THE SPECTRUM OF ISCHEMIA-INDUCED WHITE MATTER INJURY VARIES WITH AGE

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Abstract. Stroke is a neurological condition that targets the whole range of the human population, from the pre-term infant to the elderly and is a major cause of death worldwide (Ingall 2004). During its lifespan, the brain’s vulnerability to hypoxia-ischemia varies. Term infants who suffer this insult usually exhibit widespread neuronal injury in the cerebral cortex with a stroke-like distribution of damage (Deng 2008), whereas in pre-term infants immature oligodendrocytes and subplate neurons below the neocortex are most vulnerable and result in Periventricular Leukomalacia (PVL) (Back et al. 2007; McQuillen et al. 2005). The incidence of stroke decreases in young adulthood, but peaks again in the elderly. Moreover, the underlying pathological mechanisms that occur following ischemia are different at each stage.

Experimental stroke research on stroke has traditionally focused on grey matter injury, but recent evidence indicates that white matter injury is a critical part of its pathophysiology. In this debilitating condition the mechanisms of ischemia-induced damage differ with age and all cellular components of white matter (axons, oligodendrocytes and astrocytes) are affected.

This review paper focuses on the relative vulnerability to ischemia of white matter during the course of development and on our recent findings of how individual cellular components are affected during each stage.

Keywords White Matter Development; Axon; Oligodendrocyte; Astrocyte; Optic Nerve; White Matter Injury; Ageing; Ischemia; Periventricular Leukomalacia.

1 Introduction

There are currently more than 250 identified neurological diseases. These constitute a worldwide problem that affects over 2 million people in Britain alone (Brain Research Trust, 2003). Fifteen million people suffer from stroke worldwide yearly (World Health Organisation 2002), and in those who survive the initial insult, mortality during the first year is about 20% (Dewar et al., 1999). The disease affects not only the patients, but also causes considerable burden on family members and on society in general. Taking into consideration in-patient rehabilitation and follow-up care, the estimated direct and indirect costs of stroke for 2009 were 68.9 billion in the U.S.A. and 32.3 billion in the countries of the European Union (Annunziato 2009).

Age has been regarded as the most significant risk factor for stroke for several decades and to result from a combination of atherosclerosis in the cerebral arteries.
associated with age-related co-morbidities such as cardiac dysfunction, hypertension, diabetes mellitus and hypercholesterolemia (De Grey, 2005). However, ageing also alters the relationship between myelin and axons and changes the relative densities of the brain’s cellular constituents (Hinman et al. 2006; Hinman et al. 2007; Peters et al. 2002; Sandell et al. 2002). Therefore, an understanding is required of whether the ageing process underlies the susceptibility of white matter to ischemic injury and therefore whether this heightened vulnerability is associated with a change in the course of the underlying mechanisms that contribute to this selective type of injury.

The magnitude of the problem posed by ischemic injury on pre-term infants is very significant. In the U.S.A. approximately 50,000 low birth-weight infants (<1500 g) are born every year. Advances in medical treatment have led to survival of almost 90% of such infants (Deng 2008). About 10% of the survivors later develop spastic motor deficits (Doyle 2010), and about 20 – 25% later exhibit cognitive, attentional, behavioural, and/or socialisation defects that significantly impair their quality of life (Msall 2010; Johnson 2009). PVL is the predominant form of brain injury that underlies mortality and morbidity in pre-term and term infants who suffer from a hypoxic-ischemic insult and is the leading cause of cerebral palsy in premature infants (Deng 2008).

Since the establishment of the central role of the excitotoxic cascade in the neurochemical pathological process that occurs during ischemia, numerous clinical trials have been performed, but virtually every drug that conferred protection to neurons in experimental models failed in those trials (Del Zoppo 1998, Del Zoppo 1995; Dinnagl 2006; O’Collins et al. 2006). A primary reason for this was the failure of the drugs used to protect white matter. Most experimental work on stroke was performed on rodent brains, but white matter constitutes only 13% of their brain (Zhang et al. 2000) whereas it accounts for 50% of the volume of the human brain (Zhang et al. 2000). Besides, the metabolic rate of white matter is only modestly reduced in comparison to that of grey matter (Nishizaki 1988). Moreover, ischemic injury is never limited to grey matter alone, and white matter injury contributes significantly to the clinical deficits that lead to mortality and morbidity.

The mechanisms that underlie white matter injury are unique and very complex (Agrawal et al. 1997; Fern et al. 1997; Follett et al. 2000; McDonald et al. 1998; Sanchez-Gomez et al. 1999; Stys 2004; Tekkøk et al. 2001; Tekkøk et al. 2007; Wrathall et al. 1992). Two distinct mechanisms seem to operate sequentially or simultaneously following energy depletion and can be traced to intra-axonal ionic distribution and excitotoxic-ity with over-activation of AMPA and kainate receptors (Stys 2004; Tekkøk et al. 2007). However, most of the data is derived from experimental work carried out in young adult animals (Tekkøk et al. 2008). This review highlights the main difference in the mechanisms of ischemic white matter injury during different developmental stages. A preliminary account of part of our work mentioned here has been published elsewhere (Zammit et al. 2011, Alix et al. 2012).

![Figure 1: Progress of axonal injury following 30 mins of OGD](http://dx.medra.org/10.7423/XJENZA.2013.1.03)

There is progression of injury in all age groups, but features of axonal damage are first evident in P20 – 50 mice, followed by P > 50 mice, and finally P < 20 mice. Thick arrows mark axonal swelling; thin arrows mark beading formation. CT - controls; IF - immediately fixed after OGD; 1hr - 1 hour reperfusion; 2hr - 2 hours reperfusion; 3hr - 3 hours reperfusion (Zammit et al. 2011). (Magnification X60 lens - X400 digital zoom)

![Figure 2: Axons from P20 – P50 mice are extremely vulnerable to ischaemia](http://dx.medra.org/10.7423/XJENZA.2013.1.03)

Comparing axonal injury following ischemia between different age groups. There was a statistically significant difference (*p ≤ 0.001) in axonal injury score between the different age groups after each duration of OGD. P20 – 50 mice (box) were the most vulnerable to injury, and P < 20 mice (diamond) were the most resistant (Zammit et al. 2011).
2 Vulnerability of axons to ischemia and age

The central nervous system of the neonatal mammal was formerly regarded to be more resistant to ischemia than that of the adult (Duffy et al. 1975). This was often interpreted to be a safety mechanism of the neonate brain which is more prone than that of the young adult to suffer from periods of hypox-perfusion during its pre- and post-natal phases (Vannucci 1990; Volpe 1992).

Fern et al. (1998) showed that neonatal white matter to be very resistant to ischemia. Using optic nerves from neonatal mice at P2 (postnatal day 2) they showed that axons at this age group were more tolerant than older aged to anoxia, aglycaemia or their combination in maintaining evoked compound action potential (CAP), and in recovery of function during reperfusion (Fern et al. 1998). This result was supplemented by an imaging study that tested the observed preservation of CAP with maintenance of axon structural integrity (Zammit et al. 2011) during variable degrees of an ischemic insult. In this study, optic nerves from Thy-1/GFP-M mice from three different age groups (< P20, P20–P50 and > P50) were exposed to variable durations of oxygen-glucose deprivation (OGD) at 30, 60, 90 and 120 mins respectively. Quantitative scoring of axon injury revealed that the degree of structural damage in axons from neonatal mice (< P20) was significantly smaller than that in older mice (Figure 1).

The sensitivity of rat grey matter to anoxia and aglycaemia increases progressively from birth to adulthood, consistent with the rise in metabolic rate of this tissue (Cherubini et al. 1989; Crépel et al. 1992). White matter does not follow a similar pattern. Fern et al. (1998) showed that white matter in mice between P20 and P50 is most vulnerable to an ischemic insult in terms of decrease in CAP during OGD and of the rate of recovery from the insult. This vulnerability starts to decrease at P50 which is in agreement with our published observations (Zammit et al., 2011). In that study, the degree of axonal injury in P20 - P50 mice was significantly higher than in any other age group, and this sensitivity stabilised in mice older than P50 (Figure 2).

In view of the above findings the following questions arise: Why is there such a difference in vulnerability? What types of axons are present at each developmental stage? Do the underlying ischemia-induced pathological mechanisms vary at different developmental stages?

3 Mechanisms of ischemia-induced injury in axons and development

In the mouse optic nerve, myelination starts at about P7, with few axons having only one whorl of myelin at this age (Foster et al. 1982). The rate of myelin deposition thereafter peaks at P21 – P28, and from this point onward, the process of myelination is at its highest (Skoff et al. 1976). The increased tolerance to OGD-induced damage is dominated by unmyelinated axons and may be attributed to the lower metabolic rate of neonatal white matter (Duffy et al. 1975; Hansen 1985). At this developmental stage, there is also increased glycogen deposition in astrocytes (Kohle et al. 1977), and, but only transiently, in immature axons (Bruckner et al. 1981).

The mechanism of ischemia-induced injury in these axons differs from that at other stages of development. In young adult white matter, ischemic injury is mediated by AMPA/Kainate receptors (Tekkö et al. 2001; Baltan et al. 2008). However, McCarren et al. (2007) showed that ionotropic glutamate receptor agonists did not damage rodent white matter axons at P3 and damaged them only minimally at P7. Since unlike myelinated white matter, premyelinated axons do not express functional glutamate receptors on their axolemma, it was suggested that there could be a distinct mechanism of injury at this developmental stage, coupled to ionic imbalances culminating in deleterious intra-axonal Ca2+ overload (McCarran et al. 2007).

The period of low tolerance to ischemia (between P20 and P50) coincides with the process of myelination, and the increase in sensitivity to ischemia could be attributed to the onset of the associated heightened metabolic activity (Azzarelli et al. 1980; Davison et al. 1966; Wiggins 1982). Fowler and colleagues (2003) also proposed that myelination may increase axonal vulnerability to oligodendrocyte-induced damage, as perturbation of the oligodendrocyte-myelin-axon interaction in myelinated white matter decreased axonal damage after AMPA injection. Myelination is not the only contributor as Na+ -channel density in optic nerve axons also varies with age, starting from < 2 µm2 in the neonate (Waxman et al. 1989), increasing up to the age of about P25, and declining in adulthood (Xia et al. 1994). During myelination, the Na+ channels aggregate at the nodes of Ranvier and the change in their density results in a persistent non-inactivating Na+ current, which exacerbates white matter injury after anoxia (Alzheimer et al. 1993). Cu2+ may also have a role at this stage since Ca2+ currents were observed to increase in magnitude in the postnatal period (Lorenzon et al. 1995), and have been shown to contribute directly to anoxic injury in white matter (Fern et al. 1995).
The spectrum of ischemia-induced white matter injury varies with age

We have recently reported that large (> 0.4 µm in diameter) pre-myelinating axons are more sensitive to OGD than smaller pre-myelinated and myelinating axons. This heightened sensitivity could not be attributed to the myelination process per se, and blockage of intracellular Ca\(^{2+}\) was not protective during a 60 – min period of OGD (Alix et al., 2012). Blockade of NMDA and non-NMDA glutamate receptors (GluRs) alone provided only partial protection to P10 axons in rat optic nerves following 60 mins of OGD plus 60 mins of recovery and addition of L-type and P/Q-type voltage-gated calcium channel (VGCC) blockers to those GluRs blockers produced complete recovery of CAP following the same ischemic insult (Alix et al. 2009). Comparison of OGD-induced damage to small (< 0.4 µm) and to large (> 0.4 µm) premyelinating axons showed that the former were protected by GluR blockers alone, whilst the latter needed addition of VGCC-blockers to confer protection (Alix et al. 2012). That study gave direct evidence of the importance of VGCC in this age group and provided new insight on the pathophysiological mechanism of injury during ischemia in these very sensitive axons.

![Figure 3: APC +ve oligodendrocytes in mouse optic nerve after 60 mins OGD](http://dx.medra.org/10.7423/XJENZA.2013.1.03)

Figure 3: APC +ve oligodendrocytes in mouse optic nerve after 60 mins OGD. Cropped sections from high power micrographs (X60) of optic nerve sections from 3 different age groups (P < 20, P20 – 50, and P > 50) stained with APC (left) and Hoechst stain (right) after 60 mins OGD. Optic nerves from P < 20 mice had a greater number of pyknotic nuclei when compared to older age groups (Zammit et al., 2011).

4 Axonal injury and the effect of age

In young adults and in ageing white matter, glutamate excitotoxicity plays a central role in ischemia-induced injury. It is therefore not surprising that AMPA/Kainate receptors have been found to mediate excitotoxic injury in ageing white matter tracts (Tekköök et al. 2008). These authors studied ischemia-induced injury in the optic nerve of 1−, 6−, 12−, 18− and 24-month-old mice. Excitotoxic events occurred more quickly and more vigorously in ageing white matter, but were not mediated by Ca\(^{2+}\) influx (Tekköök et al. 2008). In contrast, in young adults, ischemia-induced injury could be almost entirely prevented if the OGD was performed in a Ca\(^{2+}\) -free medium (Fern et al. 1995; Tekköök et al. 2001; Tekköök et al. 2007). In this context, accumulation of Na\(^{+}\) that leads to lethal cellular swelling and reversal of Na\(^{+}\)/K\(^{+}\) ATPase activity decreases with age. This leads to inability to maintain an appropriate transmembrane ion gradient, which results in slower restoration of normal ion gradients in ageing tissue following energy deprivation. Consequently, pathological pro-

Figure 4: Oligodendrocytes vulnerability to ischaemia decreases with age. Comparing percentage dead oligodendrocytes following ischemia between different age groups. There was a statistically significant difference (\(p \leq 0.05\)) in percentage dead oligodendrocytes between the different age groups after each duration of OGD. APC +ve oligodendrocytes at P < 20 were the most vulnerable to injury, and tolerance to ischemia increased with age (Zammit et al., 2011).

![Figure 4: Oligodendrocytes vulnerability to ischaemia decreases with age](http://www.mcs.org.mt/)
cesses initiated by ion dysregulation would last longer, and produce more damage (Scavone et al. 2005). Besides, decline in mitochondrial function in brain cells (Toescu 2005) and increase in free radical generation (Droge et al. 2007) also occur with increasing age.

5 Early oligodendrocyte progenitor cells and Periventricular Leukomalacia

Periventricular Leukomalacia (PVL) is the most common cause of brain injury in premature infants (Back et al. 2004) and results from an ischemic insult through the high-risk developmental period of 23 and 32 weeks gestation (Alix 2006). The pathogenesis of PVL comprises three major interacting factors: cerebral ischemia, systemic infection and inflammation, and maturation-dependent intrinsic vulnerability of premyelinating oligodendrocytes (Volpe et al. 2011).

Oligodendrocyte injury has long been regarded as the hallmark of PVL. Oligodendrocyte development occurs in four stages: early oligodendrocyte progenitor cell (OPC), late OPC (also called premyelinating oligodendrocytes), immature myelinating oligodendrocyte, and mature myelinating oligodendrocyte (Back 2006). Back and Volpe used brain slices containing corpus callosum from P2 mice to demonstrate the relative susceptibility of early OPC, late OPC, and immature oligodendrocytes. After induction of OGD, early OPC were significantly more resistant to ischemia than late OPC (Back et al. 2002). This maturation-sensitivity of late OPC leads to preferential white matter injury in the neonate (Volpe et al. 2011) and coincides with the high-risk period for PVL in humans (Craig et al. 2003).

The major factors that underlie the maturation-dependent susceptibility of late OPC are: abundant production of reactive oxygen and nitrogen species during PVL, delayed development of glutathione antioxidant defences, acquisition of Fe\(^{2+}\), and exuberant expression of the major glutamate transporter, of AMPA receptors deficient in the GluR2 subunit (and therefore Ca\(^{2+}\)-permeable), and of NMDA receptors (also Ca\(^{2+}\)-permeable) (Volpe et al. 2011).

6 Vulnerability of immature and mature myelinating oligodendrocytes to ischemia

As indicated above, late OPCs are important contributors to PVL. Therefore several questions immediately come to mind. What role do myelinating oligodendrocytes feature in ischemic injury? Do immature and mature myelinating oligodendrocytes vary in their vulnerability to ischemic damage? Do they contribute to the continuum of white matter susceptibility during ischemia?

To gain further insight into these questions, the effect of different durations of OGD (0, 60, 90, 120 mins) on the viability of myelinating oligodendrocytes in mouse optic nerves from three different age groups (<P20, P20 – P50 and >P50), were studied by Zammit et al. 2011 and Alix et al., 2012. In these studies, the authors used anti-APC antibody in combination with Hoechst 33342 to determine the percentage number of dead oligodendrocytes in each stage. (Figure 3). Data from these experiments clearly show that 30 mins of OGD was sufficient to kill almost 70% of oligodendrocytes in all age groups, with the number of dead oligodendrocytes in neonatal mice (P < 20) being significantly higher (p > 0.05) than that in the older age groups (Figure 4).

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The spectrum of ischemia-induced white matter injury varies with age

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Figure 7: Astrocyte process damage is more pronounced and occurs earlier in older mice. Cropped sections from high power micrographs (X60) of optic nerve sections from 3 different age groups (P < 20, P20−50, and P > 50) stained with GFAP, showing detail of astrocyte processes. Astrocyte processes damage (red arrows) is visible after 2 hour of reperfusion in P < 20 mice, after 1 hour reperfusion in P 20−50 mice, and in immediately fixed slices after OGD in P > 50 mice (Zammit et al. 2011).

In mouse optic nerve, immature myelinating oligodendrocytes predominate at around P7 to P10, and mature myelinating oligodendrocytes appear at around P14 and increase in number from P20 onwards (Craig et al. 2003). Therefore, our results showed that immature myelinating oligodendrocyte (which predominates in P 20 mice) are more vulnerable than mature myelinating oligodendrocytes (which predominate in older age groups). In cell cultures, mature oligodendrocytes (A2B5+/GC+) were more resistant to ischemia than immature ones (O4+/GC-) which led Fern et al. 2000 to propose that rapid ischemic cell death of the immature oligodendrocytes was mediated by Ca2+ influx via non-NMDA glutamate receptors, and exacerbated by significant autologous feedback of glutamate from cells on their own receptors (Fern et al. 2000).

7 Astrocytes are vulnerable to ischemia

Astrocytes have long been thought to be very resistant to ischemia, probably because most of the early studies were performed in dissociated cultures (Goldberg et al. 1993). However, studies performed by Fern 2001 show that neonatal white matter astrocytes are more vulnerable to ischemic injury than axons at the same developmental stage.

The mechanism of ischemia-induced astrocyte injury varies with age. In P2 mice, significant astrocyte death was apparent just after 10 − 20 min of ischemia, with death in approximately 50% after 80 min of OGD. This high sensitivity results from Ca2+ influx through T-type channels (Fern 1998). In older mice (P10), induced Na+−K+-Cl− and HCO3− channels contribute to osmo-regulatory challenge (cell swelling), and are considered to be the main determinant of cell death (Thomas et al. 2004).

Live imaging of P7 − P14 GFP-GFAP mouse optic nerves showed approximately 50% decrease of astrocyte cell bodies and 40% decrease of astrocyte processes after 20 mins of OGD and 1 hour of reperfusion (Shannon et al. 2007). This led to the proposal that astrocytes of actively myelinating white matter have a heightened sensitivity to ischemic-type injury, especially during the period of reperfusion.

8 Astrocyte vulnerability and age

In an immunocytochemical study, Zammit et al. 2011, and Alix et al. 2012, reported a predisposition of varying vulnerability to ischemia of successive developmental stages in astrocytes from mouse optic nerve exposed to different durations of OGD. In these studies, OGD resulted in a gradual decrease in pixel intensity in all age groups, and reperfusion further exacerbated the injury induced by the initial insult. The decline in GFAP staining following ischemia in P < 20 mice was less than in P20 − P50 or in > P50 mice (Figure 5 and 6). Also, the same duration of OGD resulted in loss of structural integrity of astrocyte processes at an earlier stage in P20 – 50 and P > 50 mice than in P < 20 mice (Figure 7). These findings support the hypothesis of Shannon et al., 2007 that astrocytes in myelinated white matter are more vulnerable than those in unmyelinated white matter. The mechanism behind this difference and the reason why astrocytes from older age groups were even more vulnerable is still unclear. However, caution must be exercised in the interpretation of these results since GFAP-staining intensity as a measure of astrocyte viability is not optimal (Shannon et al. 2007) and is highly variable although widely used as an assessment tool of viability by several groups (Chen et al. 1993; Davies et al. 1998; Fern 2001; Garcia et al. 1993; Petito et al. 1993; Schmist-Kastener et al. 1993).

9 Conclusion

There is convincing evidence that the vulnerability of white matter elements is dependent on their developmental stage.

Unmyelinated axons are very resistant to ischemia; injury to these axons is mediated by ionic imbalances with intra-axonal Ca2+ overload and not via
The spectrum of ischemia-induced white matter injury varies with age

Figure 8: Cellular mechanisms of injury in white matter ischemia changes from development through ageing. Cartoon depicts the sequential and stage-specific mechanisms that are thought to contribute to white matter injury at P2, P10 and mature (> 1 year) white matter. The cellular targets are oligodendrocytes and axons in all age groups. This emphasizes the need for therapeutic approaches in white matter ischaemia to be more selective and cell-specific when considering age-related differences in neurobiology and pathophysiology.

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AMPAR/Kainate-receptor activation, which is followed by a period of heightened sensitivity to ischemia (Figure 8).

Large premyelinated axons (> 0.4 μm in diameter) are extremely vulnerable and their injury is mediated by VGCC. Axons going through the process of myelination are also very sensitive to ischemia and their increased vulnerability is thought to coincide with the increased metabolic demand needed for myelination through the redistribution of Na⁺ channels and an increase in Ca²⁺ currents. At this stage they have just initiated diameter expansion and express clusters of functional VGCC at future nodes of Ranvier.

The vulnerability of late OPCs can no longer be regarded as the sole contributor to immature white matter ischemic injury, and the central role of these axons must be appreciated. Myelination of the CNS is a timely and systematic process that occurs in an orderly spatial and temporal sequence. All CNS neurons are formed before birth, while white matter begins to develop and expand in the third trimester of gestation. White matter development is still incomplete at birth, and only 90% complete by 2 years of age. Before the onset of myelination when late OPCs and large calibre pre-myelinating axons co-exist and contribute to white matter injury, the susceptibility of a particular white matter region to ischemic injury will depend on axonal and oligodendrocyte maturation at that site. In young adult white matter, once axons are fully myelinated, their vulnerability to ischemia decreases, and ischemic injury is mediated by AMPAR/Kainate receptor activation and calcium overload. With increasing age, white matter suffers another period of increased risk in vulnerability to ischemia. In the ageing brain, excitotoxic events occur earlier and more vigorously and are not mediated by Ca²⁺ influx. Instead, there is an accumulation of intracellular Na⁺ leading to lethal swelling and reversal of Na⁺-dependent glutamate transporter (which increases in expression) and release of intracellular Ca²⁺.

PVL is the most common cause of brain injury in the premature infant with the stage specific and most sensitive developmental stage of oligodendrocytes identified as the late OPC. This maturation-dependent intrinsic vulnerability plays a vital role in the pathophysiology of PVL since the late OPC are vulnerable to free radical attack and are very sensitive to excitotoxicity. Our recent findings suggest that immature myelinating oligodendrocytes are more vulnerable than the mature myelinating ones. The immature myelinating oligodendrocytes co-exist with axons undergoing myelination, and their vulnerability might contribute to the increased sensitivity to OGD of myelinating axons. Ischemia-induced mechanisms of injury in these cells has been postulated to be mediated by Ca²⁺ influx via non-NMDA glutamate receptors, and it is exacerbated by a significant element of autologous feedback of glutamate from cells onto their own receptors.

Astrocytes are also equally vulnerable to ischemia. Ischemic injury in immature astrocytes (P2 mice) is mediated by Ca²⁺ influx via T-type channels, whilst that in more mature astrocytes (P10 mice) is mediated by Na⁺-K⁺-Cl⁻ and HCO₃⁻ channel activation. Our recent study found that astrocytes present in younger mice (P < 20) are more resistant to ischemia than those present in older age groups.

White matter injury during ischemia plays a central role in the pathophysiology of stroke in all human age groups. Future therapeutic strategies should take into consideration selective white matter protection and recognize that the mechanisms that lead to this type of injury are variable with age. Extrapolation of findings and results from one age group to another may contribute to strategy failure. A better understanding of these differences, might give new insights on developing new therapeutic modalities for such a challenging disease.

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