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TTC, Fluoro-Jade B and NeuN staining confirm evolving phases of infarction induced by Middle Cerebral Artery Occlusion

Fudong Liu^{a,b}, Dorothy P. Schafer^a, and Louise D. McCullough^{a,c,*}

^aDepartment of Neuroscience, University of Connecticut Health Center, Farmington, CT 06032, USA.

^bDepartment of Neurology, Yijishan Hospital of Wannan Medical College, Wuhu, Anhui 241001, China.

^cDepartment of Neurology, University of Connecticut Health Center, Farmington, CT, 06030 and the Stroke Center at Hartford Hospital, Hartford CT 06102

Abstract

Considerable debate exists in the literature on how best to measure infarct damage and at what point after middle cerebral artery occlusion (MCAO) infarct is histologically complete. As many researchers are focusing on more chronic endpoints in neuroprotection studies it is important to evaluate histological damage at later time points to ensure that standard methods of tissue injury measurement are accurate. To compare tissue viability at both acute and sub-acute time points, we used 2,3,5-Triphenyltetrazolium chloride (TTC), Fluoro-Jade B, and NeuN staining to examine the evolving phases of infarction induced by a 90-minute MCAO in mice. Stroke outcomes were examined at 1.5h, 6h, 12h, 24h, 3d, and 7d after MCAO. There was a time-dependent increase in infarct volume from 1.5h to 24 h in the cortex, followed by a plateau from 24h to 7d after stroke. Striatal infarcts were complete by 12h. Fluoro-Jade B staining peaked at 24 hours and was minimal by 7 days. Our results indicated that histological damage as measured by TTC and Fluoro-Jade B reaches its peak by 24h after stroke in a reperfusion model of MCAO in mice. TTC staining can be accurately performed as late as 7 days after stroke. Neurological deficits do not correlate with the structural lesion but rather transient impairment of function. As the infarct is complete by 24 hours and even earlier in the striatum, even the most efficacious neuroprotective therapies are unlikely to show any efficacy if given after this point.

Keywords

Fluoro-Jade B; Infarction; Ischemic stroke; Middle Cerebral Artery Occlusion (MCAO); NeuN; Penumbra

1. Introduction

Experimental stroke models are essential to study the pathophysiology of cerebral ischemia and to evaluate the effects of novel therapeutic interventions. The MCAO model in rodents has been widely used to study focal cerebral ischemia. This model offers a simpler and less traumatic surgical approach compared with earlier craniotomy models (Tamura et al., 1981),

*Corresponding author: Louise D. McCullough, MD, PhD, Tel: +1-860-679-2271; fax: +1-860-679-1181, E-mail address: E-mail: lmccullough@uchc.edu.

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lends itself more readily to the study of reperfusion and has been adapted for use in continuous magnetic resonance imaging (Roussel et al., 1995). However significant controversy exists due to the variability of final infarct size and debate as to the most reliable time point to measure the effects of various therapeutic agents (DeVries et al., 2001; Culmsee et al., 2005; Hoyte et al., 2006). Although transient MCAO model has been utilized to study ischemic stroke for decades, the evolution of infarct within the area blood supplied by MCA has not been well elucidated. In the present study we performed an analysis to investigate the evolution of infarct after MCAO. We performed this analysis in mice, a model system that has been less well characterized (Duckworth et al., 2005).

In order to analyze the time-dependent changes following transient MCAO, several different histochemical methodologies can be utilized. 2,3,5-Triphenyltetrazolium chloride (TTC) is one of the most common histochemical stains used to assess cerebral injury. In ischemic tissue, lack of TTC staining is considered “infarcted” and defined as core and viable tissue is stained red (Benedek et al., 2006). Although widely accepted and used, TTC staining has received criticism as TTC is a marker of tissue dehydrogenase and mitochondrial dysfunction and may not represent irreversible cell death, therefore this method may overestimate infarct size (Tureyen et al., 2004, Benedek et al., 2006). Despite this criticism, TTC is still a reliable, rapid, and inexpensive method for analyzing enzymatically dysfunctional cells, most of which will eventually degenerate (Lust et al., 2002).

Because of the caveats described above, it becomes important to assess and confirm infarct size by other methods in addition to TTC, especially at the commonly used 24-hour time point. Others have demonstrated that TTC and cresyl violet (CV) staining show a high degree of correlation in infarct area and volume at 24 hours, indicating that both methods are suitable for producing accurate measurements of cerebral infarcts (Kudret et al., 2004). However, conventional histological techniques such as Nissl, hematoxylin and eosin (H&E), or CV stains also have limitations, as false positives occur due to processing artifacts or non-lethal alterations in cellular morphology (Schmued et al., 1997) and assessment is timely and labor intensive. These stains are also not specific for neuronal degeneration, as all cell types stain with these dyes. Additional relatively subtle morphological differences exist between normal and degenerating neurons making assessment more prone to bias.

Fluoro-Jade B is an anionic dye that specifically stains the soma and neurites of degenerating neurons by binding to a currently unknown basic substance in the neuron, most likely a poly-amine. It has the advantage of being as reliable and technically simple as a conventional Nissl stain, while being as specific for degenerating neurons as the “gold-standard” suppressed silver stain. It has a higher affinity for degenerating tissue components than Fluoro-Jade, reducing non-specific staining (Schmued and Hopkins, 2000). Recently Fluoro-Jade B has been used to identify neuronal degeneration secondary to ischemia (Schmued and Hopkins, 2000; Duckworth et al., 2005). Neuronal nuclear antigen (NeuN), a widely used marker for mature neurons, is expressed in nucleus and cell body of most neurons and not in glial cells, oligodendrocytes, astrocytes, or microglial cells (Wolf et al., 1996). Immunoreactivity for NeuN has been reported to decrease dramatically following CNS injury (e.g. MCAO and traumatic brain injury) (Igarashi et al., 2001; Davoli et al., 2002; Sugawara et al., 2002). However the loss of NeuN immunoreactivity may reflect injury-induced antigenicity rather than irreversible neuronal injury in ischemic models (Unal-Cevik et al., 2004).

In this study we used TTC, Fluoro-Jade B, and NeuN staining to examine the chronology of infarct development following MCAO. Our objectives were to determine if Fluoro-Jade B was superior to TTC staining, to delineate the time course of infarct progression, and to establish the anatomic boundaries of core and penumbra in mice at several time points after transient MCAO in mice.

2. Materials and methods

2.1. Animals

Male C57BL/6 mice (Charles River Laboratories) weighing 20–25g at the time of surgery were used for all experiments. The mice were group-housed and maintained on a 12:12h light/dark cycle, with ad libitum access to water and rodent chow. All procedures with animals were in accordance with the NIH guidelines for the care and use of animals in research and under protocols approved by the Animal Care and Use Committee of the University of Connecticut.

2.2. Ischemic model

Cerebral ischemia was induced by 90 min of MCAO under isoflurane anesthesia as previously described (McCullough et al., 2003). Briefly, rectal muscle temperature were monitored with a MONOTHERM system and maintained at approximately 37°C during surgery and ischemia with an automated temperature control feedback system. A midline ventral neck incision was made, and unilateral MCAO was performed by inserting a silicone rubber coated monofilament (Doccol Corp, CA) into the right internal carotid artery 6 mm from the internal carotid/pterygopalatine artery bifurcation via an external carotid artery stump. Sham animals underwent the same procedure but the suture was not advanced into the middle cerebral artery. Laser Doppler flow (Moor Instruments Ltd, England) was measured through the skull at the right temporal fossa (Sampei et al., 2000). Only the mice whose cerebral blood flow (CBF) showed a drop of over 85% of baseline just after MCAO were included which corresponds to dense ischemia as measured by quantitative blood flow methods (McCullough et al., 2005). Intra-ischemic neurological deficit was confirmed and scored as follows: 0, no deficit; 1, forelimb weakness and torso turning to the ipsilateral side when held by tail; 2, circling to affected side; 3, unable to bear weight on affected side; and 4, no spontaneous locomotor activity or barrel rolling. Reperfusion was documented with LDF.

2.3. TTC staining and measurement of brain infarct volume

Mice were euthanized at different time points of reperfusion. The brains were chilled at –80°C for 4 min to slightly harden the tissue. Five, 2 mm coronal sections were made from the olfactory bulb to the cerebellum and then stained with 1.5% TTC (Sigma, St. Louise, MO). The stained brain sections were captured with a digital camera (MicroPublisher 5.0 RTV, QIMAGING). The infarct area of each brain was measured in a blinded manner, using an image analysis software, Sigmascan Pro 5. The infarct volume was calculated by Swanson's method (Swanson et al., 1990) to correct for edema. The total volumes of both contralateral and ipsilateral hemisphere, and the volumes of the striatum, cortex in both hemispheres were measured and the infarct percentage was calculated as % contralateral structure to avoid mis-measurement secondary to edema.

2.4. Fluoro-Jade B staining

Mice were anesthetized with pentobarbital and intracardially perfused with phosphate buffered saline (PBS) for 1 min followed by 4% paraformaldehyde in PBS for 30 min. Following perfusion, brains were dissected and post-fixed in 4% paraformaldehyde in PBS for 4 hrs. After post-fixation, brains were transferred to 20% sucrose (w/v) in 0.1 M phosphate buffer (PB) until equilibrated. The tissue was then frozen in Tissue-Tek OCT mounting medium and 30 µm coronal sections were cut and placed in 0.1 M PB. Sections were subsequently spread on microscope slides and allowed to air dry. Air dried sections were mounted on microscope slides and placed in 70% ethanol and ultrapure water for 3 min followed by 3 washes in ultrapure water for 1 min each rinse. Sections were oxidized by soaking in a solution of 0.06% KMNO₄ for 15 min then washed 3 times in ultrapure water 1 min each. Sections were subsequently stained in 0.001% Fluoro-Jade B (Chemicon International, CA) in 0.1% acetic acid for 20 min.

Slides were subsequently washed 3 times in ultrapure water for 1 min each and dried overnight at room temperature. Dried slides were cleared in xylene and coverslips were mounted using Permount (Fisher Scientific). Digital images were collected on a Zeiss (Thornwood, NY) Axiovert 200M fitted with an apotome for optimal sectioning.

For the cortex and striatum, six 10× fields/animal and three 20× fields/animal were collected/animal (n=3 per time point) respectively. Fluoro-Jade B-positive cells were subsequently counted from each field using MacBiophotonics ImageJ software (NIH). For each animal, the total number of cells was averaged across fields of view for cortex, striatum, or total (cortex +striatum). These averages (avg # cells/field of view) were used for statistical analysis.

2.5. NeuN staining

Brains were prepared and sectioned as described previously (see Fluoro-Jade B staining). Sections were subsequently mounted onto gelatin-coated coverslips and allowed to air dry. Air dried sections were blocked and permeabilized in 0.1 M PB with 0.3% TX-100 (sigma) and 10% goat serum (PBTGS) for 1 hr. Following permeabilization, primary antibody (mouse monoclonal anti-NeuN 1:200; Chemicon International, Temecula, CA) was applied overnight at room temperature. Primary antibody was removed with 3 washes in PBTGS and secondary antibody (Alexa-594 conjugated to goat anti-mouse) and Hoechst (Molecular Probes, Eugene, OR) were applied for 1 hr at room temperature. Secondary antibody was removed with 3 consecutive washes in PBTGS, 0.1 M PB, and 0.5 M PB. Coverslips were mounted onto microscope slides with mounting medium (glycerol and p-phenylenediamine in PBS pH 9.0). Digital images were collected and quantification was performed as previously described (see Fluoro-Jade staining).

2.6. Statistical analysis

Data are expressed as mean $\pm$ standard error of the mean (SEM). Statistical comparisons were made by analysis of variance (ANOVA) with post-hoc correction except for behavioral assessment which was analyzed with non-parametric tests. Values were considered to be significant when P is less than 0.05. Experimenters were blinded to groups during infarct and behavioral analysis.

3. Results

3.1. Infarct Volume Increased from 1.5h to 24h Then Remained Stable From 24h to 7d after Stroke

The infarct area measurements on TTC stained brains indicated a small infarction in striatum ($23.20 \pm 3.32\%$, n=6/gp) and even smaller infarction in cortex ($8.83 \pm 1.84\%$, n=6/gp) at 1.5h of stroke, while at 6h the infarct enlarged to include the majority of the striatum ($44.24 \pm 5.85\%$, n=6/gp) and part of the cortex ($26.14 \pm 1.86\%$, n=6/gp). At earlier time points (1.5h~6h) after stroke, the infarct core is limited to the striatum, and the penumbra begins to be seen as pink staining around this central infarct core (Benedek et al., 2006)(Fig. 1). Almost all of the striatum was infarcted by 12 hours after stroke ($72.98 \pm 3.68\%$, n=6/gp) which also enlarged in the cortex ($40.70 \pm 6.44\%$, n=6/gp). The infarct volume then peaked at 24h of stroke (cortex vs. striatum: 63.74 ± 2.34 vs. $78.41 \pm 3.60\%$, n=6/gp). The ipsilateral ventricle disappeared due to edema formation. No further infarction growth was seen at either 3d or 7d of stroke, but less edema could be discerned as seen by reappearance of the ipsilateral ventricle (Fig. 1). The differences in total infarct volumes between 1.5h, 6h, 12h, and 24h were significant ($P < 0.05$) but there was no significant difference in infarct measurement at 24h, 3d and 7d of stroke ($P > 0.05$). We found that at later time points (12h~24h) the majority of the “penumbral” cortex had evolved into core leaving only a small wedge near the boundary of the core as measured by TTC at 3 and 7 days. There was no significant difference between infarction

volumes at 12h and 24h in striatum (Fig.2 A&B&C), and tissue is unlikely to be salvageable by any pharmacological treatment at this point.

Neurological deficits were observed and scored, and there was a trend toward better recovery as reperfusion time extended. Behavioral deficits were significantly improved at 24h, 3d or 7d of stroke when compared to early time points (1.5h, 6h, or 12h) ($P < 0.01$) (Fig.3B). Correlation analysis of neurobehavioral and morphological data was also performed. A significant negative linear relationship was seen between neurological score and infarct volume ($r = -0.988$, $P < 0.01$) (Fig.3C), indicating neurological deficits mostly reflect an impairment of function (penumbra) but not necessarily a structural lesion (core) early in the course of stroke induced by MCAO (Fig.3A). There was no infarction on TTC analysis or neurological deficits in sham animals (data not shown). Mortality was 10% at 24 hours, but reached 15% at 7 days.

3.2. Fluoro-Jade B Staining Revealed Degenerating Neurons Early After Stroke

Similar to what was observed with TTC staining, there was a time dependent increase in Fluoro-Jade B and NeuN staining in the cortex and striatum after stroke that peaked at 24 hours. Fluoro-Jade B is a marker of degenerating neurons; therefore staining should increase following injury (Butler et al., 2002). While TTC staining revealed no apparent infarct in the cortex and only a small infarcted region in the striatum after 1.5h of stroke, degenerating neurons were detected in both regions by Fluoro-Jade B (Fig.4B, arrow and inset). Similar to TTC, there was a time-dependent increase in degenerating neurons, which plateaued by 24 h of reperfusion. The quantification of Fluoro-Jade B positive cells demonstrated that degenerating neurons significantly increased in both the cortex and striatum from 1.5h to 24h, remained at this level from 24h to 3d, and then diminished to near baseline by 1 week after stroke (Fig.5A&B&C). Some Fluoro-Jade B positive cells were present in the contralateral hemispheres at 72h of stroke which were not present at earlier time points, suggesting that a delayed, transcallosal degeneration occurred in the cortex contralateral to the lesion (Adkins et al., 2004) after stroke.

3.3. Loss of NeuN Immunoreactivity Was Time-dependent After MCAO

Previous studies have demonstrated that loss of NeuN immunoreactivity occurs with CNS injury (Bendel et al., 2005; Ajmo et al., 2006). Similar to previous studies, we demonstrated a clear decrease in NeuN staining following ischemic injury as compared to contralateral or sham controls (Fig.4C). Similar to TTC staining, no infarct was visible in the cortex at the earliest time point (1.5h of stroke) but a small area of NeuN staining loss was seen in the striatum. Subsequently, a time-dependent decrease in NeuN staining that peaked between 6 and 24h of stroke was seen. There was a slight increase in cortical NeuN staining 3d after stroke as compared to earlier time points (6h), which may reflect of “stunned” or transiently damaged cells.

4. Discussion

The present study revealed several findings that are important for investigators that utilize murine models of stroke. Firstly, the transient MCAO model, which is widely used in stroke research, induces a peak volume of injury as delineated by TTC staining by 24 hours and remains unchanged through day 7 of reperfusion. A spatiotemporal evolution of core and penumbra was also seen; at earlier time points the histological infarct core, as measured by TTC is in the striatum, and the viable tissue was around this core, and included the cortex. Subsequently the TTC-defined core expands to involve most of the cortex supplied by the MCA. Secondly, there is a negative correlation between volume of injury, as defined by TTC staining, and neurological dysfunction. Thirdly, the highest number of Fluoro-Jade B positive cells was seen at 24 hours after reperfusion, corresponding with the peak volume of injury by TTC. Scattered positive cells were seen in the cortex at early time points, prior to loss of TTC.

Fluoro-Jade B positive cells were seen in the contralateral hemisphere at 72h of stroke and not present at earlier time points, suggesting that a delayed, transcallosal degeneration occurred in the cortex opposite the lesion (Adkins et al., 2004) after stroke. Finally, these studies demonstrate that Fluoro-Jade B is an appropriate agent for staining acutely degenerating cells, however it may under-estimate lesion size if used at later time-points as staining levels rapidly decrease.

This is the first paper to examine the chronology of infarct up to 7 days after stroke in the transient MCAO model in mice, although transient MCAO has been used for decades. The vast majority of previous studies were done in rats; however mice are now becoming increasingly used with the development of knockout and transgenic technology. Infarct development appears to be more rapid in mice than in rats as previous studies of permanent “non-reperfusion” MCAO models demonstrated an increase in infarct volumes even as late as 3d after stroke (Garcia et al., 1993; Aspey et al., 1998), however these animals had no restoration of blood flow. In the present study, infarction was mainly seen in the striatum at 1.5h of stroke, an area that is known to be especially vulnerable to ischemia due to its lack of collateral blood supply. With prolongation of reperfusion and survival times, the infarct extended to the other parts of the MCA territory. The time-dependent increase in infarct volume to the maximum measured infarct at 24 h of reperfusion is consistent with several MRI experiments in rat models of transient MCAO (van Lookeren Campagne et al., 1999; Hoehn et al., 2001). These studies demonstrated that diffusion disturbances initially resolved after reperfusion, but reappeared several hours later despite restoration of cerebral blood flow. The infarct then evolved by 24 hours into an area of injury of similar magnitude to the initial diffusion disturbance as measured by a reduced apparent diffusion coefficient (ADC) and prolonged T2 signals. Former studies (Dereski et al., 1993; Garcia et al., 1993) also indicated that twelve hours after the onset of ischemia, lesion development slowed considerably or ceased altogether. This was confirmed by our histological analysis, as no subsequent infarct growth was seen after 24 hours. Several previous studies have also revealed that apoptosis induced by transient MCAO model reached its peak at 24–48 h of stroke (Linnik et al., 1995; Chen et al., 1997). It is possible that the peak of delayed cell death is at 48 hours, a time-point we did not directly assess. This would be best assessed with Fluoro-Jade B, as it is specific for acutely degenerating neurons (Schmued and Hopkins, 2000; Duckworth et al., 2005). However, the fact that infarct did not change between 24hrs and 3d by TTC and NeuN staining make this unlikely. With the increasing use of reperfusion therapies such as clot retrieval and thrombolytics, more patients have successful reperfusion (Juttler et al., 2006). It is crucial to understand the evolution of infarct in transient and embolic occlusion models as these translate best to the ischemia/reperfusion injury seen in clinical populations (Chopp et al., 1999). Our study emphasizes that even the most potent neuroprotective agent will not reduce injury if given 12h after stroke, and the time window is even smaller (6h) for striatal salvage. Despite this, many of our clinical studies administer agents as late as 24 h after the event, diluting any potential therapeutic effects. Of course, the human brain is vastly more complex and undoubtedly has a larger “penumbra” than that of the mouse, but this provides one explanation for the numerous clinical failures of promising neuroprotective agents (Ginsberg, 2008). As all of the MCA territory eventually infarcted in this model, the only way to determine reversibility would be to shorten the ischemic duration. It remains to be determined if “penumbra” is a useful term in preclinical stroke models using small animals that develop rapid infarction as even with reperfusion, there is often little reversibility to the area “at risk”.

In this study we also show that at early time points after stroke, the cortex surrounding the striatum is likely the molecular penumbra as only scattered neuronal loss is seen. At later time points, almost the entire penumbra has been recruited into the core as assessed by TTC staining at both 24 hours and 7 days. Therefore studies on infarct core and penumbra can only be reliably performed early after MCAO (no later than 12 hours) in mouse models. This spatiotemporal

histological delineation of core and penumbra will be of use for investigators investigating early “penumbral” changes with techniques such as Western or PCR, as these areas are often selectively dissected and examined.

In addition to TTC staining, we confirmed the presence and/or absence of infarct by Fluoro-Jade B and NeuN staining. Fluoro-Jade B (1.5h following reperfusion) staining was seen at very early time points after injury in the cortex prior to the loss of TTC staining. Neuronal degeneration increased over time in both the ipsilateral hemisphere, as well as the contralateral hemisphere (72h), reflective of the transcallosal degeneration (Adkins et al., 2004) or metabolic compromise induced by edema (Lafuente et al., 2007) which spreads to these remote areas. However, Fluoro-Jade B positive cells decreased to base-line values by 7d after stroke. This suggests that Fluoro-Jade B is a sensitive marker for acute neuronal injury, but may not be as useful for delayed assessment of damage, which is more accurately performed with TTC. This characteristic of Fluoro-Jade B has not been reported previously, and may be due to the decomposition of poly-amines in the necrotic neurons that Fluoro-Jade B binds to (Schmued and Hopkins, 2000) and is an important finding of this work. Our findings are quite different from that of a former study (Duckworth et al., 2005) that suggested that Fluoro-Jade B staining was most robust 4 days after MCAO. This is likely due to differences in the brain region examined and suture placement technique. This former study focused only on hippocampal regions, and found limited staining in the classic “MCAO” territory which includes the striatum and overlying cortex (see Figure 1). The hippocampus is mainly supplied by the posterior cerebral artery (PCA) rather than the MCA. Although this structure is variably affected by MCAO, in our hands this occurs less than 10% of the time if the suture tip is placed in the origin of the MCA. No measurements of cerebral blood flow were performed in the studies of Duckworth et al., and both TTC and Fluoro-Jade B staining patterns were atypical for MCAO. Our work demonstrates that Fluoro-Jade B may not be appropriate for assessing injury after 48–72 hours despite its high sensitivity. Fluoro-Jade B is complementary to other histological methods, but cannot substitute for TTC or standard histology for infarct analysis.

NeuN staining also demonstrated a time-dependent decrease in neurons early after stroke, though a slight increase at 72h was seen in the cortex. This is consistent with former studies (Hossmann, 1993; Unal-Cevik et al., 2004) that suggested loss of NeuN staining may indicate a change in antigenicity of NeuN protein rather than cell death, and some neurons may regain their staining pattern after repair. Therefore NeuN may be a more sensitive marker for injured neuron early after ischemic challenge. Our data emphasize the importance of characterizing histological methods prior to their use in neuroprotective studies to avoid erroneous conclusions. Importantly, all three histological methods demonstrated that the peak of damage induced by MCAO was at 24 h following reperfusion.

Apart from establishment of a precise time-course for infarct progression after transient MCAO, our data address another important aspect of injury analysis. In our study the neurological deficit scores decreased while infarct volumes increased during the early period (1h to 1d) of reperfusion, and they were negatively correlated. It is known that behavioral deficits following experimental ischemic injury often do not reliably correlate with the size of the infarct, especially with the use of very simple behavioral scoring (Bederson et al., 1986; Wahl et al., 1992; Grabowski et al., 1993; Alexis et al., 1996). Early in the course of stroke neurological deficits reflect injury to both the core and the penumbra (Baird et al., 1997). As collateral perfusion develops, brain function can be restored within the penumbra (Furlan et al., 1996). Thus, symptoms can regress while the histological lesion grows. Residual anesthetic effects may also contribute to the early behavioral deficits seen in this study. After days to weeks, neurological deficits reflect the size and location of the structural lesion more closely. Recovery at these later time points is best explained by plasticity and tissue reorganization (Dirnagl et al., 1999). Animals may also improve despite lesion evolution as the contralateral

hemisphere may compensate for deficits of the ipsilateral hemisphere (Renolleau et al., 2007). Clinical data also support such a negative correlation between symptoms and lesion evolution (Dereski et al., 1993; Jorgensen et al., 1995; Baird et al., 1997).

There are several limitations of this study. We only examined ischemic injury in the cortex and striatum, and did not look at other area of brain (hippocampus etc.) distant to the area of injury and MCAO territory. Neither did we examine these methods in a model of neuroprotection (hypothermia etc.), in which the time course of degeneration might change. Also none of the histological methods used determine whether non-injured neurons or early penumbral neurons are functional. We did not examine chronic endpoints (ie., 6 weeks), although we have previously found that cresyl violet staining and measurements of tissue atrophy are more reliable at later time points (Li et al., 2004). Additionally, Fluoro-Jade B and NeuN are neuronal stains, and other cell types clearly contribute to infarct size. It is difficult to directly compare these methods without double labeling the same tissue sections with Fluoro-Jade B, NeuN and cresyl violet.

In conclusion, our study shows a specific pattern of ischemic lesion evolution in a mouse model of 90 min transient MCAO, i.e. the infarct increases until 24 hours of stroke when it becomes stable. Furthermore, the study revealed a spatiotemporal localization of infarct core and penumbra that suggests that the penumbra around the core may only exist for several hours after stroke. This should be considered when developing clinical trials. We also show that other methods of injury assessment (i.e. Fluoro-Jade B and NeuN) may be beneficial for showing different aspects of injury, and each has strengths and weaknesses. In addition, neurological deficits on simple behavioral scoring scales do not necessarily reflect the development of ischemic lesion, and may not be useful for assessing the efficacy of a neuroprotective agent. Most importantly, neuroprotective therapies must be administered early after stroke onset if we hope to salvage ischemic tissue.

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Fig.1. TTC staining at different time points of reperfusion. White indicates infarction; red staining indicates normal tissue. Times post-reperfusion are indicated in the bottom right corner. The infarct area increases from 1.5h to 24h (**A,B,C,D**), and remains stable from 24h to 7d (**D,E,F**). The ipsilateral ventricle cannot be seen at 24h (**D**), but re-emerges by 7d (**F**).

Fig.2.

Quantification of infarct volumes based on TTC staining. Evolution of infarct volume over time in cortex (**A**), striatum (**B**), and total hemisphere (**C**). Infarct reached its peak at 24h in cortex and total hemisphere, and at 12h in striatum. N=7 animals/time point. * $P < 0.05$.

Fig.3.

Relationship between neurological deficits and evolvement of infarct. (A) Diagrammatic representation of mouse brain depicting the concept that neurological deficits mostly reflect impairment of function (penumbra, white circle) but not necessarily a structural lesion (core, black circle) early in the course of stroke. Over time the penumbra shrinks while the core grows. (B) Neurological scores decreased over time since reperfusion. Neurological deficits were significantly improved by 24h of stroke. (C) Correlation analysis of neurological scores at 6 time points and equivalent infarct volumes. A significant negative relationship was seen between infarct volume and behavioral score. N=6 animals/time point. * $P < 0.05$.

Fig.4.

Changes in Fluoro-Jade B and NeuN staining reflect TTC time course. (A) Coronal section of mouse brain stained with TTC 24 hours following transient MCAO. Boxed areas illustrate cortical or striatal regions represented in the Fluoro-Jade B and NeuN images. (B) Representative Fluoro-Jade B staining of cortex and striatum following MCAO. Arrows (1.5 h) and insets denote early Fluoro-Jade B positive neurons. (C) Representative NeuN immunostaining of cortex and striatum following MCAO. Asterisks and insets denote examples of normal NeuN immunostaining (contralateral). (B,C) Times post-reperfusion are indicated in the bottom left corner. Dotted lines represent the pial surface. N=4 animals/time point. Scale bars = 100 μ M except Fluoro-Jade striatum, scale bar = 50 μ M.

Fig.5. Quantification of the Fluoro-Jade B positive cells in cortex (**A**), striatum (**B**) and the total hemisphere (**C**) at different time points after stroke. Positive staining increased during the early period of stroke and peaked at 24 hr of stroke. Some neurons in the cortex of contralateral hemisphere also had evidence of Fluoro-Jade B positivity at 72h of stroke. N=4 animals/time point. $P^* < 0.05$.