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Stroke and Translational Research – Review of Experimental Models with a Focus on Awake Ischaemic Induction and Anaesthesia

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Abstract—Animal models are an indispensable tool in the study of ischaemic stroke with hundreds of drugs emerging from the preclinical pipeline. However, all of these drugs have failed to translate into successful treatments in the clinic. This has brought into focus the need to enhance preclinical studies to improve translation. The confounding effects of anaesthesia on preclinical stroke modelling has been raised as an important consideration. Various volatile and injectable anaesthetics are used in preclinical models during stroke induction and for outcome measurements such as imaging or electrophysiology. However, anaesthetics modulate several pathways essential in the pathophysiology of stroke in a dose and drug dependent manner. Most notably, anaesthesia has significant modulatory effects on cerebral blood flow, metabolism, spreading depolarizations, and neurovascular coupling. To minimise anaesthetic complications and improve translational relevance, awake stroke induction has been attempted in limited models. This review outlines anaesthetic strategies employed in preclinical ischaemic rodent models and their reported cerebral effects. Stroke related complications are also addressed with a focus on infarct volume, neurological deficits, and thrombolysis efficacy. We also summarise routinely used focal ischaemic stroke rodent models and discuss the attempts to induce some of these models in awake rodents.

Keywords: Ischemic stroke, Anesthesia, Rodent models, Awake, Awake stroke.

INTRODUCTION

Over the last few decades hundreds of potential therapeutics have emerged from the preclinical pipeline in ischaemic stroke (O'Collins et al., 2006; Schmidt-Pogoda et al., 2020). However, translation of the most effective preclinical agents to clinic has failed. Tissue plasminogen activator (tPA), the only currently approved pharmacological treatment, and the rapid and successful development of endovascular thrombectomy (EVT) (Campbell et al., 2015) have revolutionised the emergency standard of care of ischaemic events. Unfortunately, both thrombolysis with tPA and EVT have a narrow time window of intervention (4.5 h and up to 24 h, respectively), strict eligibility criteria, and are not available treatments for all patients. In addition, despite successful recanalisation not all patients show improved outcome. The unmet need for widely available therapeutics for ischaemic stroke therefore remains an urgent concern.

To bridge the translational gap, steps have been taken to improve translational efficacy to increase the quality and reproducibility of therapeutic studies. These include enhanced preclinical study design (Fisher et al., 2009), improved experimental settings (Percie du Sert et al., 2017) and data reporting (Kilkenny et al., 2010). Despite these initiatives, several translational pitfalls are yet to

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Abbreviations: ARRIVE, Animal Research: Reporting In Vivo Experiments; CBF, cerebral blood flow; CBFa, cerebral blood flow autoregulation; CCA, common carotid artery; CMR, cerebral metabolic rate; ECA, external carotid artery; EEG, electroencephalogram; EVT, endovascular thrombectomy; $GABA_A$, γ-aminobutyric acid type A; ICA, internal carotid artery; ICH, intracerebral haemorrhage; LSCI, laser speckle contrast imaging; MABP, mean arterial blood pressure; MAC, minimum alveolar concentration; MCA, middle cerebral artery; MCAo, middle cerebral artery occlusion; N_2O, nitrous oxide; NMDA, *N*-methyl-D-aspartate; NVC, neurovascular coupling; PSD, post stroke depression; PT, photothrombotic; SD, spreading depolarization; SN, substantia nigra; STAIR, Stroke Therapy Academic Industry Roundtable; tPA, tissue Plasminogen Activator; VCAM-1, vascular cell adhesion molecule 1.

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be fully addressed, including measuring long-term functional outcome (Rosell et al., 2013), integrating comorbidities (McCann and Lawrence, 2020), incorporating both genders in study design (Lyden, 2021) and choosing appropriate preclinical stroke models (Fluri et al., 2015).

While ischaemic stroke has been modelled in several species such as rabbits, hamsters, pigs, sheep, cats, dogs, non-human primates (Narayan et al., 2021); rodents are the most frequently used species for several reasons. Beyond the greater acceptability of ethical implications, rodents are used for modelling ischaemic stroke due to their comparable vasculature and physiology to humans (Yamori et al., 1976), suitability for genetic manipulation, great availability of tools and equipment for imaging, tissue processing and analysis, easy monitoring of physiological parameters and lower research cost compared to higher species.

Currently employed rodent stroke models vary in stroke aetiology, lesion size and mode of induction. These models have been thoroughly reviewed previously, with authors detailing the advantages and disadvantages of using each model, their clinical relevance and other translational challenges (Fluri et al., 2015; Kumar et al., 2016; Li and Zhang, 2021; Narayan et al., 2021). Both mechanistic and interventional studies imply several considerations when choosing the right combination of models to match the aim of the study and the tested outcomes. For example, uncovering underlying mechanisms of disease often requires targeting a precise area, which is possible with some available models (i.e., the photothrombotic (PT) or the endothelin-1 injection models). For interventional studies, the suitability of long-term functional assessment is an essential criterion. When testing neuroprotective drugs that target salvageable tissue, the presence of an expanding penumbra would also be an important aspect to consider. Adjuvant therapies to reperfusion aimed for co-administration with thrombolytics should also be tested in models where thrombolysis is feasible. Lastly, as the current models have different external validities mimicking different stroke aetiologies, a combination of models (permanent, transient) should be considered for improved translation. A summary of these main features for routinely used rodent focal ischaemic stroke models is provided in Table 1.

The confounding effect of anaesthesia during stroke induction in preclinical studies has been raised as an important consideration. Anaesthesia modulates numerous molecular pathways identified as relevant for stroke (Hoffmann et al., 2016). These include excitotoxicity, thermoregulation, inflammation, seizures and spreading depolarisations (SDs). Of particular importance are the effects of anaesthesia on cerebral blood flow (CBF) and neurovascular coupling (NVC).

Here, we provide a review of the anaesthetic strategies commonly utilised in preclinical rodent focal cerebral ischaemic models and compile the direct cerebral effects of anaesthesia that have been reported. We discuss anaesthetic induced complications, relating to stroke severity indicators, such as cerebral infarct volume, neurological deficits, and thrombolysis efficacy. Awake stroke induction is a rarely attempted refinement that will minimise confounding effects of anaesthesia and may improve translational relevance. Here we review the limited approaches taken so far in this direction.

INHALATION ANAESTHETICS

Volatile anaesthetics, such as isoflurane and sevoflurane, are the most used anaesthetics in preclinical stroke research, likely due to the ease of administration, depth control and easy reversal (Archer et al., 2017). Volatile anaesthetics prolong synaptic inhibition and positively modulate postsynaptic γ-aminobutyric acid type A ($GABA_A$) receptor gating to induce anaesthesia (Topf et al., 2003; Goetz et al., 2007; Saab et al., 2010). Volatile anaesthetics are also a partial antagonist of glutamatergic N-methyl-D-aspartate (NMDA) receptors (Yang and Zorumski, 1991; Berg-Johnsen and Langmoen, 1992). At the correct therapeutic concentrations, volatile anaesthetics induce a non-reflexive and deep state of unconsciousness. Whilst this is important to ensure animal welfare is optimal, the effects of volatile anaesthetics on the brain can independently alter the outcome of a preclinical stroke study. Sakai *et al.* demonstrated the neuroprotective effect of isoflurane in rats, reporting significant post intraluminal filament middle cerebral artery occlusion (MCAo) neurological and histological improvements, when compared to animals subjected to awake MCAo (Sakai et al., 2007). Yang *et al.* also reported neuroprotective properties of sevoflurane by suppression of neuronal apoptosis after ischaemia and reperfusion in rats (Yang et al., 2022). The neuroprotective properties of volatile anaesthetics are well described in preclinical focal cerebral ischaemic literature, in which cerebral infarct volume and neurological deficits improve considerably when compared to awake groups (Kimbro et al., 2000; Li et al., 2013; Seto et al., 2014; Jiang et al., 2017; Neag et al., 2020). In the sections below, case studies are provided in which infarct volume, neurological deficits and thrombolysis are discussed in further detail.

Nitrous oxide (N_2O) is a less common preclinical inhalation anaesthetic that offers a different mechanism of action to induce anaesthesia. N_2O is believed to antagonise the glutamatergic NMDA receptor (Jevtović-Todorović et al., 1998). The experimental approach to determine the effect of N_2O on stroke severity was by postconditioning using a non-anaesthetic concentration of N_2O post intraluminal filament MCAo. David *et al.* reported a reduction in neuronal death in rats and a reduction in NMDA induced calcium ion influx in cortical cell cultures (David et al., 2003). Haelewyn *et al.* also reported a neuroprotective effect of N_2O, demonstrating a reduction in infarct volume and neurological deficits in rats (Haelewyn et al., 2008). However, other groups have reported no effect of N_2O on post ischaemic stroke outcome (Reasoner et al., 1990; Yokoo et al., 2004). See Zhang *et al.* for further discussion into the effects of N_2O in the context of stroke (Zhang et al., 2017).

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Table 1. Summary of most frequently used focal ischaemic stroke models in rodents. Essential features are described above and are intended to aid choosing a model to match experimental purpose. [a] delayed spontaneous reperfusion occurring between 48 and 72 h post stroke was described with the PT model when using the ring-shaped laser irradiation system, with low laser intensity and thinner thickness beam[68,69]; [b] co-administration of thrombin (80 U/kg) and rose bengal dye (50 mg/kg) in the proximal middle cerebral artery (MCA) prior to PT stroke induction resulted in an altered thrombus composition, with increased fibrin quantity, thus, increasing the tPA sensitivity[70]; [c] an inverse penumbra has been described using the ring-shaped laser irradiation system to induce PT stroke resulting in an inwardly expanding ischaemic core[41,71]; [d] an inflammatory penumbra was described as the perilesional area expressing vascular cell adhesion molecule 1 (VCAM-1) during the subacute phase. This was shown using microparticles of iron oxide targeted to VCAM-1 with enhanced magnetic resonance imaging[42]; PSD = post stroke depression; SN = substantia nigra. References: 1.(Hata et al., 1998); 2.(Koizumi et al., 1986); 3.(Longa et al., 1989); 4.(Belayev et al., 1996); 5.(Z. Zhang et al., 1997); 6.(Chen et al., 2015); 7.(Kudo et al., 1982); 8.(Kaneko et al., 1985); 9.(R. L. Zhang et al., 1997); 10.(Sugimori et al., 2004); 11.(Watson et al., 1985); 12.(Yao et al., 1996); 13.(Cai et al., 1998); 14.(Horie et al., 2008); 15.(Tennant and Jones, 2009); 16.(Robinson et al., 1990); 17.(Sharkey, 1993); 18.(Macrae et al., 1993); 19.(Fuxe et al., 1997); 20.(Orset et al., 2007); 21.(Karatas et al., 2011); 22.(Kurz et al., 1990); 23.(Backhauss et al., 1992); 24.(Doyle and Buckwalter, 2014); 25.(Tamura et al., 1981); 26.(Welsh et al., 1983); 27.(Welsh et al., 1987); 28.(Robinson et al., 1975); 29.(Shigeno et al., 1985); 30.(Tibo Gerriets et al., 2003); 31.(Silasi et al., 2015); 32.(Bralet et al., 1979); 33.(Demura et al., 1993); 34.(Overgaard et al., 1993); 35.(De Lizarrondo et al., 2017); 36.(T Gerriets et al., 2003); 37.(Memezawa et al., 1992); 38.(Peters et al., 1998); 39.(Nagayama et al., 2000); 40.(Reid et al., 2012); 41.(Hu et al., 2001); 42.(Gauberti et al., 2013); 43.(Tyson et al., 1984); 44.(Claus et al., 2013); 45.(Balkaya et al., 2013); 46.(Yu et al., 2019); 47.(Kronenberg et al., 2012); 48.(Boyko et al., 2013); 49.(Zhang et al., 2005); 50.(Zhang et al., 2015); 51.(Alamri et al., 2018); 52.(Lui et al., 2022); 53.(Vahid-Ansari et al., 2016); 54.(Rosell et al., 2013); 55.(Syeara et al., 2020); 56.(Lubjuhn et al., 2009); 57.(Gerlai et al., 2000); 58.(Balbi et al., 2019); 59.(Nemeth et al., 2012); 60.(Sakai et al., 2007); 61.(Seto et al., 2014); 62.(Bogaert et al., 2000); 63.(Xie et al., 2016); 64.(Zhang et al., 2000); 65.(Lu et al., 2014); 66.(Brunner et al., 2023); 67.(Tsai et al., 2016); 68.(Gu et al., 1999b); 69.(Hu et al., 1999); 70.(Sun et al., 2020); 71.(Wester et al., 1995)

Model	Intraluminal Filament	Embolic	Photo thrombosis	Endothelin-1 injection or application	Thrombin intraluminal injection	Ferric chloride application	Electro-coagulation	Mechanical clip or ligature	Macrosphere induction	Microsphere induction
Species	mice[1], rats[2-4]	mice[5,6], rats[7–9]	mice[10], rats[11–13]	mice[14,15], rats[16–19]	mice[20]	mice[21], rats[22]	mice[23,24], rats[25]	mice[26,27], rats[28,29]	rats[30]	mice[31], rats[32,33]
Type of Occlusion	transient	transient, spontaneous reperfusion	permanent[a]	permanent or transient dose-dependent[18]	transient, spontaneous reperfusion	permanent	permanent	transient	permanent	permanent
If present, thrombus aetiology	–	fibrin-rich	platelet-rich[b]	–	fibrin-rich	platelet-rich	–	–	–	–
Thrombolysis studies possible	no	yes[34]	no[b]	no	yes[20]	no[35]	no	no	no	no
Targeted Area	striatum, cortex, thalamus, SN, hypothalamus	variable MCA territory	precise target of choice within cortex	precise target of choice	cortex	cortex	cortex	cortex	MCA territory, *without hypothalamus* [30,36]	multifocal, heterogeneous
Described penumbra	yes[37–40]	not defined	yes[c 41]	yes[19]	yes[d 42]	not defined	no[43]	not defined	yes[44]	not defined
Functional impairment	sensorimotor [45,46], PSD[47,48]	sensorimotor[49,50]	sensorimotor[51]	sensorimotor[15,52], PSD[53], anxiety[53]	limited [20,54]	limited [55]	sensorimotor [56,54,24]	sensorimotor[57]	sensorimotor [36]	sensorimotor[58], cognitive[58], PSD[59]
Reported effects of anaesthesia	yes[60]	–	yes[61]	yes[62]	–	–	–	–	–	–
Awake induction attempted	yes[63]	yes[64]	yes[65]	yes[62]	–	yes[66]	–	–	–	yes[33,67]

INJECTABLE ANAESTHETICS

Injectable anaesthetics, such as ketamine, barbiturates, propofol and urethane are commonly used in preclinical research. Ketamine is a non-competitive NMDA receptor antagonist and, at anaesthetic and non-anaesthetic concentrations, has been suggested to influence focal cerebral ischaemic outcome. Ketamine is commonly paired with xylazine as an anaesthetic strategy and has been reported to improve neurological deficits (Hoffman et al., 1992; Reeker et al., 2000; Chen et al., 2020), reduce cerebral infarct volumes (Chang et al., 2002; Xiao et al., 2012; Shekarforoush et al., 2016), provide apoptotic suppression (Engelhard et al., 2003) and improve recombinant tissue-type plasminogen activator efficacy (Gakuba et al., 2011). On the contrary, ketamine has also been shown to have no effect on ischaemic outcome (Jensen and Auer, 1988).

Barbiturates modulate $GABA_A$ receptor activity by prolonging channel opening duration, similar to volatile anaesthetics (Henschel et al., 2008). Many studies have shown the neuroprotective properties of barbiturates in monkeys (Gisvold et al., 1984; Nehls et al., 1987) but few in rodents. Kimbro *et al.* reported the reduction in post ischaemic excitotoxicity and cerebral infarction when 50 mg/kg pentobarbital was administered as a bolus in rats (Kimbro et al., 2000). Interestingly, this neuroprotective effect was dose dependant, a low dose of 16–17 mg/kg was ineffective, and electroencephalogram (EEG) burst suppression was key to see the improved ischaemic outcome. Propofol is a similar anaesthetic to barbiturates and is presumed to also enhance $GABA_A$ receptor activity (Peduto et al., 1991), however it also inhibits NMDA receptors (Orser et al., 1995). Propofol and barbiturates are so similar that Pittman *et al.* compared their neuroprotective effects in rats undergoing filament MCAo and found the neuroprotective outcome to be comparable (Pittman et al., 1997). The consensus on the neuroprotective properties of propofol are unclear and are believed to be dose dependent. Propofol has been shown to improve focal cerebral outcome in rodents via different mechanisms of action, such as EEG suppression and antioxidant effects (Kochs et al., 1992; Gelb et al., 2002; Engelhard et al., 2004; Wang et al., 2009; Cai et al., 2011; Wang et al., 2011). However, there are reports suggesting propofol has no effect on ischaemic outcome (Tsai et al., 1994; Young et al., 1997).

Urethane induces a long-term level of surgical anaesthesia and has minimal effects on the cardiovascular system (Soma, 1983). The mechanism of urethane anaesthetic induction is unclear, however, it has been reported to affect multiple neurotransmitter systems, including inhibition of GABAergic neuron responses (Hara and Harris, 2002; Accorsi-Mendonça et al., 2007). Urethane is not used for chronic rodent studies due to the carcinogenic properties it exhibits (Tomisawa et al., 2003).

CEREBRAL EFFECTS OF ANAESTHETICS

The fundamental problem with most anaesthetics, in stroke research, is their neuroprotective properties in which many mechanisms of the brain are altered. Anaesthetics suppress neurotransmission, thus reduce adenosine triphosphate consumption, providing a post stroke brain with more energy than if the ischaemia was induced awake (Fukuda and Warner, 2007). In recent years, the type of anaesthetic, in which the ischaemic stroke model is performed under, has shown to directly affect cerebral conditions. Here we will cover the anaesthetic effects on CBF and CBF autoregulation (CBFa), cerebral metabolic rate (CMR), SDs and NVC, summarised in Fig. 1.

CEREBRAL BLOOD FLOW AND CEREBRAL BLOOD FLOW AUTOREGULATION

CBFa is a homeostatic reflex in which CBF is maintained to account for changing metabolic demands of the brain (Wang et al., 2010). In the context of focal cerebral ischaemia, fluctuations in CBF under anaesthesia could be a potential mechanism that explains the reported improvements in ischaemic outcome. Interestingly, Li *et al.* reported the predictability of cerebral infarct volume by using CBF reduction, noting a 50% reduction in regional CBF strongly correlated with tissue that would succumb to infarct (Li et al., 2013). Volatile anaesthetics have been shown to increase CBF in rodents (Hansen et al., 1989; Hendrich et al., 2001; Li et al., 2013). Sullender *et al.* reported a 14.1% increase in vessel diameters, in anaesthetised mice (1–2% maintenance isoflurane) when compared to awake mice (Sullender et al., 2022). Similar findings were seen by Rakymzhan *et al.*, in which artery diameter increased up to 55% and vein diameter increased up to 22% depending on the vessel imaged (Rakymzhan et al., 2021). However, volatile anaesthetics inhibit CBFa in a dose dependant manner; doses of up to 1 minimum alveolar concentration (MAC) preserve CBFa, doses above 1.5 MAC inhibit CBFa (Strebel et al., 1995; Werner et al., 2005; Wang et al., 2010). There is limited work assessing the effects of inhalational N_2O anaesthesia on CBF and CBFa in rodents. Reasoner *et al.* reported an increase in CBF when N_2O was used in combination with isoflurane compared to isoflurane alone (Reasoner et al., 1990). Ketamine has been reported to regionally increase CBF in rodents (Cavazzuti et al., 1987; Burdett et al., 1995; Zeiler et al., 2016) whilst CBFa remains intact (Engelhard et al., 1997). Barbiturates are known to reduce CBF in rodents (Todd and Weeks, 1996; Hendrich et al., 2001). Suppression of bursting EEG activity was a noted biomarker that infers a reduction in CBF (Siddiqi et al., 2023). A systematic review by Wang *et al.* concluded that barbiturates, namely pentobarbital, caused CBFa breakdown (Wang et al., 2010). Similar to barbiturates, propofol has been reported to reduce CBF but maintain CBFa (Werner et al., 1993; Strebel et al., 1995). The effects of urethane are not well defined in rodent work. Drew *et al.* reported an observation in which urethane attenuated arterial diameter oscillations when compared to awake animals (Drew et al., 2011).

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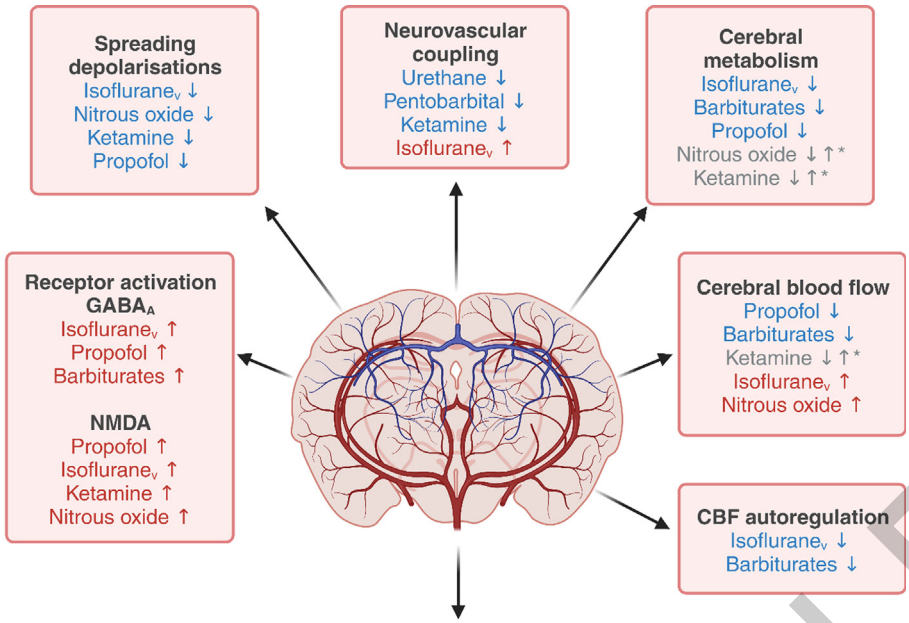

Fig. 1. Summary of cerebral effects of anaesthesia. Anaesthetic effects on physiological states and ischaemic outcome. A decrease in function or activity is indicated in blue, an increase in red and regionally diverse effects are highlighted in grey. Created with BioRender.com.

CEREBRAL METABOLIC RATE

While the brain has an intense metabolic demand, resting CMR is greatly affected by most anaesthetics with changes ranging between 30 to 60% of baseline (Hoffmann et al., 2016). As glucose is the main source of energy in the brain, most CMR measures are dependent on glucose utilisation (Gao et al., 2017). A dose-dependent decrease in CMR was reported during anaesthesia induction using isoflurane (Reasoner et al., 1990), pentobarbital (Crane et al., 1978; Warner et al., 1996), propofol and sevoflurane (Kaisti et al., 2003). However, heterogenous effects depending on dose and brain region were observed in studies using nitrous oxide (Reinstrup et al., 2008). A study in rats by Kofke *et al*. observed similar heterogenous effects using ketamine, with higher CMR recorded in the hippocampus and lower glucose utilisation rates in several other brain regions (Kofke et al., 1992). Overall, the regional coupling between CBF and CMR are important considerations when choosing and utilising anaesthetics in the context of ischaemia (Lenz et al., 1999; Franceschini et al., 2010).

SPREADING DEPOLARISATIONS

SDs are a slowly propagating wave of sustained mass neuronal and glial depolarisation, marking the near-complete breakdown of neuronal ionic homeostasis (Dreier, 2011). Soon after the induction of focal cerebral ischaemia, SDs initiate in the ischaemic tissue and propagate throughout grey matter (Nedergaard and Hansen, 1993; Hartings et al., 2003). Rodent studies of focal cerebral ischaemia have reported that an increase in SD frequency strongly correlates with an increase in infarct volume (Busch et al., 1996; Takano et al., 1996; Dijkhuizen et al., 1999). Therefore, if anaesthetics inhibit SD initiation, reducing SD frequency, or decrease SD depolarisation duration, this could be an explanation for their reported neuroprotective properties. Volatile anaesthetics, N_2O and propofol suppress SDs (Kitahara et al., 2001; Kudo et al., 2008; Dhir et al., 2012; Takagaki et al., 2014), ketamine attenuates SDs (Gorelova et al., 1987; Hernández-Cáceres et al., 1987), while barbiturates and urethane do not suppress SDs (Van Harreveld and Stamm, 1953; Kudo et al., 2008; Klass et al., 2018).

NEUROVASCULAR COUPLING

The effects of anaesthesia on NVC are complex and happen at the interface of changes in neuronal activity, vascular reactivity and CBF; and have been thoroughly reviewed previously (Masamoto and Kanno, 2012; Gao et al., 2017). The compounding effects of anaesthesia on spatial and temporal dynamics of NVC are controversial and debated in the field (Masamoto and Kanno, 2012). However, the quantitative interaction between neural and vascular response is consistently reported to reflect a decreased amplitude of the haemodynamic responses in rodents with most frequently used anaesthetics (Franceschini et al., 2010; Williams et al., 2010). For instance, haemodynamic responses evoked during wakefulness were four-fold larger and two-fold faster compared to responses measured under anaesthesia using isoflurane or urethane, as measured using optical imaging in the primary visual vortex in mice (Pisauro et al., 2013). Urethane was shown to have a suppressive effect on cortical excitability compared to measurements performed in conscious rats (Martin et al., 2006). Similar suppressive effects of cortical activity were observed

under anaesthesia induced with pentobarbital or propofol, while ketamine/xylazine had suppressive effect on the thalamocortical inputs in a comprehensive study by Franceschini *et al*. (Franceschini et al., 2010). However, an increasing dose-dependent effect of isoflurane was noted on stimulus evoked CBF responses at different stimulation frequencies (Masamoto et al., 2009). Furthermore, any of the vascular reactivity and CMR effects described in the sections above could have a compounding effect on altered cortical activity when NVC measurements are undertaken under anaesthesia.

ANAESTHETIC EFFECTS ON STROKE OUTCOME

Despite intensive efforts to demonstrate the neuroprotective effects of anaesthetics in humans, most clinical trials proved negative (Ishida et al., 2014). However, due the complexity of anaesthetic effects in the preclinical setting, the impact on ischaemic outcome must be considered (Hoffmann et al., 2016).

LESION VOLUME

Histologic neuroprotection was observed in several studies comparing awake MCAo with induction under volatile anaesthesia, including isoflurane (Sakai et al., 2007; Seto et al., 2014; Jiang et al., 2017; Neag et al., 2020), sevoflurane and halothane (Warner et al., 1993; Haelewyn et al., 2003; Yang et al., 2022). When Haelewyn *et al.* compared the neuroprotective effects of desflurane and halothane on lesion volume using the intraluminal filament MCAo model, they noted a 55% and 30% decrease respectively, only when the animals remained under anaesthesia for the whole duration of the surgery (Haelewyn et al., 2003). If animals underwent MCAo preparation under anaesthesia and then were awake for the duration of ischaemia induction, there was no difference in lesion volume (Haelewyn et al., 2003). This study highlights the potential effects of immediate cessation of anaesthesia and encourages the use of awake methodology. However, contradictory studies also exist, where isoflurane was shown to only confer initial neuronal protection, which was followed by delayed apoptosis and no effects on lesion volume compared to awake induction (Inoue et al., 2006).

In the case of injectable anaesthetics, barbiturates such as pentobarbital administered during the induction phase of intraluminal filament MCAo was shown to significantly decrease lesion volume by 25% (Warner et al., 1996), however, the effects were modest in comparison to volatile anaesthetics. Inconclusive contradictory effects were observed with propofol (Tsai et al., 1994; Pittman et al., 1997) and ketamine (Jensen and Auer, 1988; Winkelheide et al., 2009) where both positive and negative interactions were noted (Hoffmann et al., 2016). The main reason for controversy was suggested to be poor thermoregulation in some of the studies reported (Warner et al., 1993).

Importantly, preconditioning with isoflurane (1%) at 0, 12 and 24 h before MCAo using the intraluminal filament was also reported to have a neuroprotective effect leading to decreased lesion volumes by −38%, −31% and −24% respectively (Kapinya et al., 2002b). The preconditioning neuroprotective effect has been also confirmed in studies using halothane (Kapinya et al., 2002a) and sevoflurane (Payne et al., 2005; Ye et al., 2012), but not propofol (Bhardwaj et al., 2001) or ketamine (Li et al., 2007). On the other hand, postconditioning with propofol (25 mg/kg/h) immediately or 1 h after MCAo in a rat model of intracerebral injection of endothelin-1 was also demonstrated to significantly decrease lesion volumes up to 80% of the volumes resulted from awake induction (Gelb et al., 2002). Propofol postconditioning also improved lesion volume and promoted neurogenesis in the intraluminal filament MCAo model (Wang et al., 2011). Similar postconditioning effects were also observed for isoflurane (Zhao et al., 2014) and sevoflurane (Lai et al., 2016).

THROMBOLYSIS WITH TISSUE PLASMINOGEN ACTIVATOR

tPA is the standard of care in ischaemic stroke and it is administered without sedation or anaesthesia via intravenous infusion in the clinic. However, in the preclinical setting tPA is administered via a tail vein infusion predominantly under anaesthesia. Therefore, it is essential to consider any potential anaesthetic effects on thrombolysis in the preclinical setting. In this context, Gakuba *et al.* demonstrated the synergistic effects of ketamine on thrombolysis with tPA (Gakuba et al., 2011). For this study, a mouse model of *in situ* thrombotic stroke was performed under anaesthesia, upon recovery mice underwent thrombolysis with tPA awake or anaesthetised. Isoflurane/N_2O and propofol anaesthesia had no effects on thrombolysis resulting in similar lesion volumes as measured in awake mice (Gakuba et al., 2011). However, when thrombolysis was administered under ketamine (3.5 mg/kg in bolus, 47.2 mg/kg per hour) anaesthesia, a synergistic effect was noted, resulting in significantly lower lesion volumes (50–60%) compared to volumes measured in the other anaesthetic groups or the awake mice (Gakuba et al., 2011). None of the anaesthetics tested had any effect on the fibrinolytic activity of tPA *ex vivo* (Gakuba et al., 2011). On the other hand, N_2O was shown to directly inhibit tPA in a dose-dependent manner (Haelewyn et al., 2011). In an embolic model of stroke in rats, N_2O administration during thrombolysis led to lower lesion volumes but an increased rate of haemorrhage occurrence and blood–brain barrier disruption (Haelewyn et al., 2011).

Additional effects of isoflurane in the context of thrombolysis were investigated in the intraluminal filament MCAo. Postconditioning with isoflurane (1.5% for 1 h) following thrombolysis with tPA led to reduced haemorrhagic transformation, lower lesion volumes and improved neurological scores (Kim et al., 2015). The possible mechanism was further investigated *in vitro*, where Kim *et al*. suggested that postconditioning with isoflurane inhibits the induction of matrix metalloproteinases 2 and 9 via the low-density lipoprotein receptor-related protein (Kim et al., 2017).

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FUNCTIONAL OUTCOME

Neurological score assessment (Yang et al., 1994) is the most frequently used functional outcome measure in preclinical ischaemic stroke studies. For instance, the intraluminal filament model results in a significant increase in the neurological score highlighting great sensorimotor deficit (Balkaya et al., 2013; Yu et al., 2019). However, Sakai *et al*. demonstrated the significant impact of anaesthesia on neurological deficit, with MCAo induction during isoflurane being neuroprotective in comparison to awake MCAo induction (Sakai et al., 2007). These protective effects were noted up to eight weeks post MCAo (Sakai et al., 2007). Similar effects were observed with barbiturates (Warner et al., 1996) and propofol (Wang et al., 2011); most frequently these effects were directly corelated with improved histopathological findings as documented above.

Furthermore, a study by Saab *et al*. demonstrated the long-lasting effects of exposure to isoflurane (1.3% for 1 h) on healthy brains in mice. While motor and sensory functions recovered within minutes, short-term memory formation was impaired for up to 24 h post anaesthesia as tested using the fear-associated contextual and cued learning paradigms (Saab et al., 2010). This was reversed using the L-655,708, an α5GABAA receptor-selective inverse agonist, highlighting the inhibition of the receptor early post anaesthesia (Saab et al., 2010). To note, trace amounts of isoflurane were found in the brain up to 24 h post exposure (Saab et al., 2010). Additionally, exposure to general anaesthesia during infancy was shown to have long lasting effects on fear behaviour in healthy adult mice and lower emotional control in humans (Salaün et al., 2023).

AWAKE INDUCTION OF FOCAL CEREBRAL ISCHAEMIA

It is evident in the focal cerebral ischaemic literature that anaesthetics, administered via inhalation or injection in rodent models, compromise many cerebral mechanisms relevant to stroke. Furthermore, the neuroprotective benefits of anaesthetics could be an implication that reduces the efficacy of preclinical to clinical translation. To overcome the requirements of anaesthetics, when conducting ischaemic stroke models in rodents, the experimental design should be surgically engineered so that the stroke model can be completed in an awake animal. Awake induction of stroke is a relatively new strategy and has been attempted in the filament MCAo model, photothrombotic model, endothelin-1 injection model, distal MCAo via ferric chloride, microsphere-induced stroke model but also intracerebral haemorrhage (ICH) model. The specifics of these studies are discussed below.

Intraluminal filament MCAo

The intraluminal filament MCAo model is induced by inserting a monofilament into the internal or external carotid artery (ICA or ECA) depending on variations of the model (Longa et al., 1989; Schmid-Elsaesser et al., 1998). The common carotid artery is commonly clipped or ligated and the filament is advanced until it reaches the MCA bifurcation, with confirmation of decreased blood flow. The duration of ischaemic induction can vary between 15 to 180 min in the literature, and it results in a reproducible MCA territory infarction. As this surgery does not require a craniotomy, the induction of intraluminal filament MCAo using limited anaesthesia has been employed in several studies over-time (Sarraf-Yazdi et al., 1998; Sakai et al., 2007; Van Winkle et al., 2013; Xie et al., 2016). The procedure followed involved induction of anaesthesia for a short (20–45 min) preliminary surgical procedure to allow the filament insertion, temporary ligature of vessels or the optional fixation of any blood flow, mean arterial blood pressure (MABP) or other measuring probes. Anaesthesia was abruptly interrupted, and rodents were allowed to recover freely moving in a chamber during the induction phase of ischaemia. The chamber was provided with normal airflow (Xie et al., 2016) or supplemented with a gas mixture of oxygen and nitrogen dioxide (Sakai et al., 2007) and the temperature was maintained at normothermic levels. For reperfusion, brief anaesthesia was required to allow for painless removal of the filament and surgical site(s) suturing. Depending on the experimental setup available, various physiological measurements were recorded during the awake induction of ischaemia including CBF (Xie et al., 2016), core or pericranial temperature (Haelewyn et al., 2003; Sakai et al., 2007), carbon dioxide and oxygen partial pressures and arterial pH (Sakai et al., 2007) or MABP (Haelewyn et al., 2003).

Photothrombotic stroke

Photothrombotic induction of focal cerebral ischaemia is an *in vivo* photochemical reaction used to form a reproducible thrombosis that requires a laser and a photosensitising dye, such as rose bengal (Watson et al., 1985). Considering the awake stroke methodologies, photothrombosis would be a desirable choice due to the relatively minimal surgical invasiveness required compared to other models. The reported studies require a pre-stroke surgery to implant a headcap or cranial window before the induction of photothrombosis. Seto *et al.* described an experimental set up that enables through the skull cortical photothrombosis induction in freely moving awake mice (Seto et al., 2014). Days before the induction of ischaemia, brief anaesthesia (15–20 mins) was required to implant a headcap, in which an optic fibre can be connected. The study determined the neuroprotective effect of isoflurane during ischaemic stroke induction, noting a significantly larger cerebral infarct in the awake group. Furthermore, the study also reported a 'masking' effect of isoflurane by reducing the efficacy of stroke therapies which aim to reduce cortical excitotoxicity (Seto et al., 2014).

Lu *et al.* utilised a different approach to induce focal ischaemia in awake rats that required the implantation of a cranial window, which was performed under isoflurane anaesthesia (Lu et al., 2014). One day after the surgery, a specifically designed miniature CBF monitoring head stage, which also contained a 532 nm optic

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fibre, was attached to the head of the rat. This setup allowed for targeted distal MCA branch photothrombosis, with simultaneous perfusion monitoring, and no anaesthetics requirements during ischaemic induction. Balbi *et al.* modified this approach to induce focal ischaemia in the awake mouse (Balbi et al., 2017). This experimental design also required the implantation of a cranial window which was performed under isoflurane anaesthesia. There was a seven-day recovery period before the next stages of the experiment. The mice then underwent daily habituation to a head fixation set up, minimising signs of struggling and to encourage normal behaviours. Once habituated, photothrombosis was induced by head fixation and cortical illumination through the cranial window entirely without the need of anaesthetics. The described set up allows for laser speckle contrast imaging (LSCI) and calcium imaging during stroke induction through the cranial window. This strategy was repeated by Sunil *et al.* but was modified to enable targeted vessel occlusion (Sunil et al., 2020). The methods described by the authors above require brief exposure to anaesthetics during the surgical implantation of either a headcap or cranial window. During ischaemic induction, no anaesthetics were used.

Endothelin-1 model

Endothelin-1 is a potent vasoconstrictor used to induce MCAo most commonly by intracerebral injection (Sharkey, 1993), but also by direct application on the cortical surface (Fuxe et al., 1997) or the exposed MCA (Robinson et al., 1975). Some of the model advantages are precise regional targeting and dose dependent action of endothelin-1 (Macrae et al., 1993). The endothelin-1 model was induced fully awake in freely moving rats using a previously implanted cannula (Bogaert et al., 2000; Gelb et al., 2002). Briefly, animals were anaesthetised and fixed in stereotaxic apparatus where a small burr hole (2 mm in diameter) was drilled in the skull at target coordinates relative to bregma. Guide cannulas were inserted and fixed in place and the surgical site was closed. Animals were allowed to recover for a period ranging from one (Bogaert et al., 2000) to four days (Gelb et al., 2002). Using an injection cannula, endothelin-1 was then administered in freely moving awake rats resulting in reproducible infarct size significantly larger compared to anaesthetised animals (Bogaert et al., 2000; Gelb et al., 2002). It is important to note that anaesthetised rats required a four-times higher dose of endothelin-1 to induce similar lesion volumes compared to conscious rats (Bogaert et al., 2000).

Embolic model

For the embolic model, externally generated autologous or heterologous clots are injected in the ECA (Kudo et al., 1982; Kaneko et al., 1985; R. L. Zhang et al., 1997). The surgical procedure is similar to the intraluminal filament MCAo model, with the common carotid artery (CCA) and ICA being temporarily isolated and clamped to restrict blood flow. A catheter is inserted in the ECA and advanced to the MCA origin after which a clot is slowly injected. This model has also been modified for awake induction, where Zhang *et al.* allowed the rats to recover for 45 min after the catheter insertion before injecting an autologous clot (Zhang et al., 2000). This method results in variable and heterogenous infarcts in the MCA territory, however, the model has high external validity due to the formation of fibrin-rich clots sensitive to thrombolysis and recanalisation (Zhang et al., 2015).

Distal MCAo – Ferric chloride application

To induce MCAo, ferric chloride (20%) is topically applied on the MCA bifurcation activating the vascular endothelium (Karatas et al., 2011). Despite requiring an invasive craniotomy, distal MCAo induction results in a platelet-rich thrombus formation resistant to tPA thrombolysis, which poses several advantages when mimicking the lack of recanalisation in the human condition. The awake induction of this model has been described recently in rats (Brunner et al., 2023). The experimental design included the implantation of two cranial windows over intact dura mater, with a metallic headpost for fixation. These implantations facilitate ischaemic induction and imaging and were performed during two different surgical procedures under anaesthesia. The small cranial window (1 mm^2) over the left distal branch of the MCA was protected with a silicon plug. Animals were allowed to recover for one week and were progressively habituated to the head-fixed apparatus while restrained in a sling suit. For induction, the plug was removed, and a drop of ferric chloride solution (20%) was applied in the cranial window while the rats were awake, and head fixed. After confirmation of occlusion, the solution was washed out using saline (Brunner et al., 2023). Awake induction facilitated continuous brain haemodynamic measurements and CBF imaging during distal MCAo without the effects of anaesthesia.

Microspheres-induced model

The microsphere model generates microinfarcts via injection of spheres with a diameter of 20–50 μm and of various materials (Fluri et al., 2015). It is procedurally similar to the embolic model, where a catheter is inserted via the ECA and advanced to MCA, with the CCA ligated. A bolus of 1000–8000 spheres is injected and circulates passively until microspheres become lodged into capillaries of target vessels depending on sphere diameter (Fukuchi et al., 1999). The model leads to formation of variable, multifocal infracts that develop up to 48 h after injection (Mayzel-Oreg et al., 2004). Demura *et al.* first described the awake induction of this model by injecting the microspheres in fully awake freely moving rats 24 h after the catheter was implanted under anaesthesia (Demura et al., 1993).

Intracerebral haemorrhage

Although beyond the scope of this review, it is important to mention that anaesthetic effects are also observed in animal models of ICH (Wilkinson et al., 2020). The intracerebral injection of collagenase is the most frequently

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used ICH model in rodents (Rosenberg et al., 1990). The awake induction of this model was recently described (Wilkinson et al., 2020), following a similar protocol as detailed above for the endothelin-1 model. Briefly, a guide canula was inserted in the striatum under anaesthesia. After a three-day recovery period, collagenase was injected in awake or anaesthetised rats using isoflurane (2–2.5% for 25 min) (Wilkinson et al., 2020). The neuroprotective effects of isoflurane were noted for decreasing haematoma volumes, core temperature and blood pressure, while blood glucose levels were two-fold higher compared to conscious rats (Wilkinson et al., 2020).

Choosing a preclinical ischaemic stroke model should always be matched to the aim and the translational relevance of the study. Beyond the typical model considerations of stroke aetiology, size, region affected, or behavioural deficits, the effects of anaesthesia, the type of anaesthetic and the possible cerebral effects induced should also be considered. The anaesthetic effects vary depending on the model and protocol of choice. While the awake intraluminal filament MCAo model is induced upon immediate recovery from anaesthesia, other models offer the possibility of later induction after preliminary preparations. A full recovery from anaesthetic effects might be of importance for some studies, especially as trace amounts of anaesthetics were found in the brain up to 24 h (Saab et al., 2010). Finally, according to the Stroke Therapy Academic Industry Roundtable (STAIR) guidelines (Fisher et al., 2009) for any experimental setting aiming to validate interventional studies, more than one model or species should be employed.

AWAKE METHODOLOGY

While anaesthesia is a useful tool for imaging in reducing motion artifacts and background noise, the anaesthetic effects have been debated in the imaging field due to their impact on CBF, vasculature, CMR, NVC and oxygenation states (Gao et al., 2017). Therefore, the following methods have been described for awake measurements with varying amount of freedom of movement: laser-doppler flowmetry (Gu et al., 1999a; Takuwa et al., 2011), functional magnetic resonance imaging (Sicard et al., 2003; King et al., 2005), functional ultrasound (Brunner et al., 2023), two photon imaging (Yang et al., 2013; Dombeck and Tank, 2014), LSCI (Miao et al., 2011), optical coherence doppler tomography (Pan et al., 2023), optical imaging using near infrared spectroscopy (Berwick et al., 2002; Sharp et al., 2015; Brothers et al., 2021), diffuse optical imaging (Franceschini et al., 2010), telemetry devices to monitor neuronal activity (Schregardus et al., 2006) and calcium imaging (Balbi et al., 2017).

GUIDELINES AND ETHICAL CONSIDERATIONS

To improve translatability and reproducibility of awake stroke induction, it is imperative to accurately report study design and experimental procedures as per the *Animal Research: Reporting In Vivo Experiments (ARRIVE) 2.0 guidelines* (Sert *et al.*, 2020). Of the essential criteria detailed in the guidelines, the following are key to *experimental design* when employing awake stroke methods: using appropriate controls (e.g. awake sham surgery); reporting awake surgery success and mortality rates; *sample size* (considering greater effect size previously reported with awake induction (Seto et al., 2014)); *inclusion and exclusion criteria* (reporting side effects of awake induction, investigating differential blood flow response for stroke confirmation without the confounding effects of anaesthesia). Most notably, to facilitate the standardisation of awake stroke induction, *experimental procedures* should be carefully reported, emphasising the following aspects: detailed methodology (including experimental setup, stroke induction and habituation protocols); if anaesthesia was employed at any point in the study design (including anaesthetic used, dose, duration and route of administration); *physiological monitoring* (body temperature, blood flow, motion, behavioural phenotypes, etc.); environmental considerations (olfactory, visual, auditory cues). *Animal care and monitoring* should also be adapted for awake methodology, considering *housing and husbandry* (adapted to head stages if needed), the use of analgesics (type, dose, timing and route of administration). Although not specific to awake methodology, *blinding* and *randomisation* are important aspects of preclinical work and should be applied to all *in vivo* stroke work, alongside the remaining *ARRIVE 2.0* (Sert *et al.*, 2020) guidelines.

In line with the *STAIR* preclinical criteria (Fisher et al., 2009), awake induction improves the *external validity* of stroke models, as the majority of human strokes happen during an awake state. Therefore, awake preclinical stroke induction could be implemented as a key step in the drug development pipeline to avoid the confounding and neuroprotective effects of anaesthesia.

One of the main concerns of awake methodology is the ethical consideration that animals might experience elevated stress levels or pain. However, the most surgically intense part of all models is performed under anaesthesia prior to stroke induction as described above. On the other hand, for surgical interventions and imaging studies requiring immobilisation (such as using a sling suit or restraining device), it was previously shown that acclimatisation to the equipment used was successful in reducing physiological parameters indicative of stress (Parry and McElligott, 1993; King et al., 2005). After only eight days of habituation to full body restrainer for imaging, King *et al*. demonstrated that heart and respiratory rates, as well as the amount of corticosterone (stress hormone) in serum were reduced to baseline levels in rats (King et al., 2005). Performance in cognitively demanding tasks was also successful during head fixation as assessed in several studies (Komiyama et al., 2010; Mayrhofer et al., 2013) suggesting successful habituation. Other non-invasive methods can be employed to monitor pain during awake experimental setups, such as the grimace scales (Langford et al., 2010; Sotocinal et al., 2011) – to monitor animal wellbeing; recording of behavioural phenotypes (vocalizations, head scratching, aggressiveness, ambulation) (Seto et al., 2014); or pupil dynamics - to detect an altered

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state of arousal and vigilance (Sobczak et al., 2021; Zeng et al., 2022). Finally, expert training of *in vivo* researchers is a critical first step to ensure minimal animal stress and pain experienced during procedures; and to reduce experimental variability. We highly suggest surgical competency is achieved first in anaesthetised models before moving to awake induction.

FUTURE PERSPECTIVES

In this review, we have discussed the cerebral effects of anaesthesia on histopathological and functional ischaemic outcome in rodents and the potential to mask therapeutic benefits. Therefore, it is evident that anaesthesia should be carefully considered when constructing an experimental design. We aimed to outline existing methodologies which performed well established models of focal cerebral ischaemia in awake rodents. Despite the increased technical challenges and more extensive ethical considerations, the awake induction of stroke has the potential of improving the clinical translation of rodent studies.

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