HHS Public Access

Author manuscript

Exp Neurol. Author manuscript; available in PMC 2022 January 01.

Published in final edited form as:

Exp Neurol. 2021 January ; 335: 113494. doi:10.1016/j.expneurol.2020.113494.

Impact of Aging and Comorbidities on Ischemic Stroke Outcomes in Preclinical Animal Models: A Translational Perspective

Eduardo Candelario-Jalil[a], **Surojit Paul**[b]

[a]Department of Neuroscience, McKnight Brain Institute, University of Florida, Gainesville, FL 32610, USA

[b]Department of Neurology, University of New Mexico Health Sciences Center, Albuquerque, NM 87131, USA

Abstract

Ischemic stroke is a highly complex and devastating neurological disease. The sudden loss of blood flow to a brain region due to an ischemic insult leads to severe damage to that area resulting in the formation of an infarcted tissue, also known as the ischemic core. This is surrounded by the peri-infarct region or penumbra that denotes the functionally impaired but potentially salvageable tissue. Thus, the penumbral tissue is the main target for the development of neuroprotective strategies to minimize the extent of ischemic brain damage by timely therapeutic intervention. Given the limitations of reperfusion therapies with recombinant tissue plasminogen activator or mechanical thrombectomy, there is high enthusiasm to combine reperfusion therapy with neuroprotective strategies to further reduce the progression of ischemic brain injury. Till date, a large number of candidate neuroprotective drugs have been identified as potential therapies based on highly promising results from studies in rodent ischemic stroke models. However, none of these interventions have shown therapeutic benefits in stroke patients in clinical trials. In this review article, we discussed the urgent need to utilize preclinical models of ischemic stroke that more accurately mimic the clinical conditions in stroke patients by incorporating aged animals and animal stroke models with comorbidities. We also outlined the recent findings that highlight the significant differences in stroke outcome between young and aged animals, and how major comorbid conditions such as hypertension, diabetes, obesity and hyperlipidemia dramatically increase the vulnerability of the brain to ischemic damage that eventually results in worse functional outcomes. It is evident from these earlier studies that including animal models of aging and comorbidities during the early stages of drug development could facilitate the identification of neuroprotective strategies with high likelihood of success in stroke clinical trials.

Corresponding author: Eduardo Candelario-Jalil, Ph.D., Department of Neuroscience, McKnight Brain Institute, 1149 SW Newell Drive, University of Florida, Gainesville, FL 32610, USA. Phone: +1-352-273-7116. Fax: +1-352-392-8347. ecandelario@ufl.edu.

Declaration of Competing Interests

The authors declare no competing interests.

Keywords

Aging; Comorbidity; Diabetes; Hyperlipidemia; Hypertension; Inflammation; Ischemic stroke; Neuroprotection; Obesity; Stroke models

1. Introduction

Stroke is defined as a sudden disruption in blood flow to the brain due to either occlusion (ischemic stroke) or rupture (hemorrhagic stroke) of a cerebral blood vessel. Transient ischemic attacks (TIA) or mini-strokes occur when blood supply to the brain is interrupted only briefly, and they are usually a warning of a full-blown stroke. Ischemic stroke or focal cerebral ischemia is mainly caused by either a *thrombus*, blood clot formed in a major brain artery, or by an *embolus*, blood clot formed outside of the brain, most commonly in the carotid circulation. These *emboli* formed in the periphery travel to the brain and can lodge in a major cerebral artery or in a penetrating arteriole. Blood clot formation is precipitated by atherosclerosis and atrial fibrillation, which are major risk factors for ischemic stroke (Elkind, 2006; Virani et al., 2020).

Interruption of cerebral blood flow dramatically impairs energy production resulting in the collapse of ionic homeostasis and excessive release of the neurotransmitter glutamate, which in turn leads to neuronal cell death and the development of a cerebral infarct. Focal ischemic stroke is characterized by an infarcted core, where cell death occurs within minutes after arterial occlusion and brain tissue in this region is generally considered unsalvageable. The peri-infarct region surrounding the ischemic core is termed penumbra (tissue at risk), where there is partial reduction in blood supply due to the presence of collateral vessels. Salvaging of the penumbra by prompt recanalization correlates with better neurological outcomes in stroke patients (Kakuda et al., 2008; Kidwell, 2013; Legrand et al., 2016; Ma et al., 2015). Therefore, the penumbral tissue is the main target for the development of neuroprotective drugs (Adibhatla and Hatcher, 2008; Hermann et al., 2019b).

Based on recent data from the World Health Organization, stroke is the leading cause of adult neurological disability and the second cause of death worldwide after ischemic heart disease (Johnson et al., 2019). With the increase in the aging population the burden of stroke is likely to increase dramatically in the coming years. According to recent statistics from the American Heart Association (Virani et al., 2020), ischemic stroke accounts for about 80–85% of all stroke cases in the Caucasian population. Between 15–20% of all the stroke cases are hemorrhagic, which comprise intracerebral hemorrhage (ICH) and subarachnoid hemorrhage (SAH). Globally, 68% of all strokes are ischemic and 32% are hemorrhagic (Lozano et al., 2012). This is mainly due to a higher incidence of ICH in many Asian countries (Hata and Kiyohara, 2013; Khan et al., 2017; Panel, 2000; Sudlow and Warlow, 1997; Venketasubramanian et al., 2017).

With the exception of reperfusion therapies utilizing recombinant tissue plasminogen activator (rtPA) or mechanical thrombectomy, there are no other therapeutic interventions to reduce ischemic brain injury and neurological deficits in stroke patients. Over the last two decades, the stroke research community has made significant progress in understanding the

pathophysiological mechanisms of ischemic brain injury. A large number of candidate neuroprotective drugs have been identified as potential therapies based on highly promising results from studies utilizing ischemic stroke models in rodents. However, most of these drugs failed to show efficacy in randomized clinical trials conducted in ischemic stroke patients. The failure to translate preclinical studies in rodents to the clinic is due to many factors, including insufficient statistical power, poor experimental design, publication bias, lack of randomization and blinding in many preclinical studies, and unrealistic therapeutic time window (Berge et al., 2017; Dirnagl and Macleod, 2009; Sena et al., 2010; van der Worp et al., 2010). Numerous recent articles and commentaries have been published discussing the main causes of the translational roadblock in stroke research, as well as possible solutions to this problem (Bosetti et al., 2017; Fisher et al., 2009; Lalu et al., 2019; O'Collins et al., 2006; Philip et al., 2009; Savitz et al., 2019; Schmidt-Pogoda et al., 2020).

In this review, we aim to discuss the need to have better preclinical models of stroke that more accurately mimic the stroke population by incorporating aged and reproductively senescent rodents, as well as animals with comorbid conditions. We discuss recent findings highlighting the stark differences in the response to ischemic stroke between young and aged rodents, and how major comorbid conditions such as hypertension, diabetes, obesity, and hyperlipidemia dramatically alter stroke outcomes.

2. Neuroprotection in Stroke – Urgent Need of Novel Therapeutics in the Era of Thrombolysis

Clinical approaches that aimed at increasing perfusion of the ischemic territory with rtPA thrombolysis or with endovascular clot removal have been proven very efficacious when given in a timely manner upon hospital arrival. For many acute stroke patients, rtPA thrombolysis is recommended within the first 4.5 hours of symptoms onset (Demaerschalk et al., 2016; Emberson et al., 2014) or within the first 6 h for a subset of patients with large vessel occlusions (Goyal et al., 2016). However, this time line of thrombolytic treatment for acute stroke care has changed due to recent progress in perfusion imaging modalities using magnetic resonance imaging (MRI) and computed tomography (CT), which allows identification of brain infarcted tissue and surrounding penumbral tissue in stroke patients in a relatively timely manner. Data from recent studies suggest that extending pharmacological thrombolysis with rtPA or desmoteplase up to 9 h from stroke onset improves functional outcomes in patients with salvageable tissue as assessed by perfusion-diffusion MRI imaging (Campbell et al., 2019; Hacke et al., 2005; Ma et al., 2019; Thomalla et al., 2018; Zhao et al., 2019). Similarly, successful recanalization with mechanical thrombectomy has been shown to be beneficial when the endovascular procedure is performed within 24 h of stroke onset in patients with a significant amount of salvageable ischemic brain tissue (Albers et al., 2018a; Albers et al., 2018b; Casetta et al., 2020; Lansberg et al., 2005; Nogueira et al., 2018; Sarraj et al., 2019).

Despite the success of reperfusion therapies, a large percentage of stroke patients are not eligible to receive them due to several factors including late hospital arrival, potential for complications especially in older patients, and increased risk of hemorrhagic transformation

associated with delayed rtPA treatment. Moreover, endovascular clot removal (thrombectomy) is only available at specialized hospitals and successful recanalization with this approach occurs in approximately two-thirds of stroke patients subjected to thrombolytic treatment (Flottmann et al., 2018). Treatment rates with rtPA or thrombectomy vary from 3.4 to 9.1% for acute ischemic stroke patients, according to several databases from clinical centers around the world. For intra-arterial rtPA delivery, the treatment rates are significantly lower (Fassbender et al., 2017). These statistics show that a very large number of patients never receive reperfusion therapies highlighting the urgent need for neuroprotective strategies to reduce the progressive cell death associated with worse neurological outcomes in ischemic stroke patients.

It is evident from recent clinical studies that post-ischemic cell death in the brain progresses over the course of several hours or days, even in patients that have been successfully recanalized (Elijovich et al., 2016; Federau et al., 2016; Labeyrie et al., 2012; Legrand et al., 2016; Seners et al., 2015; Soomro et al., 2020; Tisserand et al., 2016). As such, there is high enthusiasm to combine reperfusion therapies with neuroprotectants to reduce delayed ischemia-reperfusion injury. This is exemplified by recent trials testing the combination of rtPA with 3K3A-APC, a recombinant variant of human activated protein C (Lyden et al., 2019), rtPA with intravenous glibenclamide (glyburide), a blocker of sulfonylurea receptor 1 (Huang et al., 2020; Sheth et al., 2016), and nerinetide (NA-1, a peptide that interferes with post-synaptic density protein 95) combined with rtPA (Hill et al., 2020). Combining reperfusion approaches with protective drugs would also facilitate the access of the drug to the ischemic territory potentially increasing its therapeutic efficacy.

Neuroprotection is defined as a therapeutic intervention or combination of therapeutic strategies aimed at blocking, preventing, or interrupting the deleterious biochemical and molecular pathways that, if left unchecked, would eventually result in irreversible ischemic brain injury (Ginsberg, 2008). Most studies have focused on protecting the neurons, but several cell types die following a stroke. Protecting all cell types vulnerable to ischemic cell death should be the focus of neuroprotective strategies (Candelario-Jalil, 2009; Dirnagl et al., 1999). Ideally, a neuroprotectant would prevent cell death in the penumbral tissue, have a low risk of adverse effects, be easily administered in the pre-hospital setting (ambulance, emergency room, triage, imaging) and be given concomitantly with reperfusion therapy by pharmacological thrombolysis or endovascular approaches (Patel and McMullen, 2017; Shi et al., 2018). Agents targeting the vascular occlusion/clot such as thrombolytics, anti-thrombotic, anti-platelets, and fibrinogen-depleting drugs are excluded from the cerebroprotective/neuroprotective category since these classes of drugs act mainly via hemodynamic mechanisms (clot removal) rather than targeting injury mechanisms of the ischemic cascade (Ginsberg, 2008).

Pathophysiological mechanisms of ischemic stroke have been extensively investigated at the vascular, cellular, and molecular levels, which led to the identification of several potential targets to diminish brain injury, improve neurological outcomes, and promote neural repair (Carmichael, 2016; Iadecola, 2017; Iadecola and Anrather, 2011a, b; Iadecola et al., 2020; Moskowitz et al., 2010). Cellular and molecular events of the ischemic cascade are highly complex and interconnected, with several cell death mechanisms occurring simultaneously

(Dirnagl et al., 1999; Kang and Yao, 2020; Malone et al., 2019; Moskowitz et al., 2010; Shi et al., 2019). A multi-pronged approach that targets multiple pathways with multifactorial drugs or a combination of drugs is expected to increase the chances of successful neuroprotection in stroke. Multiple earlier reviews have summarized some of the promising pharmacological and non-pharmacological strategies for treatment of ischemic stroke, and discussed their mechanisms of neuroprotection (Ginsberg, 2016; Grupke et al., 2015; Moretti et al., 2015a, b; Neuhaus et al., 2017). The pharmacological agents target excitotoxicity, oxidative stress, neuroinflammation, blood-brain barrier (BBB) breakdown and vasogenic edema, whereas the non-pharmacological approaches include induction of hypothermia, remote ischemic preconditioning, and cell-based therapies. In addition, growth factors and other agents aim to enhance neurorepair.

Many reasons for the failure of protective agents from preclinical to clinical studies have been extensively discussed in several review articles (Babadjouni et al., 2017; Berge et al., 2017; Bosetti et al., 2017; Cho and Yang, 2018; Fisher et al., 2009; Grupke et al., 2015; Lalu et al., 2019; O'Collins et al., 2006; Philip et al., 2009; Rajah and Ding, 2017; Savitz et al., 2019; Schmidt-Pogoda et al., 2020; Shi et al., 2018; Sutherland et al., 2012; van der Worp et al., 2010), and are summarized here in Figure 1.

In subsequent sections of this review article, we will discuss recent studies highlighting the need to perform preclinical studies in aged rodents and rodent models with comorbidities in order to increase the likelihood of translating neuroprotective strategies to the clinic. Since the incidence of stroke is more prevalent in the aged population with comorbid conditions, preclinical studies evaluating the efficacy of potential neuroprotective drugs using healthy and young rodents have low external validity and may fail as they do not mimic the clinical situation (Schmidt-Pogoda et al., 2020; van der Worp et al., 2010).

3. Response of the aged brain to ischemic stroke

Age is the most significant non-modifiable risk factor for many human diseases, and the single most important risk factor for ischemic stroke (Popa-Wagner et al., 2020; Sacco, 1997; Sacco et al., 1997; Virani et al., 2020). With every decade of life, the incidence of stroke more than doubles (Mozaffarian et al., 2016). Aging promotes the development of many vascular risk factors such as hypertension, diabetes, hyperlipidemia, and obesity. Moreover, advanced age is associated with profound pathophysiological changes in both the CNS and the periphery, which underlie the increased susceptibility of the brain to ischemic injury, resulting in worse functional outcomes after stroke (Figure 2). Therefore, performing preclinical studies with neuroprotective agents in aged rodents might provide meaningful insights into the potential protective effects of the drugs in the elderly stroke population, increasing the translational impact and relevance of the findings.

Clinical studies have shown that age is an independent predictor of neurological outcome in acute ischemic stroke patients following reperfusion therapies. Despite similar rates of arterial recanalization, aged patients perform worse than younger individuals, and the viable penumbral tissue is more rapidly recruited into the infarct in the aged population, as assessed by diffusion-weighted imaging (DWI)/Perfusion-weighted imaging (PWI) mismatch on MRI

(Ay et al., 2005; Kruetzelmann et al., 2011; Mishra et al., 2010; Sharma et al., 2020; Weimar et al., 2004). In line with the clinical findings, animal studies consistently show that aged rodents develop worse neurological deficits, impaired long-term functional recovery, exacerbated BBB damage and vasogenic edema, as well as increased mortality compared with young animals (Andersen et al., 1999; Brown et al., 2003; Buchhold et al., 2007; Chen and Sun, 2007; Crapser et al., 2016; Davis et al., 1995; DiNapoli et al., 2008; Lindner et al., 2003; Ritzel et al., 2018; Sutherland et al., 1996; Wang et al., 2003; Won et al., 2006; Zhang et al., 2005). Interestingly, there are some discrepancies in the ischemic infarct size observed in aged animal models of stroke. Compared to the young control group, some studies show that aged animals have larger stroke volumes (DiNapoli et al., 2008; Doyle et al., 2010; Kelly et al., 2009; Ma et al., 2020; Suenaga et al., 2015), while others report the opposite (Liu et al., 2010; Liu et al., 2012; Liu and McCullough, 2012). Sex seems to be an important variable in the observed differences in infarct volume between young and aged rodents. Middle-aged females display larger infarcts compared to young females or aged mice of either sex (Manwani et al., 2013). A recent study showed that aged males have greater mortality and sensorimotor impairment than aged female mice after stroke (Ahnstedt et al., 2020). These studies emphasize the complex interactions between sex, hormonal changes, and the aging process in response to focal ischemic brain injury.

3.1. Aging increases stroke-induced neurovascular damage

Aging has a profound impact on the cerebrovasculature with damaging consequences in the context of stroke. During normal human brain aging, there are structural and functional changes in cells composing the neurovascular unit (endothelial cells, neurons, astrocytes, pericytes, microglia) that result in significant alterations in brain perfusion and permeability of the BBB. Human studies have shown 25–40% reduction in cerebral blood flow (CBF) and oxygen consumption between 30 and 89 years of age (Ainslie et al., 2008; De Vis et al., 2015; Matteis et al., 1998; Pantano et al., 1984). Conflicting data exist regarding changes in the BBB permeability associated with normal aging. Compared to the young brain, some studies found the BBB to be leakier to various tracers in aged subjects, which is associated with modifications in tight junction proteins (Farrall and Wardlaw, 2009; Goodall et al., 2019; Goodall et al., 2018; Hafezi-Moghadam et al., 2007; Janota et al., 2015; Montagne et al., 2015; Popescu et al., 2009; Senatorov et al., 2019; Shin et al., 2015; Stamatovic et al., 2019; Yang et al., 2020), while others show that the BBB remains intact in the aged brain (Banks et al., 2000; Mooradian and McCuskey, 1992; Vorbrodt and Dobrogowska, 1994; Wadhwani et al., 1991).

In ischemic stroke models, there is exacerbated BBB damage leading to vasogenic edema and higher mortality in aged animals when compared to young animals (DiNapoli et al., 2008; Kelly et al., 2009; Tan et al., 2015; Yu et al., 2019). The underlying mechanisms for the increased vulnerability of the aged brain to neurovascular injury are complex and involve higher susceptibility to oxidative damage, increased pro-inflammatory cytokines (e.g., interleukin-1β and tumor necrosis factor-α) and production of matrix metalloproteinase-9 (MMP-9). The neurovascular unit is particularly susceptible to hypoxia in the aged brain with neurons and endothelial cells being most vulnerable and undergo rapid cell death (Macri et al., 2010; Ostergaard et al., 2016; Popa-Wagner et al., 2007a). This could explain

the earlier appearance of infarcted brain tissue assessed by MRI in aged animals when compared to young animals following focal ischemic injury (Canese et al., 1998; Titova et al., 2014).

3.2. Altered neurovascular coupling in aged animals

Spreading depolarization (SD) is a major cause of neuronal damage and expansion of the infarct in ischemic stroke. This phenomenon has been well documented in both animals and stroke patients (Dohmen et al., 2008; Dreier, 2011; Dreier et al., 2018; Lapilover et al., 2012; Ostergaard et al., 2015; Strong et al., 2007). Neuronal death related to SD events after stroke is thought to be caused by an insufficient hyperemic response, where tissue acidosis seems to play an important role (Menyhart et al., 2017). Compared to young animals, the magnitude of the SD-evoked CBF response is significantly reduced with aging, which could prolong tissue acidosis and increase neuronal vulnerability after ischemic injury (Balint et al., 2019; Hertelendy et al., 2019; Menyhart et al., 2015; Menyhart et al., 2017).

3.3. Impaired collateral circulation in aging

Aging significantly impairs the cerebral collateral circulation by promoting the rarefaction of collateral vessels (Faber et al., 2011). This process leads to a significant reduction in the blood flow to the penumbral tissue, accelerating tissue infarction and edema, which ultimately results in worse stroke outcomes. Leptomeningeal (pial) anastomotic connections between adjacent vascular territories serve as a ‘backup’ mechanism, or vascular redundancy, for when blood flow to a particular region is reduced due to vessel occlusion as it occurs during ischemic stroke (Ginsberg, 2016; Winship, 2015). By restoring flow to the affected region from other vascular territories, the leptomeningeal collateral circulation plays a key role in limiting the recruitment of the ischemic penumbra into the infarct core.

In a recent study using two-photon laser scanning microscopy combined with laser speckle contrast imaging, pial collaterals between the middle cerebral artery (MCA) and the anterior cerebral artery (ACA) were monitored during distal MCA occlusion in young and aged rats. Following MCA occlusion, there is a significant decline in collateral perfusion in both aged and young rats, with aged rats showing a more dramatic decline in penumbral perfusion via leptomeningeal collaterals, which translated into larger areas of ischemic brain injury (Ma et al., 2020).

3.4. Aging significantly alters the neurogenic and angiogenic responses following ischemic brain injury

Neurogenesis is significantly impaired with aging, both under normal conditions and in response to stroke (Apple et al., 2017; Cutler and Kokovay, 2019; Daynac et al., 2016). The number of proliferating neural progenitor cells (NPCs) in the subventricular zone and the dentate gyrus subgranular zone are lower in old compared to young rats after stroke. Also, the ability of newly formed progenitor cells to differentiate into neurons is significantly impaired in the aged animals (Darsalia et al., 2005; Jin et al., 2004). Surprisingly, there is increased neurogenesis in the unaffected (contralateral) hemisphere in the aged, but not young, mice after focal ischemia. In an elegant study using mice expressing luciferase in doublecortin positive cells, Adamczak *et al* showed that in middle-aged and old mice there is

a significant upregulation of neurogenesis in the contralesional hemisphere in response to stroke (Adamczak et al., 2017). Neurorehabilitation with forced limb-use is highly effective in increasing neurogenesis and neurological recovery after stroke in aged rats (Qu et al., 2015). In a very recent study, electrical stimulation was utilized to stimulate neurogenesis, leading to improved functional outcomes in aged rats following permanent cortical stroke (Balseanu et al., 2020).

Similar to what occurs to the neurogenic response, aging studies in humans and animals show that there is progressive failure of brain angiogenesis in old compared to young during both physiological conditions and in response to injury, which is associated with a significant alteration in the expression of several angiogenesis-associated genes (Black et al., 1989; Murugesan et al., 2012; Popa-Wagner et al., 2010a; Riddle et al., 2003). Impaired neurogenesis and angiogenesis could be an important contributor to poorer outcomes after ischemic injury in the aged brain. Inhibition of angiogenesis dramatically impairs the survival of migrating neuroblasts after stroke, suggesting that the neurogenic response is highly dependent on angiogenesis (Nih et al., 2012). Recent studies have shown that it is possible to therapeutically enhance neurogenesis and angiogenesis in the aged brain to improve functional recovery after stroke. Growth differentiation factor-11 (GDF11) significantly increases angiogenesis, improves white matter integrity, and reduces sensorimotor deficits in aged mice subjected to transient focal cerebral ischemia (Hudobenko et al., 2020). In other studies, post-ischemia treatment with omega-3 polyunsaturated fatty acids resulted in a significant improvement in neurological function associated with enhanced angiogenesis and reduction in white matter damage in a permanent distal middle cerebral artery occlusion (MCAO) model in aged mice (Cai et al., 2017; Jiang et al., 2019). Increased neurogenesis and differentiation of neuroblasts by granulocyte-colony stimulating factor (G-CSF) treatment significantly increases motor recovery in aged rats after stroke (Popa-Wagner et al., 2010b).

3.5. Inflammaging and gut dysbiosis exacerbate ischemic stroke outcomes

Chronic and heightened inflammation associated with aging, a phenomenon coined '*inflammaging*' (Franceschi et al., 2018; Furman et al., 2019) seems to play a key role in ischemic stroke outcomes. A large body of evidence from animal and human studies shows that inflammaging is triggered by various stimuli including viral and bacterial infections, cell debris, as well as misfolded and oxidatively-modified proteins. Gut microbiota and intestinal immune responses take center stage in inflammaging. Age-related gut dysbiosis (altered ratio of *Firmicutes* to *Bacteroidetes* and reduced bacterial diversity) and increased gut leakiness have been documented in animals and humans (Biagi et al., 2011; Franceschi et al., 2018; Thevaranjan et al., 2017). Age-related changes in microbiota composition drive gut permeability, increased systemic inflammation, and altered immune cell function (Thevaranjan et al., 2017), which could exacerbate pathological processes in many diseases including stroke. Inflammaging increases the vulnerability of the aged brain to ischemia and enhances the post-stroke inflammatory response.

Several studies have found increased intestinal permeability, enhanced translocation and dissemination of commensal bacteria, and gut dysbiosis in response to ischemic stroke

(Ahnstedt et al., 2020; Blasco et al., 2020; Chen et al., 2019; Crapser et al., 2016; Ferrara et al., 2020; Houlden et al., 2016; Kurita et al., 2020; Liu et al., 2019; Singh et al., 2016; Stanley et al., 2016; Stanley et al., 2018; Wen et al., 2019; Xia et al., 2019). Importantly, aged animals show worse gut dysbiosis and intestinal permeability after stroke (Crapser et al., 2016; Wen et al., 2019). The aged biome seems to worsen ischemic stroke outcomes, at least in part, by increasing levels of systemic pro-inflammatory mediators. Fecal transplant gavage of microbiota from young to aged mice significantly reduces mortality and improves neurobehavioral outcomes following MCAO (Spychala et al., 2018).

Production of short-chain fatty acids (SCFAs), primarily butyrate, acetate and propionate, is significantly reduced in aged animals (Lee et al., 2020; Spychala et al., 2018). SCFAs are important signaling molecules mainly produced by bacterial metabolism in the gut. Recent evidence demonstrates that increasing levels of SCFAs improves ischemic stroke outcomes in animal models (Lee et al., 2020; Sadler et al., 2020). The study by Lee *et al.* showed for the first time that the worse stroke recovery in aged mice can be reversed by post-ischemia "bacteriotherapy" with SCFA-producing bacterial strains given by oral gavage (Lee et al., 2020).

3.6. Microglial and astroglial responses to ischemic damage in the aged brain

Age-associated changes in microglia/macrophage function could have a big impact on neuroinflammation and neurovascular function after ischemic brain injury. Microglia in the aged brain show a chronic 'primed' pro-inflammatory phenotype, are less phagocytic, produce high levels of reactive oxygen species (ROS), and secrete more pro-inflammatory mediators associated with damaging pathogenic events (Frank et al., 2006; Godbout et al., 2005; Kim and Cho, 2016; Marschallinger et al., 2020; Mosher and Wyss-Coray, 2014; Salas et al., 2020; Streit et al., 2004; Streit and Xue, 2010). Overall, these microglial changes associated with aging make the microenvironment of the CNS more pro-inflammatory. Exaggerated neuroinflammation in the aged brain could worsen ischemic stroke pathology and interfere with neurorepair and functional recovery. Interestingly, the gut microbiota is a critical regulator of microglial function mainly via production of SCFAs and aryl hydrocarbon (AhR) ligands (Erny and Prinz, 2020). A very recent study showed that SCFA supplementation in the drinking water improved outcomes in models of ischemic stroke mainly through reduction in microglial activation (Sadler et al., 2020).

Using RNA sequencing, recent studies compared microglial cells isolated from 2.5-month-old and 18-month-old mice subjected to permanent distal MCAO. Significant increase in transcription of many pro-inflammatory genes was observed in microglia obtained from aged naïve mice, suggesting heightened on-going inflammation in the aged brain. In response to stroke, aged mice showed impaired transcriptional activation of genes involved in immune cell chemotaxis, tissue remodeling, cell-cell interactions, and inflammatory responses, which may contribute to enhanced vulnerability and worse recovery in aged animals after ischemic stroke (Jiang et al., 2020; Shi et al., 2020). Aged mice exhibited a reduced number of microglia/macrophages expressing phenotypic markers of alternative activation (M2 polarization) compared with young animals following focal cerebral ischemia (Suenaga et al., 2015).

Aging also accelerates astrocytic responses to ischemic injury. Earlier formation of an astroglial scar has been documented in aged rats (20-month old) compared to young animals in response to ischemic stroke (Popa-Wagner et al., 2007b; Popa-Wagner et al., 2006). This age-associated premature astroglial scarring could be an impediment for neuronal plasticity and neurorepair in the aftermath of an ischemic event.

3.7. More neutrophils infiltrate the aged brain after stroke

Infiltration of peripheral immune cells into the ischemic brain is a critical event in the ischemic cascade, having a huge impact on tissue fate, functional outcomes, and neurorepair processes. Among the infiltrated immune cells, neutrophils are the first responders to injury and they have been shown to worsen stroke pathology by releasing proteolytic enzymes (e.g., MMP-9, neutrophil elastase, cathepsin G), disrupting the BBB, producing blockage of blood vessels due to their local accumulation in the ischemic territory (no-reflow phenomenon), and releasing ROS (Lambertsen et al., 2019; Otxoa-de-Amezaga et al., 2019a; Otxoa-de-Amezaga et al., 2019b; Perez-de-Puig et al., 2015). Compared to the young, the aged ischemic brain shows a larger number of neutrophils that have a reduced phagocytic function and produce high levels of MMP-9 and ROS (Ritzel et al., 2018). Since microglial phagocytosis controls the accumulation and fate of invading neutrophils after stroke (Otxoa-de-Amezaga et al., 2019b), and aging impairs the phagocytic capacity of microglia, it is tempting to speculate that increased neutrophil infiltration in the aged ischemic brain is due to alterations in microglial-mediated clearance of extravasated neutrophils. However, this hypothesis needs to be demonstrated experimentally.

3.8. Oxidative stress in the ischemic aged brain

Shortly after ischemic brain damage, there is excessive ROS production and, at the same time, cellular antioxidant mechanisms become deficient, leading to oxidative stress (Heo et al., 2005). Compared to other organs, the brain is highly susceptible to oxidative stress, and the aged brain is more so. There is a powerful association between the antioxidant status and longevity, suggesting that increased ROS production precipitates the aging process. Aging is associated with mitochondrial dysfunction, which makes the body more susceptible to increased oxidative stress after injury. Oxidative damage to endothelial cells that line the brain vasculature contributes to vasogenic edema after stroke (Chan, 2001). Cellular antioxidant mechanisms are decreased in older individuals contributing to exaggerated tissue damage after stroke (Popa-Wagner et al., 2018).

4. Hypertension and stroke outcomes

Hypertension is defined by a systolic/diastolic arterial blood pressure above 130/80 mmHg, based on a recently-revised definition (Whelton et al., 2018). Hypertension induces endothelial dysfunction and angiopathy in large and small vessels. Arterial hypertension is more frequent in older individuals (≥ 60 years) (Benjamin et al., 2017), and it dramatically increases ischemic brain injury in both humans and animal models. Blood pressure lowering therapies significantly reduce stroke risk (Hong, 2017). Hypertension ranks at the top of modifiable risks factors for stroke and up to 75% of stroke patients have hypertension (AlSibai and Qureshi, 2016; Hong, 2017; van der Worp and van Gijn, 2007; Virani et al.,

2020). Thus, it is surprising that only about 10% of the preclinical studies use animals with hypertension when testing potential neuroprotective drugs (van der Worp et al., 2010).

In acute ischemic stroke patients, hypertension is associated with increased mortality, worse functional outcomes, and a higher risk of intracranial hemorrhage after thrombolytic therapy (Ahmed et al., 2009; Leonardi-Bee et al., 2002; Maïer et al., 2017; Maïer et al., 2018). Hypertension is also associated with cognitive decline through mechanisms involving impaired neurovascular coupling and altered cerebral blood vessel reactivity (Iadecola, 2017).

There are several preclinical rodent models to study hypertension (Maier and Kubis, 2019). These include the spontaneously hypertensive rat (SHR), Dahl salt-sensitive rats that develop different degrees of hypertension depending on salt intake, surgically-induced models involving constriction of one or two renal arteries with or without kidney ablation, and pharmacologically-induced hypertension with angiotensin II, deoxycorticosterone acetate (DOCA) or the nitric oxide synthase inhibitor L-N-nitroarginine-methyl ester (L-NAME). The spontaneously hypertensive rat (SHR) is among the most widely utilized animal model of hypertension. This inbred strain start exhibiting high blood pressure around 6 weeks of age and reach levels of 180–200 mmHg at ~18 weeks. The SHR stroke prone (SHRSP) rat is a sub-strain of the SHR and they develop spontaneous strokes. The infarct size in SHRSP rats subjected to 1h of MCAO is twice as large as that measured in normotensive Wistar-Kyoto (WKY) rats, the strain from which SHRSP was derived. In addition, SHRSP rats subjected to stroke exhibit less neurological recovery than WKY control animals (McCabe et al., 2009; McGill et al., 2005). This is associated with stronger microglial responses and enhanced neuroinflammation in SHRSP rats compared to normotensive controls (Marks et al., 2001).

Arterial hypertension is accompanied by microglial activation in the hypothalamic paraventricular nucleus (PVN), which results in increased neuroinflammation (Shen et al., 2015; Shi et al., 2010). The involvement of inflammation in the onset and maintenance of the hypertensive condition is supported by studies showing that anti-inflammatory treatment with minocycline or overexpression of interleukin-10 (IL-10), an anti-inflammatory cytokine, attenuates hypertension in both SHR rats and in the chronic angiotensin II-infused hypertensive rat model (Santisteban et al., 2015; Shi et al., 2010). Enhanced microglial activation in autonomic brain regions plays a key role in neurogenic hypertension. Increased sympathetic nerve activity seen in hypertension leads to a significant increase in bone marrow-derived peripheral pro-inflammatory cells and enhances activation of leukocytes in the spleen (Ahmari et al., 2019; Ganta et al., 2005; Santisteban et al., 2015). Collectively, these studies suggest that hypertension is characterized by a highly pro-inflammatory environment that is likely to contribute to the worse tissue injury and limited functional recovery seen in hypertensive ischemic stroke patients.

Using a photothrombotic stroke model, Möller *et al* compared the post-ischemic inflammatory response between SHR and WKY normotensive control rats. Infarct volume was significantly larger in the SHR, which showed a strong correlation with the number of invading $CD45^{high}$ leukocytes present in the ischemic hemisphere. Brain infiltrating myeloid

cells had a higher surface level of intercellular adhesion molecule-1 (ICAM-1) in SHR compared to normotensive animals. Similarly, hypertensive rats had a significant increase in the number of infiltrating neutrophils, monocytes, and macrophages compared to WKY rats, which correlated with higher expression of chemokines (CCL2, CXCL2, and CCL7) known to participate in the transmigration of immune cells to the brain after stroke (Möller et al., 2015).

Stroke patients with good collateral status have a larger penumbra region and are more likely to respond favorably to thrombolytic therapy and/or potential neuroprotectants (Ginsberg, 2016; Rusanen et al., 2015; Vagal et al., 2018). There is convincing evidence that hypertension accelerates the rate at which the penumbral tissue is incorporated into the infarct. This is mainly due to worse perfusion of the penumbra via the leptomeningeal collateral circulation (Campbell et al., 2013). In acute ischemic stroke, chronic hypertension has a detrimental effect on collateral flow in patients with large-vessel occlusions (Fujita et al., 2019). Luminal narrowing of blood vessels due to atherosclerosis is further exacerbated by hypertension (Sabbatini et al., 2001).

Remodeling capacity in hypertension seems to be age-dependent. Comparing young (3-month-old) and middle-aged SHR rats (12-month-old) that were subjected to focal cerebral ischemia, Liang *et al.* found that, while they exhibited similar infarct size, neurobehavioral recovery was significantly impaired in the 12-month-old SHR rats compared to the 3-month-old controls. This impaired recovery after stroke was associated with decreased neurogenesis and oligodendrogenesis in aged hypertensive rats (Liang et al., 2016).

The above studies highlighted the significant differences in the response to ischemic brain injury between hypertensive and normotensive animals and humans. Therefore, incorporating hypertension as a crucial comorbid condition to our preclinical models will likely increase the likelihood of translation of potential neuroprotective strategies from the bench to the bedside. Evidence from recent studies further help to emphasize the need to have better preclinical models to test neuroprotective agents. While some of these studies show that administration of adipose tissue-derived mesenchymal stem cells (MSCs) are highly protective in young animals after focal cerebral ischemia (Gutiérrez-Fernández et al., 2015; Gutiérrez-Fernández et al., 2013; Ikegame et al., 2011), similar treatment with MSCs fails to modify stroke outcomes in hypertensive animals (Diekhorst et al., 2020; Mangin et al., 2019).

5. Stroke Outcomes in Animal Models of Metabolic Disease

5.1. Diabetes

Diabetes mellitus (DM) is a metabolic disease characterized by hyperglycemia caused by defects in insulin secretion, insulin action, or both. The chronic hyperglycemic state results in organ damage, especially to the eyes, nerves, kidney, heart, and blood vessels (Gavin et al., 2003). Based on recent statistics, 9.8% of the population in the USA has been diagnosed with DM, and 37.6% have pre-diabetes. Type 1 diabetes constitutes 5–10% of DM patients, while type 2 diabetes represents 90–95% of all DM cases (Virani et al., 2020). Type 1 DM is an autoimmune disease that triggers a dramatic loss of insulin-producing β cells in the

pancreas. Type 2 DM is characterized by peripheral insulin resistance and hyperinsulinemia caused by obesity, excessive food intake, and lack of physical activity (Gavin et al., 2003).

A large percentage of ischemic stroke patients have DM, which is associated with increased brain injury and mortality, as well as worse neurological impairment (Ergul et al., 2009). Hyperglycemia at hospital admission is an independent predictor of neurological worsening and intracerebral hemorrhage after endovascular clot removal (Laredo et al., 2020; Soomro et al., 2020), rtPA thrombolysis (Alvarez-Sabín et al., 2004; Bruno et al., 2002), as well as in patients not receiving any recanalization therapy (Baird et al., 2003; Capes et al., 2001; Yong and Kaste, 2008). Similarly, in rodent models of ischemic stroke, diabetic hyperglycemia increases brain injury and worsens neurological deficits (Chen et al., 2011; Gómez-de Frutos et al., 2019; Kusaka et al., 2004; Martini and Kent, 2007; Mayanagi et al., 2008; Shukla et al., 2017; Tureyen et al., 2011). A more dramatic reduction in CBF in the peri-infarct region is seen in animals exposed to acute hyperglycemic conditions induced by infusion of glucose immediately before stroke (Kawai et al., 1997) or chronic diabetes induced by the administration of streptozotocin (STZ), a toxin that destroys β cells of the pancreatic Langerhans islets (Martini and Kent, 2007). Thus, impaired brain reperfusion after vessel recanalization is an important mechanism underlying the detrimental effects of hyperglycemia on ischemic stroke outcomes.

Diabetic hyperglycemia induces major biochemical changes within endothelial cells. These changes include overproduction of superoxide radicals by the mitochondria leading to oxidative stress. In addition, increased formation of diacylglycerol (DAG) results in increased PKC activation, which in turn has several pathogenic consequences including reduction in endothelial nitric oxide synthase (eNOS) levels, increased expression of vascular endothelial growth factor (VEGF), activation of NF-κB-dependent inflammatory gene expression, and increased NADPH oxidase levels (Brownlee, 2001). These changes contribute to the hyperglycemic endothelium being highly vulnerable to ischemia.

A dysregulated inflammatory response seems to be a hallmark of ischemic stroke under diabetic conditions. Mice fed with high-fat diet for 8 weeks to model diabetes have increased basal MCP-1 levels in the plasma and in peritoneal macrophages. In response to focal ischemia, diabetic mice have larger strokes and a marked increase in brain swelling compared to control animals (Kim et al., 2014). Interestingly, mRNA levels of MCP-1, IL-6, and CCR2 are significantly reduced in the ischemic brain in diabetic mice compared to normoglycemic controls (Kim et al., 2014). In another study using diabetic mice subjected to hypoxia-ischemia, a delayed and diminished inflammatory response, as assessed by lower levels of brain TNF-α, IL-1α and IL-1β, have been observed (Kumari et al., 2007). As speculated by Kim *et al.*, the inability of the diabetic animals to launch a proper immune response to ischemic brain injury may prolong the acute inflammatory phase, leading to more infiltration of peripheral immune cells resulting in worse outcomes (Kim et al., 2014).

Not all studies using diabetic animals have found reduced pro-inflammatory gene expression in response to ischemic injury. Due to a G-to-T point mutation in the diabetic gene (*db*) encoding for the leptin receptor, *db*/*db* mice have defective leptin signaling leading to obesity and hyperinsulinemia, resembling some aspects of human type 2 diabetes. Starting

around 12 weeks of age, these mice have dramatic hyperglycemia (Hummel et al., 1966). Using the *db*/*db* mouse model of type II diabetes, a previous study found much higher levels of IL-1β, IL-6, MIP-1α, MCP-1, P-selectin, and E-selectin in the ischemic brain of diabetic animals compared to normoglycemic controls at 12h after transient MCAO. Increased immunoreactivity for ICAM-1 in brain vasculature and marked increase in microglia/macrophage activation were seen in *db*/*db* mice compared to controls. These inflammatory changes correlated with larger strokes, worse brain swelling, and more infiltration of neutrophils into the ischemic brain of diabetic mice compared to normoglycemic controls (Tureyen et al., 2011). In another study using a rat model of permanent MCAO, acute hyperglycemia induced by intraperitoneal administration of D-glucose before MCAO induction resulted in significantly higher levels of IL-1β and cyclooxygenase-2 (COX-2) in hyperglycemic rats compared to normoglycemic rats or hyperglycemic sham controls (Bémeur et al., 2005). In Zucker Diabetic Fatty (ZDF) rats subjected to 2h of focal cerebral ischemia and 24h of reperfusion, there is a dramatic increase in the levels of ICAM-1 leading to more neutrophil adhesion to the cerebral endothelium and infiltration into the ischemic brain, which correlates with larger strokes, worse edema formation, and poorer neurological function in ZDF compared to lean normoglycemic control rats (Ritter et al., 2011). Thus, increased inflammation might be a contributing factor to the exacerbated brain injury observed under diabetic conditions (Venkat et al., 2017). However, differences between the studies in the selection of diabetes models, severity of brain injury determined by the duration of MCAO, and degree of reperfusion (transient vs permanent occlusion models) could explain some of the differences in the published literature regarding the role of inflammation in stroke outcomes in diabetes.

Impaired polarization of monocytes/macrophages to an anti-inflammatory phenotype seems to be one of the mechanisms underlying the detrimental effects of diabetic hyperglycemia in ischemic stroke. In a mouse model of permanent distal MCAO, hyperglycemia induced by intraperitoneal injection of glucose at the time of vessel cauterization significantly increased infarct volume and reduced the number of non-inflammatory monocytes (Ly-6C^{low} Ly-6G^{-}; Arginase-1^{+}) infiltrating the injured brain. Interestingly, monocyte ablation induced by diphtheria toxin (DT) administration to CD11b-DTR mice, abrogated the hyperglycemia-induced exacerbation of ischemic brain injury, suggesting that hyperglycemia produces its damaging effects on stroke, at least in part, through monocytes (Khan et al., 2016).

In animal models, diabetes increases neovascularization in the brain, as demonstrated by greater vascular density, volume and surface area, increased number and diameter of collateral vessels, as well as increased anastomoses between MCA branches. Diabetes-augmented angiogenesis is dysfunctional since new vessels are immature, as indicated by reduced pericyte coverage, increased vascular permeability, and higher percentage of nonperfused vessels in the diabetic animals compared to normoglycemic controls (Ergul et al., 2014; Li et al., 2010; Prakash et al., 2013; Prakash et al., 2012). It is important to emphasize that irrespective of infarct size, there is increased intracerebral bleeding after ischemic stroke in diabetic animals compared to controls (Ergul et al., 2007; Mishiro et al., 2014). A greater hemorrhagic transformation and brain swelling in diabetic conditions correlates with increased mortality and worse outcomes after ischemia. A higher level of MMP-9 in diabetes is critically involved in abnormal cerebrovascular remodeling that

contributes to greater hemorrhagic transformation and edema following stroke (Elgebaly et al., 2010).

Hyperglycemia induced by STZ given three days before stroke increases ROS generation and activation of MMP-9 leading to exacerbation of BBB damage and dramatically increasing vasogenic edema after focal cerebral ischemia in rats. Transgenic rats overexpressing human *SOD1*, an antioxidant enzyme, have reduced hyperglycemia-induced BBB opening, vasogenic edema, and MMP-9 activation after ischemic stroke compared with control, non-transgenic animals (Kamada et al., 2007). More recent studies have shown that MMP-3 and MMP-9 play an important role in the damage to the neurovascular unit following focal cerebral ischemia in diabetic animals (Elgebaly et al., 2011; Elgebaly et al., 2010; Hafez et al., 2016; Hafez et al., 2017; Kumari et al., 2011).

Angiopoietins are a family of vascular growth factors that modulate endothelial cell function and angiogenesis. Angiopoietin (Ang) 1 and Ang2 are the most widely studied angiopoietins and they seem to have opposing effects on vasculogenesis. Ang1 plays a critical role in vessel maturation by promoting the migration, adhesion and survival of endothelial cells, while Ang2 disrupts the connections between the endothelium and perivascular cells and promotes endothelial cell death and vessel regression (Fagiani and Christofori, 2013). Alterations in the levels of Ang1 and Ang2, as well as their receptor Tie2, seem to play an important role in the increased vascular damage in diabetic animals after stroke. The Ang1/Tie2 signaling pathway contributes to endothelial cell survival and is important in vascular stability by promoting the recruitment of pericytes to the blood vessels (Brindle et al., 2006; Teichert et al., 2017). Ang2-mediated signaling has opposing effects leading to endothelial cell apoptosis and BBB breakdown (Nag et al., 2005). Utilizing the *db*/*db* type-2 diabetes mouse model, Cui *et al.* found decreased Ang1/Tie2 and increased Ang2 levels in diabetic animals compared to controls after focal ischemic brain injury. This was associated with worse BBB disruption and loss of tight junction proteins in *db*/*db* mice compared to normoglycemic controls after stroke (Cui et al., 2011).

5.2. Obesity and stroke outcomes

Obesity is a major health concern worldwide and a well-known risk factor for diabetes, hypertension, cardiovascular disease and stroke. Obesity is considered an independent risk factor for ischemic stroke (Lu et al., 2014; Strazzullo et al., 2010), but a few epidemiological studies suggest that obesity is associated with reduced long-term mortality and better functional recovery after stroke (Doehner et al., 2013; Vemmos et al., 2011). Other studies report the opposite: worse outcomes in obese stroke patients (Bazzano et al., 2010; Yi et al., 2009). The obesity-stroke paradox and contradictory clinical data may be explained by poor design of the human epidemiological studies or the influence of other factors such as age, ethnicity, and sex-specific differences on the interpretation and analysis of the data, as discussed in recent reviews (Haley and Lawrence, 2016; Scherbakov et al., 2011). What is clear from preclinical studies is that obesity results in exacerbated brain injury and more BBB disruption and brain edema, which leads to worse neurobehavioral outcomes in rodent models of focal cerebral ischemia (Deng et al., 2014; Deutsch et al., 2009; Haley et al.,

2019; Haley and Lawrence, 2017; Haley et al., 2017; Langdon et al., 2011; Li et al., 2013; Maysami et al., 2015; Osmond et al., 2010; Ritter et al., 2011).

Mice fed a high-fat diet for 10 weeks have increased cerebrovascular tortuosity and decreased lumen diameter of the middle cerebral artery. In response to transient ischemic stroke, these animals develop larger strokes compared to lean controls on a normal diet, and have a dramatic increase in MMP-9-mediated BBB damage, edema and hemorrhagic transformation. Of interest is the finding that MMP-9 deficient mice on a high fat diet have attenuated vascular remodeling and less infarct volume and neurovascular injury compared to obese wild-type controls, suggesting that MMP-9 activation plays a key role in obesity-induced worsening of stroke outcomes (Deng et al., 2014).

Excessive accumulation of fat, impaired metabolic processes, and chronic low-grade inflammation are among the most salient features of obesity (Virani et al., 2020). Increased adipose tissue inflammation results from cellular stress, since the capacity of adipocytes to store lipids is exceeded in obesity. Inflamed adipose tissue releases several pro-inflammatory mediators and adipokines into the blood stream, which produce many effects on different organs and alter various physiological functions (Chan et al., 2019; Haley et al., 2017). Using metabolomics, Haley *et al.* showed that obesity produces marked changes in the acute metabolic and inflammatory response to ischemic stroke. In naïve *ob/ob* mice, there was a significant increase in plasma free fatty acids compared to *ob*/− control mice. Stroke induced a further increase in these metabolites only in the obese mice. Similarly, inflammatory mediators (IL-6, G-CSF, CXCL1) were increased in plasma, adipose tissue, and liver after stroke, and this increase was greater in obese mice (Haley et al., 2017).

Adiponectin, leptin, and resistin are the most important adipokines produced by the adipose tissue. Before stroke, obese *ob*/*ob* mice have lower levels of resistin and adiponectin in adipose tissue, which is further decreased by ischemic brain injury (Haley et al., 2017), suggesting that stroke dramatically alters the release of adipokines from adipose tissue under obese conditions. Reduced levels of adiponectin in obese mice after stroke could significantly impact outcomes since there is a large body of evidence indicating that adiponectin is protective in the context of ischemic stroke (Li et al., 2017; Miao et al., 2013; Nishimura et al., 2008).

5.3. Hyperlipidemia

Hyperlipidemia is associated with atherosclerosis of blood vessels in humans and is one of the main risk factors for coronary artery disease and cerebrovascular disease (Virani et al., 2020). A few animal models of hypercholesterolemia have been widely utilized to study the impact of this comorbid condition on stroke outcomes. Mice with genetic deletion of apolipoprotein E (ApoE), a fat-binding protein critically involved in cholesterol metabolism, have several fold increases in plasma cholesterol levels, which are further elevated in *ApoE*$^{-/-}$ mice on a high-cholesterol diet. Another popular mouse model of hyperlipidemia is the low-density lipoprotein receptor (*Ldlr*$^{-/-}$) knockout mice. By binding to ApoE and ApoB, the LDL receptor controls cellular uptake of LDL and VLDL lipoproteins from the blood. On a regular diet, the *Ldlr*$^{-/-}$ mice have ~2 times elevated cholesterol concentrations in blood, and this increase is greater when maintained on a high-cholesterol diet.

Several lines of evidence indicate that hyperlipidemia exacerbates ischemic brain damage through different mechanisms including increased oxidative stress, inflammation, BBB damage, impaired CBF regulation, and deficient collateral perfusion (Ayata et al., 2013; Cao et al., 2015; ElAli et al., 2011). Hypercholesterolemia also dramatically affects the cerebrovasculature under resting conditions and in response to focal cerebral ischemia (Ayata et al., 2013; ElAli et al., 2011). Using intravital microscopy, a previous study showed that high-fat diet significantly increases the interactions of platelets and leukocytes with the cerebral endothelium, a process that depends on ROS production by NADPH oxidase and increased levels of P-selectin. In response to ischemic brain injury, high-fat diet further exaggerated the platelet- and leukocyte-endothelial cell interactions, which could contribute to inflammation and focal thrombosis (Ishikawa et al., 2004). Massive neutrophil infiltration has been documented in ApoE$^{-/-}$ mice fed a high-cholesterol diet compared to wild-type controls on a normal diet in response to transient focal cerebral ischemia. Blockade of CXCR2, a neutrophil receptor that binds to the chemokines CXCL1 and CXCL2/3, significantly reduces hyperlipidemia-exacerbated neurological deficits and brain tissue infarction after experimental ischemic stroke (Herz et al., 2015). Hyperlipidemia induced by high-fat diet increases pro-inflammatory mediators in the brain including IL-6, TNF-α, ICAM-1, and VCAM-1, and these changes are greater in rats subjected to 2h of MCAO. Worse neurological deficits, larger infarct volumes and increased apoptosis were observed in stroked rats fed with high-fat diet compared to controls (Cao et al., 2015). Hyperlipidemia is associated with increased inflammation not only in the brain, but also in the periphery. These changes are even more dramatic in response to stroke (ElAli et al., 2011; Herz et al., 2014), which might explain the higher vulnerability to ischemic brain injury in hyperlipidemic conditions.

A key role for CD36/fatty acid translocase, a scavenger receptor with a high affinity for lipids, in the exacerbation of ischemic stroke pathology in hyperlipidemia has been demonstrated in previous studies. Infarct size and brain swelling were significantly increased in ApoE$^{-/-}$ mice on a high-fat diet compared to wild-type controls on a normal diet, which was associated with a significant increase in CD36 and MCP-1 in the brain and the periphery. Genetic deletion of CD36 ameliorated stroke-induced inflammation, edema, and neuronal injury (Kim et al., 2008). Infiltrating monocyte-derived macrophages are the major source of CD36 in the ischemic brain of hyperlipidemic mice (Kim et al., 2012). Effective pharmacological targeting of CD36 in hyperlipidemic stroke might be challenging based on data from a recent study which showed that only chronic administration of CD36 inhibitors *prior* to stroke was beneficial in reducing brain swelling after stroke. Treatment with CD36 inhibitors after the stroke onset, a more clinically relevant therapeutic schedule, failed to impact stroke outcomes in hyperlipidemic mice (Kim et al., 2020).

Deficits in cerebral perfusion and the status of the leptomeningeal collateral circulation are associated with hyperlipidemia (Hermann et al., 2019a; Zechariah et al., 2013), contributing to the increased susceptibility of the brain to ischemic stroke in hyperlipidemic conditions. Compared to controls, ApoE$^{-/-}$ mice fed a high-fat diet have impaired cerebrovascular responses, as demonstrated by reduced resting CBF, altered vasodilatory reflexes, and worse perfusion deficits after distal occlusion of the middle cerebral artery (Ayata et al., 2013).

Vascular dysfunction in hyperlipidemic stroked mice is ameliorated by Rho-associated kinase (ROCK) inhibition (Shin et al., 2014).

As with other comorbidities discussed before, hyperlipidemia is associated with worse stroke outcomes. However, it is important to discuss a potential caveat of utilizing genetic models combined with high-fat diet to study the therapeutic effects of potential neuroprotectants in stroke under hyperlipidemic conditions. When maintained on high-fat diet, ApoE and Ldlr knockout mice have dramatically elevated levels of cholesterol in their blood, which models familial hypercholesterolemia, a rare human hereditary condition. Thus, it is not clear how well these animal models reflect the hyperlipidemic conditions seen clinically in stroke patients. As discussed in a recent article (Hermann et al., 2019a), potentially neuroprotective strategies could be prematurely abandoned (missed opportunity) by using preclinical models that do not accurately represent the clinical situation.

6. Challenges of modeling ischemic stroke in aged and/or comorbid animals

Most preclinical stroke studies fail to include aged and/or comorbid animals. In a recent analysis of data from preclinical systematic reviews of therapeutic interventions for ischemic stroke, only 11.4% of studies included an aged or comorbid model (McCann and Lawrence, 2020). The main reasons for the lack of attention to aging and comorbidities in preclinical stroke research include the high costs and increased mortality in aged and/or comorbid animals subjected to ischemic stroke. Moreover, the time that it takes to perform the experiments is dramatically increased since animals need to be maintained for long periods of time to reach a certain age (aging experiments) or to induce a specific phenotype by maintaining the animals on a particular diet for several weeks/months (e.g., rodent models of hyperlipidemia, obesity, and diabetes).

Inducing intraluminal MCAO or embolic stroke in aged mice or rats is methodologically challenging. Aged rodents, especially outbred rat strains fed *ad libitum*, show a wide range of body weights, making the intraluminal approach of vessel occlusion more complicated due to varying diameters of cerebral vessels, which require altering the caliber of the occluding filament for each range of animal weights (Turner et al., 2013). Moreover, in heavier or obese animals, it is harder to dissect the arteries due to increased adiposity. Inbred Fischer-344 and Fischer-344/Brown-Norway hybrid rats are widely utilized in aging research. These rat strains are unsuitable for intraluminal MCAO due to kinking of the internal carotid artery, as assessed by magnetic resonance angiography. Surgical complications in these strains are mainly due to inability to advance the intraluminal filament, usually resulting in subarachnoid hemorrhage and high mortality (Dittmar et al., 2006).

Based on our experience using aged rodents to model ischemic stroke (Bennion et al., 2017; DeMars et al., 2019; Yang et al., 2017), a special attention should be paid to the level of anesthesia and breathing patterns during surgery. Furthermore, the variability in infarct volumes is higher in aged animals compared to young ones, despite similar degree of CBF

reduction during MCAO. Thus, more animals per group are needed to detect differences between treatment conditions.

Mortality rate in aged ischemic rodents is significantly higher than in young animals when ischemic stroke is induced by the intraluminal approach. In some studies, the mortality rate is as high as 50% (Crapser et al., 2016). Shortening the occlusion time helps reduce mortality in the intraluminal filament stroke model in aged and comorbid animals. Based on our own experience, aged stroked rodents require careful and more frequent post-operative monitoring to avoid dehydration and excessive weight loss, which are a major cause of mortality in the intraluminal MCAO model. Importantly, mortality rate is extremely low in aged and comorbid rodents when focal ischemia is induced by distal MCAO (DeMars et al., 2019; Hermann et al., 2019b), which makes this stroke model very useful for studies using aged and/or comorbid animals. Similar to the distal MCAO, the photothrombotic stroke model induces smaller infarcts with lower mortality rates and this model has been employed to overcome the challenge of high mortality typically seen in aged animals or in the *db*/*db* mouse model of diabetes.

An important consideration in conducting stroke preclinical work using aged rodents is the very high cost of acquiring and using these animals. When establishing an aging colony, *per diem* animal housing charges add up quickly. Similarly, procuring aged animals from commercial suppliers is expensive. Overall, the cost of using aged rodents could be several times higher than performing studies in young-adult animals.

7. Concluding remarks

Experimentally-induced focal cerebral ischemia in young and healthy rodents does not mimic the highly heterogenous and complex nature of human stroke and could lead to false conclusions regarding therapeutic efficacy of potential neuroprotective approaches. Improving the success in translating preclinical stroke research into the clinic will require incorporating better animal models to mimic human stroke. Use of aged animals and/or animals suffering from comorbidities in preclinical stroke modeling is clinically more relevant. Underlying molecular mechanisms of protection by drugs or non-pharmacological approaches could be significantly altered in aged animals compared to young ones. This is a critical factor to be considered in the road to translation from animal studies to the clinic. Moreover, our preclinical models should incorporate a more realistic therapeutic time window, as well as clinically-relevant endpoints to assess long-term recovery of neurological function.

Acknowledgements

The authors thank Jonathan Larochelle, B.S., Dr. Changjun Yang, and Dr. Lei Liu for valuable comments and suggestions.

Funding sources

Authors are supported by grants from the National Institute of Neurological Disorders and Stroke, NINDS/NIH (R01 NS103094 and R01 NS109816 to E.C.J and R01 NS059962 to S.P.).

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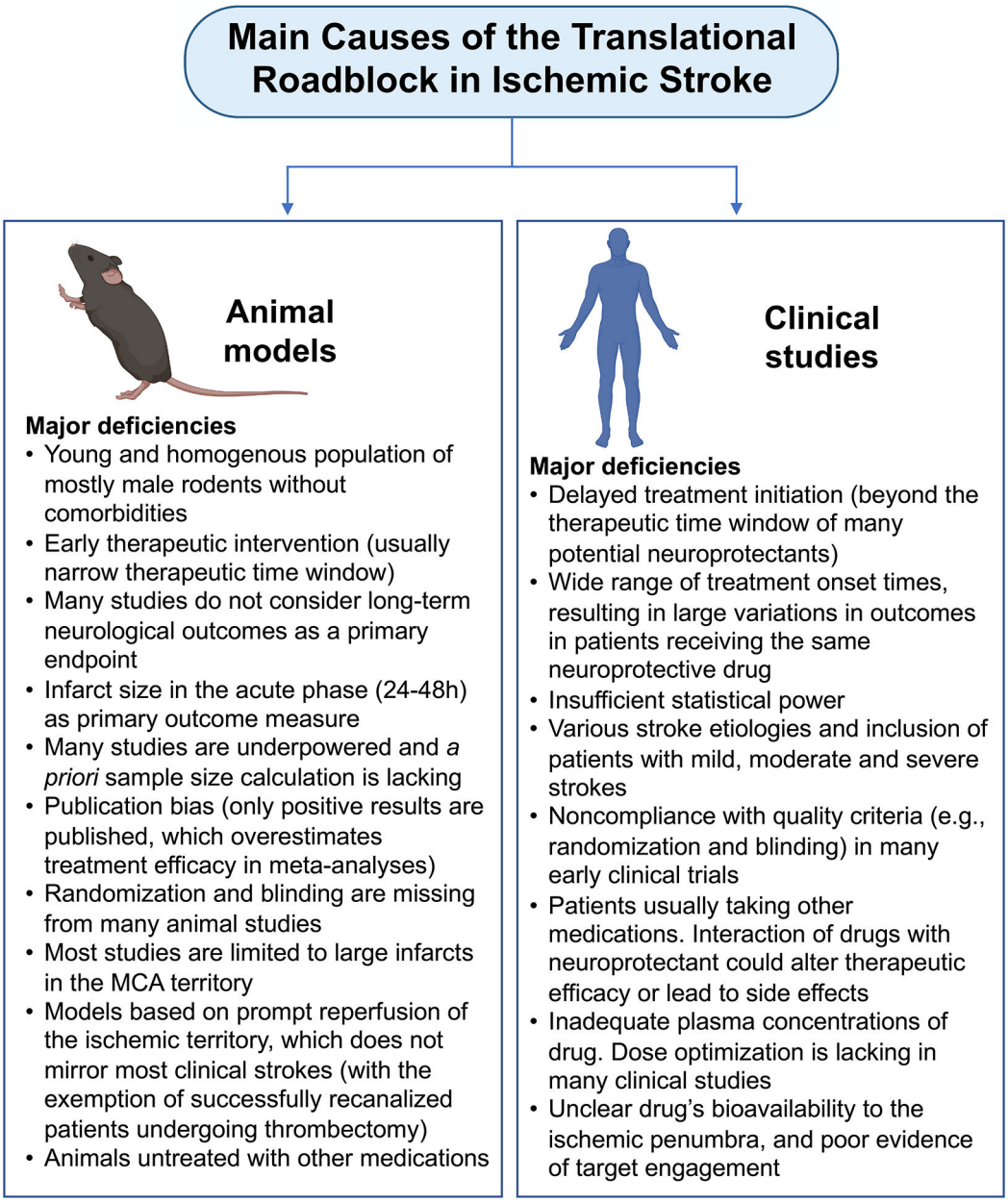

Figure 1.

Main causes for the translational failure in ischemic stroke. Deficiencies in both preclinical animal models and clinical trials account for the failure to translate potential neuroprotective strategies into the clinic.

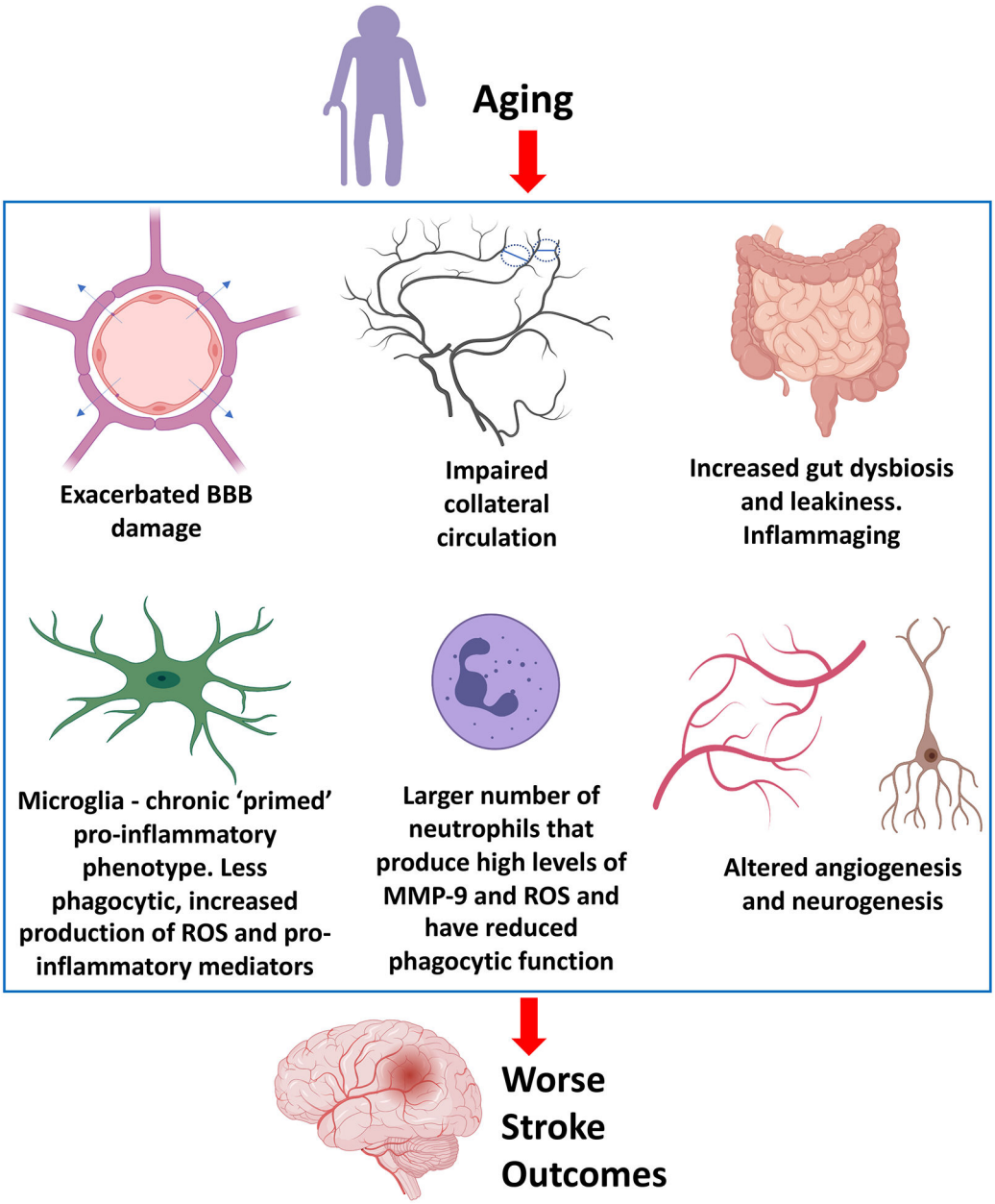

Figure 2.

Aging and Ischemic Stroke. Advanced age is associated with many pathophysiological changes in both the CNS and the periphery. These changes contribute to an altered response to ischemic brain injury, which results in worse functional outcomes in aged compared to young individuals following an ischemic stroke. BBB, blood-brain barrier. MMP-9, matrix metalloproteinase-9. ROS, reactive oxygen species.