX-111 all appeared neuroprotective histologically when assessed at 1 week, but this was not sustained at 4 weeks (Colbourne et al., 1999; Wang et al., 2002). These findings demonstrate that reliance on early end points is not sufficient and can be misleading; assessments at extended time points after ischemia are necessary to determine whether there is evidence of sustained neuroprotection (Drummond et al., 2000; Valtysson et al., 1994). Histopathological studies in animals show that infarcts evolve over time and may take many days to months to acquire their final appearance (Valtysson et al., 1994; Garcia et al., 1993-95). Thus, if single-dose neuroprotective treatment only postpones the evolution of an infarct, perhaps multidose, extended treatments or combination therapy will be required for optimal neuroprotection (Dyker et al., 1998; Coimbra et al., 1996).

Behavioral outcome as treatment end point

Traditionally, animal studies have relied on reduction in infarct size within the first few hours after stroke as the primary measure of therapeutic efficacy. In contrast, clinical trials judge efficacy by using neurological and/or functional outcomes, not infarct volume, most often at 3 months after stroke (Kidwell et al., 2001; Slikker et al., 2001). Reliance on infarct size measurement alone in animals can be misleading as an indicator of therapeutic efficacy (Hunter et al., 1998; Corbett et al., 1998). Histological end points

cannot tell whether surviving neurons are functional or dysfunctional or will go on to die in a delayed fashion, and they are less predictive of long-term histology than early behavioral assessments (Corbett et al., 1998). Moreover, some compounds [e.g., basic fibroblast growth factor (bFGF), osteogenic protein-1] have been associated with functional improvement without affecting infarct size in animals, suggesting that they act by other mechanisms, eg, enhancement of neural repair, rather than by neuroprotection (Kawamata et al., 1996-98). Therefore, assessment of therapeutic efficacy in preclinical animal studies should require, in addition to infarct size, demonstration of benefit on functional measures of motor, sensory, or cognitive deficits (Hunter et al., 1998; Hudzik et al., 2000). Examples include tests of limb placing, beam walking, grid walking, rotorod performance, grip strength, balance beam inclined plane performance, prehensile traction, and cognition eg, Morris water maze, radial maze, 1-trial passive avoidance, T-maze retention test (Corbett et al., 1998; Hunter et al., 1998-2000). These measurements are not equivalent in their reliability or predictive value. For this reason, it is better to use a series of appropriate tests rather than a single measure. The staircase test has been recommended because of its greater sensitivity in detecting persisting deficits in forepaw dexterity months after ischemia, unlike simpler sensorimotor tasks on which animals can recover quickly (Hunter et al., 1998; Corbett et al., 1998).

IV.6. Minocycline

Tetracyclines are well-known bacteriostatic antibiotics with broad-spectrum antimicrobial activity (Klein et al., 1995). Minocycline is a second generation semi-synthetic antibiotic of the tetracycline family. It was synthesized in 1967 by Martell and

Boothe and commercialized in 1972. Minocycline has a long half-life and also has a great lipophilic property. Minocycline is rapidly and completely absorbed, even in an aged population (Cunha et al., 1982; Sande et al., 1990; Kramer et al., 1978; Barza et al., 1975). Compared with many other antibiotics, it has excellent tissue penetration into the brain and cerebrospinal fluid (Cunha et al., 1982; Sande et al., 1990; Kramer et al., 1978; Barza et al., 1975; Aronson et al., 1980). Minocycline is regularly indicated in dermatological conditions such as acne and in sexually transmissible diseases such as those caused by mycoplasma, chlamydia and treponema. Because antibiotic resistance is low with minocycline compared with other tetracyclines and antimicrobials, it is the most widely prescribed systemic antibiotic for acne. Minocycline has been used for treatment of patients with severe inflammatory diseases and rheumatoid arthritis (O'Dell, 1999). It was found to be neuroprotective in models of cerebral ischemia, traumatic brain injury, Huntington's and Parkinson's disease (Yrjanheikki et al., 1998; Chen et al., 2000; Sanchez et al., 2001; Wu et al., 2002). Minocycline exerts anti-inflammatory effects that are completely separate and distinct from their antimicrobial action (O'Dell, 1999) and has neuroprotective effects in brain ischemia (Yrjanheikki et al., 1998, 1999). These effects include modulation of COX-2 activity (Patel et al., 1999), prostaglandin E2 production (Yrjanheikki et al.,1999), inhibition of induction of IL-1β- converting enzyme (ICE) mRNA mainly expressed by microglia (Fiebich et al., 2000), inhibition of matrix metalloproteinases (Golub et al., 1991), decreased T- lymphocyte transmigration, depression of oxygen radical release from polymorphonuclear neutrophils (Gabler et al., 1991-92) and inhibition of iNOS (Amin et al.,1996-97). Minocycline mediated neuroprotection is associated with inhibition of caspase-1 and caspase-3 expression

(Chen et al., 2000), cytochrome c release (Zhu et al., 2002) and iNOS transcriptional upregulation and activation (Yrjanheikki et al., 1998; Sanchez et al., 2001; Wu et al., 2002; Du et al., 2001). Inhibition of p38 MAPK and microglial activation have been associated with minocycline mediated neuroprotection (Yrjanheikki et al., Tikka et al., 2001). Despite all these neuroprotective properties of minocycline, its precise primary target is not known. Understanding the mechanism of action of minocycline will assist in the development and testing of more powerful and effective analogues. Minocycline is at present being evaluated in human trials for Huntington's disease. Although minocycline is considered a safe drug in humans (Seukeran et al., 1997; Shapiro et al., 1997; O'Dell, 1999; Sturkenboom et al., 1999) occasionally serious drug reactions can occur, including hypersensitivity syndrome, serum sickness-like reaction, drug-induced lupus and single organ dysfunction (Elkayam et al., 1999). The incidence of these is very rare (1.6 cases per million exposures) and tends to be found in subjects of African descent, a population more prone to hypersensitivity reactions.

IV.7 Targets for neuroprotection

Inflammatory reactions which occur within the brain after ischemic injury or intracerebral hemorrhage (ICH) and which may contribute to secondary cerebral damage are attractive pharmacological targets, because inflammatory reactions persists for at least a few days after brain injury. As inflammatory mechanisms are not fundamental for physiological brain functions, interference with these secondary responses may not result in intolerable side effects (Barone and Feuerstein, 1999). In previous studies, treatment of inflammation was found to reduce infarct volume and improve outcome in animal models

of ischemic stroke (Garcia et al., 1995; Matsuo et al., 1994; Zhang et al., 1995), decrease edema formation and tissue injury (Kane et al., 1992) and improve outcome (Del Bigio et al., 1999) in models of ICH. The pro-inflammatory mediators released by the inflammatory cells (neutrophils, macrophages and reactive microglia) are potential targets for human therapy with a sufficient wide therapeutic window. Compounds with anti-inflammatory properties or with an ability to inhibit microglial activation can represent potential therapies against excitotoxic brain insults. Activation of blood granulocytes and tissue mast cells is generally characterized by an influx of extra cellular calcium (Ca2+), which is essential for subsequent release of granule-derived mediators, newly generated lipid mediators and cytokines (Church et al., 1982). The mechanism by which granulocyte mediator secretion is sustained is therefore likely to include modulation of various types of ion channels. Flow of ions, including K+, might play an important role during granulocyte responses because they regulate cell membrane potential and thus influence Ca2+ influx (Lin et al., 1993).

IV.8. Ion channels

Ion channels are key molecules for signal transduction across biological membranes.

Ion channels are targets for drug treatment in many disorders involving nerve, muscle, epithelia and recently, immune cells. Voltage-gated potassium channels (Kv) are expressed in most cell types, which indicate their vital role for cell signaling. In excitable cells, potassium channels set the membrane potential contributing to ion homeostasis, helping regulate calcium levels, cell volume, and pH. In normal glia, increased Kv1.5 and Kv1.3 expression has been described in proliferating cells, and blocking these channels

resulted in decreased proliferation rates of these cells (Attali et al., 1997; Kotecha et al., 1999; Chittajallu et al., 2002). Cell-volume regulation is a fundamental homeostatic process, and one that is affected in several brain disorders, perhaps most notably in stroke. Kv1.3 has been found to be important for proliferation of T cells and microglia, and is considered as a target for immuno-suppression (Schlicter et al., 1996; Kotecha et al., 1999). Another potassium channel, human ether-a-go-go related gene (HERG) [homologous to the cardiac HERG] channel, was found to be highly up regulated during the transition of microglia to a growth-factor independent proliferative state (Zhou et al., 1998). These potassium channels expressed in microglia have been identified to be important for the NADPH- mediated respiratory burst, (Khanna et al., 2001; Colton et al., 1994; Spranger et al., 1998) and also for release of toxic reactive oxygen species (Pyo et al., 1997). Recent studies have shown that nicotinic acetylcholine receptor α7 subunit is essential for vagus nerve regulation of macrophage TNF release during the systemic inflammatory response to endotoxemia (Borovikova et al., 2000; Bernik et al., 2002; Tracey, 2002). Infiltrating neutrophils and macrophages, invading the damaged brain tissue in stroke injury producing cytokines and other proinflammatory mediators might express the α7 subunit, which can be targeted to inhibit release of cytokines and other proinflammatory cytokines. Ion channels thus expressed by inflammatory cells involved in ischemic and hemorrhagic stroke can be pharmacologically targeted to modulate cytokine activity.

IV.9. Rationale for present studies

Various studies have reported (Del Zoppo et al., 1991; Kochenek et al., 1992; Rosenberg et al., 1990; and Del Bigio et al., 1996) that there is an inflammatory response in ischemia and intracerebral hemorrhage models of experimental stroke in rats. These studies predict that while complete ischemia or hemorrhage can result in neuronal cell death, healthy adjacent brain area (penumbra) may undergo a delayed neuronal death due to release of inflammatory mediators from cells involved in neuroinflammation. Therefore, to salvage this potentially viable brain tissue from secondary inflammatory damage, several neuroprotective drugs have been studied pertaining to their potential to modulate the secondary inflammatory response by targeting neurotoxic inflammatory mediators. Minocycline was shown to be neuroprotective in models of cerebral ischemia by Yrjanheikki et al., (1998) who also further observed that minocycline mediated neuroprotection is associated with inhibition of p38 MAPK and microglial activation. The present study was undertaken to study the efficacy of minocycline in an intracerebral hemorrhage model of rat induced by bacterial collagenase injected into the striatum. Studies by Del Bigio et al., (2003) on this model reported significant inflammatory reaction around the induced lesion, which was consistently reproducible with less mortality rate. Marler et al., (1998) have indicated that the time window of drug administration is important for salvaging the potentially reversible damaged brain tissue and therefore he hypothesized that treatment started within 3 hour or 3-6 hour after the onset of stroke has a greater chance of efficacy. So, in the present study minocycline was administered at 1 hour (45 mg/kg intraperitonial at 1and 12 hour, 22.5 mg/kg ip at 24 and 36 hour) and 6 hour (45 mg/kg ip at 6 and 18 hour, 22.5 mg/kg at 30 and 42 hour) after

the induction of ICH in separate sets of animals. As any effective neuroprotective agent is clinically expected to show a permanent functional outcome and not simply delay neuronal death, obtaining knowledge of the efficacy of minocycline when administered early (3-6 hour) after stroke onset will be the first step towards designing further studies to understand its effectiveness when administered for prolonged periods of time for effecting a clinically relevant functional outcome.

Studies by Schlicter et al., (1996) have shown that ion channels expressed by resident brain microglia can be targeted for modulating the inflammatory response in the injured brain. Other studies have also indicated (Schlicter et al., 1996; Kotecha et al., 1999; and Zhou et al., 1998) that Kv1.5, Kv1.3 and HERG ion channels were involved in macrophage and microglia proliferation and that targeting them can prove to be effective neuroprotective strategy.

We tested the hypothesis that neutrophils, which are the first to infiltrate the damaged brain tissue, might also express these channels, which can make potential targets for regulating the inflammatory response. In separate studies using immunohistochemical methods employing neutrophil and microglial specific markers, we studied the ion channels expressed by these cells using anti-Kv1.5, Kv1.3, HERG and α7 antibodies in hemorrhagic and ischemic models of stroke. Data from the present study will provide insight for characterizing the dynamic roles of these channels so that novel agents can be designed to specifically block these channels for neuroinflammatory modulation.

V. MATERIALS AND METHODS

V.1. Animal preparation

All experimental and animal care procedures were performed in accordance with the guidelines of the Canadian Council on Animal Care. The local experimental ethics committee at the University of Manitoba approved the protocols followed.

V.2. Intracerebral hemorrhage

Thirty six adult male Sprague-Dawley rats weighing between 250-300 g were used in all our experiments. Rats were assigned into treatment groups (as discussed in V.4) for injections of bacterial collagenase to induce intracerebral hemorrhage (ICH) [administered by Dr.Yan in Dr.Peeling's lab]. The rats were anesthetized with somnotol (50 mg/kg intra peritoneal) and placed in a stereo tactic frame (David Kopf Instruments). A 30-gauge needle was introduced through a cranial burr hole into the caudate nucleus (3-mm lateral to midline, 0.2-mm posterior to bregma and 6-mm depth below the surface of the skull). A micro infusion pump (Sage instruments) was used to infuse 0.7 μL saline containing 0.14 U collagenase (type IV, Sigma Chemical Co. St Louis, MO) over a 5 minute period. Brain temperature was monitored and maintained (37.6°C) throughout the procedure using a tympanic membrane thermocouple probe (Cole-Parmer Inc, Montreal, Canada) and a thermostatically controlled water blanket. After the infusion, the needle was left in place for 3 minutes and then removed slowly. The bone hole was sealed with bone wax, the scalp wound was sutured and the rats were placed in a clean cage with free

access to food and water.

V.3. Endothelin-1 focal ischemia model

Six male Sprague-Dawley rats weighing between 290-320 g were anesthetized with 1% halothane in 30% oxygen and 70% nitrous oxide delivered via a nose cone. A midline incision was made, and a bur hole was drilled in the skull overlying the dorsolateral parietal cortex. Artery occlusion was induced by microinjection of the vasoconstrictive peptide endothelin-1 (ET-1; 60 pmol in 3 μl of 0.9% saline) into the striatum over 90 sec via a 30-gauge needle at steriotaxic coordinates + 0.9 mm anterioposterior; -5.2 mm mediolateral; -8.7 mm dorsoventral relative to bregma. The needle was left in place for a further 5 min to prevent backflow. Rectal temperature was monitored and maintained between 36.5 and 37.5 °C using a heated water blanket for the duration of surgery.

V.4. Minocycline treatment

To study the early (1hr) treatment effects of high and low dose of minocycline after ICH induction, rats were assigned into 3 treatment groups (n = 6). Group 1 received 45 mg/kg minocycline hydrochloride (high dose) intraperitonially after ICH induction at 1 and 12 hr followed by a dose of 22.5 mg/kg at 24 and 36 hr respectively. Group 2 received 22.5 mg/kg minocycline hydrochloride (low dose) intraperitonially after induction of ICH starting at 1 and 12 hr followed by a dose of 11 mg/kg at 24 and 36 hr. Group 3 control animals did not receive minocycline, rather they received volume equivalent injections of saline at the same time point. The rats were weighed and behavior was measured at 48 hrs after induction of ICH.

To study early (1 hr) and delayed (6 hr) treatment effects of high dose minocycline after ICH induction, animals were assigned to 3 treatment groups (6 per group). The rats were weighed and behavior was assessed before drug administration. Group 1 received 45 mg/kg minocycline hydrochloride intraperitonially after ICH induction at 1 and 12 hr followed by a dose of 22.5 mg/kg at 24 and 36 hr respectively. Group 2 received 45/kg minocycline hydrochloride intraperitonially after induction of ICH starting at 6 and 18 hr followed by a dose of 22.5 mg/kg at 30 and 42 hr. Group 3 control animals did not receive minocycline, rather they received volume equivalent injections of saline at the same time point as Group 1 animals. The rats were weighed and behavior was measured at 48 hrs after induction of ICH.

V. 5. Tissue Preparation

Rats were deeply anesthetized 2 days after ICH with equithesin (3 ml/kg) and were perfused transcardially with 40 ml of prefixative solution consisting of cold (4 °C) 25 mM sodium phosphate buffer (PB), pH 7.4, 0.9% NaCl, 0.1% sodium nitrite and heparin (1 unit/ml). This was followed by perfusion with 300 ml ice cold 0.16 M sodium PB, pH 7.4, containing 2% freshly depolymerised Para formaldehyde and 0.2% picric acid, followed by perfusion with 150 ml of phosphate buffer containing 10% sucrose. Brains were removed and cut coronally, approximately 2 to 4 mm on either side of the needle entry site, which was identifiable on the brain surface. Brain slices were cryoprotected in 30% sucrose in 0.1M PBS, pH 7.4 for 48 hr at 4 °C. The brains were sectioned in the sagittal plane at 10 μm on a cryostat microtome (Leica, CM 1800, Germany). The sections were collected on gelatinized glass slides and stored at -34 °C. Brain sections

with the lesion center, where the brain damage was maximal, were stained by immunohistochemical methods using primary antibodies directed against protein markers for neutrophils, macrophages and microglia.

V. 6. Antibodies

Primary Antibodies

Monoclonal mouse anti-rat CD68 (Serotec, UK) was used to detect ED1-positive macrophages. Monoclonal mouse anti-rat CD11 (Serotec, UK) was used to detect OX42-positive neutrophils and microglia/macrophages. OX42, also called complement receptor type 3, is found in resting, activated, phagocytic microglia as well as macrophages and regarded as the cell marker of microglia or macrophages (Matsubara et al., 1999; Streit et al., 1988). Polyclonal rabbit anti-human myeloperoxidase (Dako Diagnostics Canada Inc, Missisuaga) was used for labelling MPO-positive neutrophils. Neutrophil specific markers, monoclonal mouse anti-rat granulocytes RP3 (BD Biosciences, Sandiego, CA, USA) and monoclonal mouse anti-rat granulocytes HIS48 (BD Biosciences, Sandiego, CA, USA) were used for double labelling experiments. Monoclonal mouse anti-Kv1.5 IgG (Upstate, Lake Placid, NY,USA); polyclonal rabbit anti-Kv1.5 IgG (Upstate, Lake Placid, NY,USA) and polyclonal rabbit anti-Kv1.3 IgG (Alomone Labs Ltd, Jerusalem, Israel) were used for labelling potassium channels (Kv). For labelling nicotinic acetylcholine receptor α7channel, polyclonal rabbit anti-nAChRα7 (Santa Cruz biotechnology, Inc. CA, USA) was used.

Secondary Antibodies

The secondary antibodies employed for labelling the primary antibodies were fluoresceinisothiocyanate (FITC)-conjugated horse anti-mouse IgG (Vector laboratories, Burlingame, CA, USA) diluted 1:100, and Cy3-conjugated donkey anti-rabbit IgG (Jackson ImmunoResearch Labs, West grove, PA, USA) diluted 1:200.

V.7. Light microscopy Immunohistochemistry

Immunohistochemical staining was used to assess the infiltration of neutrophils, macrophages and reactive microglia at the lesion site. Sections were washed for 20 min in 50 mM Tris-HCl containing 1.5% sodium chloride, 0.3% Triton X-100 (TBST), pH 7.6 and 5% normal goat serum. For single immunofluorescence labelling sections were incubated with primary antibodies for 24 hrs at 4 °C. Primary antibodies employed were rabbit antihuman myeloperoxidase (diluted 1:1000); mouse anti-rat HIS48 (diluted 1:50); mouse anti-rat RP3 (diluted 1:50); mouse anti-rat OX42 (diluted 1:500); mouse anti-rat ED1 (diluted 1:100); rabbit anti-Kv1.5 (diluted 1:100); mouse anti-rat Kv1.5 (diluted 1:200); rabbit anti-Kv1.3 (diluted 1:100); rabbit anti-HERG (diluted 1:200) and rabbit anti-AChRα7 (diluted 1:500). Slides were then washed for 1 hr in TBST, and incubated with secondary antibodies for 1.5 hr at room temperature. Sections were then washed in TBST for 20 min, followed by two 20 min washes in 50 mM Tris-HCl buffer, pH 7.4, and cover slipped with anti-fade medium. Sections processed for double immunofluorescence labelling were simultaneously incubated with two different (monoclonal and polyclonal) primary antibodies for 24 hr at 4 °C before incubating with secondary antibodies.

V. 8. Inflammatory cell quantization

Using a square ocular reticule and 250 × ocular magnification (objective magnification × 40), extra vascular neutrophils and macrophages were counted in 4 fields (each area 250 × 250 μm) immediately adjacent to the hematoma site, attempting to avoid areas with large blood vessels. Cell counts were made near the margin of the lesion because the necrotic cores were devoid of viable cells.

V. 9. Photography and Imaging

Immunofluorescence staining was examined using a Zeiss Axioskop2 fluorescence microscope with filter cubes for fluorescein isothiocyanate (FITC) and Cy3. Images were acquired at sufficient magnification to discern individual immunolabeling. Images were captured using Axiovision 3.0.6 software (Carl Zeiss Canada, Toronto, Ontario, Canada) and assembled in either photoshop 6.0 (Adobe systems, Sanjose, CA, USA) or Corel Draw 8 (Corel Corp., Ottawa, Canada). Minimal adjustment to brightness and contrast were required.

V. 10. Statistics

Data are expressed as mean ± standard error mean. MR data at each time point , the behavioral data, and the histological data were evaluated using a two-tailed paired t test or an unpaired t test between groups. Statistical significance was set at $P < 0.01$.

VI. RESULTS

All rats tolerated the surgical procedures well, and there were no surgical deaths. To study the degree of tissue damage and neuronal loss in animals sacrificed 2 days after induction of ICH, sections were counterstained using a histochemistry protocol with a Nissl fluorescence counterstain. Fluorescent Nissl counterstaining (Fig. 1A,C,E) showed dying neurons with shrunken angular somata and nuclei surrounding the edematous infarct. The developed lesions were spherical and consistently located in the striatum, but sometimes extended into the adjacent white matter of the corpus callosum and internal capsule. The contra lateral sides of the same sections (Fig. 1B,D,F) was used as controls for observations of normal neuronal density and as reference for immunohistochemical background levels of immunostaining with the various antibodies employed for labelling immune cells. Neuronal densities were found to be normal at the periphery of the lesions and in the control striatum.

VI. 1. Infiltration of inflammatory cells in ICH model

Immunohistochemical staining for ED1 antigen with an antibody directed specifically against a cytoplasmic protein in tissue macrophages and monocytes was used as a marker to assess tissue infiltration of macrophages, OX42 was used as marker for both macrophages and activated microglia, and MPO (myeloperoxidase, highly expressed in neutrophils), RP3 and HIS48 (that were previously reported to be anti-rat granulocyte and neutrophil specific markers) were used to assess the infiltration of neutrophils. The inflammatory cell infiltrate in all rats was patchy, but generally maximal in a ring-like

distribution in the striatal tissue around and/or within the hematoma. ED1-positive and OX42-positive macrophages were distributed mostly at the periphery of the lesion at 2 days following hemorrhage (Fig. 2A,C). Contra lateral control striatum showed no staining for ED1 and OX42 (Fig. 2B,D). There were numerous MPO-positive neutrophils in and around the hematoma (Fig. 3A). In order to confirm and gain confidence that anti-MPO antibody produced specific labelling of neutrophils, and to allow subsequent studies involving examination of potassium channel and cholinergic receptor expression in these cells by double immunofluorescence methods using polyclonal antibodies against these proteins, two monoclonal antibodies against markers specific for neutrophils were also used to detect these cells, namely anti-RP3 and anti-HIS48. Figure 3C shows RP3-positive neutrophils and Figure 3E shows HIS48-positive neutrophils. Immunolabelling with anti-MPO, anti-RP3 and anti-HIS48 was totally absent in the contra lateral control striatum (Fig 3. B,D,F).

VI.2 Double immunofluorescence labelling

Double immunofluorescence labelling confirmed that MPO-immunoreactive cells at hemorrhage lesion sites in the striatum were neutrophils by showing co-localization relationships of labelling obtained with anti-MPO, anti-RP3 anti-HIS48, anti-ED1 and anti-OX42. Since MPO is a polyclonal antibody, neutrophil-specific monoclonal antibodies anti-RP3 and anti-HIS48 were used with polyclonal neutrophil marker anti-MPO for double labelling studies. Co-localization of labelling for MPO with RP3 (Fig. 4A), and with HIS48 (Fig. 4B3) indicated that these antibodies immunostained the same cell population. There was no co-localization between densely labelled MPO-positive

cells and ED1-positive cells (Fig. D3), indicating that MPO is at least not highly expressed or expressed at very low undetectable levels in most macrophages and activated microglia, which are labelled by anti-ED1. However, a small number of the ED1-positive cells were very faintly labelled for MPO, indicating that some macrophages and/or activated microglia contain low levels of this peroxidase enzyme. Double immunostaining with anti-MPO and anti-OX42 showed partial co-localization of MPO and OX42 as shown in Fig. 4E. Consistent with the above observations, all MPO-positive neutrophils were positive for OX42, but only a proportion of OX42-positive cells were labelled for MPO, reflecting the population of OX42-positive macrophage/activated microglial cells that were unlabelled for MPO.

To confirm that the antibodies used in the experiments lacked cross-reaction with each other or inappropriate cross reactions with the secondary antibodies used during double labelling, primary omission of one of the primary antibodies while including both secondaries (FITC-conjugated horse anti-mouse and Cy3-conjugated donkey anti-rabbit) was conducted as control. Figure. 5A shows immunofluorecence micrographs of the same field with absence of red labelling for MPO with Cy3 (Fig. 5A1) and green immunofluorescence labelling for ED1 with FITC (Fig. 5A2) after primary omission of MPO, while including primary antibody ED1 and both the secondary antibodies. The primary antibody ED1 was specifically detected by secondary FITC antibody and not by the other secondary Cy3 antibody, hence the green immunofluorescence in Fig. 5A2, and absence of labeling in Fig. 5A1 due to MPO omission. Fig. 5B shows red Cy3 immunofluorescence labelling for MPO (Fig. 5B1) and absence of labelling (Fig. 5B2) due to ED1 primary antibody omission. Similar results confirming absence of

inappropriate cross-reaction were obtained in double labelling for MPO and OX42 (Fig. 5C1-D2), as well as in studies using markers for OX42, HIS48, RP3, Kv1.3, Kv1.5, HERG and nicotinic AChRα7 subunit (Data not shown).

VI. 3. Inflammatory cell infiltration in endothelial-1 focal ischemia model

Sections from brains of animals sacrificed 2 days after endothelin-1-induced striatum ischemia were counterstained with Nissl fluorescence marker to determine the degree of neuronal loss and tissue damage. Figure 6A shows the lesion site, where there was decreased neuronal density in the lesion center compared with the lesion periphery. At higher magnifications of the same section (Fig. 6B,C), the relative density of neurons at the center and periphery of the lesion, and the loss of cells toward the lesion center is readily evident. Shrunken neuronal nuclei visualized by Nissl staining were found in the lesion core. Immunohistochemical staining for ED1 was used as a marker to assess the infiltration of macrophages, OX42 for macrophages and neutrophils, and MPO, RP3 and HIS48 were used to assess the infiltration of neutrophils. Immunohistochemical results in the endothelin ischemia model showed less intense inflammatory cell staining when compared to the ICH model, indicating that there was smaller degree of inflammatory response in the striatum after endothelin-induced ischemia. ED1-positive macrophages were distributed at the periphery of the lesion, while OX42-positive, MPO-positive, RP3-positive and HIS48-positive neutrophils were distributed more towards the centre of the lesion (Fig. 6D-H), indicating a similar distribution of cells as seen in the ICH model.

VI.4. Minocycline treatment effects in intracerebral hemorrhage

To determine the neuroprotective action of minocycline in ICH, rats were treated with low and high dose of minocycline 1 hr post ICH and with high dose minocycline 6 hr post ICH. Effects of minocycline were evaluated by immunostaining for inflammatory cell markers, and quantifying infiltration of microglia/macrophages and neutrophils around and within the injury area. Immunostaining for ED1, MPO and OX42 in the striatum of untreated rats showed a high density of intensely stained cells distributed within and around the periphery of the hemorrhage site (Fig. 7A,C,E). Although results with low dose minocycline administered 1 hr post ICH didn't show significant difference in cell density at the lesion site from untreated control rats, high dose minocycline administration beginning 1 hr post-ICH reduced the density of ED1-positive cells (Fig. 7B), suggesting that high dosage of minocycline reduced microglial activation and/or proliferation, and macrophage infiltration. The density of MPO-positive and OX42-postive cells was also reduced (Fig. 7D,F), further indicating that minocycline attenuated the infiltration of neutrophils and macrophages. Minocycline treatment beginning 6 hrs post-ICH showed little apparent difference in the density of inflammatory cell infiltration at the lesion site. Thus, the density of ED1-, MPO- and OX42-positive cells in untreated rats (Fig. 8A,C,E, respectively) was similar to that in minocycline-treated rats 6 hrs post-ICH (Fig. 8B,D,F, respectively). Results of cells counts 6 hr post-ICH minocycline are shown in Table 2. (Data in Table 1 obtained with low and high dose of minocycline administered 1 hr post ICH was gathered by Dr. Shahi T, 6 months before my arrival to the lab). Thus, minocycline treatment was effective in reducing neutrophil infiltration and the activation of microglia and/or macrophages around the area of injury after ICH when

started early at a high dose, but diminished with delayed treatment.

VI.5. Kv1.3 and Kv1.5 channel expression in inflammatory cells in collagenase model of ICH

Previous studies have reported that microglia express Kv1.3 and Kv1.5 potassium channels, which were involved in the proliferation and cytotoxic effects of these cells. Based on the rationale that these inflammatory cells express these two types of potassium channels, we tested the hypothesis that other types of leukocytes namely neutrophils also mediate cytotoxicity through their expression of these channels. Double immunohistochemical staining was used to detect the expression of Kv1.3 and Kv1.5 using polyclonal antibodies against these proteins in combination with monoclonal antibodies against inflammatory cell markers. Co-localization relationships between Kv1.3-positive cells and ED1, OX42, RP3 and HIS48 are shown in Figures 9 and 10. There was an absence of co-localization between ED1 and Kv1.3 (Fig. 9A and B), indicating that ED1-positive macrophages lacked expression of Kv1.3 two days post-ICH. There was substantial (estimated ~ 50%) co-localization between Kv1.3 and OX42 (Fig. 9C, D), suggesting Kv1.3 expression in OX42-positive neutrophils (estimated ~ 96%). As expected based on these results, together with the lack of Kv1.3 expression by macrophages/microglia and labelling of both neutrophils and macrophages/microglia with OX42, a population of cells was found to be OX42-positive, but Kv1.3-negative. In order to further confirm that Kv1.3 channel expression by neutrophils, double immunofluorescence co-localization relationships was investigated between Kv1.3 and the neutrophil-specific markers RP3 and HIS48. The results revealed substantial (estimated ~ 90%) co-localization of Kv1.3 and RP3 (Fig. 10A,B), and considerable

(estimated ~ 85%) co-localization of Kv1.3 and HIS48 (Fig. 10C,D), indicating Kv1.3 channel expression by neutrophils at the injury site 2 days post-ICH.

Double immunofluorescence of Kv1.5 and ED1 appeared to reveal partial (estimated ~ 30%) co-localization of these proteins (Fig. 11A,B), and there appeared to be greater (estimated ~ 85%) co-localization between Kv1.5 and OX42 (Fig. 11C,D). However, close examination showed that Kv1.5 antibody labelled the cell nuclei of ED1-positive cells as well as that of all neurons. The cytoplasm and plasma membrane of ED1-positive cells were not Kv1.5 positive and therefore these cells were taken as being Kv1.5 negative. More cells exhibited positive double-labelling in the lesion center where more neutrophils accumulate, suggesting Kv1.5 expression in a large proportion of OX42-positive neutrophils. On further examination of Kv1.5 relationships with other neutrophil-specific markers, a considerable (estimated ~ 75%) co-localization was observed between this potassium channel and RP3 (Fig. 12A,B) and substantial (estimated ~ 85%) co-localization with HIS48 (Fig. 12C,D), indicating that most but not all neutrophils in the ICH model expressed this channel. Results also show that Kv1.5 expression is less intense than Kv1.3 on neutrophils in the striatum 2 days post-ICH, but this may be due to the immunofluorescence procedures used to detect these channel proteins and/or the quality of the anti-channel antibodies used.

VI.6. HERG channel expression in inflammatory cells in collagenase model of ICH

Human-ether-a-go-go related gene (HERG) is a class of Kv channel found to be expressed by microglia, which were identified to be an important source of toxic reactive oxygen species. In this study, we tested the hypothesis that the HERG Kv channel may

also be expressed by inflammatory cells located at the ICH site. There was an absence of co-localization (Fig. 13A,B) between ED1-positive macrophages and HERG-positive cells, showing that macrophages at the periphery of the lesion lacked HERG at 2 days post-ICH. Double immunofluorescence co-localization relationship between OX42 and HERG showed considerable co-localization (Fig. 13C,D), suggesting that OX42 positive neutrophils expressed HERG channels at the lesion site (estimated ~ 92%). Analysis of double immunofluorescence relationships between HERG, RP3 and HIS48 showed considerable co-localization of HERG with RP3 (estimated ~ 90%) (Fig. 14 A,D) and with HIS48 (estimated ~ 75%) (Fig. 14C,D), indicating HERG expression by neutrophils at the lesion site 2 days post-ICH.

VI.7. Nicotinic AChRα7 channel expression in inflammatory cells in collagenase model of ICH

Nicotinic acetylcholine alpha 7 receptor subunit (α7 subunit) was found to be an essential regulator of macrophage TNF release via the cholinergic anti-inflammatory pathway (Hong et al., 2003). In this study, we examined the expression of α7 on inflammatory cells at the injury site 2 days post-ICH. As shown in Figure 15A-C, a large number of cells were found to be immunopositive for α7. These cells were present more towards the lesion centre. Double immunofluorescence co-localization relationships between the α7 subunit and ED1 showed an absence of co-localization (Fig. 15D), indicating that ED1-positive macrophages lacked α7 subunit expression at the site of inflammation 2 days post-ICH. There was considerable co-localization between α7 subunit-positive and OX42-positive cells, indicating that α7 subunit was expressed by OX42-positive

neutrophils (estimated ~ 99%). This was confirmed by double immunofluorescence staining for α7 subunit with RP3 and HIS48, which showed near total co localization (estimated 90-100%) (Fig. 15 F, G).

VI. 8. Kv1.3, Kv1.5, HERG and α7 expression in inflammatory cells in Endothelin model of ischemia

We tested whether Kv1.3, Kv1.5, HERG and α7 subunit channels were expressed by inflammatory cells in the ET-1 ischemia model. Immunofluorescence results confirmed the expression of these channels on inflammatory cells infiltrated in and around the lesion site 2 days post-ET-1 ischemia induction. As shown in Figure 16, cells at the lesion site exhibited a low level of labelling for Kv1.3 (Fig. 16A), but a greater density and intensity of staining for Kv1.5 (Fig. 16B), HERG (Fig. 16C), and the nAChR α7 subunit (Fig. 16D) towards the centre of the lesion suggesting their expression by neutrophils, which were usually located towards the centre of the lesion.

VII. DISCUSSION

VII.1. General conclusions

Results of the present study have shown that minocycline is neuroprotective when administered early (1 hour) after stroke onset in an experimental model of intracerebral hemorrhage. Immunohistochemical co-localization of the neutrophil marker MPO with monoclonal antibody labelling for RP3 and HIS48 by light microscopy double-labelling showed that these latter two proteins are neutrophil specific. Further, double-labelling co-localization relationships between anti-ED1, anti-OX42, anti-MPO, anti-RP3 and anti-HIS48 with markers for Kv1.3. Kv1.5, HERG and nicotinic AchRα7 subunit showed that these channels are expressed by neutrophils at the lesion site 2 days post-ICH and ischemia. These channels on neutrophils may be considered potential targets for modulating the early inflammatory response in ICH and ischemic stroke. Finally, the specificity of the antibodies used as markers in the present studies was demonstrated by their lack of cross-reaction in control experiments.

VII.2. Animal models of stroke and ICH

ICH represents a major subset of strokes, including primary ICH resulting from hypertension, vascular anomalies, bleeding diathesis and secondary hemorrhagic transformation of ischemic infarcts (Qureshi et al., 2001). The immediate mechanisms by which cellular injury occurs during ICH include mechanical compression of tissue due to blood accumulation and edema, impairment of local blood flow and extravasation into

the brain parenchyma of blood that contains potentially damaging molecules such as thrombin, complement and plasmin (Xue and Del Bigio, 2001). Although ICH represents 20% of all kinds of human stroke, there are few experimental models that recapitulate the clinical and neuropathological features of human stroke. Several experimental models have been employed in previous ICH studies, which include hemorrhage induced by autologous whole blood injection (Bullock et al., 1984; Kobari et al., 1988; Koeppen et al., 1995; Wagner et al., 1996; Xue et al., 2001) and bacterial collagenase-injection, which disrupts the basal lamina of cerebral capillaries and causes bleeding into the brain tissue (Rosenberg et al., 1993; Del Bigio et al., 1996, 2001; Xi et al., 1998). Common features in both of these models include death of neurons and glia, and inflammation with initial neutrophil infiltration followed by macrophage infiltration and activation (Del Bigio et al., 1996). Although blood vessels were disrupted due to the injection of bacterial collagenase at the injection site as indicated by previous studies, this model is still useful because it reflects many of the key aspects of human ICH, including neurobehavioral and neuropathological outcomes. Also induction of ICH by intracerebral infusion of collagenase has consistently shown a uniform, reproducible hematoma (Cregan et al., 1997; Rosenberg et al., 1990).

Previous studies indicate that inflammatory cells are activated within hours after induction of ICH, initiated by adherence of neutrophils to damaged brain endothelia and their subsequent entry into brain (Del Bigio et al., 1996). In rat brain injury studies, intense neutrophilic infiltrate has been previously documented around collagenase-induced hematomas (Rosenberg et al., 1990; Del Bigio et al., 1996), contusions (Matthew et al., 1994; Soares et al., 1995) and ischemic sites (Jean et al 1998) beginning at 6 to 12

hours and peaking at 48 to 72 hours. In human brains, neutrophil infiltration is apparent 5 to 72 hours after hemorrhage or contusion (Garcia et al., 1994; Mackenzie et al., 1999; Jean et al., 1998; Holmin et al., 1998; Loberg et al., 1989; Anderson et al., 1998). Neutrophils can release potentially harmful factors such as oxygen radicals or cytokines, including tumor necrosis factor-α, interleukin-6, and IFN-γ, which seem to play a role in brain damage (Ghirnikar et al., 1998; Munoz-Fernandez et al., 1998). Neutrophils can also exacerbate brain injury by obstructing micro vessels, which then causes local ischemia (Del Zoppo et al., 1998). Global depletion of circulating leukocytes and platelets by whole body irradiation in a rodent model of ICH was found to confer protection against both ischemia and edema formation (Kane et al., 1992), suggesting that infiltrating leukocytes may indeed play a role in brain injury following ICH.

Consistent with previous studies conducted with the collagenase model of ICH, our results indicated an inflammatory response in and around the blood clot after ICH, which was marked by the infiltration of MPO-positive neutrophils, and ED1-positive and OX42-positive macrophages and activated microglia. Although previous studies indicated that inactivated collagenase does not produce an inflammatory reaction in brain (Del Bigio et al., 1999), it cannot be ruled out that the infused collagenase in our ICH studies might have contributed to the inflammatory reaction observed. Consistent with previous observation by Xue et al. (2000), the possible cause for the observed inflammation in our study may be likely due to the extra vascular blood at the lesion core. This inflammatory response may participate in the absorption of blood clot and the removal of injured brain cells, but may also cause a secondary injury following ICH.

The mechanisms underlying the secondary brain injury and edema formation are still under investigation.

VII. 3. Minocycline

Previous studies have shown that ischemic focal damage matured and enlarged for several days, which at least partially was a result of the development of inflammatory responses during the first 2 days after ischemia (Iadecola et al., 1997). The relevance of inflammation as a late contributing mechanism in brain ischemia has been proved by previous studies, which showed that compounds that inhibit COX-2 or inducible nitric oxide synthase (iNOS) are protective in focal brain ischemia even when administered several hours after the insult (Nogawa et al., 1997; Nagayama et al., 1998). Previous studies have indicated that treatment of inflammation reduces infarct volume and improves outcome in animal models of ischemic stroke (Garcia et al., 1995; Matsuo et al., 1994; Zhang et al., 1994, 1995) and decreases edema formation and tissue injury (Kane et al., 1992) and improves outcome (Del Bigio et al., 1999) in models of ICH. Studies by Gabler et al., (1991) indicated that tetracyclines might suppress neutrophil-mediated tissue damage by inhibiting their migration and degranulation and, potentially more importantly, by suppressing synthesis of oxygen radicals. Studies by Golub et al. (1998) and Riffkin et al. (1994) have shown that minocycline and synthetic tetracycline derivatives that are devoid of antibacterial activity may be beneficial in inflammatory diseases like rheumatoid arthritis, osteoporosis and periodontal disease. Tetracyclines including minocycline, have been shown to inhibit matrix metalloproteinases (MMP) and possibly super oxide production in neutrophils (Golub et al., 1998; Gabler et al., 1991).

More recently, minocycline has been found to inhibit the activation of microglia (Tikka and Koistinaho, 2001; Tikka et al., 2001), and this has been considered to be the possible mechanism by which it is neuroprotective in focal or global ischemic models of stroke (Yrjanheikki et al., 1998, 1999). Minocycline has also been reported to inhibit the expression of caspase-1 and caspase-3, and to delay mortality in a transgenic mouse model of Huntington's disease (Chen et al., 2000). In a model of Parkinson's disease, minocycline protected against 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) induced neurotoxicity (Du et al., 2001). Recent studies indicate that minocycline is neuroprotective after cerebral ischemia, demyelination and neurotrauma (Yrjanheikki et al., 1998, 1999; Povic et al., 2002). Although past studies indicated that minocycline may down regulate microglia activation (He et al., 2001) and caspase activity (Chen et al., 2000), and block nitric oxide-induced neurotoxicity by inhibition of p38 MAP kinase (Lin et al., 2001), minocycline's primary mechanism of action remains unclear. As all these findings indicate the potential beneficial effect of minocycline as an anti-inflammatory agent, we explored the possible therapeutic efficacy of minocycline in controlling the effects of inflammation in a collagenase model of ICH. In all our experiments, rats were treated with minocycline 45 mg/kg i.p. twice a day on the first day and 22.5 mg/kg on the second day. The dosage of minocycline used in our study was comparable to those that have been used previously to ameliorate disease in models of stroke, Huntington's disease and Parkinson's disease (Yrjanheikki et al., 1999; Chen et al., 2000; Du et al., 2001). As nonneuronal cells were found to be characteristically activated in the brain in response to hemorrhagic and ischemic injury (McGeer et al., 1995; Banati et al., 1993; Giulian et al., 1993), we employed immunohistochemical

markers to detect myeloperoxidase (MPO) labelling for neutrophils, ED1 labelling for macrophages and CD11b (OX42) labelling for neutrophils and macrophages/microglia. We observed a decrease in neutrophil and macrophage cell counts in the tissue immediately adjacent to the hematoma in a concentration dependent manner in minocycline-treated animals when compared with untreated control rats. Also there was a qualitative decrease in the size of the hemorrhage site in rat striatum at the lesion site when treatment was initiated 1 hour after induction of ICH as compared to untreated controls or when treatment was initiated 6 hours following ICH. These results suggest that minocycline might be more effective in controlling neuronal cell death caused by free radicals and cytokines released by inflammatory cells around the hematoma when treatment is initiated early (i.e. within 1 hrs after ICH onset). Previous studies by Gabler et al., (1991) have indicated that tetracyclines might suppress neutrophil-mediated tissue damage by inhibiting their migration and degranulation and, potentially more importantly, by suppressing synthesis of oxygen radicals and cytokines. Consistent with these studies, we speculate that minocycline through a similar mechanism of action in a concentration dependent manner, exerts more effective neuroprotection from neutrophil-mediated damage when treatment is started early after ICH onset. Due to the current dearth in therapeutic measures for ICH, minocycline with its established safety profile in humans might be a potential neuroprotective option for ICH. Further studies focused on understanding pathogenic mechanisms after ICH and the precise mechanism of action of minocycline will be important for the success of minocycline as a neuroprotective agent.

VII. 4. Functionally selective voltage gated potassium channels modulating early inflammatory response

Potassium channels are a diverse group of ion channels present in most eukaryotic cells. Over 30 different potassium channels have been characterized biophysically. They were found to show different sensitivities to voltage and /or intracellular messengers, and have different kinetic or pharmacological properties. The wide range of potassium channel properties reflects the broad spectrum of cellular functions such as control of synaptic efficacy, heart beat, endocrine and exocrine functions. Potassium channels have also been found to play key roles in activation of microglia and macrophages, which release cytotoxic products resulting in delayed neuronal death. In excitable cells, potassium channels set the membrane potential and are crucial for regulating action potentials, calcium influx and secretion. Although microglia, macrophages and neutrophils are generally considered nonexcitable cells, they possess a variety of ion channels which contribute to ion homeostasis, calcium signaling, and cell volume regulation (Krause et al., 1993).

The Kv family of voltage-gated channels constitutes the largest family of potassium channels. To date nine different subfamilies of Kv channels named Kv1 to Kv9 have been identified. Recently the group of Kv1 or *shaker* channels have been studied more extensively among Kv channels. These channels have predominantly been found and characterized in the CNS where they participate in the control of neuronal excitability (Grissmer et al., 1994; Veh et al., 1995). Kv1.3 channels, were first described in T-lymphocytes (DeCoursey et al., 1984). Kv1.5 expression was reported in human bronchial myocytes (Sarvesh et al., 1996), human myocardium (Bertaso et al., 2002)

pulmonary vascular smooth muscle, astrocytes, glial cells of brain and spinal cord (Roy et al., 1996) and in retinal ganglion cells. Electrophysiological studies on human glioma cells in situ and in vitro demonstrated the presence of voltage-gated potassium channels on these cells (Brodey et al., 1998; Brismar et al., 1989; Labrakakis et al., 1997; Patt et al., 1996). Recent studies have shown that particular members of this voltage-gated potassium channel family are closely related to cell growth and cell cycle progression. Studies by Chttajallu et al. (2002) have demonstrated the selective increase of Kv1.3 and Kv1.5 protein expression in rat oligodendrocyte precursor cells during the transition from quiescent to G1 phase. Selective blocking of Kv1.3 containing channels alone was sufficient to elicit G1 arrest in these cells. Various studies also showed the role of Kv1.3 in hippocampal microglia proliferation (Kotecha et al., 1999; Schlichter et al., 1996), respiratory burst in cultured microglia (Khanna et al., 2000; Colton et al., 1994) and nitric oxide production (Pyo et al., 1997).

Recently, the possible use of voltage-gated potassium channel blockers (of particularly Kv1.3 and Kv1.5 channels) as immune suppressants has become the subject of much interest. Kv1.5 is regarded as a promising target for the development of atrial selective drugs to treat atrial fibrillation (AF) with fewer side effects. Previous studies indicate that selective blockade of Kv1.3 channels in T lymphocytes leads to membrane depolarization (Lin et al., 1993), inhibition of Ca2+ influx (Leonard et al., 1992) and inhibition of cytokine production and cell proliferation (Kaczorowski et al., 1994; Lewis et al., 1995; Cahalan et al., 1997; Price et al., 1989). However, Psora-4 a potent small-molecule Kv1.3 blocker exhibiting 16-to 70-fold selectivity for Kv1.3 over closely related Kv1 channels blocked Kv1.5 current in the human atrium (Fedida et al., 1993; Feng et al., 1997),

raising concerns about possible acute cardiac toxicity. These channels are speculated to be attractive molecular targets for controlling neuroinflammatory cell activation and function because these channels have restricted tissue expression and even more restricted functional contribution, thus greatly reducing the likelihood that selectively blockade will have serious side effects. Studies by Beeton et al. (2001) have indicated that selective blockade of T lymphocyte Kv1.3 channels ameliorated experimental autoimmune encephalomyelitis with no side effects despite Kv1.3 expression in other tissues such as brain, atrial myocytes, arteries, bladder, testis, kidney and colon epithelia. Kv1.3 channel blockers have also been shown recently to be effective in preventing inflammatory bone resorption in experimental periodontal disease (Valverde et al., 2004). As the relative contribution of these channels appear to be dependent on cell activation state, it may be possible to selectively inhibit over-active or inappropriately activated cells, while leaving resting cells unaffected, as in the case of inflammatory brain tissue damage in stroke.

Previous studies have indicated that neutrophil inflammation is considerable in the vicinity of collagenase-induced intracerebral hematoma (Del Bigio et al., 1996). Various other studies (Johnson et al., 1992; Shiga et al., 1991) also indicated the possibility that activated neutrophils can cause secondary tissue injury and contribute to edema formation through the release of reactive oxygen species and a variety of proteases as they migrate towards the site of lesion. Previous studies demonstrated that neutrophils express voltage-dependent potassium channels, important for the maintenance of resting potential, and a calcium-activated potassium channel, which were important for repolarization after cellular activation of these cells (Krause et al., 1990). Neutrophils

also possess voltage and pH activated H+ channels that serve to extrude protons, generated by the neutrophil respiratory burst (Krause et al., 1993). As neutrophils and macrophages are the main invaders in the hours to days following hemorrhagic or ischemic strokes and with recent evidence demonstrating the existence of Kv1.3 and Kv1.5 channels in rat cultured microglia (Kotecha et al., 1999; Khanna et al., 2001; Jou et al., 1998), we used immunohistochemical methods in 2 days post-ICH and ischemic rat stroke models to investigate the possible expression of Kv1.3 and Kv1.5 channels on inflammatory cells at the vicinity of the lesion site, and we hypothesized that these ion channels might make novel drug targets to regulate the cytotoxic activity of these cells.

Results from analysis of double immunofluorescence and co-localization relations between neutrophil markers (RP3 and HIS48) and ion channels Kv1.3 and Kv1.5 showed that these channels are expressed by neutrophils at the lesion site in 2 days post ICH and ischemic striatum. Our results indicate that most, but not all, neutrophils express Kv1.3 and Kv1.5, with intense expression of these channels in the striatum 2 days post- ICH and ischemia. Studies by Kotecha et al. (1999) demonstrated a switch from Kv1.5-like current in nonproliferating to a Kv1.3-like current in proliferating endogenous hippocampal microglia. Their studies also indicated that differential expression of Kv1.3 and Kv1.5 channels might be closely related to cell growth and cell cycle progression in microglia. Consistent with these previous studies, it appears that differential expression patterns of Kv1.3 and Kv1.5 channels in neutrophils might follow a similar expression pattern in relation to their resting and active functional state at the inflammatory site. We present, to my knowledge, the first immunohistochemical evidence of neutrophil expression of Kv1.3 and Kv1.5 channels in any inflammatory model system or disease condition.

Further studies to verify the expression and functional role of these channels in circulating neutrophils need to be determined. We indicate that these channels might represent potential functionally selective therapeutic targets for modulating the deleterious cytotoxic role of neutrophils in ICH and ischemic stroke. Therefore, future studies aimed to document the specific contribution of these potassium channels in functions of resting and active neutrophils at the inflammatory site in ICH and ischemic brain will be of importance for determining the potential benefit of selectively down regulating overactive neutrophils without compromising the beneficial functions of the resting less active neutrophils.

VII.5. HERG potassium channel

The human ether-a-go-go related gene (HERG) potassium channel is a member of the family of voltage-gated potassium channels (Warmke and Ganetzky, 1994; Trudeau et al., 1995) first cloned from a hippocampal cDNA library. In cardiomyocytes, HERG encodes the pore forming α-subunit of the rapid delayed rectifier potassium channel (Sanguinetti et al.,1995), which is central to the repolarization phase of the cardiac action potential (Spectoret al. 1996; Tseng, 2001). HERG potassium channels have been the focus of intense interest after the discovery that HERG mutations contribute to the genetic heart disease "long QT syndrome" type 2, which can cause sudden death from ventricular arrhythmia (Curran et al., 1995; Sanguinetti et al., 1995). mRNA for HERG, and the rat homologue r-erg1, have been identified in a wide variety of tissues (Wymore et al., 1997). Potassium currents closely resembling HERG have been described in mammalian neuroblastoma cells (Arcangeli et al., 1995; Faravelli et al., 1996; Hu and

Shi, 1997), Xenopus oocytes (Baueret al., 1996) and the MLS-9 cell line derived from primary cultures of rat microglia (Zhou et al., 1998). HERG or ERG channels may contribute to setting the resting membrane potential in neurons and neuronal cell lines (Arcangeli et al., 1995), modulating hormone secretion (Barros et al., 1998; Schafer et al., 1999; Schwarz et al., 1999; Pancrazio et al., 1999; Faravelli et al., 1996) and influencing neural differentiation (Pancrazio et al., 1999) and neuritogenesis (Arcangeli et al., 1995; Pancrazio et al., 1999; Faravelli et al., 1996). HERG channels may also be important in proliferating cancerous and undifferentiated cells, where there is increased HERG or ERG expression compared with that in normal cellular counterparts (Archangeli et al., 1995; Bianchi et al., 1998). As previous studies indicate that several functions common to immune cells, such as proliferation, volume regulation, production of lymphokines and cytokines, parallel commonalities in the types of ion channels expressed during these functions (DeCoursey and Grinstein, 1998), and with considerable evidence of HERG-like potassium channel expression in cultured microglia, we explored the possible expression of HERG potassium channel in infiltrated inflammatory cells in stroke models of 2 days post-ICH and ischemia employing immunohistochemical methods. Our results indicated that a considerable proportion of neutrophils expressed the HERG potassium channel at the lesion site, although macrophages distributed more at the periphery of the lesion lacked expression of these channels. This is a novel observation, providing for the first time immunohistochemical evidence of HERG potassium channel expression in neutrophils. We speculate that those inflammatory cells at the lesion site in 2 days post-ICH and ischemic stroke might have a temporal pattern of HERG potassium channel expression in response to their environment. Further studies are needed to elucidate the

expression and functional role of these channels in circulating neutrophils. In cardiomyocytes, it has been recently demonstrated that inhibition of HERG potassium currents by class III antiarrhythmic drugs caused a lengthening of the cardiac action potential, producing a beneficial antiarrhythmic effect (Thomas et al., 2004). Thus, future studies designed to better understand the role of HERG potassium channels in inflammatory cells will enable consideration of the therapeutic potential of targeting these channels for regulating toxic inflammatory reaction in ICH and cerebral ischemia.

VII.6. α7 nicotinic acetylcholine receptor

Nicotinic acetylcholine receptors are a family of ligand-gated, pentameric ion channels. The main function of this receptor family is to transmit acetylcholine signals at neuromuscular junctions and in the central and peripheral nervous systems (Lindstorm et al., 1997; Steinlein et al., 1998; Marubio et al., 2000; Leonard et al., 2001). In humans, 16 different nicotinic acetylcholine receptor subunits (α1-7, α9-10, β1-4, δ, ε, γ) have been identified (Lindstorm et al., 1997; Leonard et al., 2001).

Recent studies (Borovikova et al., 2000; Tracey et al., 2001) discovered the anti-inflammatory role of the vagus nerve in an animal model of endotoxemia and shock. Acetylcholine, an important neurotransmitter and neuromodulator in the brain, mediates neural transmission in ganglion synapses of both sympathetic and parasympathetic neurons, and is the principle neurotransmitter in postganglionic parasympathetic/vagal efferent neurons. Acetylcholine was reported to act through 2 types of receptors, namely, muscarinic (metabotropic) (Caulfield et al 1998) and nicotinic (ionotropic) (Lindstorm et al., 1997). In addition to their presence in brain, RNA for these receptor subtypes

(muscarinic) and subunits (nicotinic) has been detected on mixed populations of lymphocytes and other immune and non-immune cytokine-producing cells (Hiemke et al., 1996; Mita et al., 1996; Toyabes et al., 1997; Sato et al., 1999; Walch et al., 2001; Tayebati et al., 2002). Kawashima et al, (2000) have indicated that most of these cells produce acetylcholine.

Past studies indicate that acetylcholine significantly and concentration-dependently decreases TNF production in endotoxin-stimulated human macrophage cultures via a post-transcriptional mechanism. Acetylcholine was also found to be effective in suppressing other endotoxin-inducible pro-inflammatory cytokines, such as IL-1β, IL-6 and IL-18, by a post-transcriptional mechanism. In the peripheral nervous system, recent studies suggest that an endogenous 'cholinergic anti-inflammatory pathway' regulates systemic inflammatory responses via alpha 7 nicotinic acetyl cholinergic receptors (nAChR) found on blood-born macrophages (Wang et al., 2003). Activation of this receptor via vagus nerve stimulation (Wang et al., 2003) or cholinergic agonists (Wang et al., 2002; Han et al., 2003) suppressed TNF synthesis in wild-type mice, but failed to inhibit TNF synthesis in α7-deficient mice. The centrally acting pharmacological agent tetravalent guanylhydrazone CNI-1493 induced vagus nerve firing (Borovikova et al., 2000) and conferred anti-inflammatory effects through activation of the cholinergic anti-inflammatory pathway in both local and systemic models of inflammation (Borovikova et al., 2000; Bernik et al., 2002). In models of cerebral ischemia, CNI-1493 delivered via the intracerebroventricular (ICV) route suppressed cerebral TNF synthesis and reduced infarct volume (Meistrell et al., 1997). Based on their studies, Wang et al. (2003) indicated that acetylcholine released from vagus nerve endings, or perhaps from other

sources like lymphocytes or epithelial cells, could specifically inhibit macrophage TNF release. They further suggested that other primary immune cells (mast cells, microglial cells, kupffer cells, splenocytes), which produce cytokines during the acute, local response to infection or injury, might also express nicotinic acetylcholine receptor α7 subunits, and that these cells might be sensitive to the anti-inflammatory effects of acetylcholine. Recently, Shytle et al. (2004) demonstrated cholinergic modulation of microglial activation through alpha 7 nicotinic receptors and suggested the existence of a brain cholinergic pathway.

These previous findings led us to hypothesize that a possibly existing brain cholinergic pathway might similarly regulate neutrophil- and macrophage-mediated innate immune response to limit excessive CNS inflammation at acute stroke injury site where a considerable neutrophil and macrophage infiltration is usually present. As there was to our knowledge no previous studies indicating brain cholinergic modulation of neutrophil and macrophage activation through α7 nicotinic receptors, we used immunohistochemical methods to investigate possible expression of alpha 7 nicotinic acetylcholine receptors on neutrophils and macrophages at the vicinity of stroke lesion site in 2 days post-ICH and ischemic rat striatum.

Double immunofluorescence results indicated expression of α7 nicotinic acetylcholine receptor on neutrophils, whereas macrophages lacked α7 subunit expression at the site of inflammation 2 days post-ICH and ischemia. We thus report for the first time neutrophil expression of α7 nicotinic acetylcholine receptor at the two days post-ICH lesion site. Though our findings are preliminary, we suggest the existence of a brain cholinergic pathway that regulates neutrophil activation through α7 nicotinic receptors. As previous

studies (Wang et al., 2003) have reported that nicotinic acetylcholine receptor α7 subunit is an essential regulator of inflammation required for acetylcholine inhibition of primary macrophage TNF release, we speculate that by a possible similar cholinergic anti-inflammatory mechanism, acetylcholine released from adjacent immune cells or even cholinergic neuronal pathways, at the site of stroke lesion might have a regulatory effect on neutrophil TNF release through α7 nicotinic acetylcholine receptors. Further, studies to understand the specific role and expression pattern of nicotinic acetylcholine receptor α7 subunit on neutrophils at the inflammatory site in stroke lesion will be useful for developing possible drug targets against α7 nicotinic AchR subunits to inhibit TNF synthesis and thus regulate the excessive inflammation in acute ICH and ischemic strokes.

VII.7. Future considerations

There is now evolving evidence on the dual nature of some inflammatory mediators in reformatting brain cells for resistance or sensitivity to injury. Many studies have recently indicated the critical importance of carefully balancing pharmacological interventions for stroke treatment based on the knowledge of both beneficial and detrimental mechanisms promoted by inflammatory cells and mediators. Hence, further experiments are essential for determining hemorrhagic and ischemic conditions in which inflammatory cells are pertinent targets for stroke management.

Appropriate selection of animal stroke models has been stated to be of great priority to study the benefits of anti-inflammatory therapy by properly determining the time window for salvage of potentially reversible ischemic brain tissue (Ahmed et al., 2000). It has

often been noted that current neuroprotective approaches only delay rather than arrest cell death (Morse and Davis 1990; Li and buchan, 1995; Colbourne and Corbett, 1994; Valtysson et al., 1994; Corbett and Crooks, 1997). Therefore, it was suggested that employing a multimodal approach (Nurse and Corbett, 1994; Dooley et al., 1996; Colbourne and Corbett, 1995; Volpe et al., 1984; 1992; Grabowski et al., 1993; Okada et al., 1995; Markgraf et al., 1992) might determine true neuroprotection. A possibly desirable combination therapy recently indicated is a thrombolytic drug with a neuroprotective agent (Lindsberg et al., 2000).

It is now increasingly considered that preclinical assessment of neuroprotection based solely on measuring infarct volume or histological measures (cell counts) are not adequate. Therefore, future studies demonstrating improved functional outcomes at long-term end points measured on standard valid behavioral tests are considered mandatory for efficient stroke therapy (Corbett et al., 1998; Hunter et al., 1998; Kidwell et al., 2001; Slicker et al., 2001). To determine the efficacy of a neuroprotectant, it has been indicated that assessments should be based on delayed time windows for drug administration, in different stroke models in more than one animal species (Drummond et al., 2000; Zivin et al., 1999).

Recently, ion channels are increasingly being explored as potential targets for inhibition of inflammatory reactions and apoptosis. Further studies elucidating the role of voltage-gated potassium channels Kv1.3, Kv1.5 and HERG in effects of hemorrhage and ischemic stroke during different biological states of neutrophils and microglia at the site of brain injury will be important for specifically targeting these channels to control the toxic potential of inflammatory cells and thus regulate brain inflammation after stroke

without serious side effects. Future studies, to find more selective blockers for Kv1.3, Kv1.5, HERG and α7 which do not affect other channels and having better bioavailability and stability are important. An example of a side effect arising from imperfect selectivity is the ileal muscle twitching caused by margatoxin block of Kv1.1 channels in enteric neurons (Suarez-Kurtz et al.,1999), and since kaliotoxin also blocks Kv1.1, similar gastrointestinal side effects are predicted. Newer, small-molecule inhibitors of Kv1.3 have improved stability and bioavailability, but their ability to block other types of channels limits their use; eg., CP-339818 blocks neuronal Na+ channels (Wanner et al., 1999) and UK-78282 blocks Kv1.4 in the heart and brain (Hanson et al., 1999). In future, for successful stroke therapy, creating functionally selective channel blockers is very important. As our present studies demonstrate neutrophil expression of Kv1.3, Kv1.5 HERG, and α7 at the site of stroke lesion, we indicate that further studies investigating the effects of minocycline on activation of neutrophils based on expression of these channels might provide new insights for understanding the precise mechanism of action of minocycline in ICH and ischemic stroke.

VIII. TABLES

Table 1. Effect of minocycline administration on inflammatory cell infiltration with treatment time at one (1) hour post-ICH

Cell Type *	Control (n = 6)	Low Dose Minocycline ** (n = 6)	High Dose Minocycline** (n = 6)
ED1+ve macrophages	107 ± 11	69 ± 8.4	34.7 ± 7.8 ***
OX42+ve macrophages	180 ± 18	164 ± 18	91.2 ± 14 ***
OX42+ve neutrophils	45.1 ± 67	36.4 ± 6.9	18 ± 4 ***
MPO+ve neutrophils	157 ± 17	152 ± 16	87 ± 8.4 ***

* Values represent mean ± S.E.M. of the number of immunopositive cells counted within a representative area (250 × 250 μm) of the ICH site

** Low and high dose minocycline as defined in methods

*** $p < 0.01$ compared with control values
ANOVA/ Scheff'e

Table 2. Effect of minocycline administration on inflammatory cell infiltration with treatment time at six (6) hour post-ICH

Cell Type *	Control (n = 6)	High Dose Minocycline ** (n = 6)	
ED1+ve macrophages	26 ± 3.4	18 ± 3.1	***
OX42+ve macrophages	15 ± 22	23 ± 2.9	***
OX42+ve neutrophils	34 ± 7.4	12 ± 2.1	***
MPO+ve neutrophils	22 ± 3.3	16 ± 2.6	***

* Values represent mean ± S.E.M. of the number of cells counted within a representative area (250 × 250 μm)of the ICH site

** High dose minocycline as defined in methods

*** $p < 0.01$ compared with control values
ANOVA/ Scheff'e

Table 3. Cellular co-localization relationships between neutrophil/microglia/macrophage markers, potassium channel and α7 nicotinic Ach receptor expression

	Kv1.3 *	Kv1.5 *	HERG *	α7nAChR *
RP3 *	89 ± 6	77.3 ± 3	93.3 ± 4	99 ± 1 **
HIS48*	86.3 ± 4	86 ± 2	75 ± 4	89 ± 6 **
OX42*	96 ± 2	85.3 ± 1	92.3 ± 3	99 ± 1 **
ED1*	0	0	0	0

* Values represent percentage of RP3-, HIS48-,OX42- or ED1- immunopositive cells that are also immunopositive for potassium channels or α7 nicotinic Ach receptor, expressed as mean ± S.E.M from data obtained in 2-3 ICH animals with a counted total of 300 marker-positive cells for each of the potassium channels and α7nACh receptor

** $p < 0.01$ compared with control values
ANOVA/ Scheff'e

IX. FIGURES

Fig. 1. Nissl fluorescence (F) micrographs of adult rat striatum. (A) Low magnification Nissl fluorescence micrographs showing labelling of cells in and around the hematoma core of intracerebral hemorrhage site. (B) Low magnification Nissl fluorescence micrograph showing labelling of neurons (large cells) in the contra lateral control striatum. (C,E) High magnification Nissl fluorescence micrographs showing labelling of neurons in hematoma core and periphery of the lesion. (D,F) High magnification Nissl fluorescence micrographs showing labelling of neurons in the contra lateral control striatum. Note high density of neurons in control striatum (D), around the periphery of the hemorrhage site (C) and their absence in the lesion core. Scale bars: A-D, 200 μm; E,F, 100 μm.

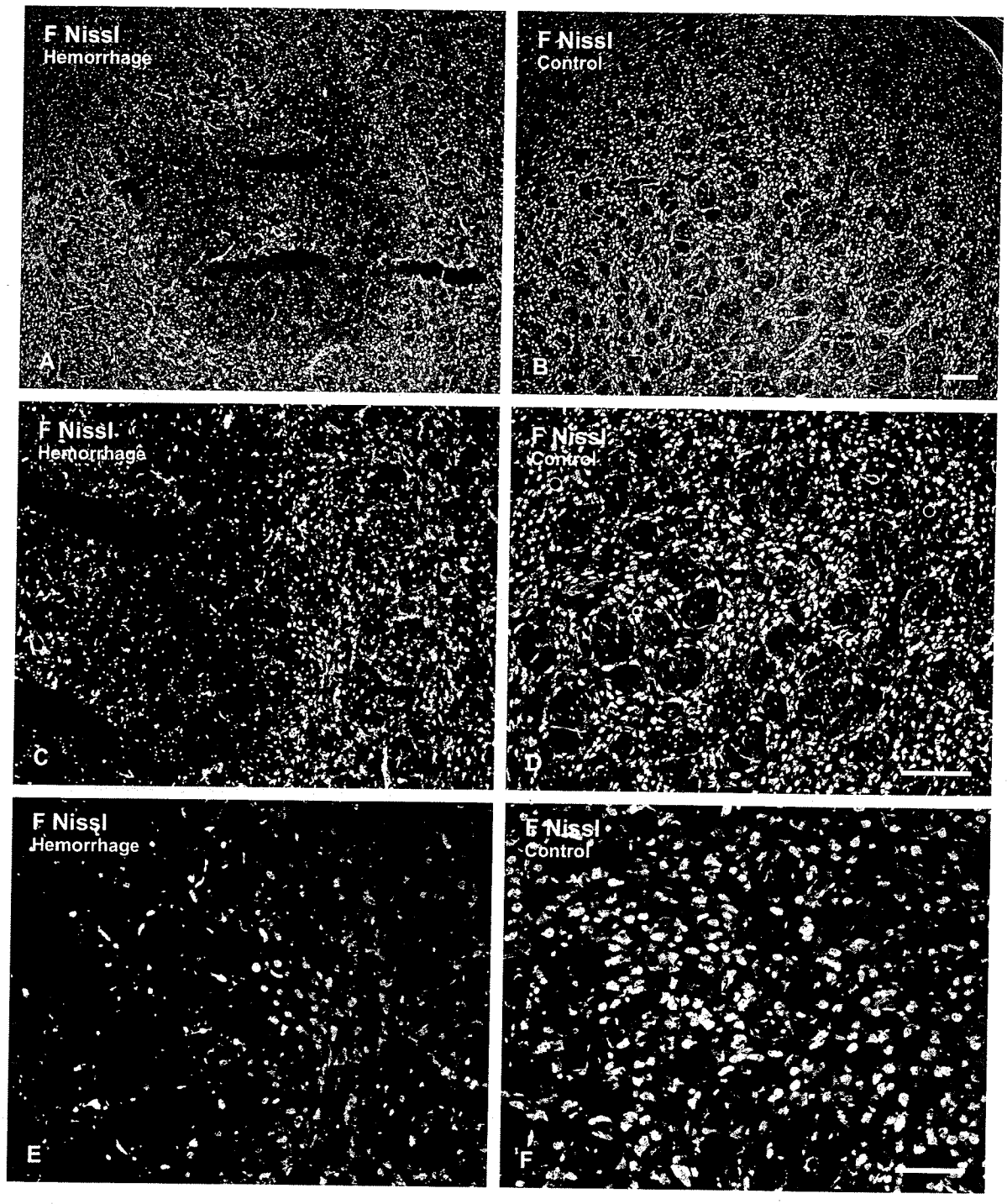
F Nissl
Hemorrhage
A
F Nissl
Control
B
F Nissl
Hemorrhage
C
F Nissl
Control
D
F Nissl
Hemorrhage
E
F Nissl
Control
F

Fig. 1

Fig. 2. Low magnification immunofluorescence micrographs showing inflammatory cells in intracerebral hemorrhage and control adult rat striatum. (A,C) immunofluorescence labelling of ED1- and OX42-positive cells respectively in ICH striatum.(B,D) Absence of labelling in contra lateral control striatum. Scale bars: A-D, 200 μm.

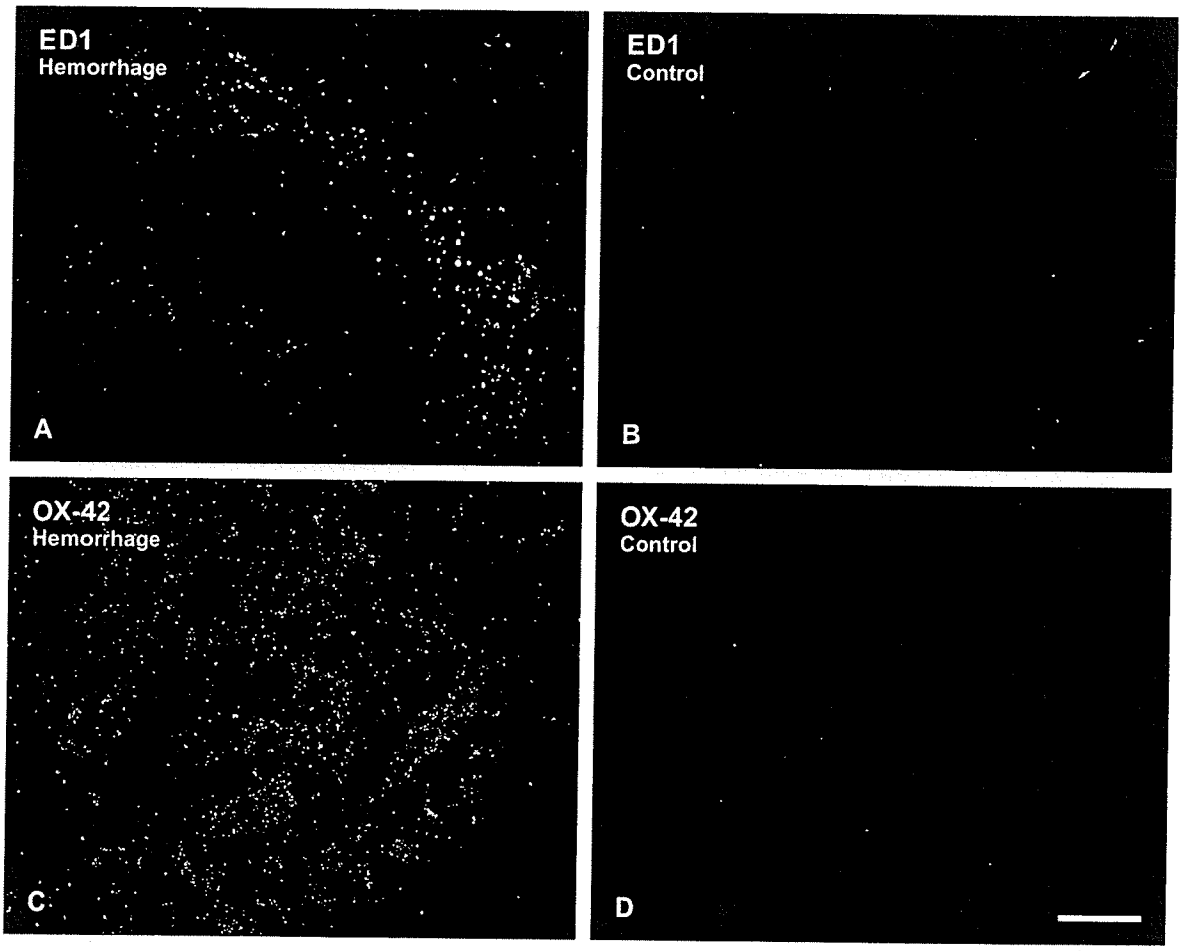
ED1
Hemorrhage
A
ED1
Control
B
OX-42
Hemorrhage
C
OX-42
Control
D

Fig. 2

Fig. 3. Low magnification immunofluorescence micrographs of neutrophils labelled with three different markers in ICH site and control adult rat striatum. (A,B) immunofluorescence labelling of MPO-positive neutrophils in ICH striatum (A) and absence of labelling in contra lateral control striatum (B). (C,D) Immunofluorescence labelling showing RP3-positive neutropils in ICH striatum (C), and absence of labelling in contra lateral control striatum (D). (E,F) Immunofluorescence labelling showing HIS48-positive neutrophils in ICH striatum (E) and absence of labelling in contra lateral control striatum (F). Scale bars: A-F, 200 μm .

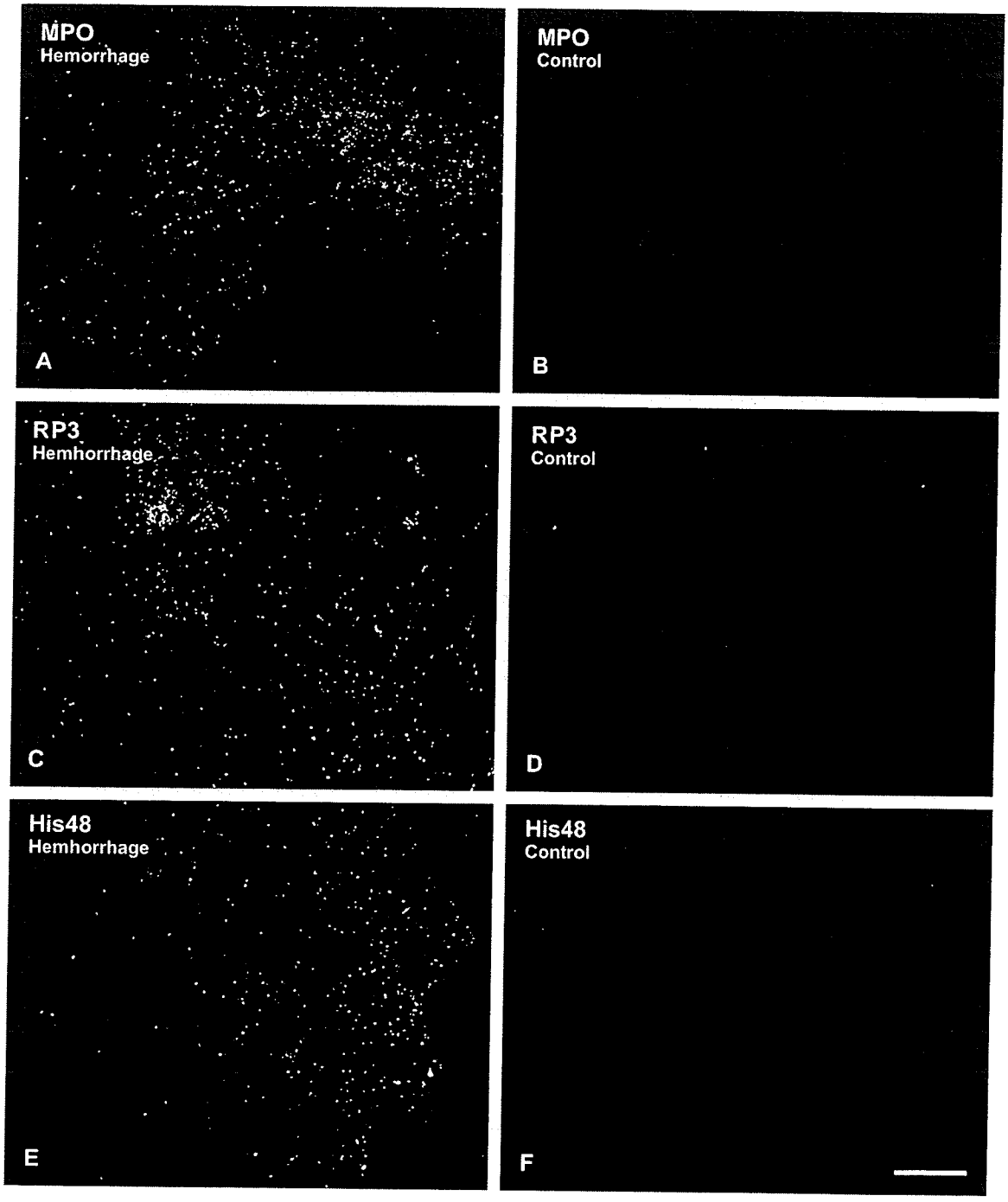
MPO
Hemorrhage
A
MPO
Control
B
RP3
Hemhorrhage
C
RP3
Control
D
His48
Hemhorrhage
E
His48
Control
F

Fig. 3

Fig. 4. Immunofluorescence co-localization relationships of MPO with RP3, HIS48, ED1 and OX42 in adult rat striatum after ICH. (A) Double immunofluorescence micrograph of the same field showing MPO- (A1) and RP3-positive neutrophils (A2), and co localized MPO/RP3 labelled neutrophils in image overlay (A3, yellow). (B) Double immunofluorescence micrograph of the same field showing MPO- (B1) and HIS48-positive neutrophils (B2), and co localization in image overlay (B3, yellow). (C,D) Double immunofluorescence at low (C) and high (D) magnification showing MPO-positive neutrophils (C1,D1) and ED1-positive macrophages (C2,D2), and image overlay showing no co-localization (C3,D3). Note large fuzzy blood clot fragments taking up FITC nonspecifically in (D2,D3). (E) Double immunofluorescence of the same field showing MPO-positive neutrophils (E1), and OX42-positive neutrophils and macrophages (E2), and partial co localization in overlay (E3, yellow). Scale bars: A-E, 100 μm.

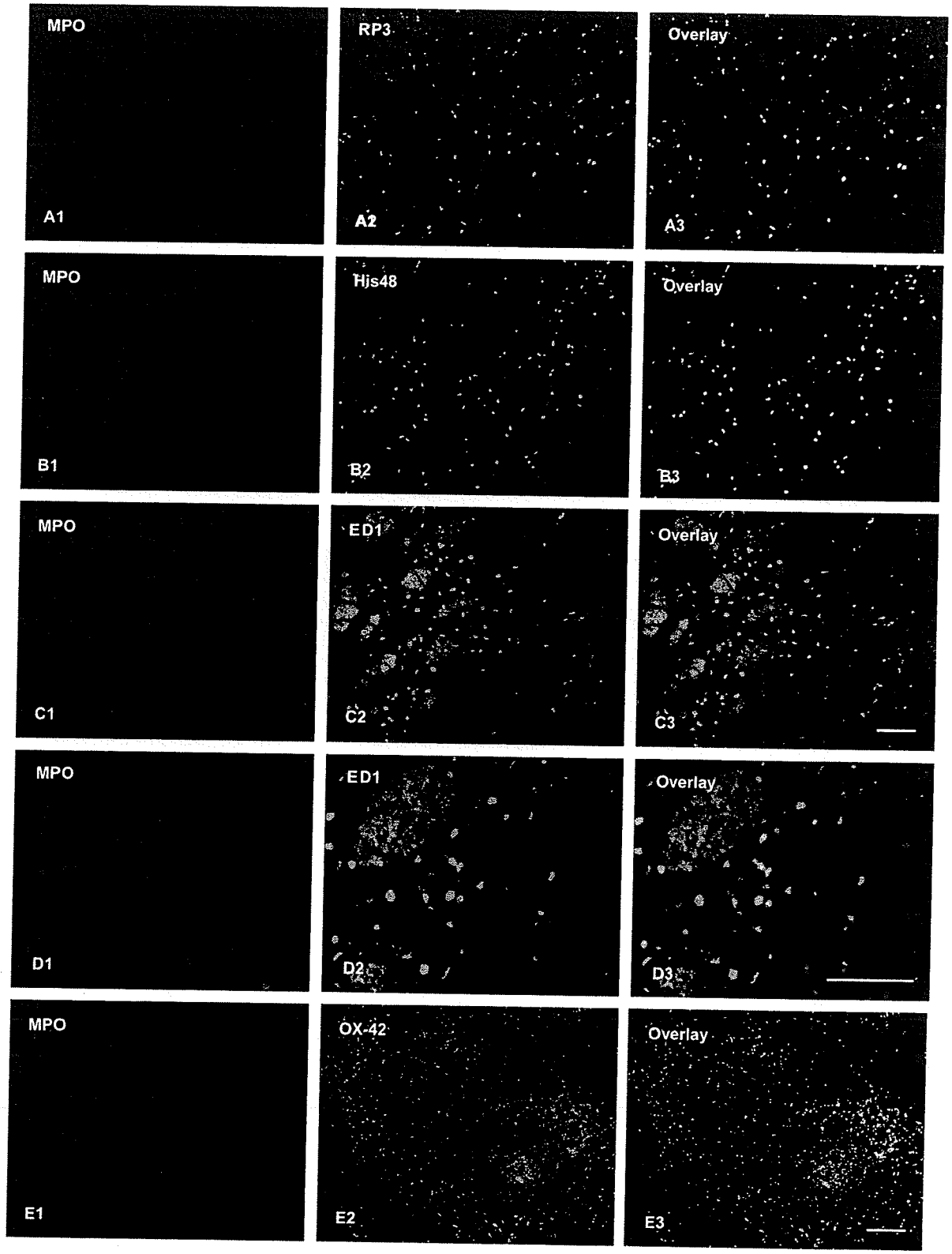
MPO
A1
RP3
A2
Overlay
A3
MPO
B1
His48
B2
Overlay
B3
MPO
C1
ED1
C2
Overlay
C3
MPO
D1
ED1
D2
Overlay
D3
MPO
E1
OX-42
E2
Overlay
E3

Fig. 4

Fig. 5. Immunofluorescence control labelling for potential inappropriate cross reactivity between primary antibodies (MPO, ED1and OX42) and secondary antibodies (FITC-conjugated horse anti-mouse and Cy3-conjugated donkey anti-rabbit). Immunofluorescence micrographs of the same field showing absence of labelling (A1) and labelling of ED1-positive cells (A2) with primary MPO omission, while including primary anti-ED1 and both secondary antibodies. Immunofluorescence micrographs of the same field showing labelling of MPO-positive cells (B1) and absence of labelling (B2) with primary ED1 omission, while including primary anti-MPO and both secondary antibodies. Immunofluorescence micrographs of the same field showing absence of labelling (C1) and labelling of OX42-positive cells (C2) with primary MPO omission, while including primary anti-OX42 and both secondary antibodies. Immunofluorescence micrographs showing labelling of MPO-positive cells (D1) and absence of labelling (D2) with primary OX42-omission while including primary anti-MPO and both the secondary antibodies. Scale bars: A-D, 100 μm.

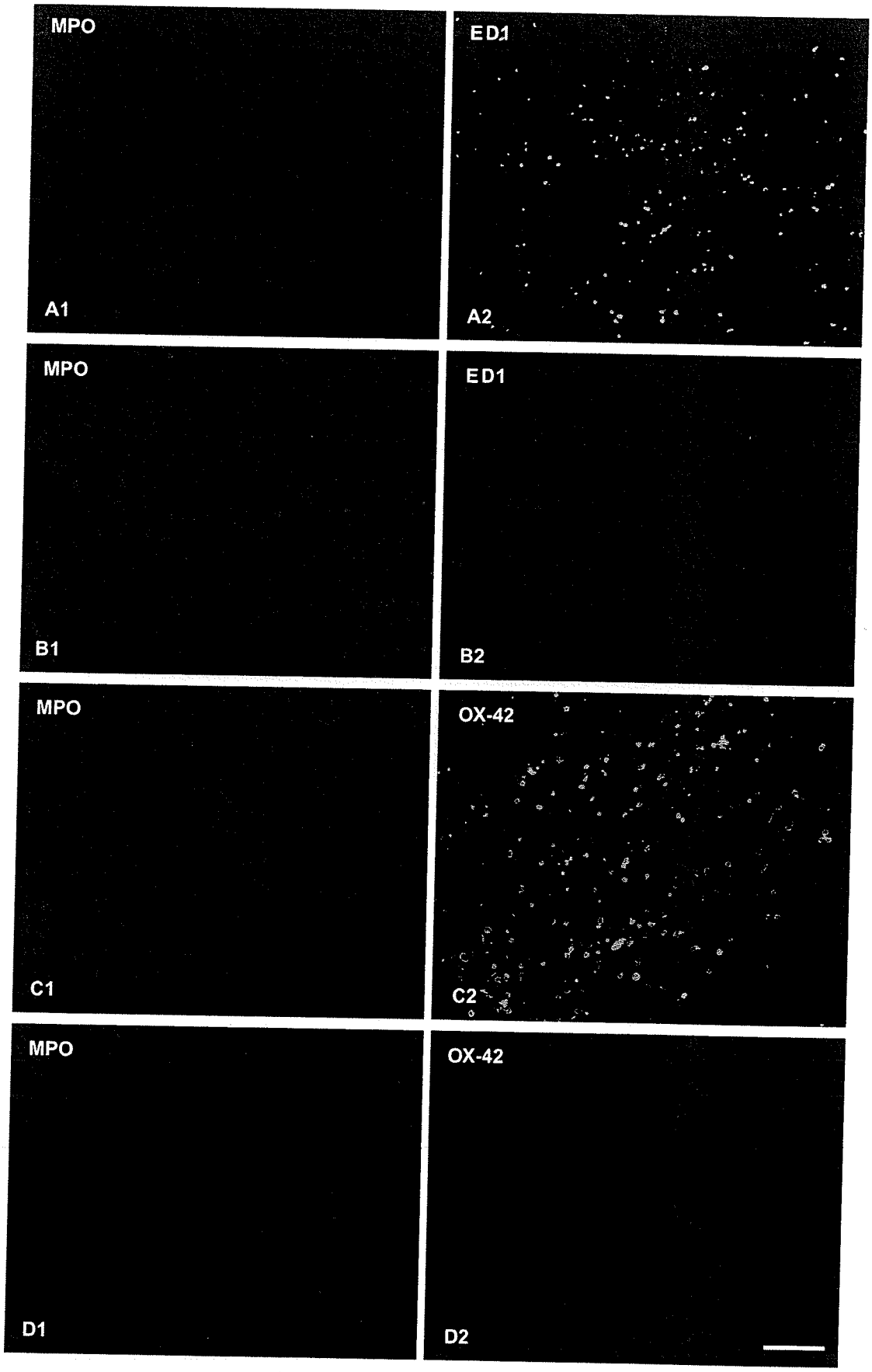

Fig. 5

Fig. 6. (A-C) Low to high magnification Nissl fluorescence micrographs of neuronal labelling in endothelin-1 induced ischemic adult rat striatum.(D-H) Low magnification immunofluorescence micrographs showing labelling of ED1-positive cells (D), OX42-positive cells (E), MPO-positive cells (F), RP3-positive cells (G), and HIS48-positive cells (H) at endothelin-1 induced ischemic site in adult rat striatum . Scale bars: A,B,D-H, 200 μm; C, 100 μm.

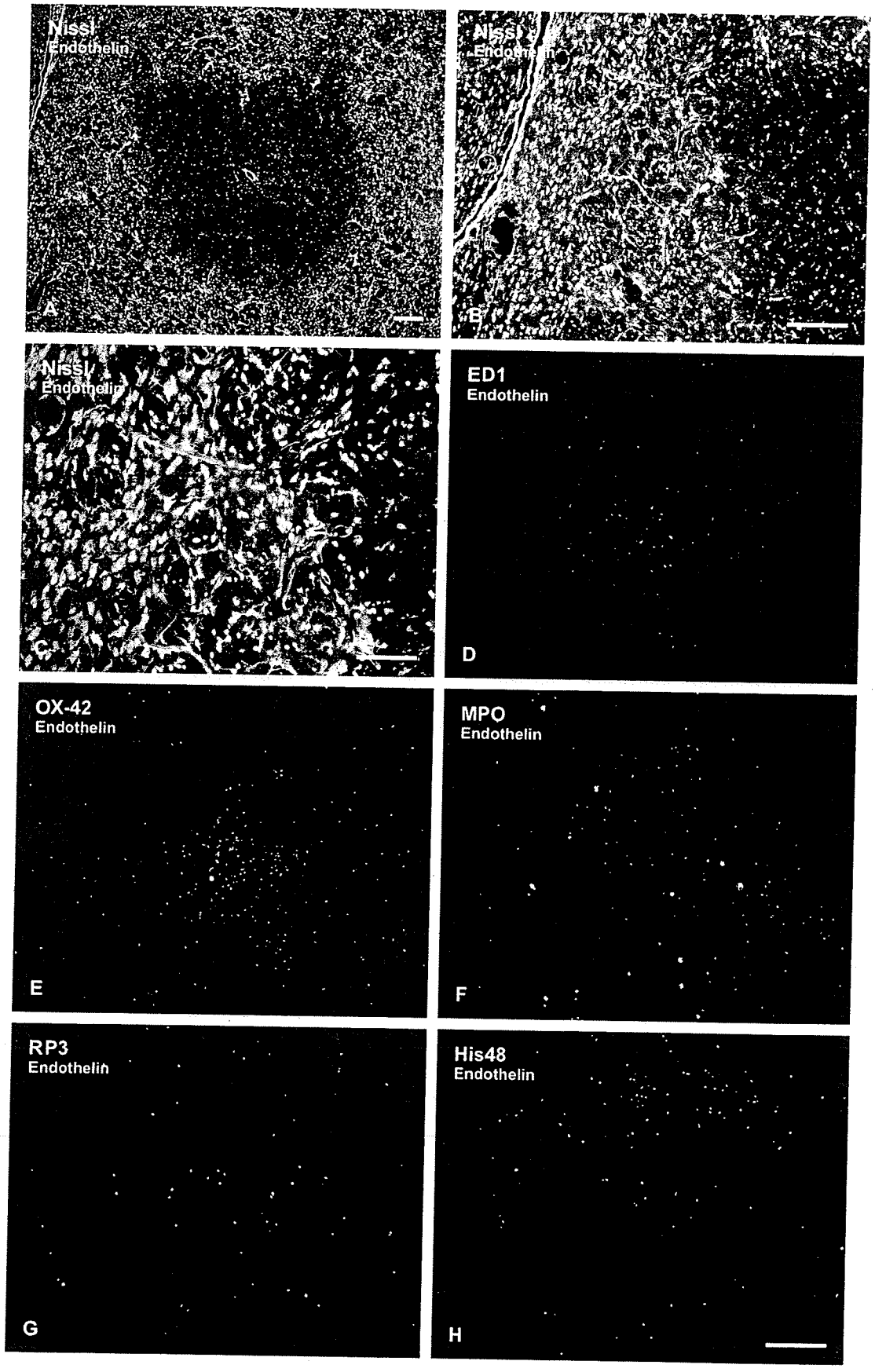
Nissl
Endothelin
A
Nissl
Endothelin
B
Nissl
Endothelin
C
ED1
Endothelin
D
OX-42
Endothelin
E
MPO
Endothelin
F
RP3
Endothelin
G
His48
Endothelin
H

Fig. 6

Fig. 7. Low magnification immunofluorescence micrographs showing inflammatory cells labelled with ED1, MPO and OX42 in saline control and minocycline treated adult rats in striatum 1 hr post ICH. Immunofluorescent labelling of ED1-positive macrophages in control (A) and minocycline treated rats (B). Immunofluorescence micrographs showing immunofluorescent labelling of MPO-positive neutrophils in control (C) and minocycline treated rats (D). Immunofluorescent micrographs showing labelling of OX42-positive neutropils and macrophages in control (E) and minocycline treated rats (F). Scale bars: A-F, 200 μm.

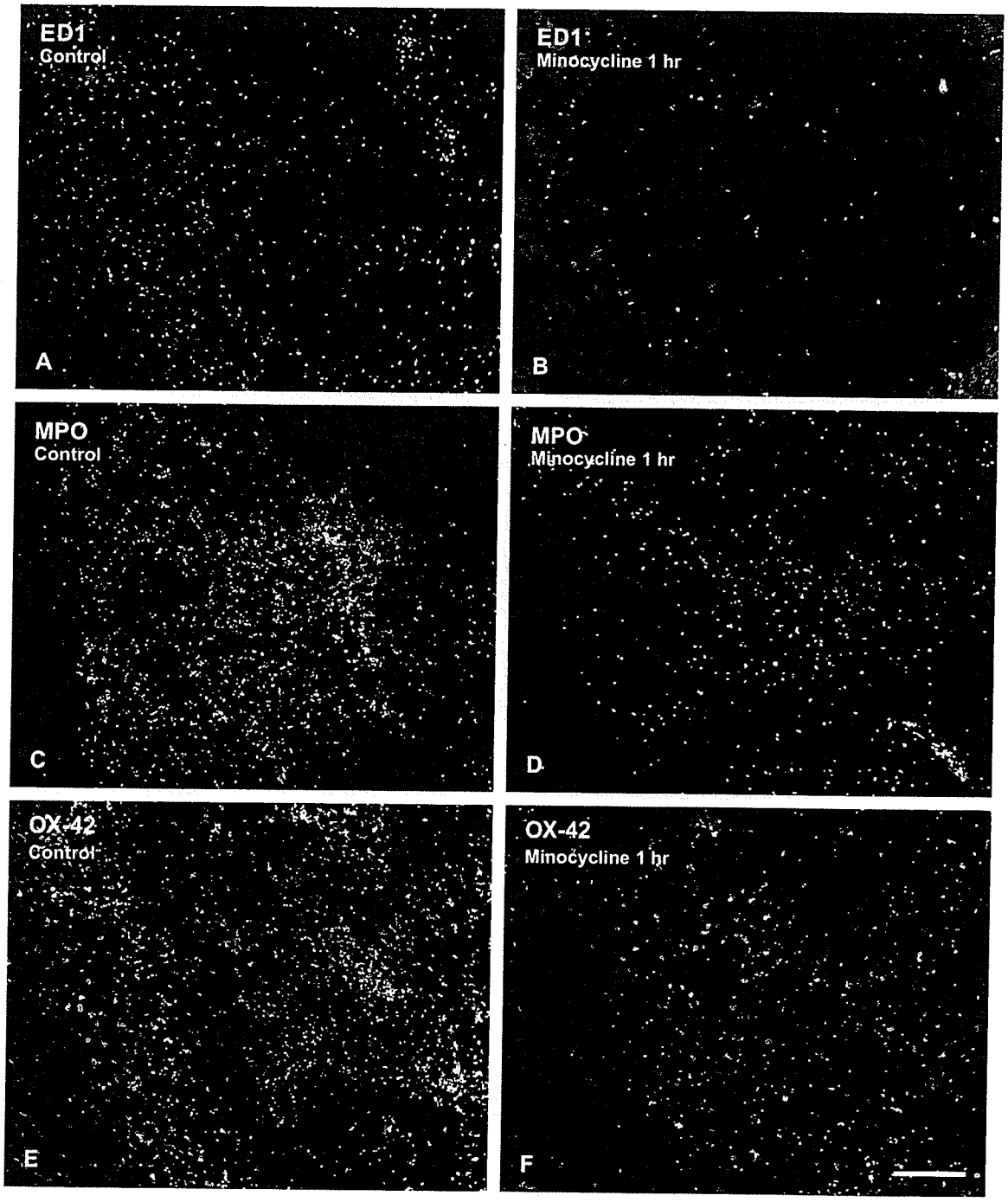
ED1
Control
A
ED1
Minocycline 1 hr
B
MPO
Control
C
MPO
Minocycline 1 hr
D
OX-42
Control
E
OX-42
Minocycline 1 hr
F

Fig. 7

Fig. 8. Immunofluorescence micrographs showing inflammatory cells labelled with ED1, MPO and OX42 in saline control and minocycline treated adult rats in striatum 6 hr post ICH. (A,B) Immunofluorescence labelling of ED1-positive macrophages in control (A) and micocycline treated rats (B). (C,D) Micrographs showing labelling of MPO-positive neutrophils in control (C) and minocycline treated rats (D). (E,F) Immunofluorescent labelling of OX42-positive neutrophils and macrophages in control (E) and minocycline treated rats (F). Scale bars: A,B, 100 μm; C-F, 200 μm.

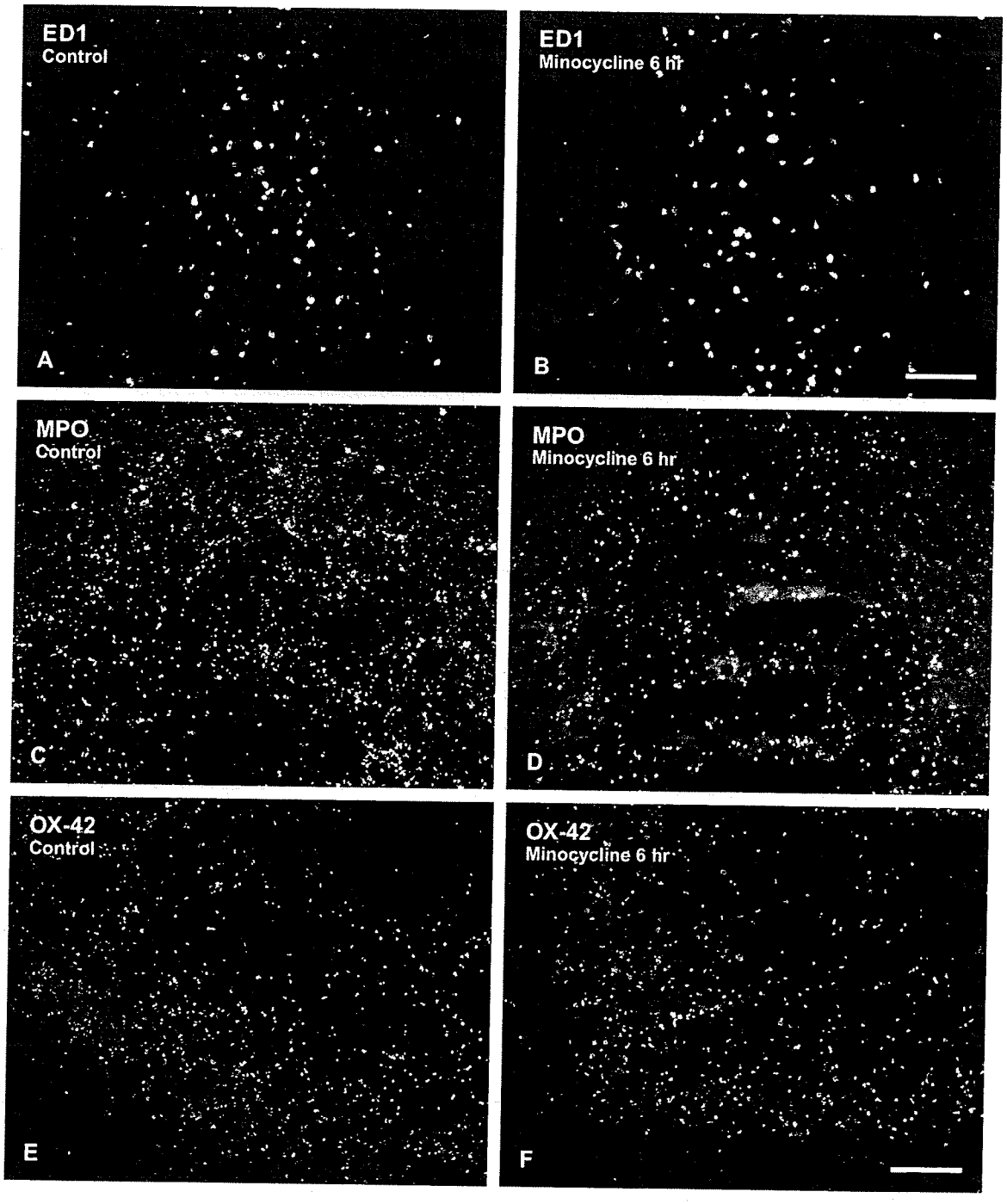
ED1
Control
A
ED1
Minocycline 6 hr
B
MPO
Control
C
MPO
Minocycline 6 hr
D
OX-42
Control
E
OX-42
Minocycline 6 hr
F

Fig. 8

Fig. 9. Immunofluorescence co-localization relationships of Kv1.3 with ED1 and OX42 at ICH site in adult rat striatum. (A) Low magnification double Immunofluorescence micrograph of the same field showing labelling of Kv1.3-positive cells (A1), and ED1-positive cells (A2), and absence of co-localization in image overlay (A3). (B) High magnification double immunofluorescence micrograph of the same field showing labelling of Kv1.3-positive cells (B1), and ED1-positive cells (B2), and absence of co-localization in image overlay (B3). (C) Low magnification double immunofluorescence micrographs of the same field showing labelling of Kv1.3-positive cells (C1), OX42-positive cells (C2) and partial Kv1.3/OX42 co-localization as yellow in image overlay (C3). (D) High magnification double immunofluorescence micrographs of the same field showing labelling of Kv1.3-positive cells (D1), and OX42-positive cells (C2), and partial Kv1.3/OX42 co-localization seen as yellow in image overlay. Scale bars: A-D, 100 μm .

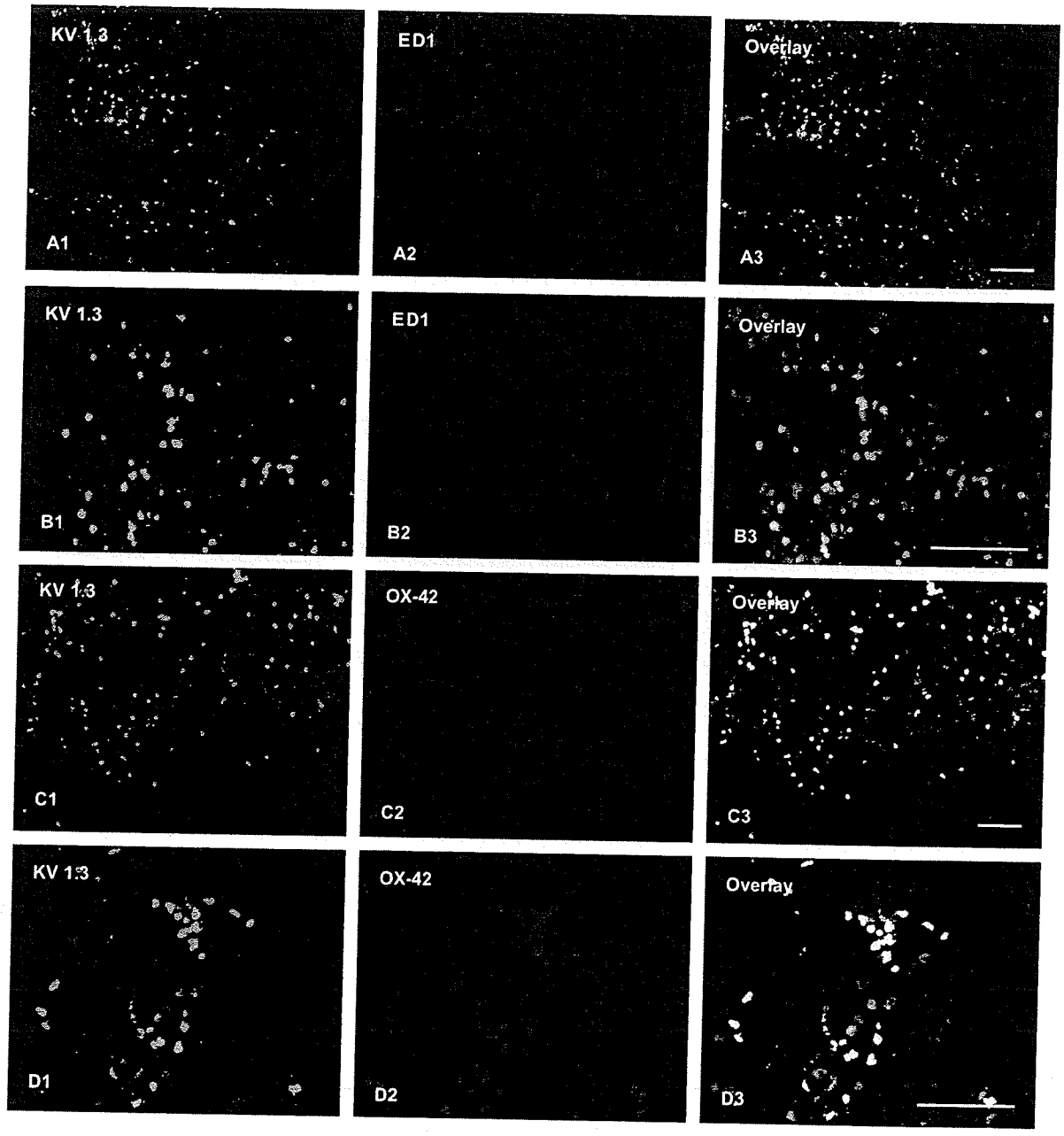
KV 1.3
A1
ED1
A2
Overlay
A3
KV 1.3
B1
ED1
B2
Overlay
B3
KV 1.3
C1
OX-42
C2
Overlay
C3
KV 1.3
D1
OX-42
D2
Overlay
D3

Fig. 9

Fig. 10. Immunofluorescence co-localization relationships of Kv1.3 with RP3 and HIS48 at ICH site in adult rat striatum. (A) Low magnification double immunofluorescence micrographs of the same field showing labelling of RP3-positive cells (A1), and Kv1.3-positive cells (A2), and yellow image overlay showing partial co-localization of Kv1.3/RP3 (A3). (B) High magnification double immunofluorescence micrograph of the same field showing labelling of RP3-positive cell (B1), and Kv1.3-positive cell (B2), and partial co-localization in image overlay (B3). (C) Low magnification double immunofluorescence micrograph of the same fields showing labelling of HIS48-positive cells (C1), and Kv1.3-positive cells (C2), with substantial HIS48/KV1.3 co-localization seen as yellow in image overlay (C3). (D) High magnification double immunofluorescence micrograph of the same field showing labeling of HIS48-positive cells (D1), and Kv1.3-positive cells (D2), with near total co-localization of His48/Kv1.3 seen as yellow in image overlay (D3). Scale bars: A-F, 100 μm.

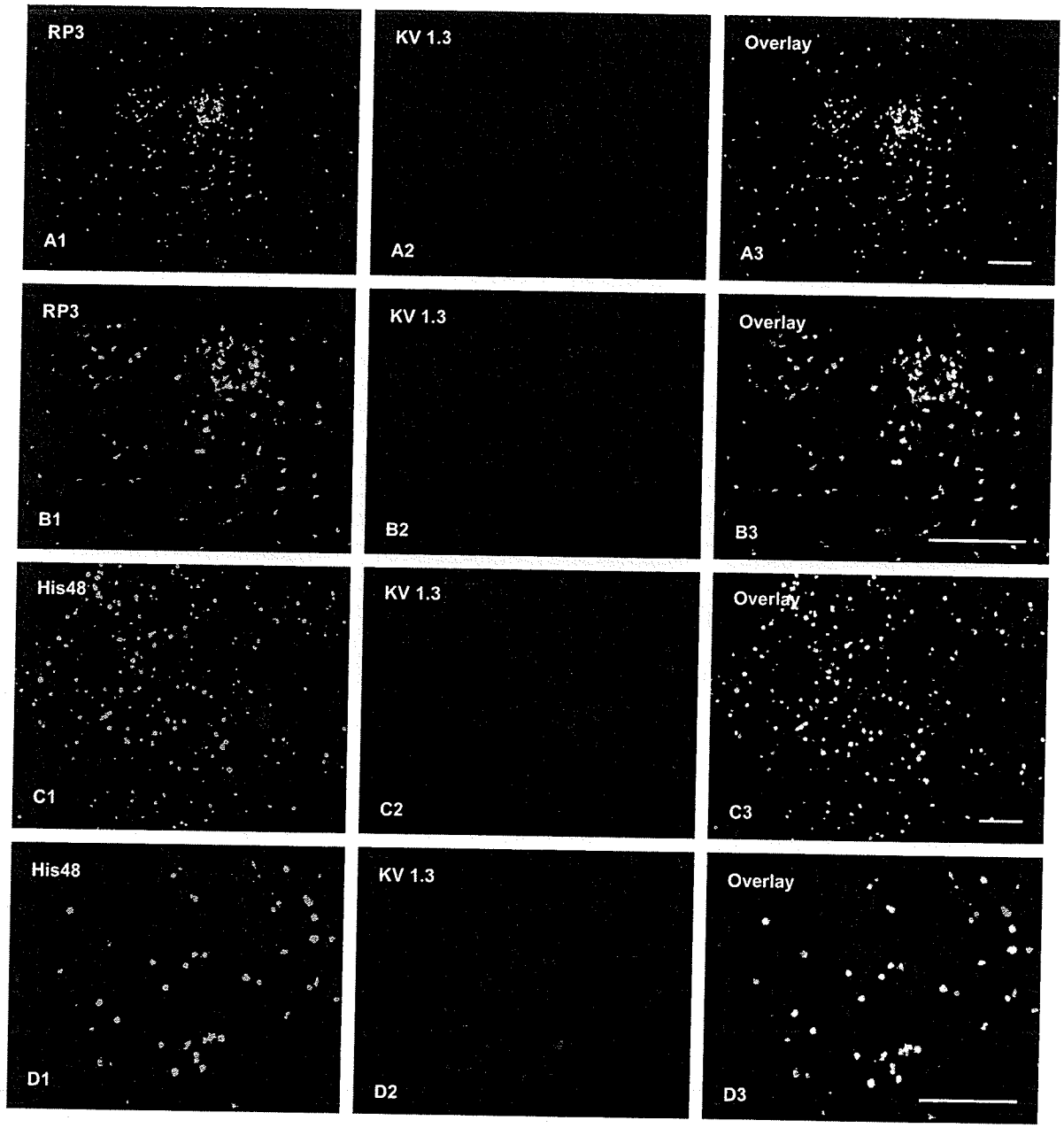
RP3
KV 1.3
Overlay
A1
A2
A3
RP3
KV 1.3
Overlay
B1
B2
B3
His48
KV 1.3
Overlay
C1
C2
C3
His48
KV 1.3
Overlay
D1
D2
D3

Fig. 10

Fig. 11. Immunofluorescence co-localization relationships of Kv1.5 with ED1 and OX42 at ICH site in adult rat striatum. (A) Low magnification double immunofluorescence micrographs of the same field showing labelling of Kv1.5-positive cell nuclei (A1), and ED1-positive nuclei (A2), and with yellow image overlay showing partial co-localization of Kv1.5/ED1 (A3). (B) High magnification double immunofluorescence micrograph of the same field showing labelling of Kv1.5-positive nuclei (B1) and ED1-positive nuclei (B2), with image overlay showing partial co-localization seen as yellow (B3). (C) Low magnification double immunofluorescence micrographs of the same field showing labelling of Kv1.5-positive nuclei (C1), and OX42-positive nuclei (C2), with partial co-localization of Kv1.5/OX42 seen as yellow in image overlay (C3). (D) High magnification double immunofluoroscence micrograph of the same field showing labelling of Kv1.5-positive nuclei (D1), and OX42-positive nuclei (D2), with subtotal co-localization of Kv1.5/OX42 seen as yellow in image overlay (D3). Scale bars: A-F, 100 μm.

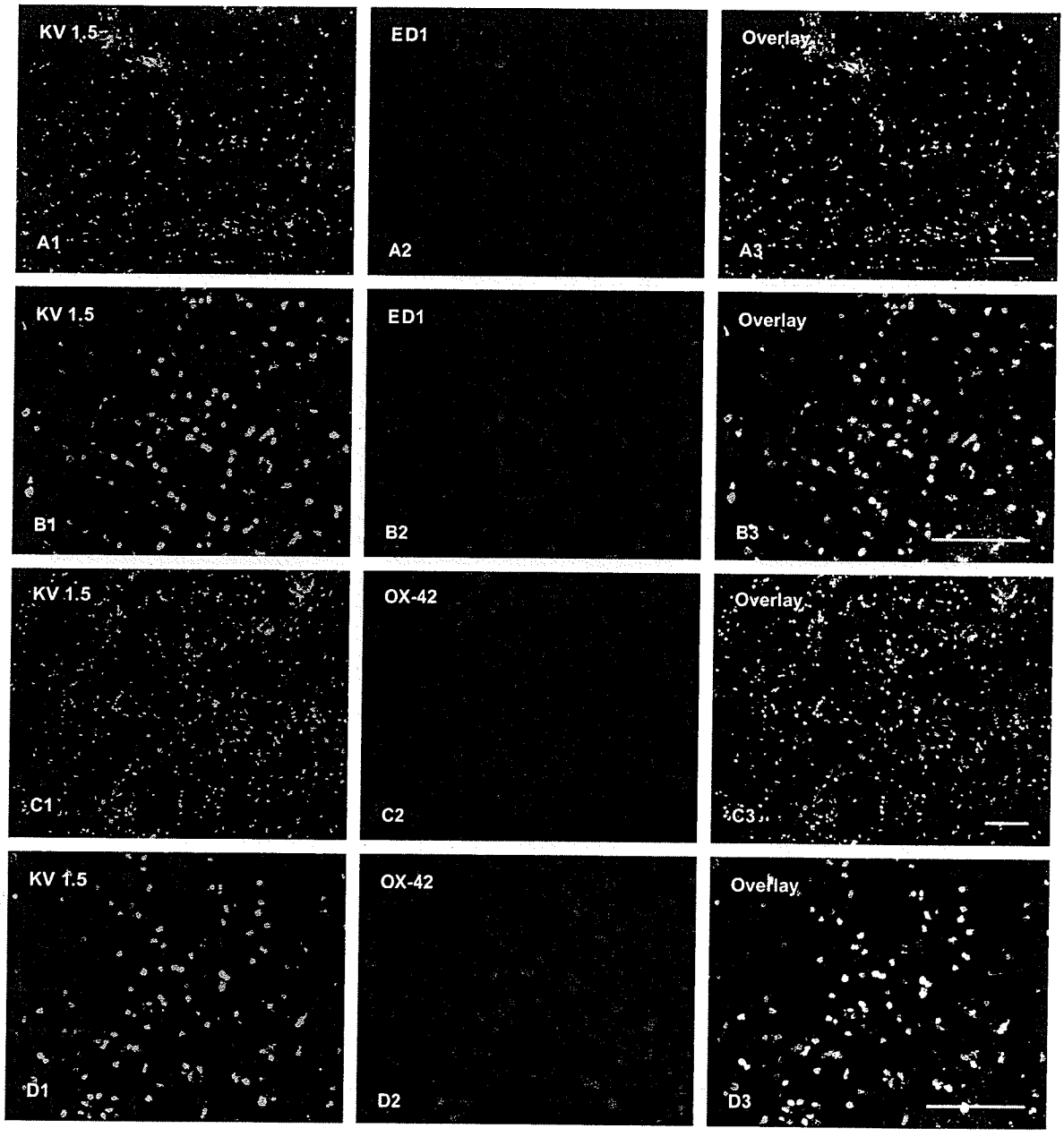
KV 1.5
A1
ED1
A2
Overlay
A3
KV 1.5
B1
ED1
B2
Overlay
B3
KV 1.5
C1
OX-42
C2
Overlay
C3
KV 1.5
D1
OX-42
D2
Overlay
D3

Fig. 11

Fig. 12. Immunofluorescence co-localization relationships of Kv1.5 with RP3 and HIS48 at ICH site in adult rat striatum. (A) Low magnification double immunofluorescence micrographs of the same field showing labelling of RP3-positive cell (A1), and Kv1.5-positive cell (A2), and image overlay showing partial co-localization of Kv1.5/RP3 seen as yellow (A3). (B) High magnification double immunofluorescence micrographs of the same field showing labelling of RP3-positive cells (B1), and Kv1.5-positive cells (B2), with overlay showing partial co-localization seen as yellow (B3). (C) Low magnification double immunofluorescence micrographs of the same field showing labelling of HIS48-positive cells (C1), and Kv1.5-positive cells (C2), and with partial co-localization of Kv1.5/HIS48 seen as yellow in image overlay (C3). (D) High magnification double immunofluorescence micrographs of the same field showing labelling of HIS48-positive cells (D1), and Kv1.5-positive cells (D2), with partial co-localization seen as yellow in image overlay (D3). Scale bars: A-D, 100 μm.

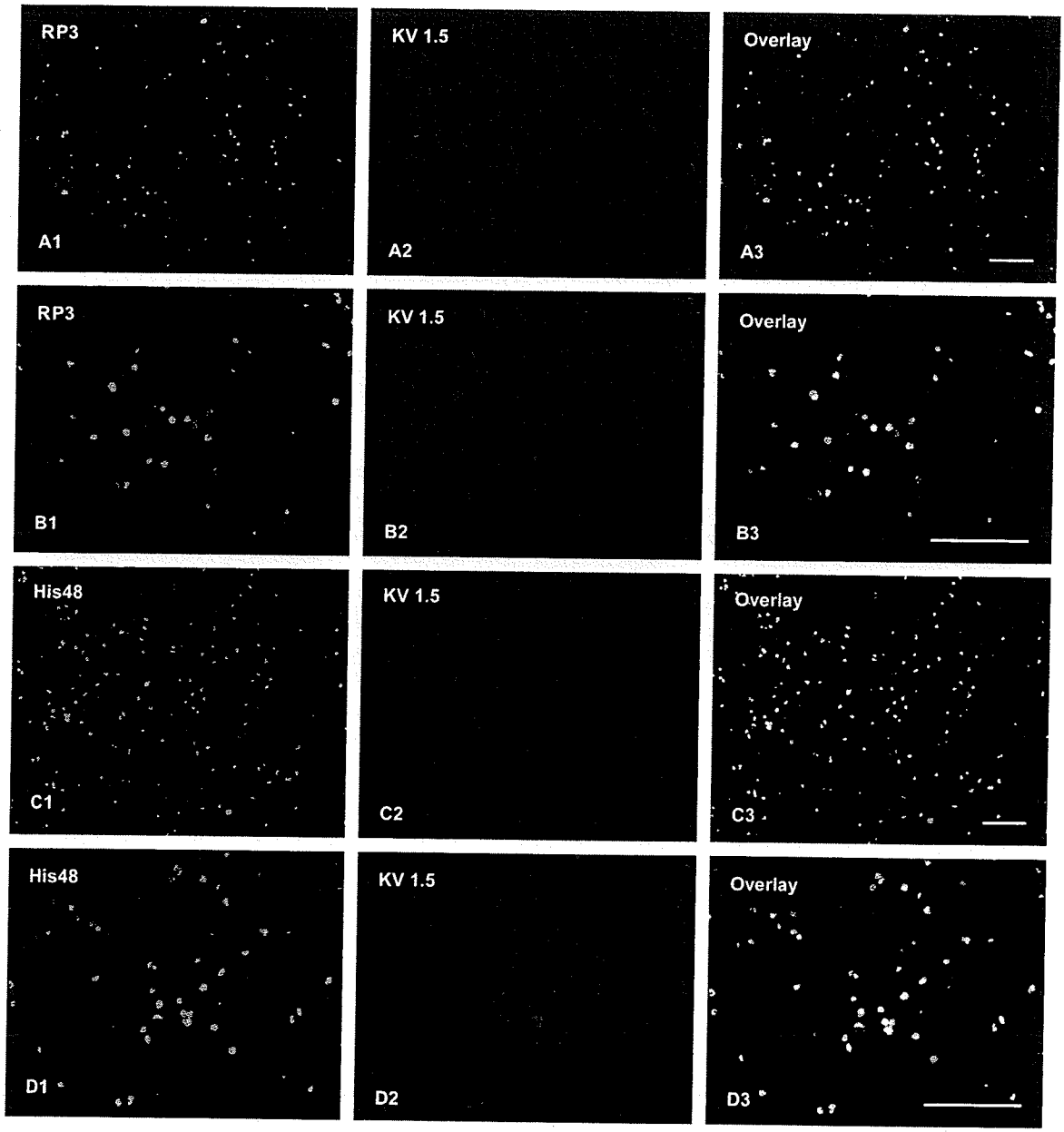
RP3
KV 1.5
Overlay
A1
A2
A3
RP3
KV 1.5
Overlay
B1
B2
B3
His48
KV 1.5
Overlay
C1
C2
C3
His48
KV 1.5
Overlay
D1
D2
D3

Fig. 12

Fig. 13. Immunofluorescence co-localization relationships of HERG with ED1 and OX42 at ICH site in adult rat striatum. (A) Low magnification double immunofluorescence micrographs of the same field showing labelling of HERG-positive cells (A1), and ED1-positive cells (A2), with image overlay showing absence of co-localization (A3). (B) High magnification double immunofluorescence micrographs of the same field showing labelling of HERG-positive cells (B1), and ED1-positive cells (B2), image overlay showing absence of co-localization (B3). (C) Low magnification double immunofluorescence micrographs of the same field showing labelling of HERG-positive cells (C1), and OX42-positive cells (C2) and partial co-localization of HERG/OX42 seen as yellow in image overlay (C3). (D) High magnification double immunofluorescence micrographs of the same field showing labeling of HERG-positive cells (D1), and OX42-positive cells (D2), co-localization seen as yellow in image overlay (D3). Scale bars: A-D, 100 μm.

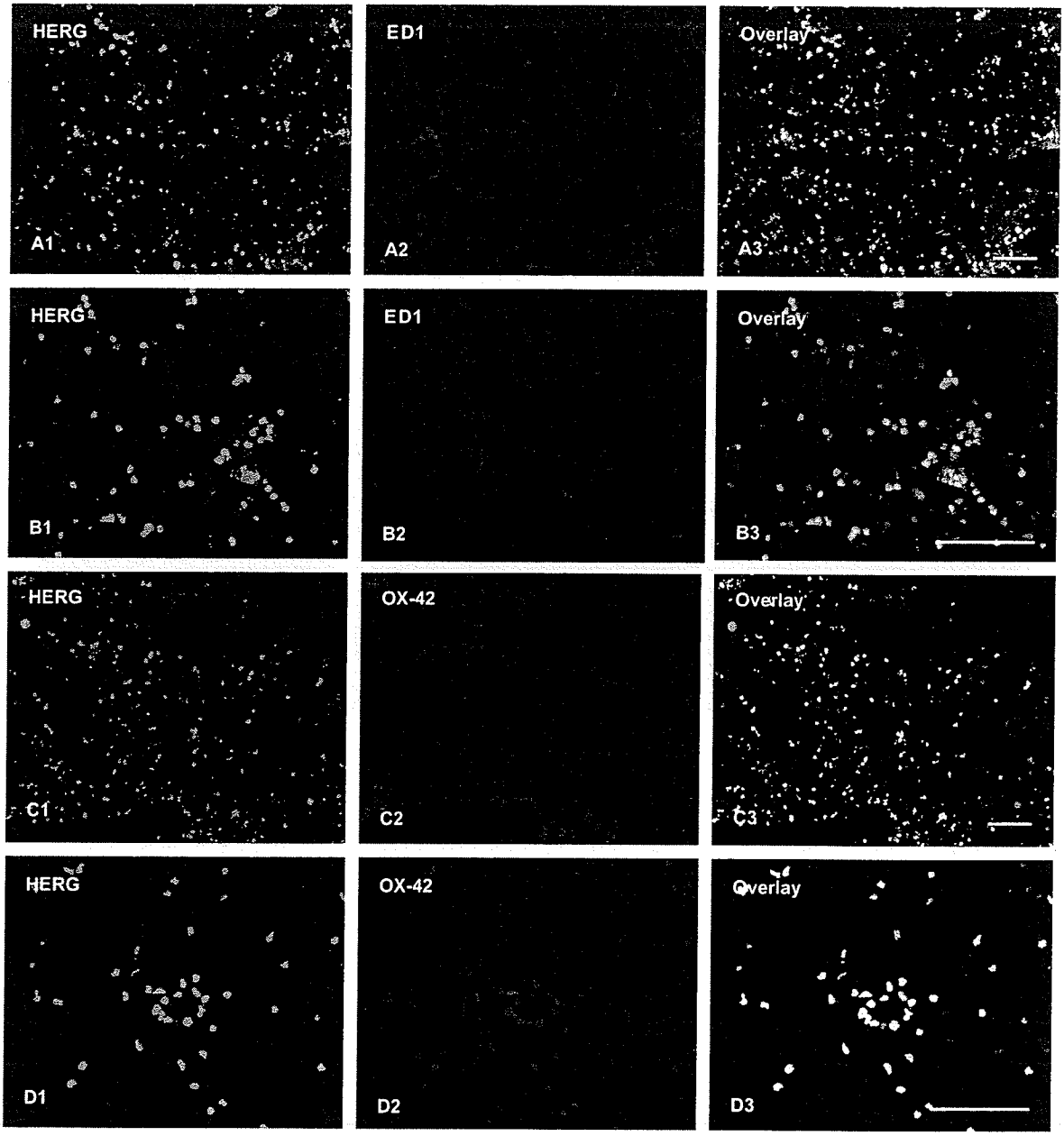
HERG
A1
ED1
A2
Overlay
A3
HERG
B1
ED1
B2
Overlay
B3
HERG
C1
OX-42
C2
Overlay
C3
HERG
D1
OX-42
D2
Overlay
D3

Fig. 13

Fig. 14. Immunofluorescence co-localization relationships of HERG with RP3 and HIS48 at ICH site in adult rat striatum. (A) Low magnification double immunofluorescence micrographs of the same field showing labelling of HERG-positive cells (A1), and RP3-positive cells (A2), with image overlay showing partial co-localization (A3). (B) High magnification double immunofluorescence micrographs of the same field showing labelling of HERG-positive cells (B1), and RP3-positive cells (B2), with image overlay showing partial co-localization (B3). (C) Low magnification double immunofluorescence micrographs of the same field showing labelling of HERG-positive cells (C1), and HIS48-positive cells (C2), with co-localization of HERG/HIS48 seen as yellow in image overlay (C3). (D) High magnification double immunofluorescence micrographs of the same field showing labelling of HERG-positive cells (D1), and HIS48-positive cells (D2), with co-localization seen as yellow in image overlay (D3). Scale bars: A-D, 100 μm.

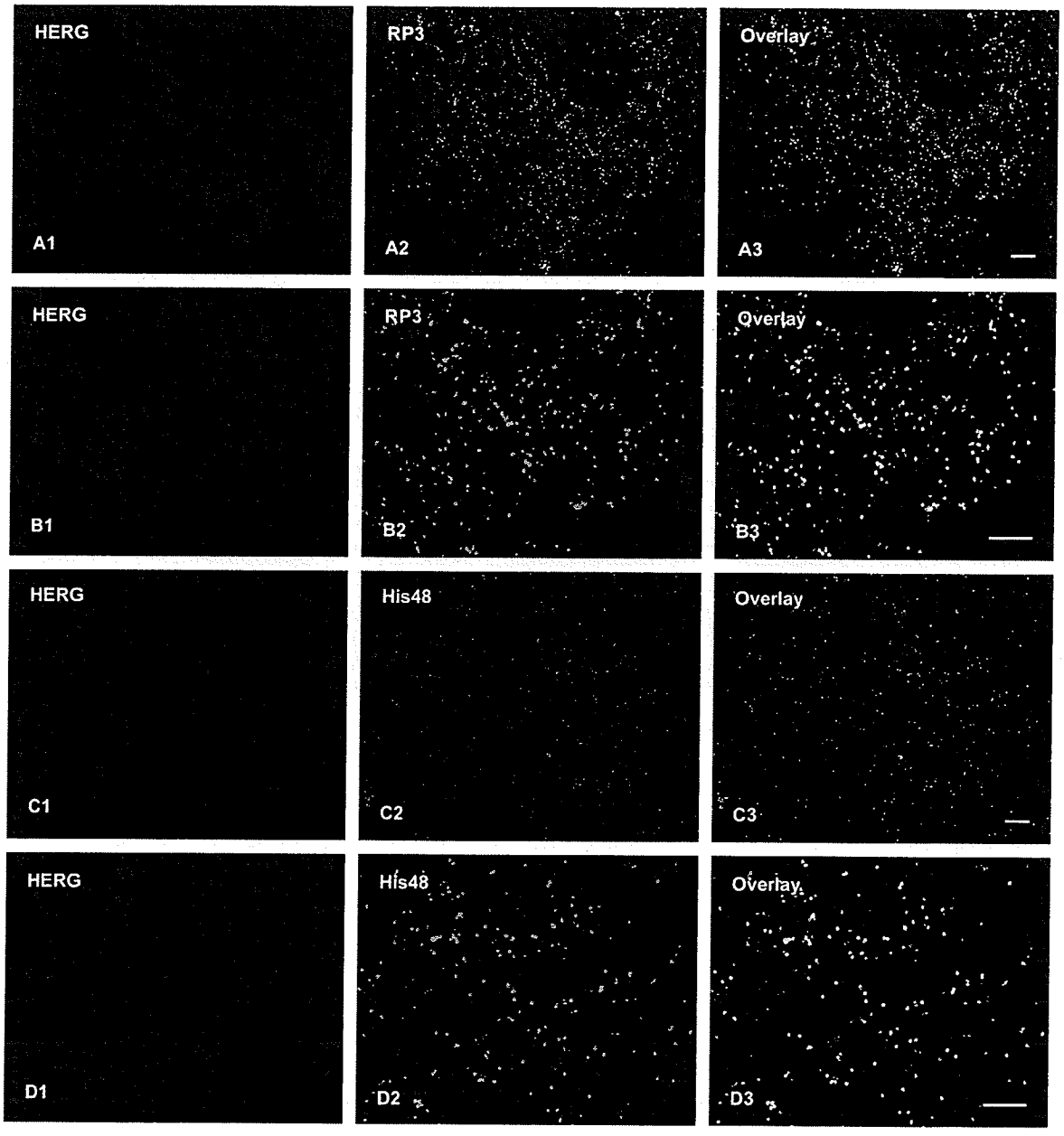
HERG
RP3
Overlay
A1
A2
A3
HERG
RP3
Overlay
B1
B2
B3
HERG
His48
Overlay
C1
C2
C3
HERG
His48
Overlay
D1
D2
D3

Fig. 14

Fig. 15. (A-C) Immunofluorescence micrographs from low to high magnification showing labelling for nicotinic acetylcholine receptor α7 subunit in ICH model of adult rat striatum. (D) Double immunofluorescence micrographs of the same fields showing labelling of α7-positive cells (D1), ED1-positive cells (D2), and absence of co-localization in image overlay (D3). (E) Double immunofluorescence micrographs of the same fields showing labelling of α7-positive cell (E1), OX42-positive cells (E2), and partial co-localization seen as yellow in image overlay (E3). (F) Double immunofluorescence of micrographs of the same fields showing labelling of α7-positive cells (F1), and RP3-positive cells (F2), and near total α7/RP3 co-localization F3). (G) Double immunofluorescence micrographs of the same field showing labeling of α7-positive cells (G1), and HIS48-positive cells (G2), and image overlay showing near total α7/HIS48 co-localization (G3). Scale bars: (A), 200 μm; B-D, 100μm.

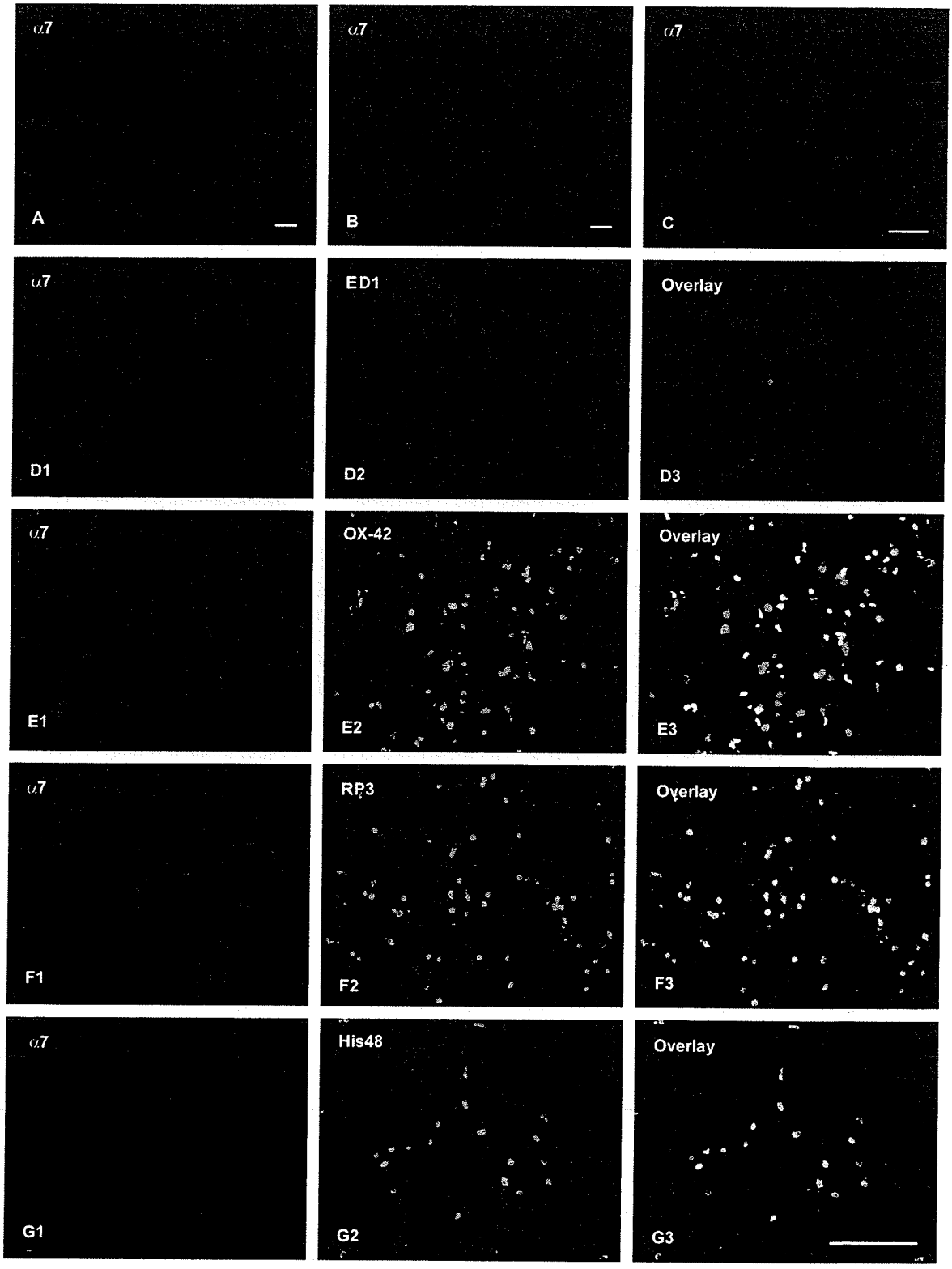
α7
A
α7
B
α7
C
α7
D1
ED1
D2
Overlay
D3
α7
E1
OX-42
E2
Overlay
E3
α7
F1
RP3
F2
Overlay
F3
α7
G1
His48
G2
Overlay
G3

Fig. 15

Fig. 16. Immunofluorescence micrographs showing cells labelled for Kv1.3, Kv1.5, HERG and α7 at endothelin-1 induced ischemic site in adult rat striatum. Low (A1) and high (A2) magnification immunofluoroscence micrographs showing Kv1.3-positive cells in ischemic striatum. Low (B1) and high (B2) magnification immunofluorescence micrographs showing cells labelled for Kv1.5 in ischemic striatum. Some Kv1.5 labelling seen around lesion periphery in B1 is due to labeling of neuronal nuclei. Low (C1) and high (C2) magnification immunofluorescence micrographs showing labelling of HERG-positive cells in ischemic striatum. Low (D1) and high (D2) magnification immunofluorescence micrograph showing cells labelled for α7 in ischemic striatum. Scale bars: A1, B1,C1,D1, 200 μm; 2,B2,C2,D2, 100 μm.

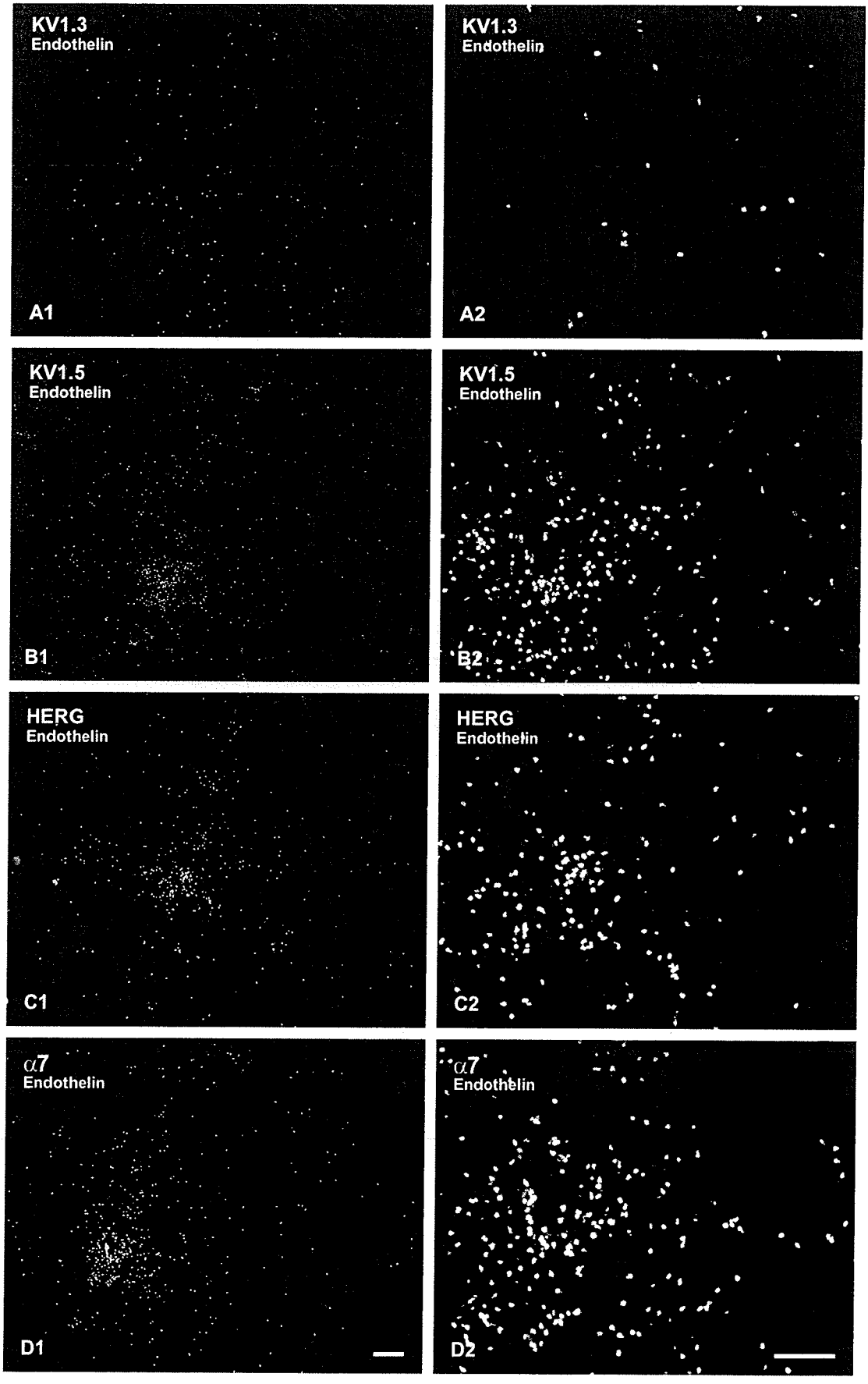
KV1.3
Endothelin
A1
KV1.3
Endothelin
A2
KV1.5
Endothelin
B1
KV1.5
Endothelin
B2
HERG
Endothelin
C1
HERG
Endothelin
C2
α7
Endothelin
D1
α7
Endothelin
D2

Fig. 16

X. REFERENCES

American Heart Association. 2001 Heart and Stroke Statistical Update. Dallas, Tex: American Heart Association; 2001.

Aarts MM, Tymianski M (2003) Novel treatment of excitotoxicity: targeted disruption of intracellular signaling from glutamate receptors. Biochem Pharmacol 66: 877-886.

Ahmed N, P. Nasman and N.G. Wahlgren (2000) Effect of intravenous nimodipine on blood pressure and outcome after acute stroke. Stroke 31: 1250–1255.

Akopov SE, Simonian NA, Grigorian GS (1996) Dynamics of polymorphonuclear leukocyte accumulation in acute cerebral infarction and their correlation with brain tissue damage. Stroke 10: 1739-1743.

Attali B, Wang N, Kolot A, Sobko A, Cherepanov V, Soliven B (1997) Characterization of delayed rectifier Kv channels in oligodendrocytes and progenitor cells. J Neurosci 17: 8234-8245

Amin AR, Attur MG., Thakker GD, Patel PD, Vyas PR, Patel RN, Patel IR & Abramson SB (1996) Proc. Natl. Acad. Sci. USA 93: 14014-14019

Anderson RM, Opeskin K (1998) Timing of early changes in brain trauma. Am J Forensic Med Pathol. 19:1–9

Arcangeli, A., L. Bianchi, A. Becchetti, L. Faravelli, M. Coronnello, E. Mini, M. Olivotto, and E. Wanke (1995) A novel inward-rectifying K+ current with a cell-cycle dependence governs the resting potential of mammalian neuroblastoma cells. J Physiol. (Camb) 489: 455-471

Aronson AL (1980) Pharmacotherapeutics of the newer tetracyclines J Am Vet Med Assoc. 10 :1061-1068.

Arvin B, Neville LF, Barone FC, Feuerstein GZ (1996) The role of inflammation and cytokines in brain injury. Neurosci Biobehav Rev 20: 445–452

Banati RB, Gehrmann J, Schubert P, Kreutzberg GW (1993) Cytotoxicity of microglia. Glia. 1: 111-118

Baron JC (1999) Mapping the ischemic penumbra with PET: implications for acute stroke treatment. Cerebrovasc Dis 9: 193-201

Baron JC (2001) Perfusion thresholds in human cerebral ischemia: historical perspective and therapeutic implications. *Cerebrovasc Dis.* 11(suppl 1): 2–8

Barone FC, Feuerstein GZ (1999) Inflammatory mediators and stroke: new opportunities for novel therapeutics J Cereb Blood Flow Metab 8:819-34.

Barza M, Brown RB, Shanks C, Gamble C, Weinstein L (1975) Relation between lipophilicity and pharmacological behavior of minocycline, doxycycline, tetracycline, and oxytetracycline in dogs. Antimicrob Agents Chemother. 6: 713-20.

Bauer CK, Falk T, Schwarz JR (1996) An endogenous inactivating inward-rectifying potassium current in oocytes of Xenopus laevis. Pflugers Arch 5: 812-820

Bernik TR, Friedman SG, Ochani M, DiRaimo R, Ulloa L, Yang H, Sudan S, Czura CJ, Ivanova SM, Tracey KJ (2002) Pharmacological stimulation of the cholinergic anti-inflammatory pathway. J Exp Med 6: 781-8.

Bernik TR, Friedman SG, Ochani M, DiRaimo R, Susarla S, Czura CJ, Tracey KJ (2002) Cholinergic anti-inflammatory pathway inhibition of tumor necrosis factor during ischemia reperfusion. J Vasc Surg. 6: 1231-1236.

Bhat NR, Zhang P, Lee JC, Hogan EL (1998) Extracellular signal-regulated kinase and p38 subgroups of mitogen-activated protein kinases regulate inducible nitric oxide synthase and tumor necrosis factor-alpha gene expression in endotoxin-stimulated primary glial cultures. J Neurosci 5:1633-41.

Bianchi M, Bloom O, Raabe T, Cohen PS, Chesney J, Sherry B, Schmidtmayerova H, Calandra T, Zhang X, Bukrinsky M, Ulrich P, Cerami A, Tracey KJ (1996) Suppression of proinflammatory cytokines in monocytes by a tetravalent guanylhydrazone. J Exp Med. 3: 927-36.

Bianchi L, Wible B, Arcangeli A, Taglialatela M, Morra F, Castaldo P, Crociani O, Rosati B, Faravelli L, Olivotto M, Wanke E (1998) herg encodes a K+ current highly conserved in tumors of different histogenesis: a selective advantage for cancer cells? Cancer Res 4:815-822.

Birdsall HH (1991) Induction of ICAM-1 on human neural cells and mechanisms of neutrophil-mediated injury. Am J Pathol. 6:1341-50.

Blalock JE (2002) Harnessing a neural-immune circuit to control inflammation and shock. J Exp Med. 6: F25-8.

Borovikova LV (2002) Vagus nerve stimulation attenuates the systemic inflammatory response to endotoxin. Nature 405: 458-462

Borovikova LV, S. Ivanova, D. Nardi, M. Zhang, H. Yang, M. Ombrellino, and K.J. Tracey (2000) Role of vagus nerve signaling in CNI-1493-mediated suppression of acute inflammation. Auton. Neurosci. 85: 141–147.

Bowersox SS, Singh T, Luther RR (1997) Selective blockade of N-type voltage-sensitive calcium channels protects against brain injury after transient focal cerebral ischemia in rats. Brain Res2: 343-7.

Brismar T, Collins VP (1989) Potassium and sodium channels in human malignant glioma cells Brain Res. 1-2: 259-267

Brismar T, Collins VP (1989) Inward rectifying potassium channels in human malignant glioma cells Brain Res. 1-2: 249-58.

Brismar T, Collins VP (1989) Potassium channels in human glioma cells. Pflugers Arch. Suppl 1: S137-138.

Bruno V, G. Battaglia, A. Copani, M. D'Onofrio, P. Di Iorio, A. De Blasi, D. Melchiorri, P.J. Flor and F. Nocoli (2001) Metabotropic glutamate receptor subtypes as targets for neuroprotective drugs. J. Cereb. Blood flow Metab. 21: 1013–1033.

Bordey A, Sontheimer H (1998) Electrophysiological properties of human astrocytic tumor cells In situ: enigma of spiking glial cells J Neurophysiol. 5: 2782-93.

Broderick J, T. Brott, T. Tomsick, J. Tew, J. Duldner and G. Huster (1994) Management of intracerebral hemorrhage in a large metropolitan population. Neurosurgery 34: 882–887.

Brown RD Jr, Ransom J, Hass S, Petty GW, O'Fallon WM, Whisnant JP, Leibson CL (1999) Use of nursing home after stroke and dependence on stroke severity: a population-based analysis. Stroke 5: 924-929.

Brown GG, Kieran S, Patel S (1989) Memory functioning following a left medial thalamic hematoma J Clin Exp Neuropsychol. 2: 206-18.

Bullock R, Mendelow AD, Teasdale GM, Graham DI (1984) Intracranial hemorrhage induced at arterial pressure in the rat, I: description of technique, ICP changes and neuropathologic findings. Neurol Res. 6:184–188.

Caulfield MP, Birdsall NJ (1998) International Union of Pharmacology. XVII. Classification of muscarinic acetylcholine receptors. Pharmacol Rev. 2: 279-290

Chao CC, Anderson WR, Hu S, Gekker G, Martella A, Peterson PK (1993) Activated microglia inhibit multiplication of Toxoplasma gondii via a nitric oxide mechanism. Clin Immunol Immunopathol 2: 178-83.

Chen H, Chopp M, Bodzin G (1992) Neutropenia reduces the volume of cerebral infarct after transient middle cerebral artery occlusion in the rat. Neurosci Res Commun 11: 93-99.

Chen M, Ona VO, Li M, Ferrante RJ, Fink KB, Zhu S (2000) Minocycline inhibits caspase-1 and caspase-3 expression and delays mortality in a transgenic mouse model of Huntington disease. Nat Med 6: 797–801.

Chittajallu R, Chen Y, Wang H, Yuan X, Ghiani CA, Heckman T, McBain CJ, Gallo V (2002) Regulation of Kv1 subunit expression in oligodendrocyte progenitor cells and their role in G1/S phase progression of the cell cycle. Proc Natl Acad Sci. U S A. 4: 2350-2355.

Church MK, Pao GJ, Holgate ST (1982) Characterization of histamine secretion from mechanically dispersed human lung mast cells: effects of anti-IgE, calcium ionophore A23187, compound 48/80, and basic polypeptides. J Immunol. 5: 2116-2121

Coimbra C and Wieloch T (1992) Hypothermia ameliorates neuronal survival when induced 2 hr after ischaemia in the rat. Acta Physiol. Scand. 146: 543–544

Colbourne F, Sutherland GR, Auer RN (1999) Electron microscopic evidence against apoptosis as the mechanism of neuronal death in global ischemia. J Neurosci. 19: 4200–4210

Colbourne F, Corbett D (1995) Delayed postischemic hypothermia: a six month survival study using behavioral and histological assessments of neuroprotection J Neurosci 11: 7250-7260

Colton CA, M. Jia, M. X. Li, and D. L. Gilbert (1994) K+ modulation of microglial superoxide production: involvement of voltage-gated Ca2+ channels. Am J Physiol 266: C1650-C1655

Corbett D and Crooks P (1997) Ischemic preconditioning: a long term survival study using behavioural and histological endpoints. Brain Res 760: 129–136

Corbett D, Nurse S (1998) The problem of assessing effective neuroprotection in experimental cerebral ischemia. Prog Neurobiol 54: 531–548

Cregan EF, Peeling J, Corbett D, Buchan AM, Saunders J, Auer RN, Gao M, Mccarthy DJ, Eisman MS, Campbell TM, Murray RJ, Stagnitto ML, Palmer GC (1997) -(S)-Alpha-phenyl-2-pyridine-ethanamine Dihydrochloride-, a low affinity uncompetitive N-methyl-D-aspartic acid antagonist, is effective in rodent models of global and focal ischemia. J Pharmacol Exp Ther 3:1412-14124.

Cunha BA, Comer JB, Jonas M (1982) The tetracyclines. Med Clin North Am.1: 293-302

Del Bigio MR, Yan HJ, Buist R, Peeling J (1996) Experimental intracerebral hemorrhage in rats: magnetic resonance imaging and histopathological correlates. Stroke. 27: 2312–2320.

Del Bigio MR., HJ. Yan, T. M. Campbell and J. Peeling (1999) Effects of fucoidan on collagenase-induced intracerebral hemorrhage in rats. Neurol. Res. 21: 415–419

Davis MH, Meunier F, Marslen-Wilson WD (2004) Neural responses to morphological, syntactic, and semantic properties of single words: an fMRI study. Brain Lang 3: 439-449.

DeCoursey, T. E., and S. Grinstein (1998) Ion channels and carriers in leukocytes. In: Inflammation: Basic Principles and Clinical Correlates, edited by J. I. Gallin, R. Snyderman, D. T. Fearon, B. F. Haynes, and C. Nathan. New York: Raven.

Del Zoppo GJ (1991) Polymorphonuclear leukocytes occlude capillaries following middle cerebral artery occlusion and reperfusion in baboons. Stroke 22: 1276–1283.

Del Zoppo GJ, GW. Schmid-Schonbein, E. Mori, B. R. Copeland and C. M. Chang (1999) Polymorphonuclear leukocytes occlude capillaries following middle cerebral art ery occlusion and reperfusion in baboons. Stroke 22: 1276–1283.

Dirnagl U, Iadecola C, Moskowitz MA (1999) Pathobiology of ischaemic stroke: an integrated view. Trends Neurosci 22: 391-397

Domingo Z, Bradley JK, Blamire AM, Brindle K, Styles P, Rajagopolan B (2000)

Diffusion weighted imaging and magnetic resonance spectroscopy in a low flow ischaemia model due to endothelin induced vasospasm. NMR Biomed 13: 154-162

Drummond JC, Piyash PM, Kimbro JR (2000) Neuroprotection failure in stroke. Lancet. 356: 1032–1033.

Du Y, Ma Z, Lin S, Dodel RC, Gao F, Bales KR (2001) Minocycline prevents nigrostriatal dopaminergic neurodegeneration in the MPTP model of Parkinson's disease. Proc Natl Acad Sci USA 98:14669–14674

Dubois JM, Rouzair-Dubois B (1993) Role of potassium channels in mitogenesis. Prog Biophys Mol Biol 59:1-21

Dubois B, D'Hooghe MB, De Lepeleire K, Ketelaer P, Opdenakker G, Carton H (1998) Toxicity in a double-blind placebo-controlled pilot trial with D-penicillamine and metacycline in secondary progressive multiple sclerosis. Mult Scler 4: 74–78.

Dubois B, Masure S, Hurtenbach U, Paemen L, Heremans H, van den Oord J (1999) Resistance of young gelatinase B-deficient mice to experimental autoimmune encephalomyelitis and necrotizing tail lesions. J Clin Invest 104: 507–515.

Duncan PW, Jorgensen HS, Wade DT (2000) Outcome measures in acute stroke trials: a systematic review and some recommendations to improve practice. Stroke 31: 1429–

1438

Dyker AG, Lees KR (1998) Duration of neuroprotective treatment for ischemic stroke. Stroke. 29: 535–542.

Elkayam O, Yaron M, Caspi D (1999) Minocycline-induced autoimmune syndromes: an overview. Semin Arthritis Rheum 28: 392–397

Fagan SC, Bowes MP, Berri SA, Zivin JA (1999) combination treatment for acute ischemic stroke: a ray of hope? J stroke Cerebrovasc Dis 8: 359-67

Faravelli L, A. Arcangeli, M. Olivotto, and E. Wanke (1996) A HERG-like K+ channel in rat F11 DRG cell line: pharmacological identification and biophysical characterization. J. Physiol (Camb) 496: 13-23

Feuerstein GZ, T. Liu and F.C. Barone (1994) Cytokines, inflammation, and brain injury: role of tumor necrosis factor-alpha. Cerebrovasc. Brain Metab. Rev. 6: 341–360

Feuerstein GZ, Wang X, Barone FC (1997) Inflammatory gene expression in cerebral ischemia and trauma. Potential new therapeutic targets. Ann N Y Acad Sci. 825: 179-93.

Feuerstein GZ, Wang X, Barone FC (1998) Cytokines in brain ischemia--the role of TNF alpha. Cell Mol Neurobiol. 6: 695-701

Fiebich BL, Mueksch B, Boehringer M, Hull M (2000) Interleukin-1beta induces cyclooxygenase-2 and prostaglandin E(2) synthesis in human neuroblastoma cells: involvement of p38 mitogen-activated protein kinase and nuclear factor-kappaB. J Neurochem. 5: 2020-2028.

Fisher M (1997) Characterizing the target of acute stroke therapy. Stroke. 28: 866–872.

Fisher M, Schaebitz W (2000) An overview of acute stroke therapy: past, present, and future. Arch Intern Med. 160: 3196–3206

Fisher M (1999) Neuroprotection of acute ischemic stroke: where are we? Neuroscientist. 5: 392–401.

Fogelholm R, Nuutila M, Vuorela AL (1992) Primary intracerebral haemorrhage in the Jyvaskyla region, central Finland, 1985-89: incidence, case fatality rate, and functional outcome. J Neurol Neurosurg Psychiatry 7: 546-552.

Furlan A, Higashida R, Wechsler L, Gent M, Rowley H, Kase C, Pessin M, Ahuja A, Callahan F, Clark WM, Silver F, Rivera F (1999) Intra-arterial prourokinase for acute ischemic stroke: the PROACT II study: a randomized controlled trial: Prolyse in Acute Cerebral Thromboembolism. JAMA 282: 2003–2011.

Gabler WL, Creamer HR (1991) Suppression of human neutrophil functions by tetracyclines. J Periodontal Res 1: 52-8.

Garcia JH, Ho KL, Caccamo DV. Intracerebral hemorrhage: pathology of selected topics. In: Kase CS, Caplan LR, eds. Intracerebral Hemorrhage. Boston, Mass: Butterworth-Heinemann: 45–72.

Garcia JH., K. F. Liu and J. K. Relton (1995) Interleukin-1 receptor antagonist decreases the number of necrotic neurons in rats with middle cerebral artery occlusion. Am J Pathol 147: 1477–1486

Garcia ML, Hanner M, Knaus H-G, Koch R, Schmalhofer W, Slaughter RS, Kaczorowski GJ (1997) Pharmacology of potassium channels. Adv Pharmacol 39: 425-471

Giroud M, P. Gras, N. Chadan, P. Beuriat, C. Milan, P. Arveux and R. Dumas (1991) Cerebral haemorrhage in a French prospective population study. J. Neurol. Neurosurg. Psychiat. 54: 595–598

Giulian D, and C. Robertson (1990) Inhibition of mononuclear phagocytes reduces ischemic injury in the spinal cord. Ann. Neurol. 27: 33–42.

Giulian D (1993) Reactive glia as rivals in regulating neuronal survival. Glia 7: 102–110.

Giulian D, K. Vaca and M. Corpuz (1993) Brain glia release factors with opposing actions upon neuronal survival. J. Neurosci. 13: 29–37.

Giulian D and K. Vaca (1993) Inflammatory glia mediate delayed neuronal damage after ischemia in the central nervous system. Stroke 24: (Suppl I) 184–194.

Giulian D, K. Vaca and M. Corpuz (1993) Brain glia release factors with opposing actions upon neuronal survival. J. Neurosci. 13: 29–37.

Ghirnikar RS, Lee YL, Eng LF (1998) Inflammation in traumatic brain injury: role of cytokines and chemokines. Neurochem Res. 23: 329–340

Gladstone DJ, Danells CJ, Black SE (2002) The Fugl-Meyer Assessment of motor recovery after stroke: a critical review of its measurement properties. Neurorehabil Neural Repair.

Golub LM, Ramamurthy NS, McNamara TF, Greenwald RA, Rifkin BR (1991) Tetracyclines inhibit connective tissue breakdown: new therapeutic implications for an old family of drugs. Crit Rev Oral Biol Med. 3: 297-321

Grabowski M, Brundin P, Johansson BB (1993) Paw-reaching, sensorimotor, and rotational behavior after brain infarction in rats. Stroke 6: 889-95.

Grotta JC (1999) Acute stroke therapy at the millennium: consummating the marriage between the laboratory and bedside: the Feinberg lecture. Stroke. 30: 1722–1728.

J.M. Hallenbeck (1996) Significance of the inflammatory response in brain ischemia. Acta Neurochirurgica. 66 (Suppl): 27–31

Hakim AM (1998) Ischemic penumbra: the therapeutic window. Neurology. 51: S44–S46

Han JL (2003) Cholinergic suppression of cytokine release from human macrophages. Shock 19 (S1): 22

Hara H, Sukamoto T, Kogure (1993) Mechanism and pathogenesis of ischemia-induced neuronal damage. Prog Neurobiol. 6: 645-70

Hara H, Huang PL, Panahian N, Fishman MC, Moskowitz MA (1996) Reduced brain edema and infarction volume in mice lacking the neuronal isoform of nitric oxide synthase after transient MCA occlusion. J Cereb Blood Flow Metab 16:605-611

Hara H, Harada K, Sukamoto T (1993) Chronological atrophy after transient middle cerebral artery occlusion in rats. Brain Res. 2: 251-260.

He Y, Appel S, Le W (2001) Minocycline inhibits microglial activation and protects nigral cells after 6-hydroxydopamine injection into mouse striatum. Brain Res. 1-2: 187-193.

Heinel LA, Rubin S, Rosenwasser RH, Vasthare US, Tuma RF (1994) Leukocyte involvement in cerebral infarct generation after ischemia and reperfusion. Brain Res Bull. 2: 137-41.

Holmin S, Biberfeld P, Mathiesen T (1998) Intracerebral inflammation after human brain contusion. Neurosurgery. 42: 291–299

Hu Q and YL Shi. (1997) Characterization of an inward-rectifying potassium current in NG10815 neuroblastoma×glioma cells. Pflügers Arch 433: 617-625

Hunter AJ, Mackay KB, Rogers DC (1998) To what extent have functional studies of ischaemia in animals been useful in the assessment of potential neuroprotective agents? Trends Pharmacol Sci. 19: 59–66

Hunter AJ, Green AR and Cross AJ (1995) Animal models of acute ischemic stroke: can they predict clinically successful neuroprotective drugs?. Trends Pharmacol. Sci. 16: 123–128

Hiemke C (1996) Expression of alpha subunit genes of nicotinic acetylcholine receptors in human lymphocytes. Neurosci Letters 214: 171-174

Iadecola C, Ross ME (1997) Molecular pathology of cerebral ischemia: delayed gene

expression and strategies for neuroprotection. Ann N Y Acad Sci. 835: 203-217.

Jander S, Kraemer M, Schroeter M, Witte OW, Stoll G (1995) Lymphocytic infiltration and expression of intercellular adhesion molecule-1 in photochemically induced ischemia of the rat cortex.J Cereb Blood Flow Metab. 1: 42-51

Jean WC, Spellman SR, Nussbaum ES, Low WC (1998) Reperfusion injury after focal cerebral ischemia: the role of inflammation and the therapeutic horizon. Neurosurgery. 43: 1382–1396.

Jones TA, Schaller T (1994) Use-dependent growth of pyramidal neurons after neocortical damage. J Neurosci. 14: 2140–2152

Jonas S, Sugimori M, Llinas R (1997) Is low molecular weight heparin a neuroprotectant? Ann N Y Acad Sci. 825: 389-93.

Jonas S, Tran AQ, Eisenberg E, Azam M, Viera D, Grumet S (1997) Does effect of a neuroprotective agent on volume of experimental animal cerebral infarct predict effect of the agent on clinical outcome in human stroke? Ann N Y Acad Sci. 825: 281-7

Jou I, Pyo H, Chung S, Jung SY, Gwag BJ, Joe EH (1998) Expression of Kv1.5 K+ channels in activated microglia in vivo. Glia 24:408-414

Kane PJ, P. Modha, R. D. Strachan, S. Cook, I. R. Chambers, C. B. Clayton and A. D. Mendelow (1992) The effect of immunosuppression on the development of cerebral oedema in an experimental model of intracerebral haemorrhage: whole body and regional irradiation. J Neurol Neurosurg Psych 55: 781–786.

Kawamata T, Alexis NE, Dietrich WD, Finklestein SP (1996) Intracisternal basic fibroblast growth factor (bFGF) enhances behavioral recovery following focal cerebral infarction in the rat. J Cereb Blood Flow Metab 16: 542–547

Kawamata T, Ren J, Chan TCK, Charette M, Finklestein SP (1998) Intracisternal osteogenic protein-1 enhances functional recovery following focal stroke. Neuroreport 9: 1441–1445.

Kawashima K, Fuji T (2000) Extraneural cholinergic system in lymphocytes. Pharmacol Ther 86: 29-48

Khanna R, roy L, Zhu XP, Schilchter LC (2001) Am J physiol cell Physiol 280: C796-C806

Khanna R, Chang MC, Joiner WJ, Kaczmarek LK, Schlichter LC (1999) hSK4/hIK1, a calmodulin-binding KCa channel in human T lymphocytes: roles in proliferation and volume regulation. J Biol Chem 274:14838-14849

Kidwell CS, Liebeskind DS, Starkman S, Saver JL. Trends in acute ischemic stroke trials through the 20th century. Stroke. 2001; 32: 1349–1359.

Klein NC, Cunha BA (1995) Tetracyclines. Med Clin North Am. 4: 789-801

Kieseier BC, Seifert T, Giovannoni G, Hartung HP (1999) Matrix metalloproteinases in inflammatory demyelination: targets for treatment. Neurology 53: 20–25.

Kloppenburg M, Dijkmans BA, Breedveld FC (1995) Antimicrobial therapy for rheumatoid arthritis. Baillieres Clin Rheumatol 9: 759–769.

Kloppenburg M, Dijkmans BA, Verweij CL, Breedveld FC (1996) Inflammatory and immunological parameters of disease activity in rheumatoid arthritis patients treated with minocycline. Immunopharmacology 31: 163–169.

Koj A (1997) Initiation of acute phase response and synthesis of cytokines. Biochim Biophys Acta 1317: 84-94

Koeppen AH, Dickson AC, McEvoy JA (1995) The cellular reactions to experimental intracerebral hemorrhage. J Neurol Sci. 134:102–112

Kotecha SA, Schlichter LC (1999) A Kv1.5 to Kv1.3 switch in endogenous hippocampal microglia and a role in proliferation. J Neurosci. 24: 10680-10693.

Kotecha SA, Schlichter LC (1998) Early expression of a novel K+ current in rat microglia. Soc Neurosci Abstr 24:830

Kramer PA, Chapron DJ, Benson J, Mercik SA (1978) Tetracycline absorption in elderly patients with achlorhydria. Clin Pharmacol Ther 4: 467-72.

Krause KH, Welsh MJ (1990) Voltage-dependent and Ca2 (+)-activated ion channels in human neutrophils. Clin Invest 2: 491-98

.

Krause KH, Demaurex N, Jaconi M, Lew DP (1993) Ion channels and receptor-mediated Ca2+ influx in neutrophil granulocytes 1: 165-73

Labrakakis C, Patt S, Weydt P, Cervos-Navarro, Meyer R, Kettenmann H (!997) Action potential- generating cells in human glioblastomas, J. Neurophysiol. Exp. Neurol. 56: 243-254.

Lassmann H, Zimprich F, Rossler K, Vass K (1991) Inflammation in the nervous system. Basic mechanisms and immunological concepts. Rev Neurol (Paris)12: 763-81.

Lassmann H (1991) Inflammation and the blood-brain barrier Brain Pathol 2: 88.

Lassmann H, Rossler K, Zimprich F, Vass K (1991) Expression of adhesion molecules and histocompatibility antigens at the blood-brain barrier. Brain Pathol. 2: 115-123.

Lee MA, Palace J, Stabler G, Ford J, Gearing A, Miller K (1999) Serum gelatinase B, TIMP-1 and TIMP-2 levels in multiple sclerosis: a longitudinal clinical and MRI study. Brain 122: 191-197

Lee KR, Colon GP, Betz AL, Keep RF, Kim S, Hoff JT (1996) Edema from intracerebral hemorrhage: the role of thrombin. J Neurosurg 84: 91–96

Lees KR (2001) Advances in neuroprotection trials. Eur Neurol 45: 6–10.

Leonard S and Bertrand D (2001) Neuronal nicotinic receptors: from structure to function. Nicotine Tob. Res. 3: 203-223

Lin S, Zhang Y, Dodel R, Farlow MR, Paul SM, Du Y (2001) Minocycline blocks nitric oxide-induced neurotoxicity by inhibition p38 MAP kinase in rat cerebellar granule neurons.Neurosci Lett.1-2: 61-4

Lin TN, He YY, Wu G, Khan M, Hsu CY (1993) Effect of brain edema on infarct volume in a focal cerebral ischemia model in rats. Stroke 24:117-121

Lindstrom J (1997) Nicotinic acetylcholine receptors in health and disease. Mol Neurobiol. 2: 193-222

Lindstrom JM (1995) Hand Book of Receptors and Channels: Ligand- and Voltage-Gated Ion Channels (ed. North, A.) 153-175 (CRC Press, Boca Raton, Florida).

Lindsberg PJ, Roine RO, Tatlisumak T, Sairanen T, Kaste M (2000) The future of stroke treatment. Neurol Clin. 2: 495-510.

Liu J, Marino MW, Wong G, Grail D, Dunn A, Bettadapura J (1998) TNF is a potent anti-inflammatory cytokine in autoimmune-mediated demyelination. Nat Med 4: 78–83.

Lo YK, CH Yiu, H. H. Hu, M. S. Su and S. C. Laeuchli (1994) Frequency and characteristics of early seizures in Chinese acute stroke. Acta Neurol Scand 90: 83–85.

Lyden PD and Zivin JA (1993) Hemorrhagic transformation after cerebral ischemia: Mechanisms and incidence. Cerebrovasc. Brain Metab Rev 5: 1–16

Mackenzie JM, Clayton JA (1999) Early cellular events in the penumbra of human spontaneous intracerebral hemorrhage. J Stroke Cerebrovasc Dis 8:1–8

Marler JR, Walker MD (1998) Progress in acute stroke research. Stroke 8: 1491-2.

Markgraf CG, Green EJ, Hurwitz BE, Morikawa E, Dietrich WD, McCabe PM, Ginsberg MD, Schneiderman N (1992) Sensorimotor and cognitive consequences of middle

cerebral artery occlusion in rats Brain Res.2: 238-46

Matsubara T, Pararajasegaram G, Wu GS, Rao NA (1999) Retinal microglia

differentially express phenotypic markers of antigen-presenting cells in vitro. Invest Ophthalmol Vis Sci. 13: 3186-93.

Matsuo Y, Onodera H, Shiga Y, Shozuhara H, Ninomiya M, Kihara T, Tamatani T, Miyasaka M, and Kogure K (1994) Role of cell adhesion molecules in brain injury after transient middle cerebral artery occlusion in the rat. Brain Res 656: 344–352.

Matsuo Y, Onodera H, Shiga Y, Nakamura M, Ninomiya M, Kihara T and Kogure K (1994) Correlation between myeloperoxidase-quantified neutrophil accumulation and ischemic brain injury in the rat: Effects of neutrophil depletion. Stroke 25: 1469–1475.

Mathew P, Graham DI, Bullock R, Maxwell W, McCulloch J, Teasdale G (1994) Focal brain injury: histological evidence of delayed inflammatory response in a new rodent model of focal cortical injury. Acta Neurochir Suppl. 60: 428–430

McGeer PL, McGeer EG (1995) The inflammatory response system of brain: implications for therapy of Alzheimer and other neurodegenerative diseases. Brain Res Brain Res Rev. 2: 195-218

Meistrell M.E, III, G.I. Botchkina, H. Wang, E. DeSanto, KM. Cockroft, O. Bloom, J.M. Vishnubhakat, P. Ghezzi, and KJ. Tracey (1997) TNF is a brain-damaging cytokine in cerebral ischemia. Shock 8: 341–348

Mita Y, Dobashi K, Suzuki K, Mori M, Nakazawa T (1996) Induction of muscarinic receptor subtypes in monocyte/macrophagic cells differentiated from EoL-1 cells. Eur J Pharmacol. 297: 121-127

Morse JK, Davis JN (1990) Regulation of ischemic hippocampal damage in the gerbil: adrenalectomy alters the rate of CA1 cell disappearance Exp Neurol 1: 86-92

Mucke L, Eddleston M (1993) Astrocytes in infectious and immune-mediated diseases of the central nervous system FASEB J.13: 1226-32.

Munoz-Fernandez MA, Fresno M (1998) The role of tumour necrosis factor, interleukin 6, interferon-gamma and inducible nitric oxide synthase in the development and pathology of the nervous system. Prog Neurobiol. 56: 307–340

National Institute of Neurological Disorders and Stroke rt-PA Stroke Study Group. Tissue plasminogen activator for acute ischemic stroke. N Engl J Med. 1995; 333: 1581–1587

Nagayama M, Zhang F & Iadecola C (1998) J Cereb Blood Flow Metab. 18: 1107-1113

T. Narahashi, C.S. Huang, J.H. Song and J.Z. Yeh (1997) Ion channels as targets for neuroprotective agents. Ann. N.Y. Acad. Sci. 825: 380–388.

Nilius B, Sehrer J, Droogmans G (1994) Permeation properties and modulation of volume-activated Cl(-)-currents in human endothelial cells. Br J Pharmacol 4: 1049-56.

Nilius B, Oike M, Zahradnik I, Droogmans G (1994) Activation of a Cl- current by hypotonic volume increase in human endothelial cells. J Gen Physiol 5: 787-805.

Nogawa S, Zhang F, Ross ME, Iadecola C (1997) Cyclo-oxygenase-2 gene expression in neurons contributes to ischemic brain damage. J Neurosci. 8: 2746-55.

Nurse S and Corbett D (1994) Direct measurement of brain temperature during and after intraischemic hypothermia: correlation with behavioral, physiological and histological endpoints. J. Neurosci. 14: 7726–7734

O'Dell JR (1999) Is there a role for antibiotics in the treatment of patients with rheumatoid arthritis?. Drugs 57: 279–282.

Pancrazio JJ, Ma W, Grant GM, Shaffer KM, Kao WY, Liu QY, Manos P, Barker JL, Stenger DA (1999) A role for inwardly rectifying K+ channels in differentiation of NG108-15 neuroblastoma x glioma cells. J Neurobiol. 4: 466-74

T.R. Patel, G.P. Schielke, J.T. Hoff, R.F. Keep and A.L. Betz (1999) Comparison of cerebral blood flow and injury following intracerebral and subdural hematoma in the rat. Brain Res. 829: 125–133.

Perry VH, Gordon S (1997) Microglia and macrophages. In: Immunology of the nervous system (Keane RW, Hickey WF, eds), p 824. New York: Oxford UP.

Pyo H, Chung S, Jou I, Gwag B, Joe EH (1997) Expression and function of outward K+ channels induced by lipopolysaccharide in microglia. Mol Cells 7:610-614

Quershi AI, Suri AI, Ling MF, Khan GS, Guterman J, Hopkins LN (2001) Absence of early proinflammatory cytokine expression in experimental intracerebral hemorrhage. Neurosurgery, 49: 416-420

Rifkin BR, Vernillo AT, Golub LM. & Ramamurthy NS (1994) Ann. N.Y. Acad. Sci. 732: 165-180

Rosenberg GA, S. Mun-Bryce, M. Wesley and M. Kornfeld (1990) Collagenase-induced intracerebral hemorrhage in rats. Stroke 21: 801–807

Sanguinetti MC, C. Jiang, M.E. Curran, and M.T. Keating (1995) A mechanistic link between an inherited and an acquired cardiac arrhythmia: HERG encodes the IKr

potassium channel. Cell 81: 299-307

Sato KZ, Fujii T, Watanabe Y, Yamada S, Ando T, Kazuko F, Kawashima K (1999) Diversity of mRNA expression for muscarinic acetylcholine receptor subtypes and neuronal nicotinic acetylcholine receptor subunits in human mononuclear leukocytes and leukemic cell lines. Neurosci Lett 1: 17-20

Saunders DE, Howe FA, van den Boogaart A, McLean MA, Griffiths JR, Brown MM (1995) Continuing ischemic damage after acute middle cerebral artery infarction in humans demonstrated by short-echo proton spectroscopy. Stroke 26: 1007–1013

Schafer R, Wulfsen I, Behrens S, Weinsberg F, Bauer CK, Schwarz JR (1999) The erg-like potassium current in rat lactotrophs. J Physiol. 518 (Pt 2): 401-416

Schlichter LC, Sakellaropoulos G, Ballyk B, Pennefather PS, Phipps DJ (1996) Properties of K+ and Cl channels and their involvement in proliferation of rat microglial cells. Glia 17:225-236

Schwarz S, Hafner K, Aschoff A, Schwab S (2000) Incidence and prognostic significance of fever following intracerebral hemorrhage. Neurology 54: 354–361.

Seukeran DC, Eady EA, Cunliffe WJ (1997) Benefit-risk assessment of acne therapies. Lancet 9060: 1251-1252.

Sherman DG, Atkinson RP, Chippendale T, Levin KA, Ng K, Futrell N, Hsu CY, Levy DE (2000) Intravenous ancrod for treatment of acute ischemic stroke: the STAT study: a randomized controlled trial: Stroke Treatment With Ancrod Trial. JAMA. 283: 2395–2403

Shapiro LE, SR Knowles and NH Shear (1997) Comparative safety of tetracycline, minocycline, and doxycycline. Arch Dermatol 133 : 1224–1230.

Shiga Y, Onodera H, Kogure K, Yamasaki Y, Yashima Y, Syozuhara H, Sendo F (1991) Neutrophil as a mediator of ischemic edema formation in the brain Neurosci Lett 2: 110-112.

Shytle RD, Mori T, Townsend K, Vendrame M, Sun N, Zeng J, Ehrhart J, Silver AA, Sanberg PR, Tan J (2004) Cholinergic modulation of microglial activation by alpha 7 nicotinic receptors. J Neurochem 2: 337-343

Siesjo BK, Siesjo P (1996) Mechanisms of secondary brain injury. Eur J Anaesthesiol 3: 247-68

Slikker W, Saran J, Auer RN, Palmer GC, Narahashi T, Youdim MBH, Maynard KI, Carbone KM, Trembly B (2001) Neuroprotection: past successes and future challenges. Ann N Y Acad Sci. 939: 465–477

Sloan MA, Gore JM (1992) Ischemic stroke and intracranial hemorrhage following thrombolytic therapy for acute myocardial infarction: a risk-benefit analysis Am J Cardiol. 2: 21A-38A

Small DL, Buchan AM (2000) Animal models. Br Med Bull. 56: 307–317.

Soares HD, Hicks RR, Smith D, McIntosh TK (1995) Inflammatory leukocytic recruitment and diffuse neuronal degeneration are separate pathological processes resulting from traumatic brain injury. J Neurosci.15: 8223–8233

Spector PS, Curran ME, Keating MT, Sanguinetti MC (1996) Class III antiarrhythmic drugs block HERG, a human cardiac delayed rectifier K+ channel. Open-channel block by methanesulfonanilides. Circ Res 3:499-503.

Spector PS, M.E. Curran, A. Zou, M.T. Keating, and M.C. Sanguinetti (1996) Fast inactivation causes rectification of the IKr channel. J. Gen. Physiol 107: 611-619

Spranger M, Kiprianova I, Krempien S, Schwab S (1998) Reoxygenation increases the release of reactive oxygen intermediates in murine microglia. J Cereb Blood Flow Metab. 6: 670-4.

Sato KZ (1999) Diversity of mRNA expression for muscarinic acetylcholine receptor

subtypes and neuronal nicotinic acetylcholine receptor subunits in human mononuclear leukocytes and leukemic cell lines. Neurosci Lett 266: 17-20

Steinlein O (1998) New functions for nicotine acetylcholine receptors ? Behav brain Res 95: 31-35

Streit WJ, Graeber MB, Kreutzberg GW (1988) Functional plasticity of microglia: a review. Glia 5: 301-7.

Sturkenboom MC, Meier CR, Jick H, Stricker BH (1999) Minocycline and lupuslike syndrome in acne patients Arch Intern Med. 5: 493-497

Stys PK (1998) Anoxic and ischemic injury of myelinated axons in CNS white matter: from mechanistic concepts to therapeutics. J Cereb Blood Flow Metab 18: 2–25

Tayebati SK, El-Assouad D, Ricci A, Amenta F (2002) Immunochemical and immunocytochemical characterization of cholinergic markers in human peripheral blood lymphocytes. J Neuroimmunol 132: 147-155

Thomas D, Karle CA, Kiehn J (2004) Modulation of HERG potassium channel function by drug action. Ann Med. 36 (Suppl 1): 41-6.

Tikka TM, Koistinaho JE (2001) Monocycline provides neuroprotection against N-methyl-D-asparate neurotoxicity by inhibiting microglia. J Immunol 166: 7521–7533.

Tikka T, Fiebich BL, Goldsteins G, Keinanen R, Koistinaho J (2001) Minocycline a tetracycline derivative, is neuroprotective against excitotoxicity by inhibiting activation and proliferation of microglia. J Neurosci 21: 2580–88

Tracey K J (2002) The inflammatory reflex. Nature 420: 853-859

Tracey KJ (1998) Suppression of TNF and other proinflammatory cytokines by the tetravalent guanylhydrazone CNI-1493. Prog Clin Biol Res 397: 335–343.

Tracey KJ, Czura CJ, Ivanova S (2001) Mind over immunity FASEB J. 159: 1575-6.

Toyabe S, Iiai T, Fukuda M, Kawamura T, Suzuki S, Uchiyama M, Abo T (1997) Identification of nicotinic acetylcholine receptors on lymphocytes in the periphery as well as thymus in mice. Immunology. 2: 201-5.

Trudeau MC, Warmke JW, Ganetzky B and Robertson GA (1995) HERG, a human inward rectifier in the voltage-gated potassium channel family. Science 269: 92-95

Tseng GN (2001) I(Kr): the hERG channel J Mol Cell Cardiol. 5: 835-49.

Valtysson J, Hillered L, Andine P, Hagberg H. and Persson L (1994) Neuropathological end points in experimental stroke pharmacotherapy: the importance of both early and late

evaluation. Acta Neurochir. 129: 58–63

Walch L, Brink C, Norel X (2001) The muscarinic receptor subtypes in human blood vessels. Therapie 56: 223-226

Wagner KR, Xi GH, Hua Y, Kleinholz M, de Courten-Myers GM, Myers RE, Broderick JP, Brott TG (1996) Lobar intracerebral hemorrhage model in pigs: rapid edema development in perihematomal white matter. Stroke 27: 490–497

Wang H, M. Zhang, M. Bianchi, B. Sherry, A. Sama, and K.J. Tracey (1988) Fetuin (-2-HS-Glycoprotein) opsonizes cationic macrophage-deactivating molecules. Proc. Natl. Acad. Sci. USA. 95:14429–14434

Wang H, Yu M, Ochani M, Amella CA, Tanovic M, Susarla S, Li JH, Wang H, Yang H, Ulloa L, Al-Abed Y, Czura CJ, Tracey KJ (2002) Nicotinic acetylcholine receptor alpha7 subunit is an essential regulator of inflammation. Nature. 6921: 384-388.

Warmke JW, and B. Ganetzky (1994) A family of potassium channel genes related to eag in Drosophila and mammals. Proc. Natl. Acad. Sci. USA 91: 3438-3442

Wolf PA, Cobb, J. L. & D'Agostino RB (1997) in Stroke, Pathophysiology, Diagnosis and Management, eds. Barnett, H. J. M., Mohr, J. P., Stein, B. M. & Yatsu, F. M. (Churchill Livingstone, New York), pp. 3-27.

Wonderlin WF, Strobl JS (1996) Potassium channels, proliferation and G1 progression. J Membr Biol. 2: 91-107

Wu B, Iwakiri R, Tsunada S, Utsumi H, Kojima M, Fujise T, Ootani A, Fujimoto K (2002) iNOS enhances rat intestinal apoptosis after ischemia-reperfusion. Free Radic Biol 5: 649-58

Wymore RS, Gintant GA, Wymore RT, Dixon JE, McKinnon D and Cohen IS (1997) tissue and species distribution of mRNA for the Ikr-like K+ channel, erg. Circ Res 80: 261-268

Xue M, Del Bigio MR (2000) Intracerebral injection of autologous whole blood in rats: time course of inflammation and cell death. Neurosci Lett 283: 230–232

Yrjanheikki J, Keinanen R, Pellikka M, Hokfelt T, Koistinaho JA (1998) Tetracyclines inhibit microglial activation and are neuroprotective in global brain ischemia. Proc Natl Acad Sci USA 95: 15769–15774.

Yrjanheikki J, Tikka T, Keinanen R, Goldsteins G, Chan PH, Koistinaho JA (1999) Tetracycline derivative, minocycline, reduces inflammation and protects against focal cerebral ischemia with a wide therapeutic window. Proc Natl Acad Sci USA 96: 13496–13500.

Yrjanheikki J, Koistinaho J, Copin JC, de Crespigny A, Moseley ME, Chan PH (2000) Spreading depression-induced expression of c-fos and cyclooxygenase-2 in transgenic mice that overexpress human copper/zinc-superoxide dismutase. J Neurotrauma 8:713-8.

Zivin JA (1999) Thrombolytic stroke therapy: past, present, and future. Neurology. 1: 14-19.

Zhang RL, M Chopp, N Jiang, WX. Tang, J Prostak, AM. Manning and DC. Anderson (1995) Anti-intercellular adhesion molecule-1 antibody reduces ischemic cell damage after transient but not permanent middle cerebral artery occlusion in the Wistar rat. Stroke 8: 1438-1442

Zhang RL, M. Chopp, Y. Li, C. Zaloga, N. Jiang, M. L. Jones, M. Miyasaka and P. A. Ward (1994) Anti-ICAM-1 antibody reduces ischemic cell damage after transient middle cerebral artery occlusion in the rat. Neurology 44: 1747–1751.

Zhou W, Cayabyab FS, Pennefather PS, Schlichter LC, DeCoursey TE (1998b) HERG-like K+ channels in microglia. J Gen Physiol 111:781-794