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Pre-clinical stroke research - advantages and disadvantages of the most common rodent models of focal ischaemia.

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Short title: **Animal models of focal cerebral ischaemia**

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Summary

This review describes the most commonly used **rodent** models and outcome measures in pre-clinical stroke research and discusses their strengths and limitations. **Most models involve** permanent or transient middle cerebral artery occlusion with therapeutic agents tested for their ability to reduce stroke-induced infarcts and improve neurological deficits. Many drugs have demonstrated pre-clinical efficacy but, other than thrombolytics, **which restore blood flow**, none have demonstrated efficacy in clinical trials. This failure to translate efficacy from bench to bedside is discussed alongside achievable steps to improve the ability of preclinical research to predict clinical efficacy: 1. **Improvements in study quality and reporting**. Study design must include randomisation, blinding and pre-defined **inclusion/exclusion criteria and journal editors have the power to ensure statements on these and mortality data are included in** pre-clinical publications.; 2. Negative and neutral studies must be published to enable pre-clinical meta-analyses and systematic reviews to more accurately predict drug efficacy in man; 3. **Pre-clinical groups should work within networks and agree on standardised procedures for assessing final infarct and functional outcome. This will improve research quality, timeliness and translational capacity ;** 4. G**reater uptake and improvements in non-invasive diagnostic imaging** to detect and study potentially salvageable penumbral tissue, the target for acute neuroprotection. **Drug effects on penumbra lifespan studied serially, followed by assessment of behavioural outcome and infarct within in the same animal group, will increase the power to detect drug efficacy pre-clinically. Similar progress in detecting drug efficacy clinically will follow from patient recruitment into acute stroke trials based on evidence of remaining penumbra.**

Abbreviations:

ADC, apparent diffusion coefficient
AMPA, Alpha-Amino-3-Hydroxy-5-Methyl-4-Isoxazole Propionic Acid
BCCAO, bilateral common carotid artery occlusion
CBF, cerebral blood flow
CCAO, common carotid artery occlusion
$CMRO_2$, cerebral metabolic rate of oxygen
DWI, diffusion-weighted imaging
HMPAO, Hexamethylpropyleneamine Oxime
MCAO, middle cerebral artery occlusion
NMDA, N-Methyl-D-Aspartate
OEF, oxygen extraction fraction
PET, positron emission tomography
PWI, perfusion-weighted imaging
rt-PA, recombinant tissue plasminogen activator
TTC, Triphenyltetrazolium chloride

Stroke – some facts & figures

Stroke is a common and devastating disease. It is the second leading cause of death after coronary heart disease in developed countries (Donnan et al., 2008) and is the greatest cause of disability, leaving 50% of survivors permanently disabled. Identification of risk factors such as arterial hypertension, high cholesterol, diabetes and obesity have led to successful stroke prevention strategies (McArthur and Lees 2010). **Some progress has been made in effective rehabilitation following the acute stroke period (Langhorne, et al., 2009, Kalra, 2010) with evidence for improvements in motor recovery associated with high intensity and repetitive task-specific practice (e.g. constraint-induced movement therapy).   However, there is insufficient data at present to identify specific interventions which would be widely applicable**.   **The area which has progressed least in the last 15 years, despite a significant pre-clinical and clinical research effort is identification of acute therapies which can limit stroke-induced brain damage and disability. There is still no clinically effective neuroprotective drug licensed for stroke.**

Strokes are classified as haemorrhagic or occlusive/ischaemic, with the majority (80%) falling into the latter category (Heiss, 2010).   **For occlusive stroke, thrombolysis with recombinant tissue plasminogen activator (rt-PA), and care provided by specialised acute stroke units, have been proven to improve outcome (Donnan et al., 2008).** Rt-PA is directed at the occluding blood clot and by enhancing the endogenous formation of plasmin from plasminogen, helps dissolve the clot by disruption of fibrin thereby enabling restoration of blood flow to ischaemic tissues. However, it does not offer any

direct protection to ischaemic tissues, and in fact has neurotoxic potential within the parenchyma if not confined within the vasculature (Yepes et al., 2009). Since thrombolysis with rt-PA, also carries a risk of symptomatic intracranial haemorrhage (~2%), and probability of benefit declined rapidly with time beyond 4.5 post- stroke (Lees et al., 2010, Hacke et al., 2008, NINDS & Stroke rt-PA Stroke Study Group, 1995) guidelines recommend treatment restricted to the first 4.5 hours following stroke onset **(del Zoppo et al., 2009, ESO, 2008)**. **In England, Wales and Northern Ireland, only 3.8% of patients are reported to receive the treatment (Royal College of Physicians, 2010 National Sentinel Audit of Stroke). Similar figures are reported in the US (~2% of eligible patients, Kleindorfer, et al., 2008) leaving the majority of stroke patients with no access to effective drug treatments.**

Targeting the ischaemic cascade

The last 30 years have seen significant progress in our understanding of the pathophysiology of ischemic stroke and the identification of mechanisms that contribute to tissue damage. **The ischaemic cascade, starting with a severe focal reduction in cerebral blood flow (CBF) and culminating in cell death and infarction, has many intervening and interlinking steps which have provided a number of potential drug targets (reviewed in Moskowitz, et al., 2010). Pharmacological agents have been developed to block the major mediators of injury: high, toxic concentrations of extracellular glutamate, intracellular calcium and free radicals. Ischaemia causes widespread cellular depolarisation, with Na^+, K^+, Ca^{2+} and Cl^- fluxes through activated ionotropic receptors and gated ion channels, loss of ionic concentration gradients across cell membranes, uncontrolled neurotransmitter release and**

glutamate-induced excitotoxicity.   Calcium dysregulation is the trigger for enzymatic destruction of the cell and its components with activation of proteases, lipases and nucleases. Activation of enzymes such as neuronal nitric oxide synthase, NADPH oxidase, cyclo-oxygenase and lipo-oxygenase generates free radicals which attack cellular membranes and dismantle key functions of the cell such as ATP production, protein synthesis and the sequestration and storage of intracellular calcium within intracellular organelles. Inflammation represents another key component of the ischaemic cascade with pro-inflammatory phospholipase A2 liberating arachidonic acid, a substrate for cyclo-oxygenase and lipo-oxygenase and precursor of prostaglandins and leukotrienes.   The culmination of this complex, self generating programme of events is either rapid necrotic cell death or delayed apoptotic cell death depending on the severity and duration of ischaemia. D**rug targets include neurotransmitter receptors and ion-channels, free radicals, cytokines and inflammatory mediators, enzymes and membranes. **Drugs developed to block these targets are often referred to as "neuroprotective". However, their role is not simply to protect the neurone (i.e. cell body, axon and nerve terminals), but rather the neurovascular unit: the neurone plus the supporting glial and vascular cells within its immediate environment which includes astrocytes, pericytes, microglia, oligodendrocytes and the endothelial cells of microvessels (del Zoppo,2010, Moskowitz et al, 2010).** A number of drugs have demonstrated neuroprotective efficacy in pre-clinical studies **where focal ischaemic insults produce reproducible infarcts and behavioural deficits**(O'Collins et al., 2006). **However, human stroke is a highly heterogeneous condition and treatment targeted at a single mechanism in the**

ischaemic cascade is unlikely to be universally effective. Combination therapy, or single drugs with multiple targets and actions are more likely to be effective.

Pre-clinical stroke research

Pre-clinical stroke research is carried out using *in vitro* and *in vivo* models. I*n vitro* studies use neuronal or mixed cell cultures and organotypic slice preparations as model systems which recreate some of the consequences of a focal ischaemic insult. Cells and tissues are exposed to excitotoxic concentrations of glutamate, NMDA, AMPA, Kainate, hypoxia or oxygen glucose deprivation. **Compounds are tested for their ability to reduce cell death and inhibit the targeted deleterious mechanism** (Richard, et al., 2010) and the most effective are then taken forward for *in vivo* evaluation.

Stroke is studied in animal models by permanently or transiently occluding a cerebral artery which causes a severe reduction in CBF in the territory of that artery. **In other words, a *focal ischaemic insult* is induced in a defined region of brain tissue. This is different from a *global ischaemic insult*, where the entire brain or forebrain is exposed to a severe reduction in its blood supply (e.g. as a consequence of cardiac arrest, severe hypotension or peripheral haemorrhage, strangulation or drowning). Animal models of both global and focal ischaemia have been developed and reveal differences in the mechanisms of injury and patterns of brain damage (reviewed in Ginsberg & Busto, 1989; Traystman, 2003).**

Models of focal cerebral ischaemia have been established in a number of species, most commonly in lysencephalic species such as rats and mice. Guidelines for drug

development recommend that once efficacy is established in rodents, studies are carried out in gyrencephalic species such as cats, pigs and non-human primates before taking the drug through to studies in man (STAIR, 1999). The current review focuses specifically on rodent models of focal cerebral ischaemia. Rodents are the species of first choice for a number of reasons. I**t is generally considered ethically** more acceptable to use rodents rather than higher mammals. Animal and maintenance costs are low and the **vascular anatomy is similar to man.** Rodent neuroanatomy and the cascade of molecular mechanisms leading to ischaemic cell death are well characterised **with additional information from stroke studies in transgenic mice where specific genes are overexpressed or knocked out/down. Rodent strains and transgenic mice developed to display comorbidities such as hypertension, atherosclerosis, diabetes and obesity are also available.** Relevance to human stroke is supported by the knowledge that these mechanisms are similar between rodents and man and that **thrombolysis, the one treatment known to be efficacious in man, is equally efficacious in rodent focal ischaemia models** (Back et al, 2007).

Selection of the model

A wide range of rodent focal cerebral ischaemia models are available for preclinical drug development and selection of the most pertinent model will depend on the class of drug under study and its perceived mechanism of action. For example, embolic stroke models are used to test new thrombolytic drugs, models of transient focal ischaemia are commonly used to test free radical scavengers and anti-inflammatory

agents and the first glutamate antagonists to be developed were tested in models of permanent focal ischaemia. However, given the heterogeneity of human stroke, drugs should demonstrate preclinical efficacy in a range of different models and species before being considered for translation through to clinical trials.

Middle cerebral artery occlusion (MCAO).

The middle cerebral artery (MCA) is the most commonly affected blood vessel in human occlusive/ischaemic stroke (Mohr et al., 1986) and is the artery most commonly targeted in rodent stroke models (see Figure 1). A range of MCAO models have been developed and there are advantages and disadvantages associated with each approach (Table 1). Following surgical exposure of the blood vessel via a craniectomy, the MCA can be occluded in a number of ways: by electrocoagulation of the blood within it and destruction of the blood vessel *per se* (Tamura et al, 1981); mechanically, by applying an occluding device such as a clip or ligature (van Bruggen et al, 1999); or pharmacologically by applying a potent and prolonged vasoconstrictor such as endothelin-1 (Macrae et al., 1993). MCAO models which do not require a craniectomy include stereotaxic injection of endothelin-1 into parenchyma adjacent to the MCA which results in a longer lasting vasoconstriction (Sharkey et al., 1993).   Alternatively, the skull is left intact and an intravascular approach via the carotid artery used to advance a filament or embolus to the point where it blocks the origin of the MCA (Koizumi et al., 1986; Longa et al., 1989; Zhang et al., 1997).

MCA occlusion by electrocoagulation or application of an occluding device

All the models within this category require a craniectomy and section of the dura mater to expose the MCA. Models which require a craniectomy are associated with low or absent mortality **(Table 2)** since the craniectomy prevents ischaemia-induced increases in intracranial pressure. This can be a significant advantage, particularly where large strokes are studied over a number of days. Oedema and brain swelling correlate with infarct size and the increases in brain volume associated with large MCA strokes over the first 24-48 hours post-stroke would cause significant mortality if the skull was intact.

Following surgical exposure, the MCA is permanently occluded by coagulating the blood within it using an electric current passed through the tips of fine diathermy forceps (Tamura et al., 1981). The occluded portion of the artery is then cut confirming complete occlusion. **Electrocoagulation models have been successfully adapted for use in larger species such as the cat (Bullock et al., 1990), miniature pig (Imai et al**, **2006**) **and baboon (Yonas et al., 1981).** Maintaining sterile conditions and careful post–stroke care of the wound is particularly important to avoid any infection associated with the surgery **which will increase ischaemic damage and confound the ability to determine drug-induced effects on infarct size and functional recovery**. The main advantages of the model are good reproducibility in infarct size and functional deficit, low mortality, visual confirmation of successful MCAO and the ability to adapt the model to produce infarcts of different size and location. The main disadvantages are that the model

induces permanent focal ischaemia and is not therefore suitable for investigation **of thrombolytic agents** or drugs designed to target the reperfusion phase following ischaemia. Inducing MCAO is technically demanding. Exposing the artery and applying electrocoagulation without rupturing the blood vessel or damaging the underlying cortex requires significant surgical skill. The surgery required for proximal MCA occlusion can also cause jaw alignment problems in rats requiring replacement of standard chow with soft diet and regular monitoring and tooth filing to avoid overgrowth.

Alternative MCA occlusion models use devices such as microaneurysm clips, hooks (used to lift the artery from the cortical surface until flow ceases), ligatures and in, larger species, inflatable cuffs to occlude the artery remotely (Shigeno et al., 1985; van Bruggen et al., 1999). These models have the advantage of control over the duration of ischaemia and allow subsequent reperfusion of ischaemic tissue. They provide visual confirmation of successful MCAO and reperfusion when the occluding device is removed. However, microaneurysm clips are too small to apply by hand in rats and have to be loaded into a special applicator for attachment to the MCA. Applying and removing the clips without damaging the artery is technically difficult, particularly when targeting the proximal MCA. Other disadvantages include greater variability in infarct size compared to electrocoagulation models, particularly when a single point on the MCA is occluded. Reproducibility is improved by occluding the artery at more than one site, combining MCAO with hypotension or ipsilateral common carotid artery occlusion, or using rat strains with poor collateral supply (e.g. the spontaneously hypertensive rat and the spontaneously hypertensive stroke-prone rat, see Coyle & Jokelainen, 1983) to increase

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the severity of the ischaemic insult. However, when considering neuroprotection studies, it is worth considering that steps such as these taken to improve reproducibility in infarct size are also likely to result in less potentially salvageable penumbral tissue being available for rescue.

Modifications to the model

The position and length of MCA occluded provides some control over the severity of the ischaemic insult, the amount of penumbral tissue available for rescue, and the neurological deficit produced. Proximal occlusion of a long segment of the MCA including the lenticulostriate branches gives rise to a large consistent ischaemia and infarct in cortical and subcortical structures (see electrocoagulation, Figure 1) and a reproducible neurological deficit. However, the greater the volume of ischaemic tissue within the MCA territory the less penumbral tissue will be available for the drug under test to rescue. Occlusion of a shorter (1-2 mm) segment of the MCA, distal to the inferior cerebral vein, will induce a smaller region of ischaemia and infarct, mainly confined to the cortex, and provide a larger volume of target penumbral tissue. However, the neurological deficit produced will be much milder and more difficult to detect.

MCA occlusion induced pharmacologically

The peptide endothelin-1 (ET-1) lends itself to cerebral ischaemia research because it induces profound and prolonged vasoconstriction of cerebral vessels (Asano et al. 1989, Robinson and McCulloch 1990). ET-1 topically applied to the abluminal surface of the exposed MCA induces an ischaemic insult of sufficient severity and duration to produce

a reproducible infarct (Figure 1). This model was developed in the rat (Macrae et al. 1993) and has been adapted for use in marmosets (Virley et al., 2004). Advantages include visual confirmation of ischaemia, with some control over the severity and duration by adjusting the ET-1 concentration, a gradual reperfusion and low mortality. Disadvantages include stability and potency issues relating to peptides which can increase variability. An alternative model, where ET-1 is administered by stereotaxic injection into piriform cortex immediately adjacent to the proximal MCA, is less demanding surgically, and induces a more persistent ischaemia that topical application (Figure 1, Sharkey et al. 1993). This model is more useful for fast throughput screening of drugs and has been adapted for induction of stroke in the conscious animal by injecting ET-1 via a previously implanted guide cannula. **If endothelin -1 is replaced with endothelin-3, it is possible to reverse vasoconstriction and downstream ischaemia with a second injection of an endothelin-A receptor antagonist (Henshall et al., 1999). Intraparenchymal injection of ET-1 has been adapted for anterior cerebral artery occlusion (Ward et al., 1998)** and selective white matter ischaemia in the internal capsule (Frost et al., 2006; Lecrux et al., 2008).

MCA occlusion by intraluminal filament

The intraluminal filament (or suture) method is currently the most widely used model of focal ischaemia in rats and mice, and is used to induce both permanent and transient ischaemia. No craniectomy is required as the occluding device, a flexible monofilament, is introduced directly into the internal carotid artery (or with modification, via the external carotid artery) and advanced until it blocks the origin of the MCA (Figure 1).

The method was introduced by Koizumi (Koizumi et al. 1986) with the first of many

modifications described by Longa (Longa et al. 1989). The advantages of this model are

the ability to precisely control the duration of ischaemia and the fact it is technically

easier to master than the craniectomy models. The disadvantages mainly relate to

difficulties in reproducibility and mortality. Modifications to improve reproducibility

include filament construction, coating, & design of the tip, and are covered in a recent

review (Durukan and Tatlisumak, 2007). Filament diameter is carefully matched to a

defined body weight range in rodents to ensure adequate occlusion of the origin of the

MCA. Laser Doppler flowmetry probes, placed on the skull above the sensorimotor

cortex, are commonly used to guide correct placement of the filament, to assess the

severity of the drop in CBF and to avoid advancing the filament too far, thereby risking

blood vessel puncture and haemorrhage. Filament insertion to block the origin of the

MCA should induce cortical and subcortical ischaemia but tissue infarction may be

confined to subcortical structures with minimal cortical involvement if the rodent strain

used has good collateral blood supply to the cortex (Coyle and Jokkelainan, 1983;

Duverger and Mackenzie, 1988). Increasing the duration of ischaemia, tandem occlusion

of the carotid artery(s) or hypotension are used to increase cortical involvement.   Since

the filament is advanced along the internal carotid artery to reach the origin of the MCA,

depending on filament construction, other arteries which branch off the internal carotid,

proximal to the origin of the MCA, may also be occluded by the filament. Therefore,

although this model is employed for MCA occlusion, other arteries, such as the anterior

choroidal and hypothalamic arteries may also be blocked, leading to damage beyond

MCA territory, and corresponding associated deficits (e.g. hyperthermia associated with ischaemic damage to hypothalamus, Li et al. 1999).

For longitudinal studies, problems with morbidity and mortality are encountered when ischaemia is permanent or prolonged (e.g. $\geq$ 90 mins). This is manifest within the first 24-48 hours in animals with large MCA territory strokes due to brain swelling and increased intracranial pressure (**Table 2**).

Embolic MCA occlusion models

Embolic models fall into two main categories: 1. Embolisation induced by the introduction of blood clots or artificial emboli; 2. Localised chemically initiated thromboembolism. Thromboemboli cause most human strokes and therefore models that mimic this type of occlusion are useful for testing new thrombolytic agents. Blood drawn from the animal to form autologous clots of specific size and composition *ex vivo* are subsequently (e.g. 24 hours later) introduced into the cerebral circulation via a cannula inserted and advanced along the internal carotid artery (using a similar approach to intraluminal filament insertion). Longa et al. (1989) first described the induction of thromboembolic stroke in the rat, with subsequent modifications published by Busch et al.(1997). Similar models have been established in mice (Zhang et al., 1997), rabbits (Lapchak et al., 2000), cats (Yamaguchi et al., 2000) and non-human primates (Kito et al., 2001, Watanabe et al., 1977). The advantages of embolic models include their clinical relevance, the fact that the surgery required is straightforward and does not involve a craniectomy. When the clot is correctly positioned, large infarcts can be induced which include both cortical and sub-cortical structures, and give rise to

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pronounced behavioural deficits. The blood clot is broken down and reperfusion induced

by administration of thrombolytics such as recombinant tissue-type plasminogen

activator (rt-PA). **Meta-analysis of the pre-clinical literature demonstrates**

similarities with the clinical literature: rt-PA administered within the first 3-4 hours

post-stroke in rodents reduces infarct volume and improves neurobehavioural

scores in rats, with an increased probability of haemorrhage (Perel et al., 2007).

However, quality scores for the 113 studies identified was poor with evidence of

publication bias. There are a number of pertinent disadvantages associated with embolic

models. Intravascular introduction of emboli can result in multifocal ischaemia with

significant variability in infarct size and location as well as early autolysis, depending on

emboli composition (see Busch et al. 1997). Brain haemorrhage is frequent with embolic

models and mortality rates are generally much higher (e.g. ≥30% in rat) than for other

models (**Table 2**).

Composition and stability of the embolus are important in preventing spontaneous clot

disintegration. Thrombolytic breakdown of the blood clot and recanalisation of the

occluded artery is related to the amount of red cells in the emboli and inversely related to

the volume of the emboli, fibrin content and density of the clots. (Overgaard et al., 1994).

Since early *in vitro* studies demonstrated that the rat's fibrinolytic system was 10-fold

less sensitive to rtPA than the human system (Korninger and Collen, 1981), the majority

of *in vivo* studies have used rt-PA at a 10 fold higher dose (10mg/kg) than the human

dose (0.9 mgs/kg). Successfull thrombolysis and reperfusion achieved with 10 mg/kg rt-

PA administered within 2-4 hours of stroke reduced infarct size, and improved survival

and neurological deficits in rodent models. However, the higher dose may not be

necessary or recommended for future pre-clinical research since a recent study,

comparing the two doses administered 45 minutes after stroke in a rat embolic model, has

demonstrated that the lower (human) dose was equally effective in inducing reperfusion

(although slower in onset) and significantly reduced infarct size and oedema. (Haelewyn

et al, 2010). The 10mg/kg dose is no longer effective beyond 4 hours post-stroke and

increases infarct size and haemorrhagic transformation (Kano et al, 2000, Lapchak et al.,

2010).

Artificial emboli

A variety of materials have been used to form non-clot emboli (see Durukan and

Tatlisumak 2007), the most common being microspheres of defined diameter.

Suspensions of calibrated microspheres injected into the internal carotid artery produce

microembolisation and slowly evolving ischaemic lesions and a model in rabbits has been

used for the study of stroke pharmacotherapy (Zivin et al. 1987). However, the utility of

microspheres is more limited than clot-based emboli in that vessel occlusion is permanent

with no potential for recanalisation, either spontaneously or interventionally (Mayzel-

Oreg et al. 2004).

Chemically initiated thromboembolism.

Chemically initiated thromboembolism is induced photochemically by systemic injection

of a photosensitive dye (e.g. Rose Bengal or erythrosin B) in combination with irradiation

through the exposed skull with light of a specific wavelength (Watson et al. 1985, rats;

Sugimori et al. 2004, mice). The reaction between the light and the intravascular dye

generates oxygen radicals causing peroxidation of endothelial lipids and blood elements, thereby inducing platelet aggregation and thrombosis (Ginsberg and Busto 1989). **The model is minimally invasive, produces a reproducible cortical photothrombosis in both rats and mice and uses specialist laser equipment which provides precision over the exact location of ischaemia.** Mortality associated with the model is low and the size and depth of ischaemic damage can be controlled by adjusting the plasma concentration of the dye and the intensity and duration of light. Limitations of the model include its end-arterial occlusive nature which makes the lesion resistant to flow enhancement strategies. **The rapid progression of ischaemic damage is associated with significant early cytotoxic and vasogenic oedema formation which is different from the situation in human stroke.** Early versions of the model induced a severe insult with rapidly evolving ischaemic damage and no salvageable penumbral tissue but more recent modifications, using a ring model and modified laser parameters, display "region-at-risk" in the ring-encircled interior region (Wester et al. 1995, Hilger et al. 2004). **Further developments include the use of optical fibres stereotaxically implanted or directed at the surgically exposed proximal MCA to produce photothrombosis in subcortical sites. These models demonstrate evidence of penumbra on MRI scans and reversal of ischaemia with rt-PA (Kuroiwa et al., 2009, Chen et al., 2007).**

Recently, a new model of thromboembolic stroke has been developed in the mouse where *in situ* microinjection of thrombin into a branch of the MCA is used to trigger local clot formation (Figure 1, Orset et al. 2007, 2010). A craniectomy is required to expose the

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MCA branch and insertion of the tip of a thrombin-filled micropipette into the blood vessel. Thrombin injection induces the formation of a clot *in situ,* a persistent downstream ischaemia and a reproducible cortical infarct. Recanalisation of the occluded branch is achievable using intravenous rt-PA (10 mg/kg i.v. administered 20 minutes later) with restoration of cortical cerebral blood flow and reduced final infarct size. The advantages of this thromboembolic model include more precise control of the location of ischaemia and the ability to visually determine the permanency of vessel occlusion, and successful recanalisation in thrombolysis studies. The size and location of cortical ischaemia and infarction is more reproducible than for the autologous clot models and mortality is minimal. Disadvantages include the requirement for a craniotomy, the possibility of spontaneous thrombus disruption and reperfusion with microclot formation (although this can be identified visually and built into exclusion criteria), and the lack of a robust neurological deficit, due to the small cortical lesion.

Outcome measures and endpoints

The major preclinical outcome measures for stroke pharmacotherapy studies are final infarct size (i.e. the amount of permanent brain damage produced by the stroke) and neurological deficit (generally sensorimotor deficits induced by MCAO). The latter is the more clinically relevant but also the more challenging to accurately assess in animal models **as these deficits can rapidly resolve in rodent models.** For flow enhancing and thrombolytic drugs, successful recanalisation and the quality of reperfusion are also assessed using CBF techniques.

Infarct volumes are quantified from histologically stained brain sections (e.g haematoxylin and eosin, cresyl violet or tetrazolium salts such as TTC) or non-invasively with T_2-weighted MRI (Sommer, 2010, Durukan and Tatlisumak, 2007, see Figure 2). The infarct develops over a number of hours (permanent MCAO) or days (transient MCAO). Consequently, it is fundamentally important in drug efficacy studies that the time point at which the measurement is made is late enough for the infarct to have fully evolved. Otherwise drugs which delay rather than stop the progression of ischaemic damage could be wrongly assessed as neuroprotective. **Infarct area is first quantified on sufficient numbers of coronal sections throughout the MCA territory to allow accurate determination of infarct volume (e.g. 8 slices described in Osborne et al., 1987 for proximal MCAO induced by electrocoagulation). However,** since brain oedema also develops within and around the infarct over the first 2-3 days post-stroke, **infarct volume calculations should be corrected for the space-occupying effect of brain oedema, to avoid overestimation of infarct volume. Corrections for brain swelling are made using published formulae (e.g. Swanson et al, 1990). Infarct volume is assessed indirectly as the volume of the contralateral hemisphere minus the non-infarcted volume of the ipsilateral hemisphere and is based on the assumption that oedema develops almost exclusively within the infarct. However, there are limitations associated with this type of correction. Tissue sections examined at the peak of stroke-induced oedema, clearly show brain swelling in both infarct and surrounding peri infarct tissue. Therefore, infarct volume correction using these methods may still be confounded by oedema (Dirnagl, 2010). Alternative approaches to correct for oedema involve identifying ischaemic damage**

**on tissue sections and transcribing the information onto line diagrams of standard
size from a stereotaxic atlases (Osborne et al, 1987).**

**Infarct size can be expressed either as an absolute volume in mm^3 or as a relative
value (e.g. percentage of the contralateral hemisphere, or brain volume). Relative
values allow more straightforward comparison between studies as absolute brain
size will vary depending on animal sex and age. Tissue dehydration and processing
also affects brain size with different techniques causing markedly different degrees
of shrinkage of the tissue** (e.g. greater in paraffin embedded that in frozen tissue). For
chronic studies, where rodents survive >7 days post-stroke, the removal of dead tissue by
macrophages and microglia results in the infarct being gradually replaced by a fluid-filled
cyst. The difference between the volume of the remaining ipsilateral and the contralateral
hemisphere provides an alternative measurement of *tissue loss* as a consequence of the
stroke.

Diffusion-weighted MRI is non-invasive and is particularly useful for serial scanning in
the acute stroke period (first 3-4 hrs post-stroke). Affected tissue appears hyperintense,
allowing tracking of the evolution of ischaemic injury and the consequences of
therapeutic intervention. **However, acute hyperintensity on DWI scans does not
signify irreversible ischaemic damage as this abnormality can disappear on early
reperfusion of ischaemic tissue.** Following serial scanning, and anaesthetic withdrawal
animals can be recovered for behavioural assessment of neurological deficits at later

time points and re-anaesthetised for assessment of final infarct with T_2-weighted MRI (Ebisu et al, 2001).

Functional outcome represents an essential component of the preclinical testing of drugs targeting the acute (neuroprotection) and chronic (repair and recovery) stages of stroke. Testing functional outcome is extremely time consuming and labour intensive. It is still an evolving science with groups developing new and modifications of existing tests, with no consensus as yet on a prescribed battery of tests to determine stroke outcome. **Not only are there many different tests being used, but there is no consistency in the number and frequency of tests applied within a study. The current lack of consensus is a weakness in the field that needs to be addressed if we are to improve the ability of animal models to predict therapeutic efficacy in man. There has to be agreement amongst preclinical researchers and guidelines drawn up on the specific sensorimotor tests which offer the best opportunity to identify long-term deficits in rodents, the optimum (i.e. minimum) number required and the frequency with which the animals should be tested.**

The range of behavioural tests developed for quantifying the severity of the sensorimotor deficit and the extent of subsequent recovery are covered in recent reviews (see Metz, 2010, Schallert, 2006). **Specific tests, such as the Morris water maze, are used to determine cognitive deficits, which may arise when artery territories other than the MCA are affected** and more general neurological scoring systems have been developed to provide an overall score of the animal's condition. They range from simple 0-3 point

scores based on forelimb flexion, resistance to lateral push and circling   (Bederson et al.,

1986) to more detailed (3-18 point or 21 point) scoring systems incorporating sensory

and motor assessments (Garcia et al., 1995, Hunter et al., 2000). However, scoring

systems have limited value and tend to be either fast to carry out but limited in ability to

differentiate between different levels of deficit or time consuming and over detailed, with

some of the component parts contributing little to the sensitivity of the method. **Hence,**

each group tends to apply further modifications to the published systems to reduce

the time required to score each animal or to increase sensitivity to pick up a deficit.

Specific behavioural tests assessing skilled motor function appear more informative and

clinically relevant.   The most commonly used tests include sensorimotor asymmetry

(sticky label or dot test), and fore/hindlimb use (e.g. cylinder test, ledged/tapered beam,

horizontal ladder walking task, Figure 3). Animals are assessed for ability to undertake

and complete the tasks (e.g. time taken and order in which sticky labels are removed from

the affected and normal forelimbs), scored for preference of use of uninjured versus

injured limb (e.g. cylinder test) or number of foot faults made in completing a motor task

(e.g. tapered beam, ladder walking test). Assessment of functional outcome in rodents is

challenging as neurological deficits are often not overt, even when the animal sustains a

large infarct. Difficulties may be encountered in animal compliance to undertake the

task, and the deficit may be short-lived, as rodents often exhibit learned compensatory

behaviour which may be wrongly identified as recovery. **However, chronic deficits post**

stroke have been identified with behavioural testing and used to assess the effects of

therapy on functional outcome. For example, the whiskers reflex, rotameter test

with amphetamine and bilateral asymmetry (sticky label) test continue to detect

sensorimotor deficits out to 12 weeks from stroke onset (60 minute ischaemia using the intraluminal thread model, Modo et al., 2000) and the sticky label and rotameter tests were sensitive enough to detect improvements in sensorimotor deficits following stem cell injection into the putamen at 4 weeks post-stroke (Stroemer et al, 2009).

Cerebral blood flow (CBF) measurement is useful for **determining blood vessel recanalisation in thrombolytic studies,** identifying drug effects on stroke severity or quality of reperfusion, and for confirmating comparable severities of ischaemic insult between groups prior to drug administration. The main techniques employed in pre-clinical research are laser Doppler flowmetry and hydrogen clearance, which provide good temporal resolution, $[^{14}C]$ iodoantipyrine and $[^{99m}Tc]$ HMPAO autoradiography, terminal techniques with good spatial resolution, and MRI perfusion-weighted imaging (PWI) which provides spatial and temporal information on CBF. Laser Doppler flowmetry probes are placed on the surface of the skull or stereotaxically implanted into discrete neuroanatomical sites to provide a semi-quantitative read out of change in blood flow over time, but data are generated in relative (flux), not absolute units with data restricted to a limited volume of tissue around the probe tip. Autoradiographic techniques provide fully quantitative ($[^{14}C]$-iodopyrine) or semi-quantitative ($[^{99m}Tc]$ HMPAO) information on CBF throughout the brain but restricted to a single time point. Non-invasive MRI perfusion techniques such as arterial spin labelling requires no exogenous contrast agent and can be calibrated to provide fully quantified serial maps of CBF (Figure 2d).

The importance of the ischaemic penumbra in pre-clinical and clinical stroke research

Occlusion of a cerebral artery causes a severe reduction in CBF within the brain territory the occluded artery normally supplies. However, because the territories of the major cerebral arteries are linked via collateral blood vessels (anastomoses), there is heterogeneity of tissue perfusion within the ischaemic region. Tissue furthest from collateral supply and exposed to the most severe reduction in CBF (<20% of baseline) is referred to as the "ischaemic core" and becomes irreversibly damaged within minutes as ATP supplies are rapidly exhausted. Surrounding the ischaemic core is a border zone where collateral flow results in a less severe reduction in CBF. However, CBF levels are not sufficient to sustain normal function. This zone, referred to as the "ischaemic penumbra", has preserved structural integrity but a limited lifespan (of hours), due to the inadequate supply of oxygen and glucose, limiting its capacity to generate ATP. It is referred to as "tissue at risk" and "potentially salvageable" as prompt restoration of CBF leads to penumbral tissue survival and recovery of function (Symon, 1987, Heiss, 2010). Without restoration of blood flow, penumbral tissue gradually becomes incorporated into the irreversibly damaged ischaemic core. **Penumbral tissue, detected and followed over time using MRI perfusion/diffusion (PWI/DWI) mismatch, becomes gradually incorporated into the ischaemic core over a period of 4-6 hours in rodent permanent MCAO models (Meng et al., 2004; McCabe et al, 2009).** Penumbra therefore represents the therapeutic target for acute stroke therapies.

In the past, clinical trials were compromised by the lack of penumbra imaging, patients being recruited on the basis of time from stroke onset, rather than detection of

potentially salvageable tissue. This combined with unrealistically long therapeutic time windows meant inclusion of patients with no remaining penumbra who were unlikely to benefit from acute stroke therapies, diminishing the power to identify effective drugs. The amount of salvageable penumbra varies widely among acute stroke patients. Some have no remaining penumbra (or chance to benefit from treatment) even within a few hours of stroke, while others may have large amounts of penumbra persisting far longer than average. Consequently, some drugs which demonstrated pre-clinical efficacy but subsequently failed in clinical stroke trials may have had clinical efficacy which was missed due to the lack of appropriate patient selection (e.g. inclusion of patients with large strokes and no remaining penumbra, or small strokes). In clinical trials, using very simple brain scanning, the thrombolytic rt-PA reduced death and disability when given within 4.5 hours of stroke, with probability of benefit declining rapidly with time thereafter (Lees et al., 2010, Hacke et al., 2008, NINDS & Stroke rt-PA Stroke Study Group, 1995). However, with more sophisticated imaging, targeting rt-PA treatment to those patients with persisting penumbra, the time frame for benefit could be potentially extended at least out to 6 hours, since outcomes in the 3-6 hour window with MRI selection did not differ from treatment within 3 hours (Kohrmann et al., 2006).

PET imaging (CBF,$CMRO_2$ OEF) is also used to detect penumbra but low availability, high cost and poor spatial resolution limit its utility for routine clinical use and pre-clinical rodent studies. MRI perfusion/diffusion mismatch and CT perfusion imaging are being validated for penumbra detection in animal and clinical scanners and will be key in identifying effective acute stroke therapies in the future. MRI provides not only longitudinal data on survival of penumbra, but also complementary multiparametric data

on the evolution of stroke **(Table 3). However, like the induction of stroke, the use of anaesthesia to limit movement during scanning has to be acknowledged as a limitation of MRI-based stroke research in animals.**

Additional confounding factors and general limitations of stroke studies in rodents

Generally in neuroprotection studies the drug under test is administered either before, or within the first 1-2 hours post-stroke, in order to provide the best opportunity to identify efficacy. Although this provides a good first step in identifying promising drugs, there are clear limitations in relying on this type of study design to identify drugs/therapies which have the potential to translate successfully to the clinic. The available time window for delaying treatment should also be determined.

In the past, most drug efficacy studies were carried out on inbred young, healthy male animals expressing none of the comorbidities commonly associated with stroke. The use of such animals limits costs and helps control variability in outcome measures such as infarct size and sensorimotor deficits. The failure of drugs, tested only in healthy animals, to show efficacy in clinical trials has challenged the relevance of these stroke models and highlighted the need for additional pre-clinical efficacy studies in aged (i.e. ~18 month old for normotensive rat), and female animals, and in animals with relevant co-morbidities (i.e. hypertension, diabetes, obesity, infection, inflammation and atherosclerosis). However, it is important to recognise that in addition to cost, stroke severity, morbidity and mortality are likely to be increased in these models. The severity of the ischaemic insult should therefore be adjusted to a level where morbidity and

mortality are within ethically acceptable levels.   Equally important for stroke pharmacotherapy studies is the fact that the amount of penumbra is also likely to be less than in young, healthy animals. This will need to be incorporated into power analysis for sample size calculation so that studies are not underpowered to detect drug efficacy.   A range of rodent disease models incorporating comorbidities and risk factors are available (see McColl et al., 2010):- 1. Hypertension: genetically determined hypertensive strains such as the spontaneously hypertensive (SHR), spontaneously hypertensive stroke-prone rat (SHRSP, also insulin resistant), and models of induced hypertension such as the DOCA salt rat; 2. Diabetes: models displaying diabetes/hyperglycaemia include the streptozotocin and Biobreeding (BB) rat models and the non-obese diabetic (NOD) mouse models of type-1 diabetes, Zucker and Goto-Kakizaki rat models of type 2 diabetes and the fructose fed rat model of metabolic syndrome (insulin resistance, hyperinsulinemia and hypertension); 3. Obesity: diet-induced obesity can be generated with high-fat and high-sugar diets in Sprague-Dawley and Long Evans rats and C57B16/J mice. Models incorporating both diabetes and obesity include the Zucker diabetic fatty rat, the *db*/*db* (spontaneous mutation in leptin receptor gene) and *ob*/*ob* (spontaneous mutation in Lep gene encoding the protein leptin) mouse models; 4. Atherosclerosis: genetically modified mouse models are most prevalent. The early foam cell/fatty streak stage of the disease can be induced in wild-type mice but requires aggressive and prolonged (4-5 month) dietary manipulation. Advanced atherosclerosis can be induced in genetically modified murine strains with the addition of dietary modification (e.g. Paigen or Western diets). The best characterised of these are the apolipoprotein E (apoE)$^{-/-}$ and low density lipoprotein receptor (LDLR)$^{-/-}$ mouse models.

Irrespective of the model to be used, the stroke must be induced in as sterile an environment as possible. Although fairly resistant to infection, wound infections can occur in rodents, particularly in chronic studies. **This can be avoided by keeping the operating area clean and as sterile as possible. Surgical sites should be shaved and disinfected, and instruments and occluding devices (e.g. intraluminal filaments, clips etc.) sterilised prior to use.** Surgery-related infections can lead to meningitis and significant recruitment of inflammatory cells into the brain, thereby influencing the severity of the ischaemic insult and increasing the final infarct size. Infections will also increase variability in outcome measures and confound interpretation of drug study results. Since inflammation is also a natural downstream consequence of focal ischaemia (Moskowitz et al, 2010, review) it is important to ensure that any inflammation identified in the brain is a consequence of the ischaemic insult and not an infectious agent introduced as a result of poor aseptic technique.

The lack of white matter in rodents compared to gyrencephalic species and man represents another important limitation. However, small ischaemic lesions can be induced in the larger white matter tracts (e.g. internal capsule) by stereotaxic injection of vasoconstrictor endothelin-1 (Frost et al., 2006, Lecrux et al., 2008). Quantification of white matter damage is achievable using immunohistochemistry (e.g. amyloid precursor protein accumulation at sites of injury, Gentleman et al., 1999, Imai et al., 2002) and MRI (e.g. diffusion tensor imaging, Sotak, 2002).

Finally, it is important to be aware that certain mouse strains display a high frequency of cerebrovascular anomalies leading to an incomplete circle of Willis (Kitagawa et al., 1998; Kelly et al., 2001). This will influence the severity of focal ischaemic insults by compromising collateral flow and represents a major source of variability in outcome measures.

Recommendations for the future

The failure to predict acute neuroprotective drug efficacy from pre-clinical data has led to the relevance of animal stroke models being questioned. There is some merit in this viewpoint. What are described in this review are NOT in fact rodent models of stroke, they are models of focal cerebral ischaemia. They model the consequences of an ischaemic insult but, in the main, do not recreate the background pathophysiology which would give rise to an endogenous stroke. Therefore, we have to accept the limitations inherent in these models and, follow early proof of efficacy studies in young healthy animals with more rigorous studies in models incorporating age and co-morbidities. Only drugs which demonstrate efficacy in a range of these models should be considered for further investigation in larger gyrencephalic models and subsequent translation to clinical trials.

For drugs targeting the brain, ability to cross the intact blood brain barrier is crucial and should be established in animals before the drug progresses through to human stroke trials.

Translational problems will be reduced with further improvement in pre-clinical study design and conduct. A number of substandard practices have been identified

and recommendations published to improve the quality and reporting of pre-clinical stroke research (STAIR I, 1999 subsequently updated by Fisher et al., 2009, Macleod et al., 2009, Kilkenny et al., 2010): study design should include randomisation and blinding to limit bias and sample size calculations to ensure studies are adequately powered to detect real differences between treatment and control groups. Oedema-corrected infarct size should be measured when the lesion has reached its maximum size, and not before (i.e. when the infarct is still evolving). If significant mortality is encountered prior to this time point, the severity of the ischaemic insult should be reduced to limit morbidity and improve survival. Information on inclusion/exclusion criteria, excluded animals and mortality should be included in pre-clinical stroke publications. Despite the fact that the first STAIR recommendations have now been in press for more than a decade, issues relating to preclinical study design and quality are still evident in the literature. A recent systematic survey examined all cerebrovascular research studies published in the Journal of Cerebral Blood Flow and Metabolism in 2008. "Few studies reported a primary research hypothesis, statement of purpose, or measures to safeguard internal validity (such as randomization, blinding, exclusion or inclusion criteria). Many studies lacked sufficient information regarding methods and results to form a reasonable judgment about their validity." (Vesterinen et al., 2010). The deficiencies identified are in no way exclusive to the Journal of Cerebral Blood Flow and Metabolism. They are evident throughout the neuroscience literature, and are just as prevalent, if not more so, for other neurodegenerative diseases (Jucker, 2010, Rooke, et al. 2011, Macleod 2011). The survey by Vesterinen and colleagues

exemplifies both the major problem and the achievable solution to the pre-clinical end of the translational roadblock in stroke research. There is an onus on researchers and manuscript referees to ensure that the basic principles of good study design and reporting are adhered to, to bring the pre-clinical literature up to the same standards as the clinical literature. Journal editors have the power to improve the standard of pre-clinical publications and should be update instructions to authors to make specific statements on study design mandatory.

Journal editors also have the powers to reduce publication bias, another issue which has influenced our ability to predict drug efficacy from pre-clinical data. Reluctance to publish negative or neutral drug studies has resulted in overestimation of drug efficacy and has severely limited the ability of pre-clinical meta-analyses and systematic reviews to predict drug efficacy in man (Dirnagl and Macleod, 2009). However, this failing is now being addressed with some journal editors recognising the importance of publishing properly conducted studies which fail to identify drug efficacy (e.g. a new Negative Results article type in the Journal of Cerebral Blood Flow & Metabolism, for data that fails to identify a difference between the experimental groups and/or reproduce published findings).

All the focal ischaemia models described require induction of anaesthesia and some surgery, both of which can give rise to variability in outcome in neuroprotection studies. Anaesthesia can affect blood pressure, blood gases and body temperature, all of which influence stroke severity and outcome. In addition, many anaesthetics (e.g. isoflurane, halothane, xenon, propofol, ketamine) have inherent neuroprotective characteristics (Kawaguchi et al., 2005, Traystman, 2010). Duration of anaesthesia should therefore be

as short as possible. In studies where the whole experiment is carried out in the anaesthetised animal, physiological monitoring is recommended to ensure physiological parameters stay within normal limits. This will help to control variability in outcome and thereby increase the power to detect a therapeutic effect. Physiological monitoring can also be useful in detecting any drug-induced influences on these parameters which could either limit or enhance efficacy.

Using a similar format to multi-centre clinical trials, networks of preclinical researchers establishing and using standardised techniques and confirming results across centres will achieve much more than individual groups competing for funds. A standardised set of tests for assessment of functional outcome from stroke in each model, would be a priority to reach agreement on.

In summary, since the positive rt-PA NINDS trial in 1995, many neuroprotective drugs have been tested in >114 clinical trials (http://www.strokecenter.org/trials/) but none has shown clinical efficacy. This dismal lack of progress has led to a devastating reduction in acute stroke research and development programmes within the major pharmaceutical companies. Published guidelines provide the steps required to address the limitations in study design and reporting on the pre-clinical side and problems with patient selection for trials on the clinical side of the translational roadblock.   Addressing these limitations is eminently achievable with the establishment of preclinical stroke networks, more widespread use of diagnostic imaging and better links between preclinical and clinical scientists. Progress will have significant cost implications, requiring greater numbers of

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animals (to include aged and comorbidities) and increased use of both pre-clinical and clinical imaging. However, identification of just one neuroprotective /neurorepair therapy with clinical efficacy for stroke would more than justify the costs, providing an alternative to rt-PA for the majority of patients who are not currently treated, and reversing the roadblock by restoring confidence in the pharmaceutical industry for further drug development.

Table 1.   Advantages and disadvantages of the different focal ischaemia models. CCAO, common carotid artery occlusion, MCAO, middle cerebral artery occlusion.

Table 2. Published mortality data for different rodent focal ischaemia models. Some data displayed represent figures for vehicle-treated or control groups in pharmacotherapy studies. Mortality levels encountered are dependent not only on the model employed but also the survival period and the level of experience and skills of the surgeon. BCCAO. Bilateral common carotid artery occlusion, f, female, m, male, PMCAO, permanent middle cerebral artery occlusion, SD, Sprague Dawley, TMCAO, transient middle cerebral artery occlusion, # Small burr hole on dorsal surface of the skull for insertion of stereotaxic needle and/or guide cannula.

Table 3. MRI techniques used in pre-clinical stroke research. ADC, apparent diffusion coefficient, CBF, cerebral blood flow

Figure Legends

Figure 1   Middle Cerebral Artery Occlusion Models in Rats & Mice. Pink shading on MCA territory diagram represents the tissues supplied by the MCA. Pink shading on the

electrocoagulation diagrams represents ischaemia and demonstrates the ability to induce purely cortical ischaemia with a distal MCAO versus cortical and subcortical ischaemia with a proximal MCAO including the lenticulostriate branches.

Figure 2 **Histology & MRI measures of outcome. a) Haematoxylin and eosin (H&E) stained section illustrating the ability to discriminate between irreversibly damaged and morphologically normal tissue (▬ . ▬ . ▬ . ▬ ） in perfusion fixed paraffin embedded tissue. With this level of tissue processing irreversible ischaemic damage is distinguished from changes in neuronal morphology and background neuropil which becomes irregular and vacuolated as early as 4 hours after permanent MCAO. Higher magnification inset panels show the characteristic morphology of dark, shrunken, pyknotic, ischaemically damaged neurone cell bodies (top left), compared to the pale stained, round morphology of normal neuronal cell bodies (bottom right). b) by 24 hours after permanent MCAO, a low magnification image of a fresh frozen cryostat section stained with H&E will clearly display the boundary between infarct (area of palor on RHS of section) and non-ischaemic tissue. Note also the brain swelling evident in the ipsilateral hemisphere. c) by 24 hours after permanent MCAO, triphenyltetrazolium chloride (TTC) staining of fresh tissue slices will also reveal a clear boundary between infarct (white) and non-iscaemic tissue (red). d-f these MRI scans are non-invasive and do not require administration of contrast agents. d) arterial spin labelling provides serial scans of cerebral blood flow to map the severity and location of ischaemia. e) diffusion-weighted imaging (DWI) provides serial scans of ischaemic injury (hyperintensity),**

which in the first hours after stroke may be reversible on reperfusion. f) T$_2$-weighted imaging identifies infarct (hyperintensity) from 24 hours post-stroke.

Figure 3. Behavioural tests in rodent stroke models. **Illustrations of four commonly used sensorimotor tests. The cylinder test reveals forelimb preference when the animal rears to explore its environment by making forelimb contact with the cylinder walls. The horizontal ladder and tapered beam tests reveals foot faults (orange arrows) as the animal traverses the ladder, which has irregularly spaced rungs, or the beam which gets gradually narrower as the animal approaches its home cage.**

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Model	Advantages	Disadvantages
MCA permanently occluded by electrocoagulation	Good reproducibility in outcome measures achievable. Successful MCAO confirmed visually by section of the occlusion site. Some control over infarct size and location possible by occluding different segments of the MCA. Infarct matures quickly and is maximal within 24-48 hours. Low mortality.	Requires craniectomy, technically challenging. Model not suitable for transient ischaemia thrombolysis studies.
MCA occluded by an intraluminal filament	Good reproducibility in outcome measures achievable. Suitable for permanent or transient ischaemia. Technically straightforward surgery (no craniectomy)	Successful MCAO cannot be confirmed visually Reproducibility dependent on identifying the optimal filament dimensions and construction for the rodent strain and size used. Increased haemorrhage risk with certain filament types. Significant mortality may be encountered with large strokes ($\geq$ 24 hours) & before infarct has fully evolved Model not suitable for thrombolysis studies
MCA occluded by endothelin-1 (topical or intraparenchymal)	Good reproducibility in outcome measures achievable. Successful MCAO can be confirmed visually (topical model). Modifications to intraparenchymal model allow ischaemia induction in conscious rat.	Dependent on endothelin-1 vasoconstrictor potency being consistent from batch to batch Topical administration requires craniectomy. Models not suitable for thrombolysis studies
MCA occluded by clip/mechanical device	Successful MCAO is confirmed visually. Suitable for permanent or transient ischaemia.	Requires craniectomy, technically challenging and likely to require more than one occlusion site, hypotension or CCAO to achieve reproducibility
MCA occluded by autologous blood	Most closely mimics human ischaemic stroke.	Less reproducible than other models.

clot	Model suitable for thrombolysis studies.	Less control over ischaemia location and duration More variability in outcome measures. Secondary microclot formation possible with clot breakdown Higher mortality than for other models
MCA branch occluded by intravascular thrombin injection	Reproducibility in cortical ischaemia and infarct volume achievable. Position and persistence of clot confirmed visually. Model suitable for thrombolysis studies. Low mortality compared to autologous clot models.	Requires craniectomy. Secondary microclot formation possible with clot breakdown Limited sensorimotor deficit due to small size and location of infarct .
Photochemical ischaemia	Reproducibility in cortical ischaemia and infarct volume achievable. Blood vessel thrombosis can be achieved through intact skull. More recent modifications of the model are suitable for thrombolysis studies	Photocoagulation insult causes severe damage to affected blood vessels and substantial early vasogenic oedema uncharacteristic of human stroke. Limited sensorimotor deficit due to small size and location of infarct

Table 1   Advantages and disadvantages of the different focal ischaemia models. CCAO, common carotid artery occlusion, MCAO, middle cerebral

artery occlusion.

MCAO Model (duration of ischaemia)	Species, Strain (m/f)	Craniectomy	Mortality (time period examined)	References
Proximal MCAO (permanent) Electrocoagulation	Rat, Wistar (m)	Yes	0% (10 weeks)	Yonemori et al., 1999
Proximal MCAO (permanent) Intraluminal filament	Rat, SD (m) Rat SD (m) Rat SD (m) Rat Wistar (m) Rat Fischer 344 (m)	No No No No No	18.2% (24 hrs) 42% (72 hrs) 0% (24 hrs), 12.5-33%* (48 hrs) 75% (24 hours) 8% (24 hours)	Lu et al., 2009 Schöller et al. 2004 Aspey et al., 1998 Aspey et al., 1998 Aspey et al., 1998
Distal MCAO (permanent) Electrocoagulation	Mouse, C57BL/6 (m)	Yes	0% (6hrs-8 days)	Kuraoka , et al, 2009
TMCAO (proximal, 60 mins) Ligature + BCCAO	Rat SD (m) young, 3-4 months Rat SD (m) old, 22-24 months	Yes Yes	6.3% (24hrs), 6.3% (28 days) 35% (24 hrs), 43.5% (28 days)	Wang et al., 2003
TMCAO Intraluminal filament (proximal, 60 mins) (proximal, 45 mins)	Mouse, SV129/J (m) Mouse, 129S6SvEv (m)	No No	13, 37 & 57% (4, 5 & 6 days, resp) 33% (72 hrs)	Meisel et al., 2004 Braun et al., 2007
Thromboembolic (autologous clot)	Rat SD Rat SD (m)	No No	30-50% (24 hrs) 44% (14 days)	Orset et al. 2010 Rasmussen et al., 2008
Thromboembolic (intra-arterial thrombin injection)	Mouse, Swiss (m)	Yes	<1% (24hrs and 3 months)	Orset et al. 2010 and Vivien D, personal communication
Photochemical stroke	Mouse and rat	No	Low (not defined)	Witte (2010)
Endothelin-1 topical application to MCA Endothelin-1 injection into piriform cortex adjacent to proximal MCA	Rat SD (m) Rat SD (m)	Yes #	<5% (4 hrs) 7% (24 hrs), thereafter 0% out to 3 months 15% (4 hrs)	Macrae IM , unpublished Sharkey J (personal communication) Nikolova et al., 2009

Table 2. Published mortality data for different rodent focal ischaemia models. Mortality levels encountered are dependent not only on the model

employed but also the survival period and the level of experience and skills of the surgeon. BCCAO. Bilateral common carotid artery occlusion, f,

female, m, male, MCAO, middle cerebral artery occlusion, SD, Sprague Dawley, TMCAO, transient middle cerebral artery occlusion, * dependent

on suture used, # Small burr hole on dorsal surface of the skull for insertion of stereotaxic needle and/or guide cannula.

MRI Technique	Potential use in pre-clinical stroke research
MR Angiography	Occlusion/patency status of the targeted blood vessel
Perfusion-weighted imaging PWI	Serial CBF maps reveal severity and location of ischaemia, and any spontaneous or induced reperfusion or drug-induced changes in CBF
Diffusion-weighted imaging DWI	Serial DWI and ADC maps reveal acute and evolving brain injury, brain swelling
PWI/DWI mismatch	Serial assessment of penumbra and its incorporation into ischaemic core over time.
Functional MRI (fMRI) combined with a stimulus such as forepaw stimulation	Serial fMRI studies combined with a specific stimulus will reveal ischaemia-induced loss of function in response to the stimulus, any compensatory changes or subsequent return of function
Contrast-enhanced T_1-weighted imaging	Blood-brain barrier breakdown
T_1, T_2 and T_2*-weighted imaging	Brain haemorrhage
Diffusion tensor imaging -DTI	White matter damage
T_2-weighted imaging	Infarct, brain swelling

Table 3 MRI techniques used in pre-clinical stroke research. ADC, apparent diffusion coefficient, CBF, cerebral blood flow.
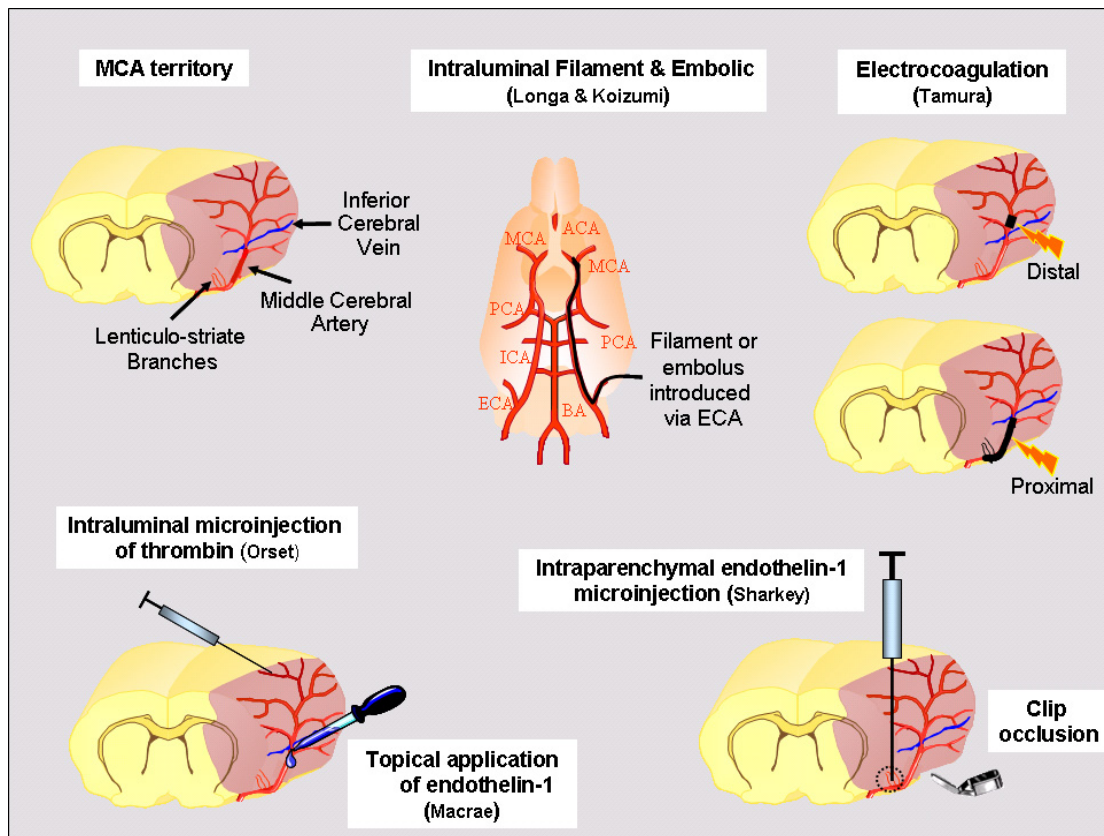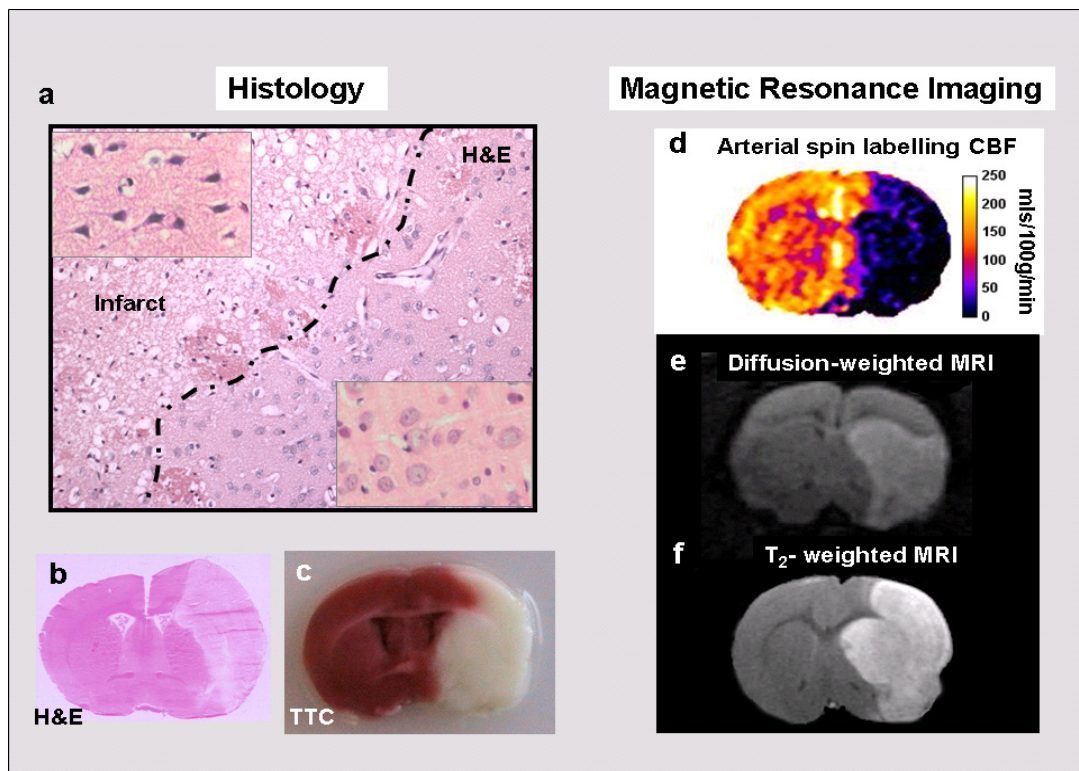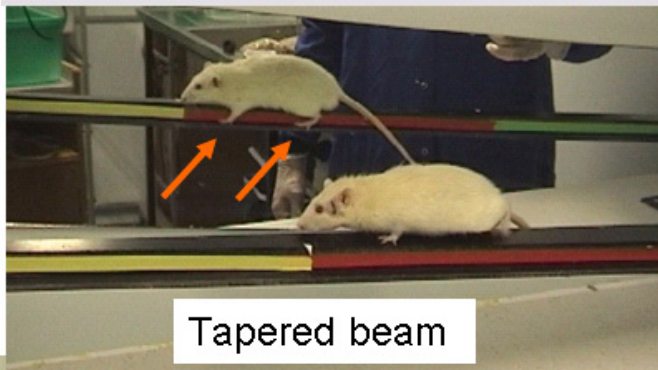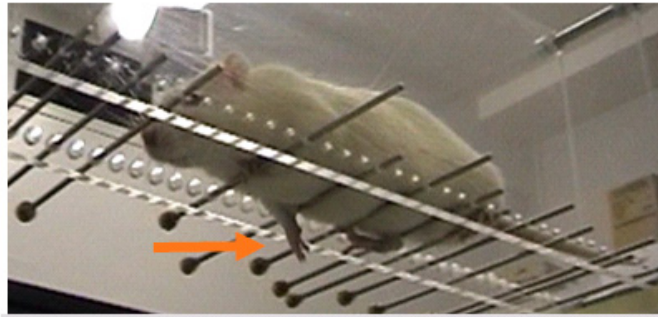
Figure 1:

Figure 2:

Figure 3:

Cylinder test

Horizontal ladder

Sticky label

Tapered beam

Home cage

2 cm wide under-hanging
ledge on either side of beam

Platform

1.5 cm

6 cm

14 cm

130 cm

14 cm

Tapered beam