Ischemic Injury to White Matter: An Age-Dependent Process

Sylvain Brunet, Chinthasagar Bastian, and Selva Baltan

Abbreviations

AMPA/KA	AMPA/kainate
CKA	7-Chlorokynurenic acid
CNS	Central nervous system
GS	Glutamate synthetase
KB-R	2-[2-[4(4-Nitrobenzyloxy)phenyl]ethyl]isothiourea mesylate
MON	Mouse optic nerve
NCX	Na^+–Ca^{2+} exchanger
NMDAR	NMDA-type receptors
OGD	Oxygen glucose deprivation
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
WM	White matter

S. Brunet, B.Sc., Ph.D. • S. Baltan, M.D., Ph.D. (✉)
Department of Molecular Medicine, Cleveland Clinic Lerner College of Medicine of Case Western Reserve University, 9500 Euclid Avenue (NB21), Cleveland, OH 44195, USA

Department of Neurosciences, Lerner Research Institute, Cleveland Clinic Foundation, 9500 Euclid Avenue (NB30), Cleveland, OH 44195, USA
e-mail: brunets@ccf.org; baltans@ccf.org

C. Bastian, M.B.B.S., Ph.D.
Department of Neurosciences, Lerner Research Institute, Cleveland Clinic Foundation, 9500 Euclid Avenue (NB30), Cleveland, OH 44195, USA
e-mail: bastiac@ccf.org

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1 Introduction

In the United States, someone experiences a stroke every 40 s [1]. Axonal injury and dysfunction are responsible for most of the disability observed after a stroke [2] and aging is one of the most significant risk factors for stroke. The human brain comprises equal proportions of gray matter and white matter (WM) and WM is injured in most strokes [2]. However, most research efforts have traditionally been dedicated to protecting the gray matter. While effective in rodents, this approach has failed to translate to humans. Many reasons may underlie this failure, but major differences between humans and rodents include the greater proportion of WM in the human brain and the lack of significant WM involvement following middle cerebral artery occlusion in rodents, which is one of the most widely used animal models of stroke. Thus, over the past several years we and others have focused on examining how WM responds to ischemic injury, with an emphasis on the impact of aging [3–5].

2 WM Is Sensitive to Ischemic Injury

WM axons are dependent on a constant supply of oxygen and glucose to transmit signals. Central nervous system (CNS) WM electrical function is remarkably tolerant to anoxia [6], while there is regional heterogeneity in the ability to function and survive anoxia [7]. On the other hand, young adult WM is susceptible to ischemia induced by combined oxygen and glucose deprivation (OGD, Fig. 1). Mechanisms underlying ischemic WM injury proved to be unpredictably complex (Fig. 2) [8–16]. WM is composed of axons, oligodendrocytes, microglia, and astrocytes [2, 17]. Axons are myelinated by oligodendrocytes and exhibit patterns/gaps called nodes of Ranvier. Astrocytes support axons metabolically and restore the extracellular ionic environment following axonal activity. Microglia is partly responsible for immune surveillance. Thus, WM is composed of a complex cellular environment in which glial cell–cell interactions intricately maintain axon function. During ischemia, WM cellular elements are individually under attack but remain interactive with each other in intricate mechanisms that are currently under investigation.

3 Mechanisms of WM Ischemic Injury

It is now well-established that ischemia in WM sequentially activates three different injury pathways: the ionic, the excitotoxic, and the oxidative stress injury pathways. The ionic pathway attacks axons by collapsing their ionic homeostasis, which is initiated by the failure of the Na^+–K^+ pump, cell membrane depolarization, Na^+ channel activation, reversal of the Na^+/Ca^{2+} exchanger, and Ca^{2+} channel activation, resulting in the accumulation of intracellular Na^+ and Ca^{2+} (Fig. 2, yellow) [18–23]. This increased intracellular Na^+ leads to the reversal of the Na^+-glutamate transporter and the release of glutamate from astrocytes [16].

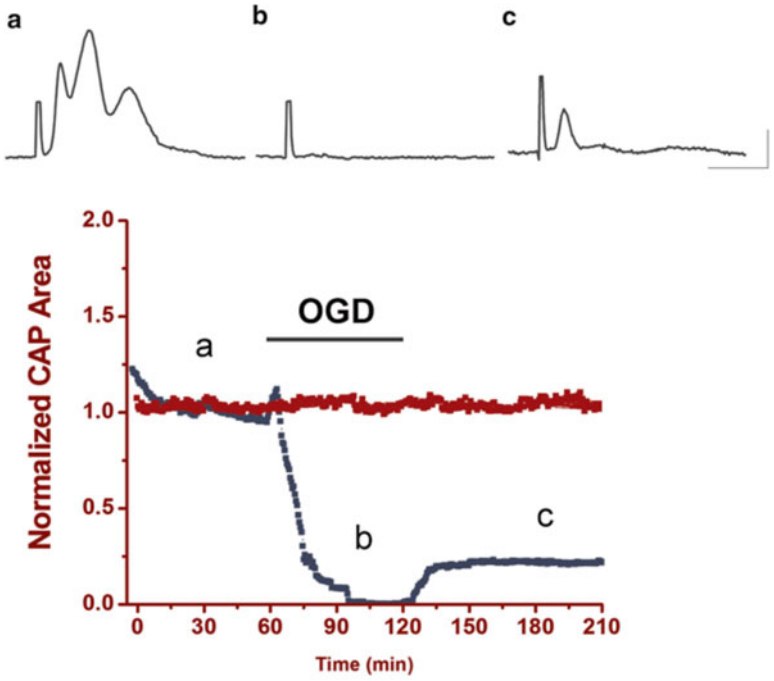

Fig. 1 WM is susceptible to ischemic injury. Axon function is quantified as the area under the compound action potential (CAP), normalized to control area, and plotted against time. Under normal conditions, CAP area is stable for long periods of time (*brown*). A 60 min period of OGD gradually depresses CAP area until conduction along axons is completely lost (*gray*). Restoring oxygen and glucose leads to ~25 % axon function recovery. Sample traces from control (*a*), OGD (*b*), and recovery (*c*) periods are shown above the graph (Reproduced in part from Baltan (2014) [18])

Subsequently, the excitotoxic pathway is initiated by an increase in extracellular glutamate (Fig. 2, green). The excitotoxic pathway mainly targets oligodendrocytes by overactivating AMPA/KA receptors [11, 14, 16, 24–27] (redox) [28], which leads to increases in intracellular Na^+ and Ca^{2+} and the activation of downstream toxic intracellular pathways to mediate WM injury.

In parallel with the excitotoxic pathway, increased extracellular glutamate also leads to the activation of the oxidative stress pathway (Fig. 2, blue). The oxidative pathway damages WM components due to the formation of reactive oxygen species (ROS), which arises because the increased extracellular glutamate competes with cysteine at the glutamate-cysteine pump [29], depleting intracellular cysteine to reduce glutathione levels [30] and to cause mitochondrial dysfunction. In addition, the increase in intracellular Ca^{2+} activates NOS to produce nitric oxide [31, 32], which readily reacts with ROS to produce reactive nitrogen species (RNS). ROS and RNS can then attack multiple cellular elements (phospholipids, proteins, DNA, RNA) to mediate injury.

The ionic pathway triggers the injury process, which subsequently reverses the glutamate transporter; however, it is the accumulation of glutamate that dictates the threshold for irreversible injury. Therefore, if the injury is short and only involves

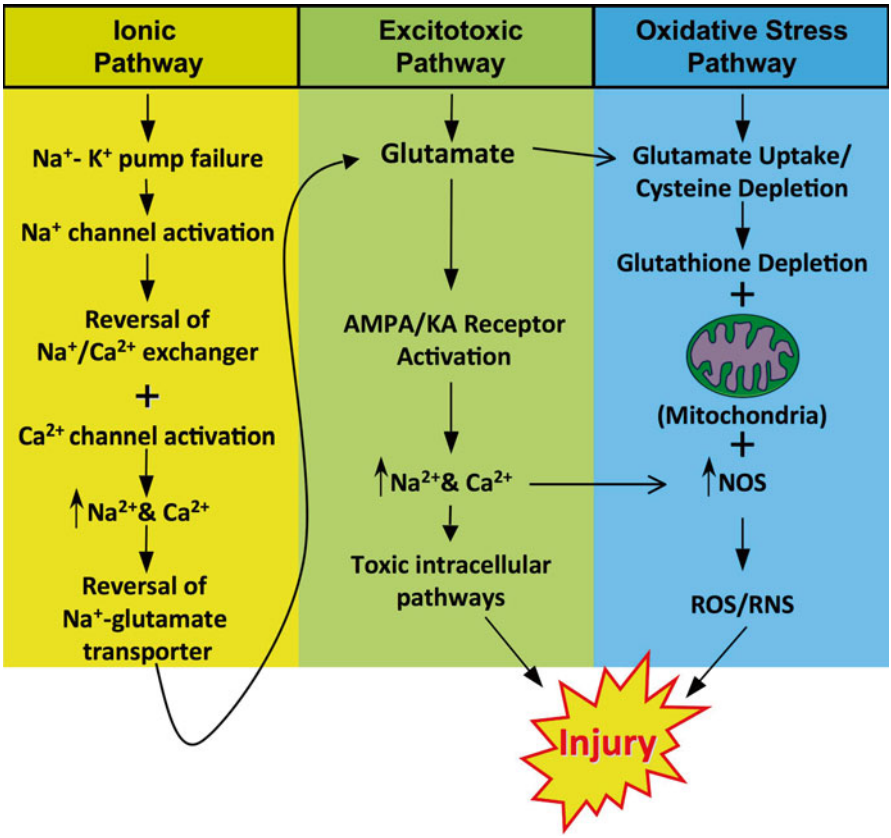

Fig. 2 Ischemia activates three pathways to mediate WM injury. Ischemia leads to the activation of the ionic pathway, which then leads to the sequential activation of the excitotoxic and oxidative stress pathways, which converge to cause irreversible injury to WM during ischemia. Note that glutamate release due to reversed Na^+-dependent transport dictates the irreversible nature of the injury. *ROS* reactive oxygen species (Reproduced in part from Baltan (2009) [2])

the ionic pathway, then the ischemic injury is completely reversible. Unlike gray matter, this sequential order of events is necessary for the injury to develop, such that bypassing the ionic pathway and applying exogenous glutamate (or glutamate analogues) fails to cause WM injury [16].

4 Mouse Optic Nerve: An Ideal Model to Investigate WM

The mouse optic nerve (MON) is ideal for ischemic studies of WM. The optic nerve, the second cranial nerve, is a purely myelinated central nervous system WM tract and is sensitive to ischemia and to the aging process [33, 34]. In addition, tissue

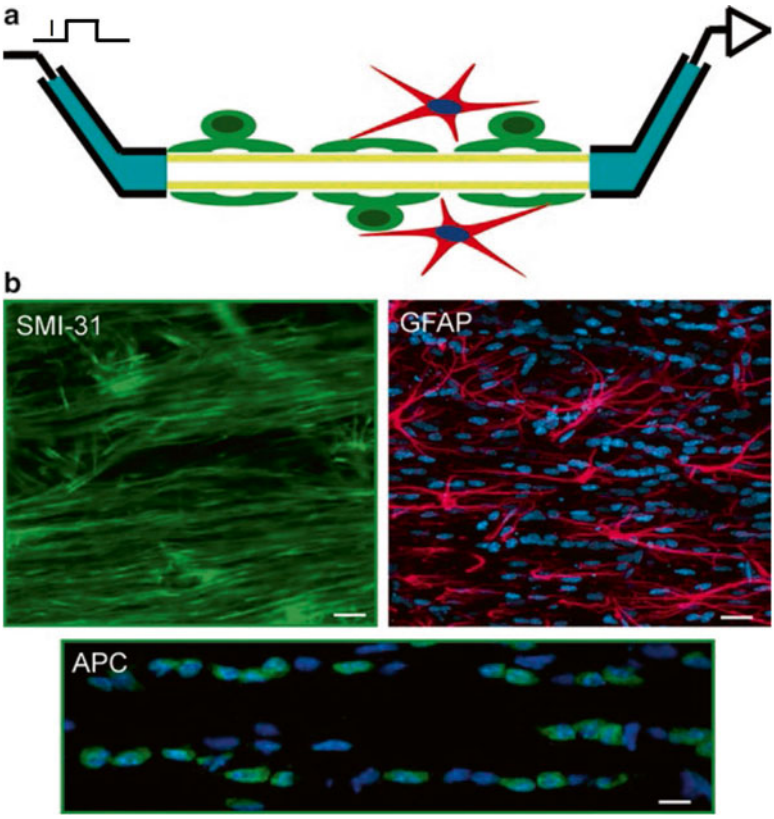

Fig. 3 The MON model is ideal for monitoring WM electrical function and cellular architecture. (**a**) Use of suction electrodes allows all axons to be stimulated and a CAP to be recorded. Cartoon of mouse optic nerve between two suction electrodes, where the left suction electrode stimulates and the right suction electrode records the CAP. Axons are represented in *yellow*, oligodendrocytes are in *green*, and astrocytes are in *red*. *I* current. (**b**) Using cell-specific antibodies, WM axons are labeled with SMI-31 for neurofilament (*green*), GFAP for astrocytes (*magenta*), and APC for mature oligodendrocyte cell bodies (*green*). Sytox (+) glial nuclei are in *blue*. Scale bar = 50 μm for SMI-31 and GFAP, 10 μm for APC (Reproduced in part from Baltan (2014) [18])

isolation does not require extensive surgical interventions; therefore there is negligible surgical injury, its small diameter allows sufficient glucose diffusion [6, 35], there are no neurons or synapses to contribute indirectly to the ischemic injury, and electrical function can be monitored by recording evoked compound action potentials (CAPs). MONs are stable both structurally and electrically for long durations (18 h) and glial cells and axons retain their native relationships to one another within a three-dimensional spatial organization (Fig. 3a). Furthermore, the cellular components can reliably be identified using immunohistochemistry (Fig. 3b), glutamate release can be measured by HPLC [36], proteins of interest can be quantified by Western blot analysis [3], and intravitreal injections provide a path for axonal

delivery [37]. Finally, the current and future availability of genetically engineered mice to experimentally test the role(s) of specific injury pathway components make the MON a model ideally suited to study WM injury mechanisms.

5 Why Is Aging WM More Susceptible to Ischemic Injury?

5.1 A Ca^{2+}-Independent Excitotoxicity Pathway in Aging WM

In aging WM, there is an enhanced contribution of a Ca^{2+}-independent excitotoxicity pathway to ischemic injury. In young WM, intracellular Ca^{2+} accumulation is important in the development of WM ischemic injury, and removal of extracellular Ca^{2+}, or blockade of Ca^{2+} entry via reversal of the Na^+–Ca^{2+} exchanger (NCX) with 2-[2-[4(4-nitrobenzyloxy)phenyl]ethyl]isothiourea mesylate (KB-R), improves WM electrical function (Fig. 4A, left). In contrast, in older WM these interventions fail to improve injury. In fact, OGD applied in Ca^{2+}-free conditions harms recovery, suggesting that extracellular Ca^{2+} entry does not significantly contribute to ischemic injury in aging WM (Fig. 4A, right). In addition, the lack of protection afforded by blockade of Ca^{2+} entry secondary from the reversal of the NCX confirms the Ca^{2+}-independent nature of ischemic injury in aging WM. On the other hand, in aging MONs, it is possible that Ca^{2+} release from intracellular Ca^{2+} stores becomes accentuated during ischemia. However, the exact relationship between transmembrane Ca^{2+} influx and intracellular Ca^{2+} concentration, and the role of intracellular Ca^{2+} stores in aging WM, all remain to be established. Though these results suggest an attenuated role for the ionic pathway with aging, whether WM injury can be triggered directly at the excitotoxic pathway needs to be tested.

Overactivation of AMPA/kainate (AMPA/KA) receptors mediates excitotoxic injury in both young and aging WM. Thus, blockade of these receptors protects axon function against OGD in both young and aging WM. Interestingly, unless combined with a brief period of OGD [16], activating AMPA/KA receptors with glutamate (or glutamate agonists) does not cause WM injury. This is presumably because of efficient uptake of glutamate from the extracellular compartment by Na^+-dependent transporters, such as GLT-1, as long as energy supply is maintained (Figs. 2 and 5). These findings suggest that OGD needs to activate ionic dysfunction to prime WM to the toxic effects of glutamate [16]. In aging WM, the Ca^{2+}-independent nature of excitotoxicity raises the question as to how activation of AMPA/KA receptors, which plays a prevalent role during ischemia in older WM, mediates injury. Either OGD-induced injury in older WM is specific to Ca^{2+} entry through AMPA/KA receptors or Na^+ entry through AMPA/KA receptors. The Ca^{2+}-mediated neurotoxicity requires distinct signaling pathways, such that some pathways are more efficiently triggered when Ca^{2+} ions enter at specific entry points such as the Ca^{2+}-permeable glutamate receptors [38]. In addition, overload of neurons by intracellular Na^+ exhibits irreversible neurotoxic swelling in the absence of extracellular Ca^{2+} [39]. Moreover, an increase in intracellular Na^+ leads to the reversal of the Na^+-dependent glutamate transporter, leading to an increase in extracellular

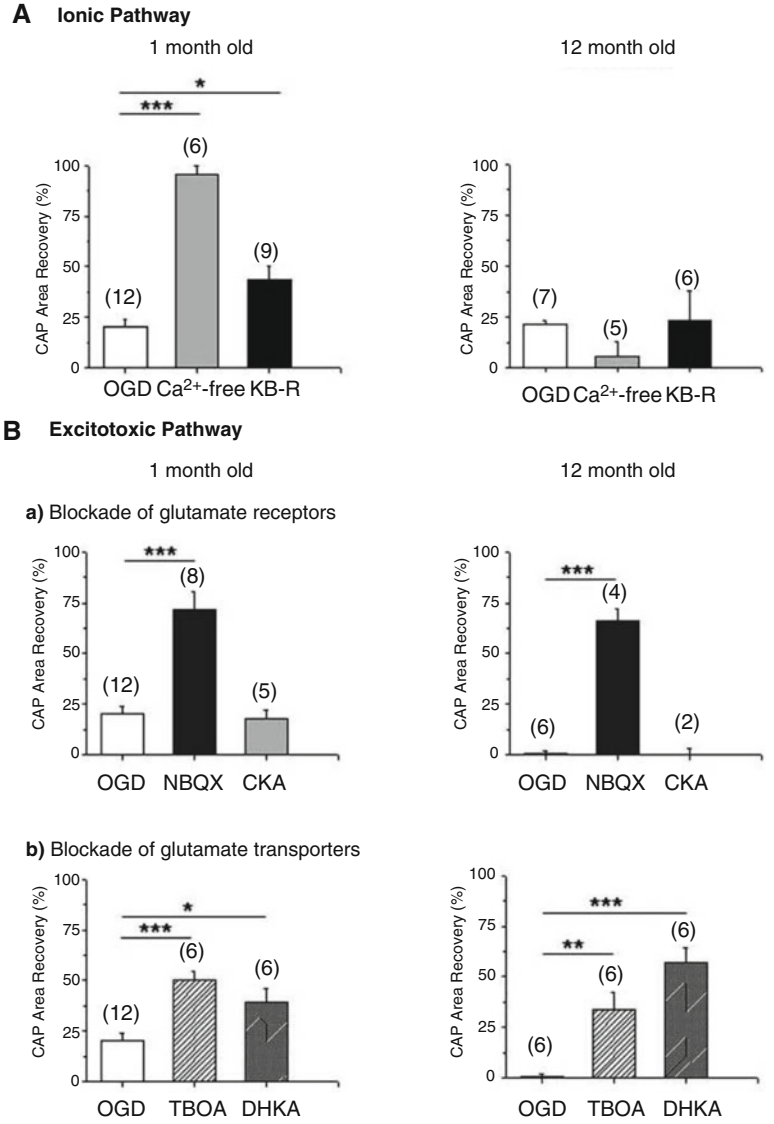

Fig. 4 Ca^{2+}-independent excitotoxicity mediates ischemic injury in older WM. (**A**) The role of the ionic pathway in ischemic injury diminishes in older WM. Removal of extracellular Ca^{2+} or blockade of Ca^{2+} entry via reversing the Na^+–Ca^{2+} exchanger (NCX) with KB-R (2-[2-[4(4-nitrobenzyloxy) phenyl]ethyl]isothiourea mesylate) is protective of axon function in 1-month-old WM after 60 min of OGD (*left*). KB-R failed to improve axon function in 12-month-old WM, even after 45 min of OGD (*right*). Note the reduced recovery in Ca^{2+}-free conditions of 12-month-old WM (right). (**B**, **a**) Blockade of AMPA/KA receptors with NBQX (2,3-Dioxo-6-nitro-1,2,3,4-tetrahydrobenzo[*f*] quinoxaline-7-sulfonamide) provides identical protection to axon function in 1- and 12-month-old WM after 60 min of OGD. Blockade of NMDA-type receptors (NMDARs) with chlorokynurenic acid (CKA) does not improve axon function recovery in 1- or 12-month-old WM. (**b**) Blockade of Na^+-dependent glutamate transporters improves axon function recovery in 1- and 12-month-old WM. Note that dihydrokainic acid (DHKA) afforded greater protection in aging axons. CAP compound action potential; TBOA (DL-threo-β-benzyloxyaspartate). $*p<0.05$, $**p<0.01$, $***p<0.001$, two-way ANOVA. Data replotted from Tekkok et al. (2007) [16] and Baltan et al. (2008) [3]) (Reproduced from Baltan (2009) [2])

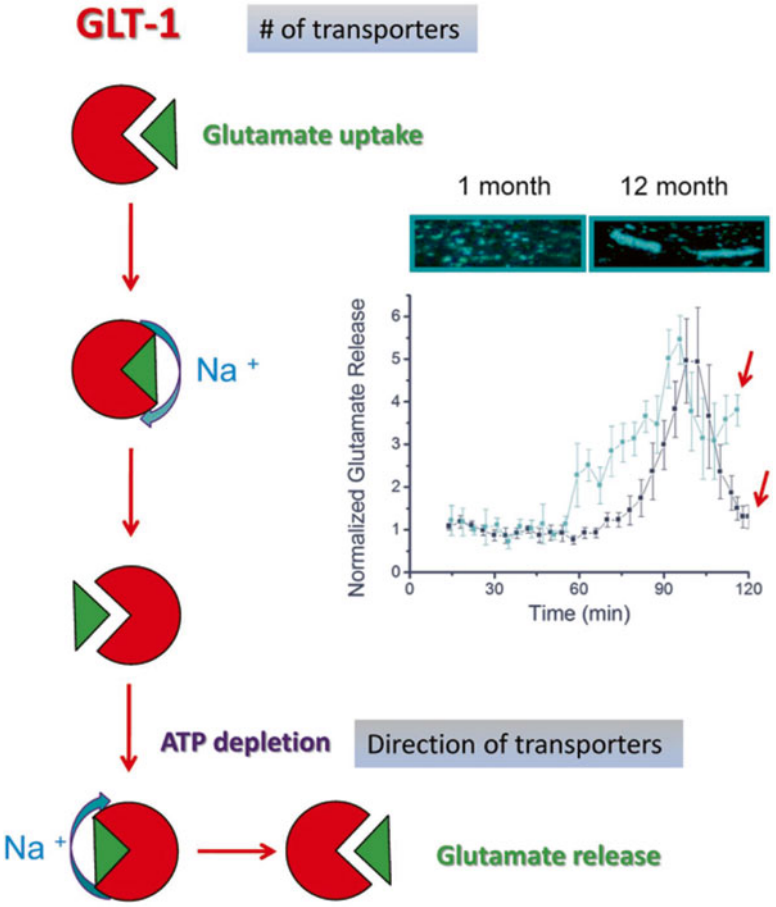

Fig. 5 In aging WM, enhanced excitotoxicity due to impaired mitochondrial function leads to early and robust glutamate release. The principal Na^+-dependent glutamate transporter, GLT-1, usually takes up glutamate with co-transport of Na^+. During ATP depletion, due to increased intracellular Na^+ levels, the transporter reverses and releases glutamate. Therefore, the number of transporters determines the capacity of the system, but it is the ATP levels that determine the direction of the transport (to either remove or release glutamate). Mitochondria in aging axons become longer and thicker compared to young axons, which may hinder ATP production and drive GLT-1 in reverse mode. Consistent with this, there is an early and robust glutamate release in aging WM (*blue*) compared to young MONs (*gray*). Note that the glutamate levels return to baseline in young WM but remain elevated in aging WM (*red arrows*) (Reproduced from in part from Baltan (2008) [3] and Baltan (2014) [18])

glutamate [40]. Together with the upregulation in GLT-1 expression in aged WM [3], increased intracellular Na^+ may cause increased and early release of glutamate, overactivating AMPA/KA receptors and creating a vicious cycle that underlies the vulnerability of aging WM to ischemia.

The NMDA-type receptors (NMDAR) are activated during ischemia by glutamate [41], and the resultant increases in intracellular Ca^{2+} lead to myelin injury [42] or detachment of oligodendrocyte processes [43]. Consequently, axon function recovery would be predicted to improve following OGD if NMDARs are blocked. However, blockade of NMDAR in 1-month-old MONs (Fig. 4B, left) [16] or corpus callosum [14] does not improve axon function recovery following OGD. Likewise, blockade of NMDARs with 7-chlorokynurenic acid (CKA) does not protect axon function in older animals (Fig. 4B, right). Moreover, even after shorter durations of OGD (30–45 min), blockade of NMDARs worsens axon function recovery in older WM (data not shown). These results suggest that NMDAR activation during OGD does not contribute to failure of axon function and raises caution for the therapeutic use of NMDAR antagonists during ischemia, particularly in aging WM. These results do not negate activation of NMDARs under ischemic conditions, but suggest that their activation does not specifically contribute to axonal injury.

6 Reorganization of Glutamate Homeostasis in Aging WM

WM glutamate homeostasis and related regulatory proteins also go through age-related remodeling (Fig. 6). Expression of GLT-1, the dominant glutamate transporter, is upregulated in aging WM [3]. GLT-1 plays a key role in the removal of glutamate from the extracellular space to maintain glutamate below neurotoxic levels [44, 45]. Even though GLT-1 is predominantly expressed on astrocytes in young WM, it extends to additional structures with aging, implying that additional WM elements may contribute to the toxic glutamate accumulation in aging WM [3].

In addition to GLT-1, other essential elements for maintaining glutamate homeostasis include GLAST, glutamate, and glutamate synthetase (GS). WM glutamate content increases significantly with aging, which correlates with increased GS levels (Fig. 6). Together with a two-fold increase in GLT-1 levels in older WM [3], these adjustments may support an age-dependent adaptive mechanism in WM to remove glutamate from the extracellular space and to convert excessive glutamate to glutamine to maintain glutamate homeostasis. As a result, glutamate levels [3] and axon conduction across aging axons are stable under normal conditions.

The number of GLT-1 transporters determines the capacity of WM to move glutamate between the intracellular and extracellular space (Fig. 5). However, the direction of the GLT-1 transporter acts to either protect or injure WM. During ischemia, GLT-1 transporter reversal acts to injure the WM due to an accelerated Na^+ overload as a result of decreased tolerance to energy deprivation in aging WM (Fig. 7). Therefore, in old WM, more GLT-1 transporters are reversed, leading to earlier and more robust release of glutamate and enhanced excitotoxicity (Fig. 7). Moreover, in young WM, glutamate levels return to baseline after the end of OGD, which suggests efficient uptake of glutamate by astrocytes. However, in aging WM,

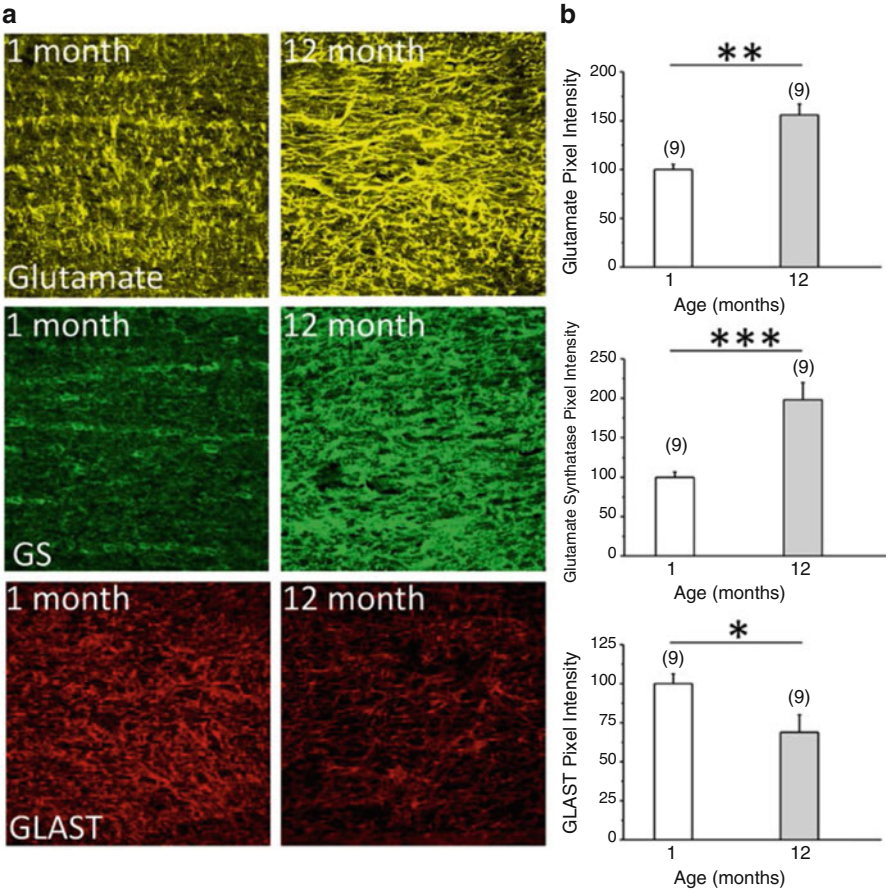

Fig. 6 Glutamate and glutamate synthetase (GS) expression are increased in 12-month-old MONs. (**a**) Immunolabeling and (**b**) quantification of glutamate, GS, and GLAST immunolabeling revealed that glutamate and GS labeling intensity is increased by 156 ± 11 % and 198 ± 22 %, respectively, in 12-month-old MONs. Note that GLAST-labeling intensity decreased to 69 ± 11 % in 12-month-old MONs. *$p = 0.0278$, **$p = 0.004$, ***$p = 0.0006$, two-tailed Student's *t*-test (Reproduced from Baltan (2014) [18])

glutamate levels remain elevated, suggesting that aging astrocytes cannot take up the excess glutamate and thereby extending the duration of excitotoxicity into the recovery period [3]. Despite the possibility that glutamate may be released from multiple sources in aging WM, astrocytes are expected to remove and store glutamate efficiently. Therefore, these results suggest a prominent change in aging astrocyte capacity to remove glutamate. On the other hand, GLAST expression in WM decreases with aging, raising the possibility that glutamate transporters can functionally substitute for one another with aging (Fig. 6).

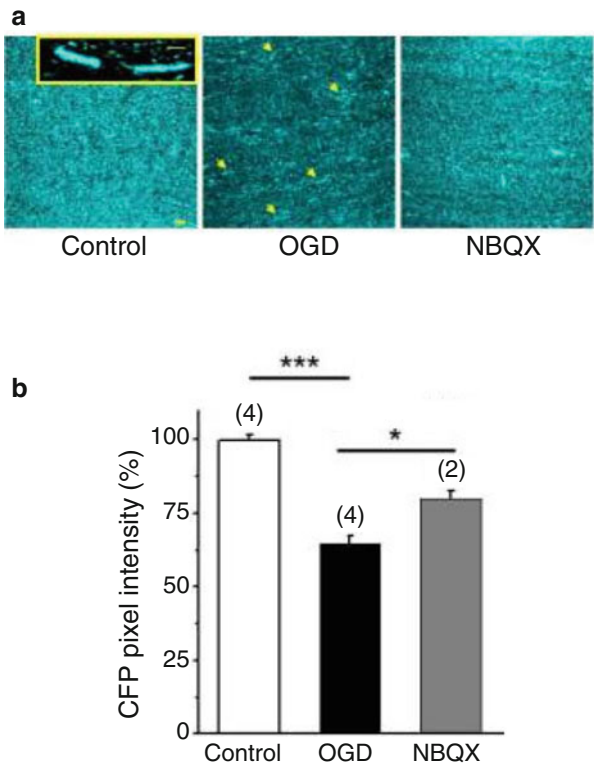

Fig. 7 In aged MONs, blockade of excitotoxicity preserves CFP (+) axonal mitochondria. (**a**) CFP (+) mitochondria were longer and thicker in MONs from 12-month-old compared to 1-month-old Thy-1 CFP mice (inset; scale bar = 2 μm). OGD reduced CFP pixel intensity in 12-month-old MONs despite longer and brighter CFP (+) mitochondria (*yellow arrows*). (**b**) Blockade of AMPA/KA receptors with NBQX (30 μM) preserved CFP pixel intensity during OGD. $*p < 0.05$, and $**p < 0.0001$, one-way ANOVA. Calibration bar = 10 μm (Reproduced in part from Baltan (2012) [4])

7 Mitochondria-Enhanced Excitotoxicity Underlies the Increased Vulnerability of Aging WM to Ischemic Injury

Mitochondria contribute to the enhanced excitotoxicity of aging WM and underlie the increased vulnerability of aging WM to ischemia. Mitochondria bioenergetics in neurons (gray matter) and their role in glutamate excitotoxicity are well-described [46]. Mitochondrial dysfunction and excitotoxicity share common features and are believed to act synergistically by potentiating one another [47–49]. Mitochondria are dynamic organelles that travel using axonal transport, in both the anterograde and retrograde directions, to reach peripheral locations and provide local energy supply [50, 51]. They constantly undergo fission and fusion events [52] and the relative rates of mitochondrial fusion and fission are implicated in the regulation of their size, number, and shape [53–55]. The balanced delivery of mitochondria to cell bodies, dendrites, axons, and axon terminals helps them serve multiple functions, including energy generation, regulation of Ca^{2+} homeostasis, cell death, and synaptic transmission and plasticity [56]. Expectedly, associations between many

neurological diseases, including aging, and defects in mitochondrial dynamics are emerging [55, 57].

In gray matter, aging neurons become more susceptible to glutamate excitotoxicity because of collapsed mitochondrial membrane potentials and increased generation of reactive oxygen and nitrogen species (ROS/RNS), leading to further reductions in mitochondrial function and energy production [58]. Mitochondrial function appears to decline in older animals, presumably causing reduced ATP production. This has been shown in the heart [59], liver [60], and brain [61]. Ion homeostasis accounts for ≈50 % of all ATP consumption, for which the Na^+/K^+ ATPase, the key enzyme to maintain ion homeostasis, is responsible for the majority of this consumption [62]. Therefore, the combined loss of ATP reserves and the high energy requirements of the Na^+/K^+ ATPase, diminishing the activity of this enzyme with aging, may heighten the sensitivity of aging WM to injury [63]. Consistent with this possibility, axon function in aging MONs, when transiently challenged with OGD, was slower to restore normal ion gradients, permitting pathological processes related to disruption of ion homeostasis to operate for a longer time (earlier reversal of the Na^+-dependent glutamate transporter, Fig. 7), thus producing more injury in aging MONs [3]. In addition, aging MONs showed greater recovery of WM function in older animals when OGD was applied at a lower temperature [3], supporting the hypothesis that ATP reserves are compromised in aged WM. Finally, axons with higher ATP requirements have many more mitochondria per unit length of process [64]; therefore, these axons would be preferentially targeted by low ATP reserve conditions.

In neurons, excitotoxicity and elevated Ca^{2+} induce marked changes in mitochondrial morphology, stopping their movement [56, 65, 66] and generating ROS [46]. In young WM, activation of either AMPA or kainate receptors [3] loads mitochondria with Ca^{2+} and fission is enhanced, associated with loss of fluorescence of mitochondria genetically tagged with CFP (Fig. 8) [67]. Ca^{2+} overload activates NOS to produce nitric oxide and ROS/RNS, which could act either directly on oligodendrocytes [68–70] to cause injury or as diffusible second messengers linking oligodendrocyte excitotoxicity to axonal injury [31, 32]. Axon function directly correlates with WM energy reserves, since Na^+–K^+ ATPase activity is dependent on ATP levels. As a result, OGD causes a reduction in ATP levels and CFP (+) mitochondria, which is prevented by AMPA/KA receptor blockade in young and old WM (Figs. 8 and 9). Despite the structural and functional changes in aging mitochondria that enhance oxidative stress and amplify glutamate-mediated excitotoxicity during OGD, blockade of AMPA/KA receptors proved to be a successful strategy in improving WM function after stroke.

In summary, we and others have identified WM as an important therapeutic target for stroke [3, 5, 13, 15, 16, 41]. We have proposed the optic nerve as an ideal model for the study of ischemic WM injury and have described, step-by-step, ischemic injury pathways and the glial cells that are impacted. We have identified that aging modifies these ischemic injury pathways to render WM more susceptible to injury, such that in aging WM, the removal of extracellular Ca^{2+} is injurious, while

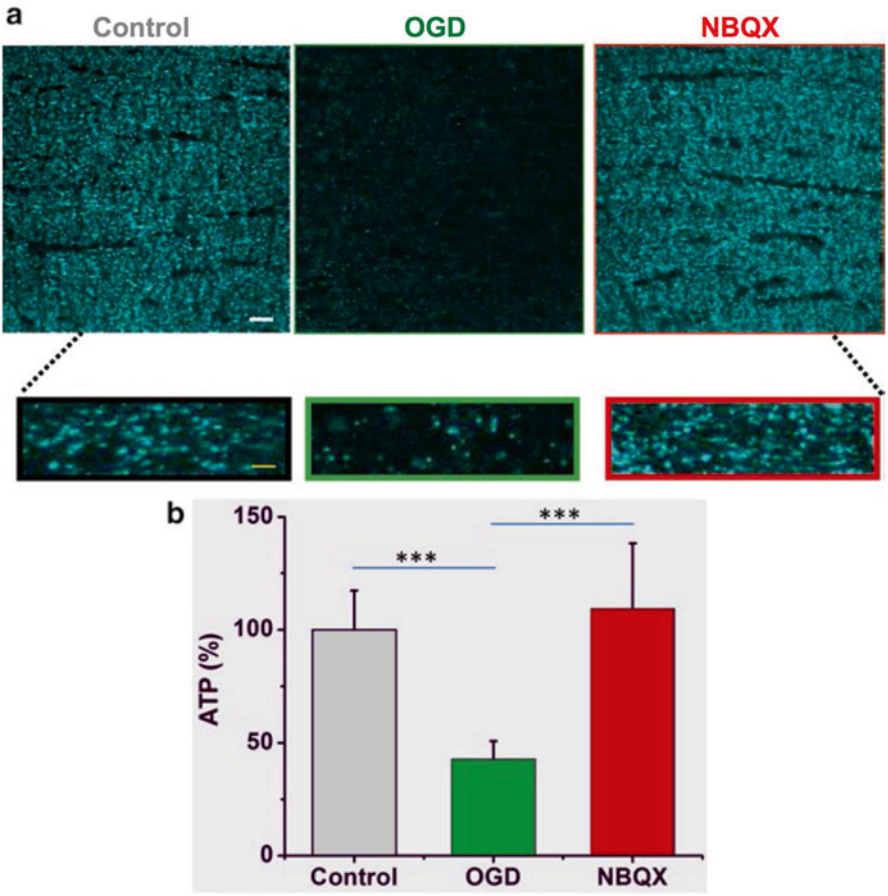

Fig. 8 In young MONs, Blockade of excitotoxicity preserves CFP (+) axonal mitochondria and ATP levels in response to OGD. (**a**) OGD severely reduced CFP fluorescence in MONs from mito CFP (+) mice and pretreatment with NBQX (30 μM) protected against this loss. Note the change in mitochondrial morphology from small and tubular under control conditions to tiny and punctate following OGD. Calibration bar = 10 μm (insets = 2 μM) (**b**) NBQX pretreatment conserved ATP levels in MONs. ***$p < 0.0001$, one-way ANOVA (Reproduced in part from Baltan et al. (2011) [36])

it was protective in young WM (Fig. 9). In addition, aging alters WM glutamate homeostasis and mitochondrial dynamics, which lead to an enhanced glutamate excitotoxicity period with ischemia, which starts earlier and extends into the recovery period. Furthermore, we have identified that AMPA/KA receptor blockade protects both young and old WM, whereas NMDA receptor blockade is not protective in neither young nor old WM. Unexpectedly, NMDA receptor blockade worsened OGD recovery in old WM. Our results suggest that NMDAR activation during OGD

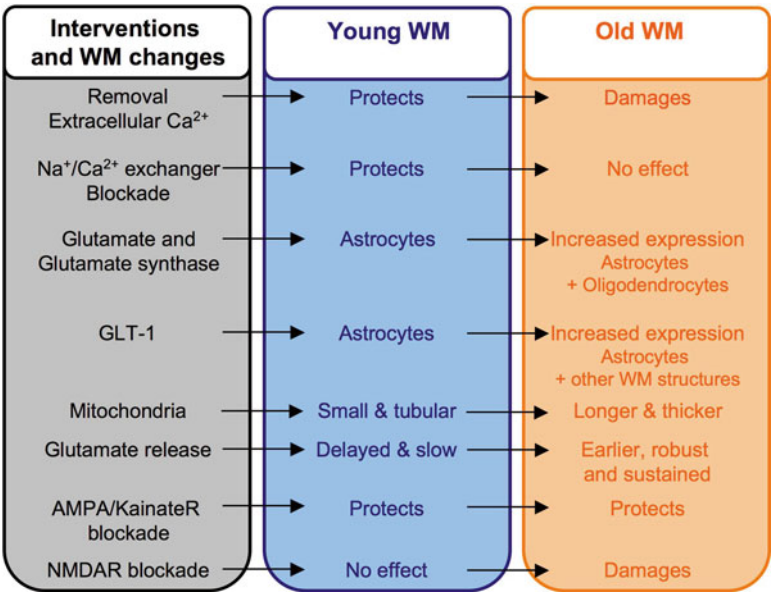

Fig. 9 Working molecular and cellular mechanisms responsible for ischemic WM injury. Age-dependent cellular remodeling of WM elements modifies the injury mechanisms and functional outcome to increase the sensitivity of aging WM to ischemic injury (Modified in part from Baltan 2014 [18])

does not contribute to failure of axon function and raises caution for the therapeutic use of NMDAR antagonists during ischemia, particularly in aging WM. Overall, our research suggests that for the development successful of future stroke therapies, we must tailor our approach to protect both gray matter and white matter as a function of age.

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