Development of a Model of Recurrent Stroke Consisting of a Mild Transient Stroke followed by a Second Moderate Stroke in Rats

M. Qiao[1], Z. Zhao[2], P.A. Barber[2], T. Foniok[1], S. Sun[3], and U. I. Tuor[1,2,3].

[1]MR Technology, Institute for Biodiagnostics (West), National Research Council Canada, Calgary, Alberta, Canada,

[2]Department of Clinical Neurosciences, University of Calgary, Calgary, Alberta, Canada

[3]Experimental Imaging Centre and Hotchkiss Brain Institute, Faculty of Medicine,

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Correspondence to: **Ursula I. Tuor, PhD**
Institute for Biodiagnostics (West)
National Research Council of Canada
B153, 3330 Hospital Dr. NW
Calgary, Alberta
T2N 4N1
Tel: 1-403-2213227
Fax: 1-403-2213230
E-mail: **Ursula.Tuor@nrc-cnrc.gc.ca**

Abstract

Recurrent stroke often consists of a transient ischemic attack or mild stroke followed by a moderate stroke. Lacking is knowledge of the mechanisms of interaction of such multiple ischemic insults. Our aim was to develop a rat model of recurrent stroke and to test whether such multiple insults would enhance brain injury. A mild focal ischemic insult was produced by transient (40 min) occlusion of the middle cerebral artery (MCAO) and this resulted in scattered necrosis and areas of increased labeling of astrocytes with glial fibrillary acidic protein. Additional animals were subjected to a moderate stroke alone or a recurrent stroke - a mild stroke followed three days later by a moderate stroke (60 min MCAO). Damage was dependent on the proximal or distal cerebral cortical location from the occlusion ($P<0.007$) and the type of stroke insult (mild, moderate or recurrent, $P<0.002$). Following recurrent stroke, the cumulative injury score was similar to a mild stroke in distal parietal cortex but enhanced proximally. Recurrent stroke also resulted in changes in magnetic resonance imaging T2, in neuronal microtubule associated protein2, in reactive astrocytes and in microglia/macrophages that were enhanced in proximal but not distal parietal cortex. This model demonstrates that when a minor stroke is combined with a second stroke, both within the same middle cerebral artery territory, there are different injury processes regionally. Proximally, damage exceeds that of the first insult whereas distally the response is consistent with a tolerance to the second insult.

Key Words - *Transient Ischemic Attack; Recurrent Stroke; Rat; Focal Cerebral Ischemia; Magnetic Resonance Imaging, Cell Necrosis, Preconditioning*

Introduction

A transient ischemic attack (TIA) is usually characterized by a sudden, focal neurological deficit that lasts for less than 24 hours (Albers et al., 2002). Initially, it was assumed that a TIA was associated with a complete recovery of symptoms and tissue disruption. However, with the increased use of non-invasive imaging techniques such as CT and MRI, it has become evident that TIA's can be accompanied by permanent brain injury indicative of a minor stroke. Between 30 to 50% of TIA patients have lesions on diffusion-weighted MRI within 6 to 12 hrs of symptom onset (Ay et al., 2002; Hill et al., 2004;Winbeck et al., 2004;Coutts et al., 2005). It is also possible for MR imaging changes to normalize following transient cerebral ischemia despite permanent cell death and brain injury as detected histologically (Li et al., 2000; Sicard et al., 2006).

In addition to the increased understanding that TIA or minor stroke is often associated with a degree of permanent brain injury, there is increasing evidence that such insults are associated with increased risk of stroke recurrence (Hill et al., 2004;Coutts et al., 2005; Rothwell et al., 2006; Nguyen-Huynh and Johnston, 2007; Fagan, 2008). A second stroke has been reported to occur within 90 days in 10-20% of patients who have had an initial TIA and in half of these patients the recurrent stroke occurs in the first 2 days after the TIA (Hill et al., 2004; Johnston and Hill, 2004; Coutts et al., 2005; Correia et al., 2006; Nguyen-Huynh and Johnston, 2007). Factors associated with an increased risk of stroke after a TIA include elderly age, diabetes mellitus, symptoms for more than 10 minutes, and weakness and impaired speech (Nguyen-Huynh and Johnston, 2007; Della et al., 2008).

Whether the ischemic episode associated with an initial TIA or minor stroke affect the second recurrent stroke is not clear, in part because there are few animal models of TIA or recurrent stroke. One approach has been to use an initial photothrombotic stroke, produced by a 10 minute irradiation of the common carotid artery, followed by either a second thromboembolism or global ischemia (Dietrich et al., 1999; Danton et al., 2002;Urrea, et al., 2004). However, the intial stroke in these studies is large compared to strokes seen in patients with TIA or minor stroke. Milder initial insults have been studied in animals but in the context of a preconditioning-induced tolerance that can be initiated by a relatively mild hypoxia or ischemia against a subsequent lethal ischemic insult (Gidday, 2006; Steiger and Hanggi, 2007;O'Duffy et al., 2007). Clinically, evidence is sparse supporting that a TIA

prior to a second ischemic insult provides a preconditioning neuroprotection. There are a few reports that patients with a history of a TIA or minor stroke prior to a more severe ischemic stroke have milder clinical deficits and/or smaller infarct size compared to those without a history of TIA (Moncayo et al., 2000;Wegener, Gottschalk et al., 2004; Zsuga et al., 2008). However, confounding factors such as small sample size, high rate of recanalization and lower incidence of a cardioembolic infarct etiology in TIA patients may affect the interpretation of neuroprotective effects observed in patients with a TIA history (Wegener et al., 2004). Indeed, there are reports that TIA-induced neuroprotection does not occur in human brain (Johnston, 2004; Della et al., 2008).

Presently, we hypothesized that a TIA or mild stroke sufficiently severe to cause scattered cell death would exacerbate the effect of a subsequent moderate ischemic stroke. In order to test this we developed a rat model of recurrent stroke. In this model, a relatively short middle cerebral artery occlusion (MCAO) producing scattered neuronal necrosis was followed by a moderate focal ischemia three days later. Brain injury was assessed noninvasively with magnetic resonance (MR) imaging and then with histology and the results demonstrate that, depending on location, injury following a moderate stroke was either reduced or enhanced by a prior mild stroke.

Materials and Methods

Model of Recurrent Stroke

Wistar rats (250-300g) were obtained from Charles River Laboratories (Montreal, Canada). All animals were treated in accordance with the guidelines provided by the Canadian Council on Animal Care and experiments were approved by the local Animal Care Committees. A total of 26 animals were used and randomized to subgroups of :1) a single mild stroke (MCAO for 40 minutes, n=10), 2) a single moderate stroke (MCAO for 60 minutes, n=8) and 3) a group with recurrent stroke consisting of a combination of a mild stroke followed 3 days later by a moderate stroke (n=8). Three days was selected for the interval between surgeries considering that substantial (e.g. up to 64%) of stroke recurrence clinically has been reported to occur during the first 2-3 days after the first event (Hill et al., 2004; Coull et al., 2004; Coutts et al., 2005).

MCAO was produced by placing an aneurysm micro clip (Codman, size #1) on the middle cerebral artery of the rat generally using techniques described previously and modified for an experimental design involving two occlusions (Tuor et al., 2007). Briefly, animals were anesthetized with isoflurane (4% for induction, 2-2.5% for maintenance). The tail or femoral artery was cannulated with PE-50 tubing for monitoring arterial blood pressure and obtaining arterial blood samples for monitoring blood gases during the surgical procedure prior to and then during cerebral ischemia. Rectal body temperature was maintained at 37.5C during the surgery and MCAO using a servo-controlled heating lamp. The animals were placed in the lateral position and a 10 mm long vertical incision was made between the right eye and the external ear. The skin and underlying temporalis muscle were retracted and a small burr hole was made on the surface of the temporal bone at the point where the MCA crossed the rhinal fissure using a drill. Bone wax was applied to stop bleeding if necessary. The meninges over the MCA was divided by a 30-gauge fine needle. The MCA was exposed and occluded with the clip and concurrently both left and right common carotid arteries were occluded with 3-0 silk sutures. Prior to occlusion, another burr hole was drilled 3 mm dorsal to the site of the MCA and the dura was left intact. A laser doppler probe (Periflux 5000, Perimed) was placed on the burr hole to measure cerebral blood flow changes during MCAO in the ischemic parietal cortex within the infarct core. Only animals with a cerebral perfusion decrease to < 10% of baseline level were included for

further study. To terminate the MCAO, the microclip was removed and the ligatures on both carotid arteries were released. For the group of recurrent stroke, a 3x3mm dura substitute (Gore Preclude MVP, Better Hospital Supplies Corp., Miami, FL) was inserted between the temporalis muscle and brain at the surgical site to minimize attachment and development of fibrotic connective tissue between muscle and brain. Analgesia (buprenorphine, 0.03 mg/kg s.c.) was administered following closure of the surgical site and recovery of the animal from anesthesia. Three days later, a second transient MCAO was produced using procedures similar to those described above for the 1st surgery.

MR imaging

At 2 days and/or 7 days following the 1st or 2nd stroke, MR scans were acquired to document ischemic injury using a 9.4T MR system (Magnex, UK) equipped with a Biospec Avance II Bruker console (Bruker, Germany). The animals were anesthetized with isoflurane (1.5-2.5%) and restrained with an incisor bar in a chamber designed to fit the bore of the magnet. T_2-weighted MR images were obtained from a spin-echo multi-slice imaging sequence. Briefly, the T_2 map was acquired from a 32-echo train with a 6 ms echo spacing and repetition time (TR) of 5000 ms within multiple coronal brain slices through the cerebrum of thickness of 1.5 mm and data matrix of 256×128. The T_2 relaxation time was measured within ipsilateral and contralateral parietal cortex using image analysis software (Marevisi, Institute for Biodiagnostics, National Research Council of Canada).

Histology

Brains were processed for histology 7 days after either the single stroke or the 2nd stroke in the recurrent stroke group. The animals were anaesthetized with pentobarbital (120mg/kg) and perfused with 10% formalin and brains were embedded in paraffin. Sections (6μm thick) were cut from blocks proximal (anterior cerebrum) and distal (mid cerebrum) to the MCAO to assess tissue injury.

Sections from blocks proximal to the MCAO containing striatum and blocks more distally containing hippocampus were stained with hematoxylin and eosin and inspected microscopically for signs of cell death or infarct. A cumulative score for brain injury at each level was assessed by an investigator blinded to animal identity using a scoring system

similar to that described previously (Barber et al., 2004). The cortical hemisphere was divided into 4 regions of interest (Fig. 1A) and a score of injury was assigned to each region as follows: 0 for normal, 1 for <10% of neuronal injury, 2 for 10-50% of neuronal injury, 3 for > 50% neuronal injury and 4 for confluent areas of pan-necrosis.

Neuronal injury was also assessed in proximal and distal sections stained immunohistochemically with microtubule associated protein 2 (MAP2). Sections were incubated with 10% goat serum for 30 minutes after antigen retrieval by boiling the sections in citrate buffer for 10 minutes and then were incubated with mouse anti-rat MAP2 (1:100, Sigma) overnight at 4 ºC. After washing with 0.01M PBS, sections were incubated with Biotin-conjugated Goat anti Mouse IgG (1:400, Jackson-ImmuoResearch Lab.) for 1 hour at room temperature followed by incubation with Peroxidase-Conjugated Streptavidin (1:400, Dako) for 30 minutes. Finally, staining was visualized with diaminobenzidine (DAB, Sigma). Stained sections were converted to digital images using a Nikon scanner (CoolScan VED, Nikon Canada). Areas of decreased MAP2 staining were quantified using Image J software (NIH) by measuring the total area of the section and the area of MAP2 staining below the corresponding contralateral level of staining used as threshold.

The glial response to injury was also assessed immunohistochemically by staining for increases in glial fibrillary acidic protein (GFAP) using rabbit - anti-rat GFAP (1:500, SIGMA). Increased staining for activated microglia/macrophages was assessed by staining immunohistochemically with mouse anti-rat ED1 (1:100, SEROTEC). The areas of increased staining for GFAP and ED1 were measured from digital scanned sections as described for MAP2.

The T2, histological scores and staining area changes at different proximal and distal levels and for the 3 different types of stroke were analyzed statistically using a two way measures analysis of variance whereas scores were analyzed using a Kruskal Wallis ANOVA on ranks. Post-hoc comparisons of groups were analyzed using a Student-Neuman-Keuls Multiple comparison of means or a Mann-Whitney Rank Sum test (SigmaStat; SPSS Inc, Chicago, IL). Left-right differences in T2, presented as ratios, were also compared using a paired Students t-test. Differences were considered significant at $P < 0.05$. Data are presented as mean ± SD.

Results

Several technical procedures were optimized in pilot experiments performed in 53 rats during the initial stage of the development of the recurrent stroke model. Fibrosis that developed between the temporalis muscle and brain or MCA, was found to cause substantial bleeding upon the second exposure and dissection of the MCA. Insertion of a sterile piece of artificial dura soaked in sterile saline between the temporalis muscle and the exposed brain/MCA at the end of 1st surgery prior to wound closure improved healing of the cut dura and prevented adhesion of brain to overlying tissues. Surgical procedures were also optimized. Thus, in the current study, of the 26 animals randomized to the treatment groups, only three were euthanized as a result of surgical complications during the production of either a single mild (n=2) or moderate stroke (n=1). One additional animal was excluded as the cortical perfusion reduction during MCAO did not reach criteria. Physiological variables including arterial blood gas measures, blood glucose values and mean arterial blood pressure were similar in all groups (Tables 1 and 2). The rats recovered well from either a single or multiple stroke, generally with minimal weight loss between surgeries and weight gain by 7 days post surgery.

Within 5 minutes of MCAO, the average cerebral blood flow measured within the parietal cortex ipsilateral to the occlusion decreased precipitously to similar baseline values of 5 ± 4%, 6 ± 4% and 5 ± 4% in the single mild, single moderate and recurrent stroke groups, respectively. Five minutes following removal of the clip and ligatures on the arteries, the MCA distal to the clip position appeared patent under the microscope. Also, blood flow in the ipsilateral parietal cortex quickly returned to pre-occlusion levels (i.e. to 105±59%, 84±59%, 100±86% baseline, (n.s.) for the single mild, single moderate and recurrent stroke groups, respectively).

The T2 increases observed following stroke were dependent both on proximal – distal location ($P<0.05$) and severity of the stroke ($P<0.05$) (Two way measures ANOVA). Two days after a mild stroke, there were subtle (2-5%) increases in intensity or T_2 values (Fig.1 B and Fig 2 A) irrespective of whether animals were randomized to a single or multiple stroke group. In contrast, at 2 days following a single moderate stroke, there was a substantial increase in image intensity or T_2 in the ipsilateral parietal cortex ($P<0.02$, Student-Newman-

Keuls Method). At 7 days after a recurrent stroke, T_2 was also significantly elevated in proximal parietal cortex but less so in the distal parietal cortex ($P < 0.05$, for location; Student-Newman-Keuls Method).

The extent of injury assessed histologically in hematoxylin and eosin stained sections demonstrated the presence of scattered necrosis following a mild stroke and more extensive injury following either a moderate or multiple stroke (e.g. Fig 1 B and Fig 2 A-F). Seven days following a mild insult, scattered eosinophilic or pyknotic cells were observed in proximal and distal regions of the parietal cortex including areas adjacent to the location of clip placement whereas subcortical gray matter such as striatum or thalamus were generally not affected. Following a single moderate stroke, regions of extensive cell death or pannecrosis were observed accompanied by edematous changes distributed throughout the parietal cortex (e.g. Fig 2E). In the group of animals with a recurrent stroke, the majority of the parietal cortex was pannecrotic and edematous in proximal sections (e.g. Fig 2 F) with often less injury distally. Quantitatively, the cumulative histological score for neuronal injury at the proximal level was dependent on stroke severity ($p<0.002$) and greater following a recurrent or moderate stroke than a single mild stroke (Fig 2, lower panel) ($P<0.05$) (two way ANOVA and Mann-Whitney Rank Sum Test). At posterior cerebral levels distally, the cumulative injury scores were increased compared to the contralateral hemisphere but similar between groups. Total infarct volume estimated in the moderate and recurrent stroke groups were similar, at 9.7 ± 3.5 and 9.5 ± 3.7% of the hemisphere, respectively.

Tissue injury was also assessed using immunohistochemical staining to demonstrate regional reductions in MAP2 within neurons, to demonstrate increased GFAP in reactive astrocytes and to demonstrate activated microglia/macrophages using ED1 (Fig 3). At 7 days following a mild stroke, there was normal intense positive labeling for MAP2 and sparse labeling for ED1 and GFAP in the uninjured cortex contralateral to the stroke. In contrast, within the area of ischemic injury in the ipsilateral parietal cortex, there was increased labeling of astrocytes with GFAP but minimal change in MAP2 or ED1 labeling. Following either a moderate or recurrent stroke, a loss of neuronal MAP2 staining and increased ED1 staining of microglia/macrophages was evident within the ipsilateral cortex whereas GFAP staining was increased in peri-infarct regions with a loss of labeling within the core. Quantitatively, both proximal-distal location or type of ischemic insult affected the extent of

neuronal loss (MAP2) or changes in astrocyte (GFAP) or microglial/macrophage reactivity (ED1) (Fig 4). The percentage area of altered stain within brain sections was greater following a moderate than mild stroke and was significantly greater following a recurrent stroke ($P<0.05$). For injury markers, the extent of altered staining following recurrent stroke was greater at proximal than distal levels where differences were significant statistically for the ED1 and GFAP stains ($P<0.007$, two way ANOVA).

Discussion

In the present study, we have developed a novel model of recurrent stroke in the rat that will help us investigate mechanisms of multiple ischemic insults and potential therapeutic or preventive injury strategies for recurrent ischemic stroke. The protocol established involves a transient mild ischemic stroke followed by a moderate ischemic stroke three days later in the same supply distribution of the middle cerebral artery. There are several novel aspects of the animal model and the results obtained. First, in contrast to pre-conditioning studies, the initial minor stroke produces a mild brain injury detected histologically as permanent damage consisting of selective neuronal necrosis. Secondly, the interaction of the first and second stroke differs regionally. In the proximal cortex, situated in the core of the ischemia, the second moderate stroke produces substantial injury that appears enhanced whereas, in the distal cortex, the second moderate stroke produces a cortical injury similar to a mild stroke alone. Thus, the manner in which multiple ischemic insults interact and evolve is complex even when both strokes affect the same arterial distribution. Our new model of recurrent stroke will allow investigation of basic aspects of multiple ischemic episodes as would occur following thrombolysis of an arterial clot or a transient ischemic attack resulting in very mild damage followed by a second stroke in the same territory.

Experimental models of TIA and multiple stroke are limited in number. The majority of previous studies with multiple episodes of ischemia have focused on the pre-conditioning neuroprotective effects of a short period of global or focal cerebral ischemia. In such studies, a mild ischemia (generally < 30 minutes duration) not resulting in pathological damage produces an ischemic tolerance against a subsequent lethal episode of ischemia or major stroke (Gidday, 2006; Steiger and Hanggi, 2007; O'Duffy et al., 2007). Relatively few

experimental studies of recurrent stroke have used an initial TIA or minor stroke producing mild tissue damage. One laboratory has used a thromboembolism for the first stroke and a thromboembolism or global ischemia for the second stroke (Dietrich et al., 1999; Danton et al., 2002; Urrea et al., 2004). In these studies, the initial photothrombotic stroke, produced by a 10 minute irradiation of the common carotid artery, causes quite large multiple infarcts (e.g. in cortex, hippocampus, thalamus and striatum) that are generally larger than TIA's or minor stroke observed clinically. Also, reproducibility is an issue as infarcts are inconsistent in location and overall size, with recurrent thromboembolic strokes being generally larger than the damage following the initial insult. The present model produces a consistent mild ischemic insult, sufficiently severe to produce a pathological change such as scattered cellular death resembling a TIA or minor stroke and a reliable moderate second stroke. Although our repeated MCAO with a clip is technically challenging, surgical skills improve with a high success rate achievable. Another challenge may be potential variations between laboratories regarding the optimal duration of ischemia needed to produce mild and moderate insults, considering the high sensitivity of ischemic injury to factors such as method of temperature regulation, depth of anesthesia and surgical approach. Nevertheless, within an experiment, the present method has the advantages of excellent control of ischemia duration and severity along with good reperfusion accompanied by minimal mechanical or chemical damage to the cerebral inflow vessels. Beyond the scope of the current study and a topic for future investigation are the corresponding behavioural correlates and effects of chronic recovery times in addition to the effect of different intervals between strokes and/or their duration. Note that although there have been reports of continued ongoing cell death for weeks following an insult, in studies where a hypoxic/ischemic insult is confirmed to be mild (e.g. with MRI) the overall final lesion also is modest (Tuor et al, 2008; Qiao et al, 2009).

The initial transient ischemia in the present study is considered to model a type of TIA or minor stroke. In clinical studies, TIA or minor stroke can result in the appearance of long-lasting lesions detected using non-invasive imaging such as MRI and CT. Indeed diffusion weighted MR imaging has been particularly useful for detecting brain injury in patients within 6-12hrs of onset of TIA or minor stroke (Winbeck et al., 2004; Coutts et al., 2005; Coutts et al., 2005). In the present study, we used T_2 imaging to identify stroke severity following the first mild stroke, because this imaging sequence is well known to

demarcate well areas of infarct in animal models of transient focal ischemia (Weber et al., 2006; Tuor et al., 2007). Diffusion weighted imaging changes in brain, although often sensitive in acute stroke, can resolve or normalize following a mild transient ischemia (van Lookeren et al., 1999;Weber et al., 2006). However, in the present study following minor stroke, there were also no detectable changes in T_2-weighted images at either 2 or 7 days post stroke despite evidence of gliosis and scattered cell necrosis. Whether similar cell death undetectable with MRI occurs following TIA in patients is not clear and will need to be investigated in good quality postmortem samples of patients following TIA. The present findings indicate that is possible that in some patients with a TIA there is diffuse or scattered permanent cell damage undetectable with current noninvasive brain imaging methods.

Another unique observation in the present study is the demonstration that the injury produced by multiple ischemic episodes is dependent on the proximal versus distal location of the parietal cortex from the occlusion site. Despite ischemia being produced within the same distribution of the middle cerebral artery territory for the first and second stroke, there were different combined brain injury outcome measures (e.g. altered T_2, MAP2, GFAP and ED1 staining ipsilaterally) in proximal versus distal regions. Proximally, brain injury was enhanced following recurrent stroke compared to mild stroke whereas distally, injury following a recurrent stroke was similar to a mild stroke. It is not entirely unexpected that a mild injury sufficient to cause brain damage prior to a moderate stroke would enhance injury. Novel is the finding that at levels more distal to the MCAO, when the first focal ischemia was still sufficiently severe to produce scattered neuronal necrosis, there was a tolerance against injury from the second insult. This was particularly evident in the less extensive area of activated microglia/macrophages observed with recurrent stroke and is consistent with a preconditioning type of protection normally observed with mild insults not producing permanent damage (Gidday, 2006; Steiger and Hanggi, 2007;O'Duffy et al., 2007).

Summary

The present recurrent stroke in the rat will allow the study of the mechanisms of injury that occur with multiple strokes. Clinically, the recurrent stroke modeled would be the ischemic injury occurring following effective thrombolysis therapy or a transient ischemic attack with mild damage which is then followed by a second stroke in the same territory.

The results demonstrate that with the current 3 day timing between strokes, a short transient stroke had dual effects on the histopathological consequences of a second ischemic insult. In the brain, proximal to the occlusion there was an enhanced injury whereas more distal to the occlusion there was evidence of neuroprotection. We anticipate that this new recurrent stroke model will be useful for investigating further the mechanisms contributing to overall injury following TIA or minor stroke when accompanied by a subsequent stroke. In addition, this recurrent stroke model will provide a useful platform for the testing of therapies designed to treat TIA or minor stroke in order to prevent or reduce injury caused by a subsequent recurrent stroke.

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Figure Legend

Figure 1. Schematic representation of distal and proximal sections used to grade brain damage (score of 0-4) within 4 areas of cerebral cortex (A). Also shown are representative T_2 weighted magnetic resonance (MR) images (2 days and 7 days post stroke) and representative schematic diagrams of areas of altered cell staining (scattered necrosis or infarct (black) within hematoxylin and eosin (HE) stained sections (B). The quantitation of MR images provided T_2 relaxation times presented as a ratios of ipsilateral/contralateral values (C). Shown are mean values in proximal and distal levels of the cerebrum for experimental groups consisting of rats exposed to a single mild middle cerebral artery occlusion (MCAO), a single moderate stroke or a recurrent stroke (a combined mild +moderate MCAO). Quantitatively, the ratio of T_2 relaxation times was not increased ipsilaterally despite the occurrence of scattered cell necrosis following a single mild stroke. Ipsilateral vs contralateral increases in T_2 were observed following either a single moderate stroke or a recurrent stroke corresponding to infarct in HE stained sections (*$P<0.05$; ** $P<0.001$; paired Students t-test). In the recurrent stroke, the increase in T_2 in proximal brain was higher than that in distal brain ($^{++}P<0.005$, Two way ANOVA).

Figure 2. Representative micrographs of haematoxylin and eosin stained sections of parietal cortex contralateral (A-C) or ipsilateral (D-F) to the transient occlusion. Sections are from rats either 7 days after a single mild stroke (A, D), a single moderate stroke (B, E) or a recurrent stroke (C, F). Pyknotic cells (e.g. arrows) are observed in all groups and in addition vacuolation associated with edema is apparent following a moderate or recurrent stroke (E,F). The cumulative injury score, assessed as a sum of grades 0-4 within 4 regions of either proximal or parietal cerebral cortex is presented as the median value along with the first and third quartile for each group. The injury score was greatest following a combined recurrent stroke proximal to the occlusion. *$P<0.05$, **$P<0.005$, ipsilateral different from contralateral; $^{\#}P<0.05$, different from a single mild stroke and $^{+}P<0.03$ different from proximal (ANOVA followed by Mann-Whitney Rank Sum Tests).

Figure 3. Micrographs of proximal parietal cortex stained immunohistochemically for MAP 2 (A-C) and GFAP (D to F). Altered staining is observed ipsilateral to the middle cerebral artery occlusion (right hemisphere) following either a mild, moderate or recurrent (mild+moderate) stroke. Additional sections (H-I) from proximal cortex one week following a recurrent stroke, demonstrate ischemic alterations in regions of core and peri-infarct in hematoxylin and eosin (HE), GFAP and ED1 stained sections. At higher magnification there are also modest increases in GFAP observed following a mild stroke and increases in peri-infarct regions following a moderate or recurrent stroke (J-L).. ED1 labeling at higher magnification is similar to the contralateral hemisphere following a mild stroke (M) and increased in both core and peri-infarct areas following either a moderate or recurrent stroke (N,O). Bar = 50μ in GFAP (J-L) and ED1 (M-O) stained sections.

Figure 4. Quantitative analysis of the areas of altered staining for microtubule associated protein 2 (MAP2, A), for astrocytes labeled with glial fibrillary acidic protein (GFAP, B) and for microglia/macrophages labeled with ED1 (C). Areas are a percentage (mean ±SD) of the total area. Areas of altered staining for MAP2, GFAP and ED1 were observed following either a single moderate stroke or a recurrent stroke (mild+moderate stroke). *$P<0.05$, **$P<0.01$, different from 0; $^{\#}P<0.05$, $^{\#\#}P<0.005$ different from the mild group; $^{++}P<0.007$ distal different from proximal, Two way ANOVA and Student-Newman-Keuls Method.

Table 1. Measures* of Cortical Perfusion, Arterial Blood Pressure, Glucose and Body Weight during Middle Cerebral Artery Occlusion for the Mild, Moderate and Recurrent Stroke Groups.

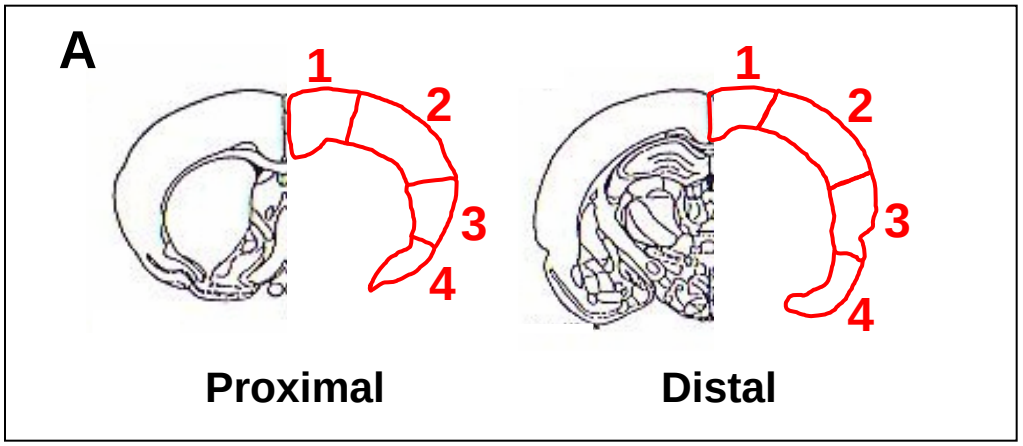
Type of Stroke	**Laser Doppler Flow (% Baseline)**		**Mean Arterial Blood Pressure** (mm Hg)	**Glucose** mM	**Weight** gm
	During Occlusion	Reperfusion			
Mild	5.5 ± 3.8	105 ± 87	80.0 ±12	11.0±1.1	258±39
Moderate	5.7 ± 4.0	84 ± 59	83.1 ± 16	12.1±2.5	276±53
Recurrent- First Mild	4.9 ± 3.7	101 ± 86	78.0 ± 15	12.0±1.5	280±37
Recurrent – Second Moderate	6.0 ± 4.6	110 ± 100	73.9 ± 15	12.3±2.1	275±35

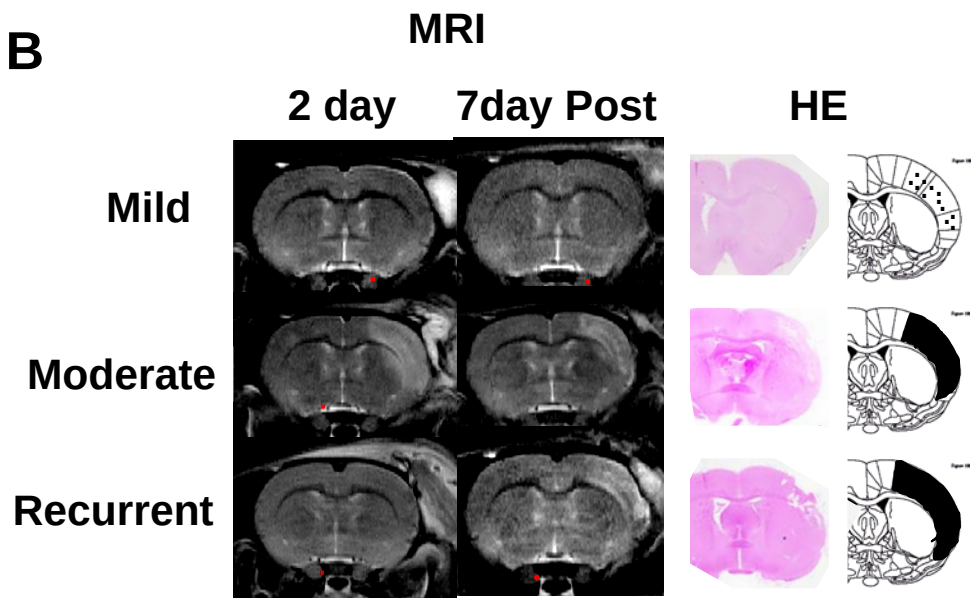
* Data presented as mean±SD.

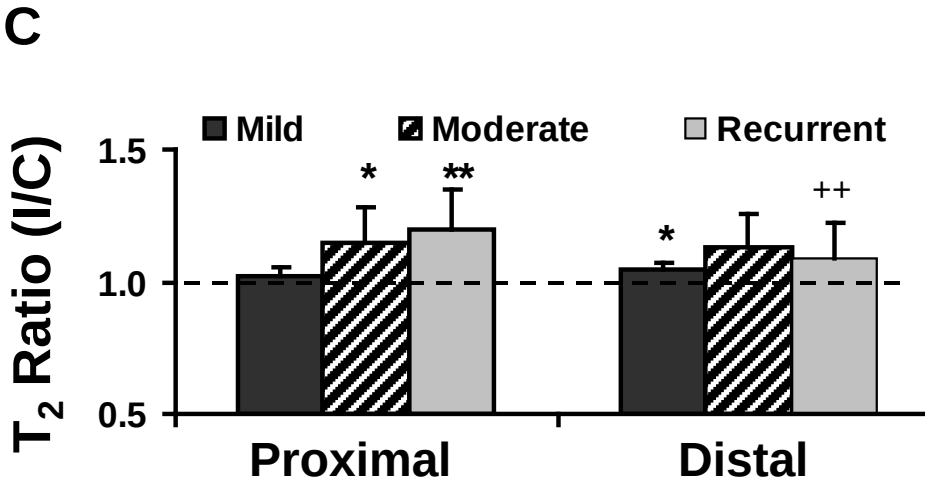
Table 2. Measures* of PCO2, PO2 and pH in arterial blood taken prior to and during middle cerebral artery occlusion (MCAO) or five minutes after reperfusion in the Mild, Moderate and Recurrent Stroke Groups.

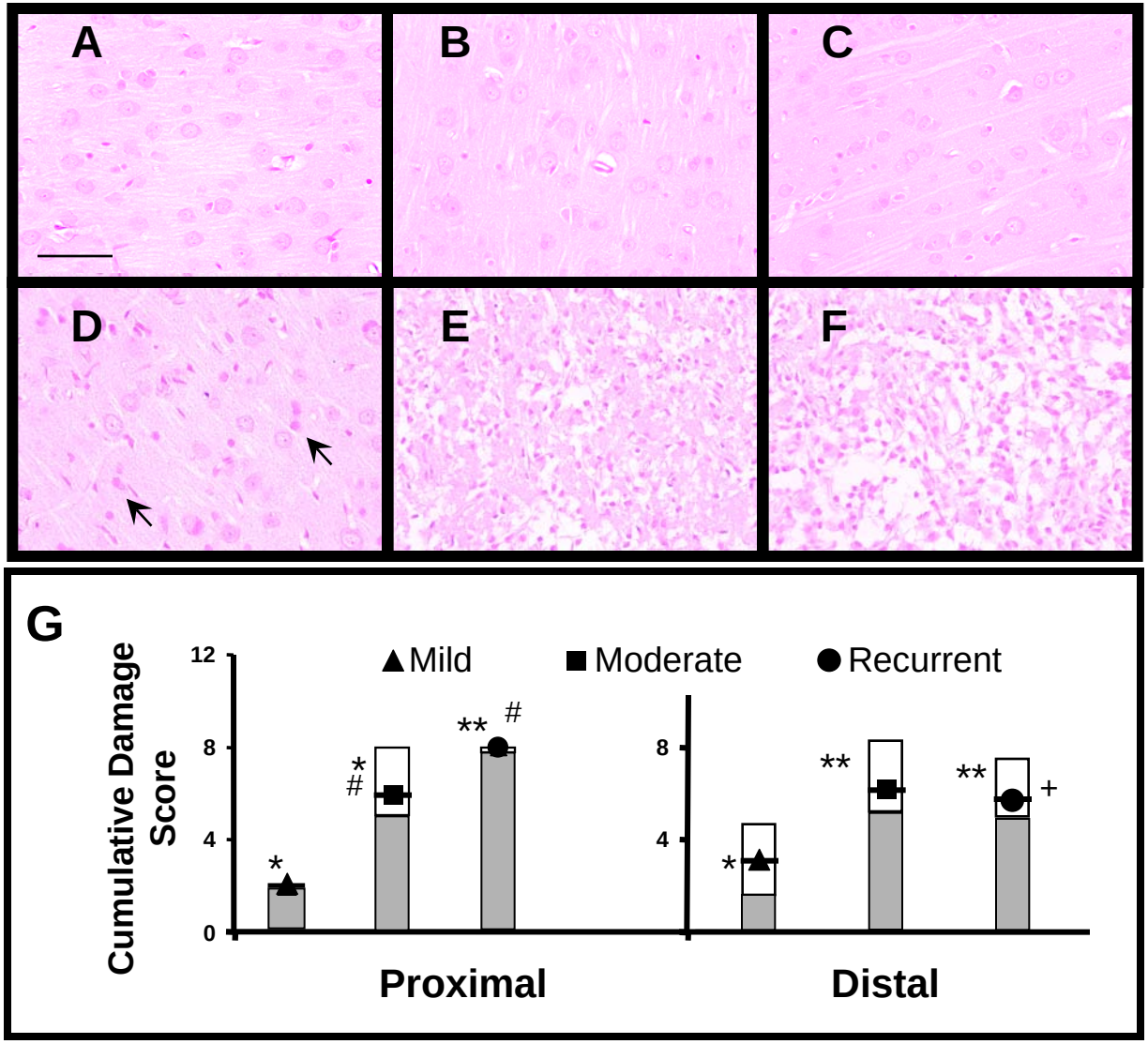
Type of Stroke	Prior to MCAO			During MCAO			Post Reperfusion		
	PCO2 mmHg	PO2 mmHg	pH	PCO2 mmHg	PO2 mmHg	pH	PCO2 mmHg	PO2 mmHg	pH
Mild	36.1±4.5	96±9	7.44±.05	35.6±6.5	109±15	7.47±.04	33.4±3.7	99±6	7.46±.03
Moderate	35.5±5.8	100±14	7.45±.04	33.4±3.0	97±13	7.47±.03	34.7±4.9	108±15	7.45±.02
First Mild	38.0±4.8	95±8	7.44±.04	35.6±5.8	111±8	7.47±.08	36.0±56.1	103±11	7.43±.04
Mild plus Moderate	38.6±3.0	101±9	7.42±.05	35.5±4.0	104.4±11	7.45±.04	35.4±3.9	104±13	7.45+.04

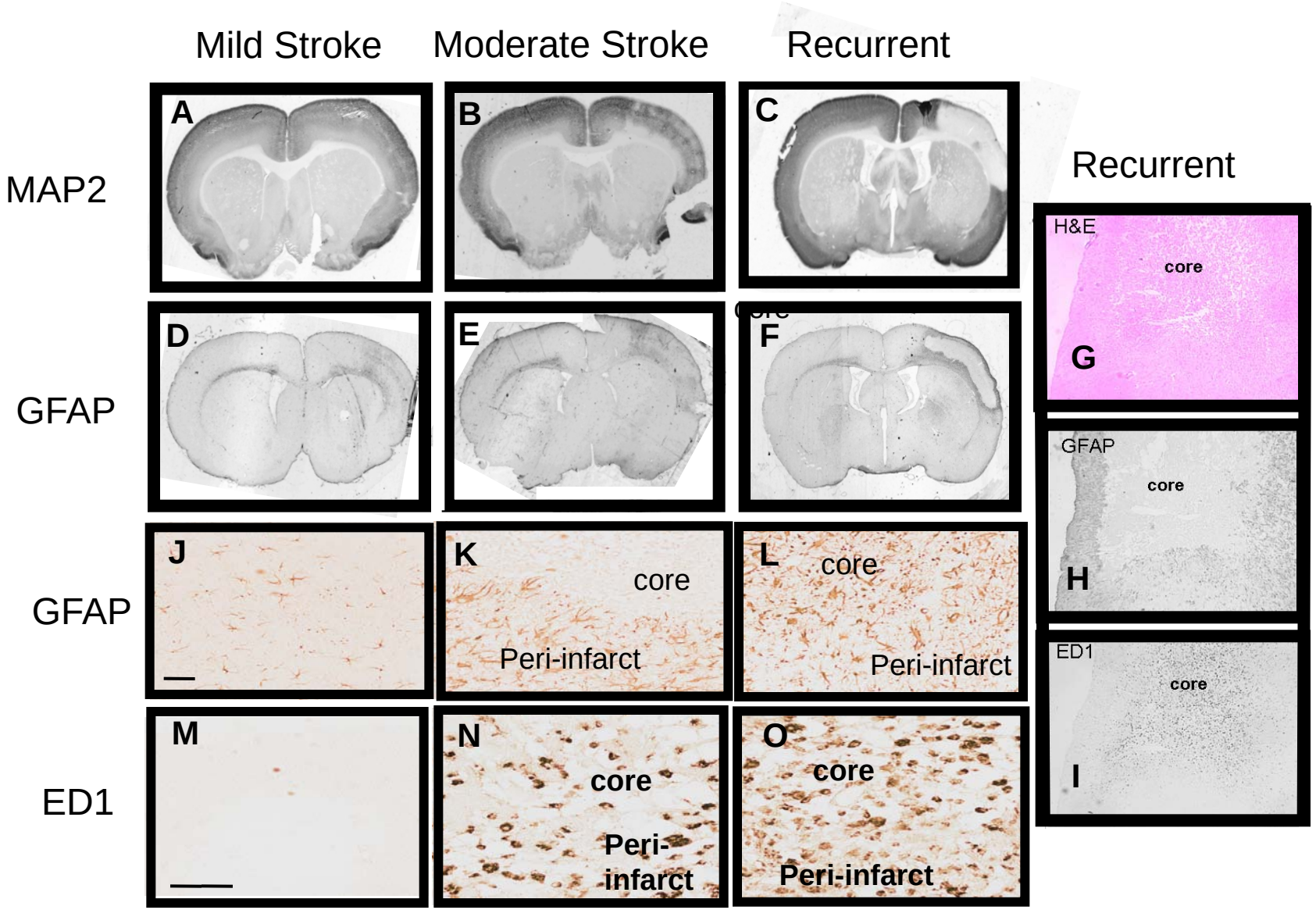
* Data presented as mean±SD.

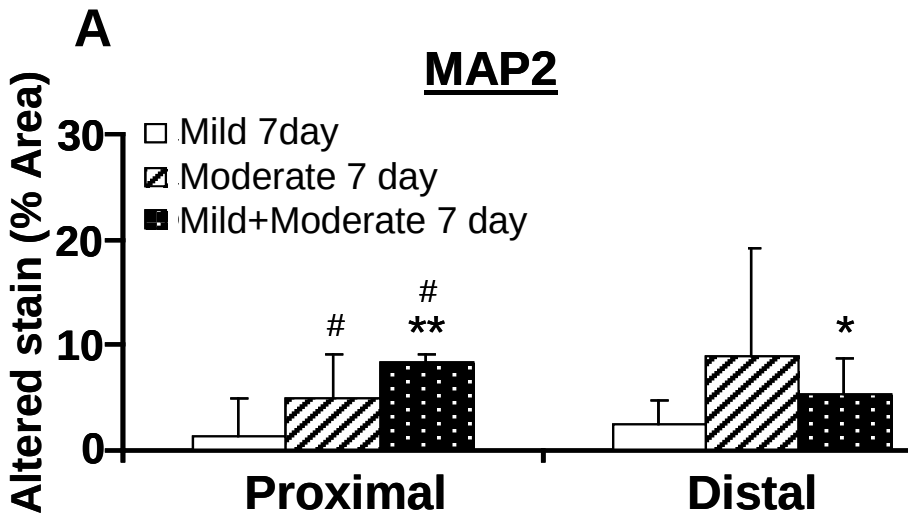

A
1
2
3
4
1
2
3
4
Proximal
Distal

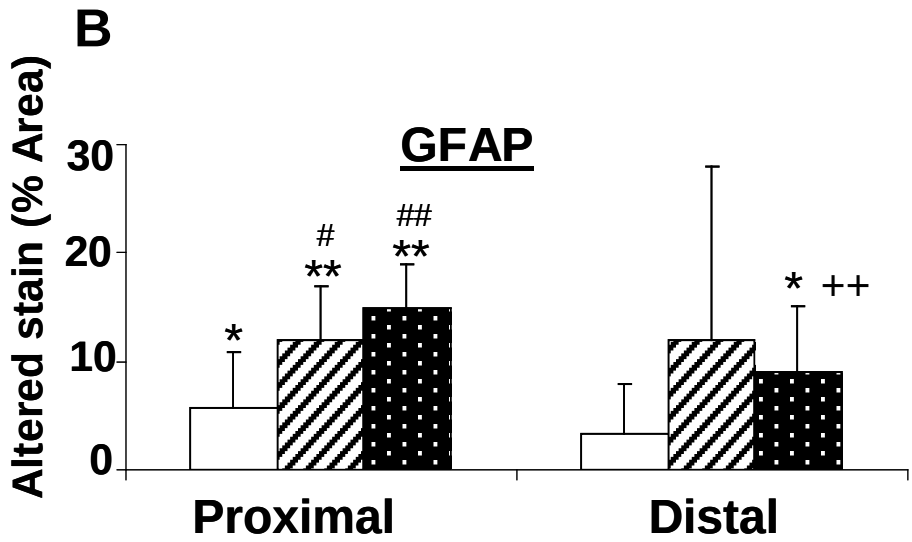

B
MRI
2 day
7day Post
HE
Mild
Moderate
Recurrent

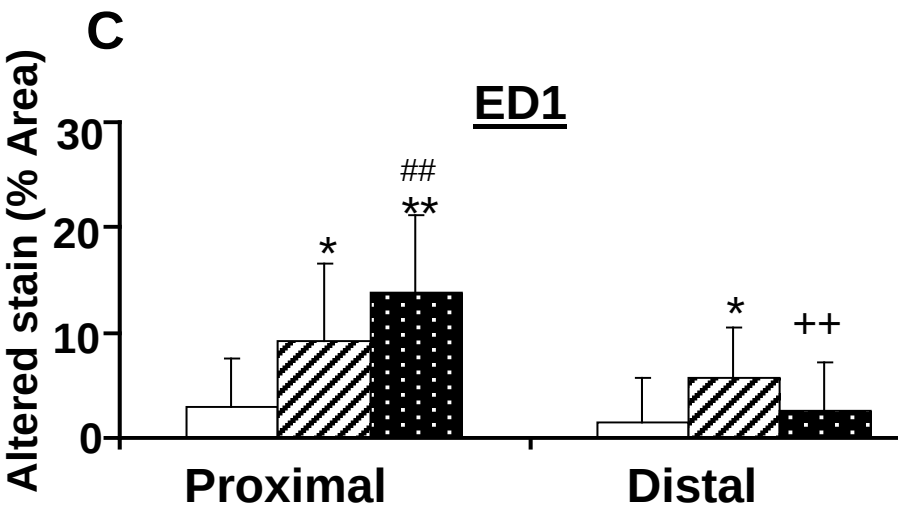

C
Mild
Moderate
Recurrent
T2 Ratio (I/C)
1.5
1.0
0.5
*
**
*
++
Proximal
Distal


A
B
C
D
E
F
G
Cumulative Damage Score
12
8
4
0
▲Mild
■Moderate
●Recurrent
*
*
#
**
#
*
**
**
+
Proximal
Distal


Mild Stroke
Moderate Stroke
Recurrent
MAP2
GFAP
GFAP
ED1
A
B
C
D
E
F
J
K
core
Peri-infarct
L
core
Peri-infarct
M
N
core
Peri-infarct
O
core
Peri-infarct
Recurrent
H&E
core
G
GFAP
core
H
ED1
core
I


A
MAP2
Mild 7day
Moderate 7 day
Mild+Moderate 7 day
Altered stain (% Area)
30
20
10
0
#
#
**
*
Proximal
Distal


B
GFAP
Altered stain (% Area)
30
20
10
0
*
#
**
##
**
*
++
Proximal
Distal


C
ED1
Altered stain (% Area)
30
20
10
0
*
##
**
*
++
Proximal
Distal