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AIF, reactive oxygen species, and neurodegeneration: a “complex” problem

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Abstract

Apoptosis-inducing factor (AIF) is a flavin-binding mitochondrial intermembrane space protein that is implicated in diverse but intertwined processes that include maintenance of electron transport chain function, reactive oxygen species regulation, cell death, and neurodegeneration. In acute brain injury, AIF acquires a pro-death role upon translocation from the mitochondria to the nucleus, where it initiates chromatin condensation and large-scale DNA fragmentation. Although *harlequin* mice exhibiting an 80–90% global reduction in AIF protein are resistant to numerous forms of acute brain injury, they paradoxically undergo slow, progressive neurodegeneration beginning at three months of age. Brain deterioration, accompanied by markers of oxidative stress, is most pronounced in the cerebellum and retina, although it also occurs in the cortex, striatum, and thalamus. Loss of an AIF pro-survival function linked to assembly or stabilization of electron transport chain complex I underlies chronic neurodegeneration. To date, most studies of neurodegeneration have failed to adequately separate the relative importance of the mitochondrial and nuclear functions of AIF in determining the extent of injury, or whether oxidative stress plays a causative role. This review explores the complicated relationship among AIF, complex I, and the regulation of mitochondrial reactive oxygen species levels. It also discusses the controversial role of complex I deficiency in Parkinson’s disease, and what can be learned from the AIF- and complex I-depleted *harlequin* mouse.

Keywords

mitochondria; electron transport; complex I; *harlequin*; oxidative stress; Parkinson’s disease

1. Introduction

AIF was identified in the late-1990s as a factor released from isolated mitochondria following calcium overload that causes fragmentation of purified nuclei (Susin et al., 1996). Although dubbed “apoptosis-inducing factor” for its ability to induce apoptotic nuclear morphology, early studies using AIF-deficient stem cells found that AIF was not required for classical caspase-dependent apoptosis induced by the DNA-damaging agent etoposide, the broad-spectrum protein kinase inhibitor staurosporine, or the pro-oxidant *tert*-

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butylhydroperoxide (Joza et al., 2001), among other stimuli. However, AIF-deficient cells were resistant to death due to the reactive oxygen species (ROS)-inducing compound menadione, but only in the presence of caspase inhibitor (Joza et al., 2001). They were also resistant to death due to growth factor withdrawal (Joza et al., 2001). Based on these studies it was suggested that AIF mediates a caspase-independent programmed cell death pathway required for death in response to certain stimuli. At the time, the early embryonic lethality of the AIF knockout was explained by the loss of a pro-death function required during cavitation of embryoid bodies (Joza et al., 2001).

The AIF protein was cast in a new light when it was discovered that the *harlequin* mutant mouse harbors a retroviral insertion in the *pcd8* gene encoding AIF (Klein et al., 2002). The intron insertion causes an ~80–90% global reduction in AIF rather than a change in the protein sequence (Klein et al., 2002, Vahsen et al., 2004, Chinta et al., 2009, Perier et al., 2010). Although the residual AIF overcomes embryonic lethality, *harlequin* mice suffer from slow, progressive neurodegeneration, ataxia, and loss of vision (Klein et al., 2002, El Ghouzzi et al., 2007). Evidence of oxidative stress was observed in the cerebellum and retina, including elevated lipid hydroperoxides and increased staining for 8-hydroxydeoxyguanosine, a marker for oxidatively damaged DNA. Cultured cerebellar granule neurons but not cortical neurons from *harlequin* mice were sensitized to hydrogen peroxide toxicity (Klein et al., 2002). A cerebellum-specific upregulation of catalase activity and glutathione content was noted relative to the whole brain that was presumably compensatory for the loss of AIF (Klein et al., 2002). Based on these results, it was suggested that AIF is an antioxidant enzyme essential for the survival of certain cell types. Despite the fact that *harlequin* mice undergo chronic neurodegeneration, they are protected from acute brain injury due to focal ischemia (Culmsee et al., 2005), global ischemia (Cao et al., 2007), neonatal hypoxia-ischemia (Zhu et al., 2007), and trauma (Slemmer et al., 2008).

The goal of this review is to re-evaluate the connection between AIF and mitochondrial ROS production and discuss the implications for neurodegenerative conditions, with an emphasis on Parkinson's disease. Rather than providing a comprehensive overview of the literature, focus is placed on a few key areas of research.

2. Is AIF an antioxidant enzyme?

AIF is an FAD-containing flavoprotein localized to the mitochondrial intermembrane space, with its N-terminus anchored to the inner membrane (Sevrioukova, 2011). It has significant sequence homology to both vertebrate and non-vertebrate oxidoreductase enzymes (Mate et al., 2002, Ye et al., 2002, Sevrioukova, 2011). When AIF was purified with an N-terminal affinity tag, the recombinant protein exhibited an NAD(P)H oxidase activity that generates superoxide anion under cell-free conditions (Miramar et al., 2001). This finding is opposite to the expected behavior of an antioxidant enzyme; however, it is consistent with the behavior of many FAD-containing enzymes such as alpha-ketoglutarate dehydrogenase (Gazaryan et al., 2002, Starkov et al., 2004, Tretter and Adam-Vizi, 2004, Zundorf et al., 2009), succinate dehydrogenase (Zhang et al., 1998, Quinlan et al., 2012), and glycerol 3-phosphate dehydrogenase (Tretter et al., 2007b, Orr et al., 2012) that generate ROS rather than exhibiting antioxidant activity. In addition to generating superoxide, recombinant AIF could also transfer electrons to the typical NAD(P)H dehydrogenase electron acceptors 2,6-dichlorophenolindophenol or ferricyanide, but only at a slow rate relative to known NAD(P)H dehydrogenases (Miramar et al., 2001).

A physiological electron acceptor for the putative electron transfer function of AIF has not been identified. However, Churbanova and Sevrioukova found that when recombinant AIF was purified using a C-terminal tag that was subsequently excised following purification, the

properties of the protein differed markedly from the previously characterized N-terminally tagged variant (Churbanova and Sevioukova, 2008). A significant difference was that FAD was naturally incorporated into C-terminally tagged AIF upon expression of the recombinant protein, whereas FAD had to be added to the N-terminally tagged AIF protein, which was then refolded to accommodate the flavin moiety. Recombinant AIF with naturally incorporated FAD formed stable FADH₂-NAD(P) charge-transfer complexes upon reduction with NAD(P)H, with only very low electron transferring ability (Churbanova and Sevioukova, 2008). Although findings did not support an electron transfer function for AIF, additional evidence indicated that AIF reduction by NAD(P)H causes a monomer-to-dimer transition, raising the possibility that AIF acts as a redox-sensitive signaling molecule (Churbanova and Sevioukova, 2008). Importantly, when AIF was purified with naturally incorporated FAD, AIF was incapable of transferring electrons from NADH to superoxide anion, hydrogen peroxide, ascorbate free radical, or dehydroascorbate (Churbanova and Sevioukova, 2008). These results indicate that at least under cell-free conditions, AIF does not act as an NADH-dependent antioxidant enzyme.

3. What is the mitochondrial function of AIF?

A breakthrough in our understanding of the function of AIF came in 2004 when Vahsen et al. discovered that AIF deficiency influences the stability of the electron transport chain (Vahsen et al., 2004). The abundance and activity of both complex I and complex III were reduced in embryonic stem cells completely deficient in AIF, whereas complex I was exclusively impaired in the brains of *harlequin* mice expressing 10–20% of normal AIF protein. Notably, complex I was not reduced in the heart or liver of *harlequin* mice despite a large decrease in AIF (Vahsen et al., 2004). However, complete conditional knockout of AIF in these tissues led to complex I deficiency (Joza et al., 2005, Pospisilik et al., 2007). This finding suggests that different organs differ in their ability to tolerate loss of AIF function before the electron transport chain is compromised, with the brain being one of the most sensitive.

How AIF leads to changes in the electron transport chain is still not clear. The decreased expression of complex I subunits in AIF-deficient embryonic stem cells was not accompanied by changes in mRNA levels (Vahsen et al., 2004) and only modest mRNA transcript reductions were observed in a muscle-specific AIF knockout (Joza et al., 2005). Therefore, regulation of complex I integrity by AIF occurs primarily at the post-transcriptional level. Although complex I is reduced in AIF-depleted cells and tissues, the complex I that is assembled appears to have normal protein subunit stoichiometry and function. Most studies have failed to find a direct association of AIF with complex I subunits (e.g. (Vahsen et al., 2004), but see (Palmisano et al., 2007)). Consequently, it has been proposed that AIF indirectly affects complex I assembly or stability (Vahsen et al., 2004, Sevioukova, 2011). Complex I is rate-limiting for oxygen consumption by isolated synaptic mitochondria or isolated nerve terminals (synaptosomes), with only 10–25% complex I inhibition leading to impaired respiration (Davey et al., 1997, Telford et al., 2009). Consequently, the reduction in assembled complex I associated with AIF deficiency is anticipated to have a profound effect on mitochondrial function in neurons, which rely heavily on oxidative phosphorylation for meeting the energy demand associated with frequent depolarization (Davey et al., 1998, Nicholls, 2009).

3. How does AIF influence ROS in cells?

Both complex I and AIF are thought to modulate the levels of mitochondrial ROS; complex I as a generator and AIF as either a generator or suppressor of ROS. However, surprisingly, ROS levels in AIF-deficient embryonic stem cells were not altered (Vahsen et al., 2004).

Similarly, no alteration in ROS production was found in liver- or muscle-specific AIF knockout mitochondria (Pospisilik et al., 2007). However, a detailed study of ROS in immortalized Hep3B cells and HeLa cells supported the original conclusion by Klein et al. that AIF has an antioxidant role in cells. In this study, small interfering RNA (siRNA) knockdown of AIF led to a 1.5 to 2-fold increase in ROS levels, regardless of whether ROS were measured by oxidation of the fluorescent dyes amplex red, dihydroethidium, or 2',7'-dichlorodihydrofluorescein (DCFH-DA) (Apostolova et al., 2006). Basal ROS levels were reduced in electron transport chain-deficient Hep3B rho0 cells depleted of mitochondrial DNA compared to control Hep3B cells. In contrast to Hep3B control cells, knocking down AIF in these rho0 cells did not increase the amount of detectable ROS. This finding suggested that the normal function of AIF is to decrease or detoxify ROS originating from the electron transport chain but not ROS originating from elsewhere in the cell. A respiratory deficit in complex I-dependent but not complex II-dependent respiration was detected in AIF-knockdown Hep3B cells, as might be expected following a decrease in the level of assembled complex I. However, surprisingly, 16-hour antioxidant treatment rescued the deficit in respiration without restoring the level of complex I subunits. This finding suggested that oxidative damage to the residual complex I, rather than the reduced complex I level *per se*, was responsible for the respiratory deficiency. Although this study of ROS in intact cells provided support for the hypothesis that AIF decreases mitochondrial ROS levels, another detailed AIF knockdown study in immortalized cell lines yielded opposite results. In this study, siRNA knockdown of AIF in five different cell lines, HCT116, DLD-1, SW480, MCF-7, and A549, led to a decrease rather than an increase in ROS levels (Urbano et al., 2005), suggesting that AIF normally contributes to ROS production. ROS was measured by three different methods, by DCFH-DA oxidation, as in the other study, by lucigenin, which forms light upon reaction with superoxide, and by activity of aconitase, a mitochondrial matrix enzyme which can undergo superoxide-dependent inactivation. Despite exhibiting lower basal ROS, AIF knockdown cells were sensitized to *tert*-butylhydroperoxide and hydrogen peroxide-induced death (Urbano et al., 2005). This finding indicates that sensitization to pro-oxidants following AIF loss does not necessarily imply a direct AIF antioxidant activity. As in other studies, complex I was decreased by AIF knockdown. Expression of a DNA-binding mutant of AIF lacking the nuclear apoptotic function rescued complex I levels. However, additionally mutating two key amino acids in the oxidoreductase domain of AIF abrogated the ability to rescue complex I. This observation represents the first molecular evidence directly linking the putative redox function of AIF to complex I assembly or stability.

ROS levels in primary neurons following AIF knock down have not been investigated. However, Higgins et al. silenced AIF using siRNA in primary cortical neurons in an attempt to investigate its role in H₂O₂-induced death. They reported that AIF-deficient neurons exhibited poor (<20%) viability in antioxidant-free medium irrespective of H₂O₂-treatment (Higgins et al., 2009). This finding suggests an overall antioxidant function for AIF in neurons, but does not necessarily imply direct antioxidant activity.

How can the discrepant effects of AIF knockdown/knockout on cellular ROS levels be explained? It appears unlikely that AIF simply acts as an antioxidant protein, both from the failure of recombinant AIF to display antioxidant activity and from the fact that several independent studies have yielded disparate findings on how AIF removal influences the amount of detectable ROS. A more likely possibility is the influence of AIF on the integrity of the electron transport chain. Complexes I and III are both thought to contribute to physiological and pathological ROS production (Andreyev et al., 2005). A number of factors in intact cells can lead to changes in electron flux through the respiratory complexes and affect how much ROS are produced. Substrates entering the electron transport chain, as well as electron flux through the complexes, differ from cell type to cell type. The locations

where electrons enter the respiratory chain, as well as whether mitochondria are closer to a resting state or an ADP-stimulated state, influence how much ROS are produced (Andreyev et al., 2005). The control of complex I over respiration also differs among cell types, and even between synaptic mitochondria and non-synaptic brain mitochondria that are derived from neuronal cell bodies and glia (Davey et al., 1997, Pathak and Davey, 2008). Because cellular energy utilization was not studied in parallel to ROS production in AIF knockdown studies, it is impossible to know the extent to which cell type-to-cell type variability in substrate utilization and energy demand influenced conclusions on the role of AIF in mitochondrial ROS regulation.

To investigate how AIF and the concomitant complex I deficiency influence mitochondrial ROS production independent of cellular energy demands, we studied ROS using non-synaptosomal brain mitochondria isolated from AIF-depleted *harlequin* mice and their wild type littermates (Chinta et al., 2009). The use of isolated mitochondria allowed us to examine mitochondrial ROS release in the presence of defined electron transport chain substrates and inhibitors. If AIF functions primarily as an antioxidant enzyme, an elevation of ROS emission by *harlequin* brain mitochondria would be expected, regardless of mitochondrial substrate. However, if the dominant effect on ROS is due to AIF-dependent modulation of complex I levels, changes in ROS in proportion to changes in complex I would be expected.

ROS production by isolated mitochondria oxidizing succinate is thought to occur in large part by reverse electron transfer from coenzyme Q to complex I (Cino and Del Maestro, 1989, Hinkle et al., 1967, Kushnareva et al., 2002, Lambert and Brand, 2004, Tretter et al., 2007a, Votyakova and Reynolds, 2001, Liu et al., 2002). This happens only in mitochondria with a very high membrane potential and it is not known whether reverse electron transfer occurs in cells (Andreyev et al., 2005). However, ROS generated by this mechanism are very well established to originate from complex I. We found that ROS emission by *harlequin* brain mitochondria oxidizing succinate was decreased by half compared to wild type, in close proportion to the reduction in the complex I subunit NDUFA9 (Chinta et al., 2009). Indeed, ROS production in the presence of succinate was blocked by the complex I inhibitor rotenone in both wild type and *harlequin* mitochondria, consistent with complex I being the primary source of ROS emission by brain mitochondria oxidizing succinate.

Surprisingly, we and others found that basal ROS emission did not differ between wild type and *harlequin* brain mitochondria oxidizing complex I substrates (Chinta et al., 2009, Perier et al., 2010). In our hands, complete inhibition of complex I by rotenone increased ROS release by both wild type and *harlequin* mitochondria by >10 fold. We did not find a significant difference between the two (Chinta et al., 2009), while another group found a very modest ~18% elevation in ROS release by *harlequin* mitochondria (Perier et al., 2010). In both our study and the study by Perier et al., there was no difference in either resting or ADP-stimulated respiration between WT and *harlequin* non-synaptosomal brain mitochondria despite a 40–50% decline in the complex I subunit NDUFA9. Therefore, it is possible that complex I is normally in excess, and electron flux through complex I was similar in wild type and *harlequin* brain mitochondria due to a limitation in tricarboxylic acid cycle dehydrogenases that produce NADH. This might have caused similar rates of ROS generation when electrons flowed through complex I in the forward direction. It is also possible, but unlikely, that a decrease in ROS production by complex I is perfectly counterbalanced by a loss of AIF antioxidant function, leading to no change in mitochondrial ROS release. Alternatively, it is possible that most of the ROS generated by mitochondria oxidizing complex I subunits are generated at site(s) other than complex I. In support of this hypothesis, ROS levels were unaltered in intact primary neurons from mice deficient in complex I activity due to the knockout of complex I subunit NDUFS4 (Choi et

al., 2008). ROS stimulation in response to rotenone was actually greater in NDUF54 knockout dopaminergic neurons compared to wild type, but this was due to an effect of rotenone on microtubule depolymerization rather than complex I since it was blocked by the microtubule stabilizing agent taxol (Choi et al., 2011). Considerable evidence links the common dihydrolipoamide dehydrogenase subunit of alpha-ketoglutarate dehydrogenase and pyruvate dehydrogenase to ROS production (Gazaryan et al., 2002, Starkov et al., 2004, Tretter and Adam-Vizi, 2004, Zundorf et al., 2009). This enzyme generates more ROS when there is a high NADH/NAD⁺ ratio, such as when complex I is inhibited by rotenone. Therefore, it is possible that some of the ROS originally attributed to complex I originate from dihydrolipoamide dehydrogenase or other sources. Parallel studies of energy utilization and ROS production by intact *harlequin* neurons would be expected to yield further insight into the roles of complex I and AIF in mitochondrial ROS production and/or elimination; however such studies have yet to be performed.

4. Why don't *harlequin* mice get Parkinson's disease?

Complex I impairment/deficiency is one of the prevailing hypotheses for the occurrence of idiopathic Parkinson's disease (Fiskum et al., 2003, Abou-Sleiman et al., 2006). Oxidative stress resulting from impaired complex I is thought to contribute to the pathology (Fiskum et al., 2003). Post-mortem samples from human Parkinson's disease patients revealed a complex I deficiency in the substantia nigra, the brain region that demonstrates selective degeneration in the disease (Mizuno et al., 1989, Schapira et al., 1990). Rotenone or 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) intoxication causes complex I inhibition and rapid development of Parkinsonian symptoms in animals, as well as selective degeneration of dopaminergic neurons in the substantia nigra (Betarbet et al., 2000, Panov et al., 2005, Burns et al., 1983). Consequently, AIF-depleted *harlequin* animals, which exhibit a 40–50% deficiency in brain complex I, would be expected to develop similar pathology. Nevertheless, 6–9 month old *harlequin* mice already displaying cerebellar and retinal degeneration do not display any deficits in the nigrostriatal dopaminergic pathway (Perier et al., 2010).

One possible explanation is that *harlequin* animals have acquired secondary changes in gene expression in the substantia nigra that compensate for the reduction in complex I. If so, study of *harlequin* mice could prove insightful for identifying pathways that protect against the development of Parkinson's disease. An alternative possibility is that complex I deficiency alone is insufficient to cause dopaminergic neurodegeneration but increases the susceptibility of the neurons to other impairments. Consistent with the possibility that complex I deficiency alone is insufficient to cause dopaminergic death, the survival of cultured dopaminergic neurons from NDUF54-deficient embryos lacking complex I activity was equivalent to wild type. Remarkably, these neurons were still selectively killed by the complex I inhibitors rotenone and 1-methyl-4-phenylpyridinium (MPP⁺, the active metabolite of MPTP), displaying actually increased sensitivity to rotenone (Choi et al., 2008). A follow up study identified rotenone-induced microtubule depolymerization and associated accumulation of cytosolic dopamine and ROS as an explanation for rotenone-induced toxicity to complex I-deficient dopaminergic neurons (Choi et al., 2011). Thus, although complex I deficiency alone did not cause the death of dopaminergic neurons, it sensitized them to death induced by rotenone. Interestingly, MPP⁺, in addition to inhibiting complex I, was also shown to destabilize microtubules (Cappelletti et al., 2005). Therefore, the combined actions of MPP⁺ and rotenone on complex I and microtubules appear to contribute to their extreme toxicity to dopaminergic neurons.

The absence of nigrostriatal pathology in AIF-deficient *harlequin* mice with diminished complex I adds to the evidence from NDUF54-deficient mice suggesting that impaired

complex I alone is insufficient to explain selective dopaminergic cell death in Parkinson's disease. Although displaying no basal nigrostriatal impairment, *harlequin* mice were more sensitive to dopaminergic neuron degeneration caused by MPTP intoxication (Perier et al., 2010), similar to the increased sensitivity of NDUFS4-deficient neurons to rotenone. The antioxidant tempol ameliorated MPTP toxicity in *harlequin* mice, implicating ROS as a causative factor in dopaminergic neurotoxicity. As further support for ROS as a primary mode of MPTP-induced death, the same group showed that the transgenic expression of mitochondrially-target catalase, an H₂O₂ detoxifying enzyme, preserved dopaminergic neurons in the substantia nigra of wild type mice (Perier et al., 2010). On the surface, these results would again suggest that AIF plays an antioxidant role. However, in this study the expression of *bona fide* antioxidant enzymes in *harlequin* substantia nigra at the time of MPTP administration was not examined. Notably, while most studies have found that several antioxidant enzymes such as MnSOD were unaltered in the *harlequin* brain (Zhu et al., 2007, Chinta et al., 2009), one study found that catalase was decreased by 30% at postnatal day 9 (Zhu et al., 2007). Because either no difference (Chinta et al., 2009) or only a very minor increase (Perier et al., 2010) in MPP⁺-induced ROS release from AIF- and complex I- deficient *harlequin* brain mitochondria was observed compared to controls, it seems unlikely that elimination of a direct AIF antioxidant role explains the increased sensitivity of *harlequin* mice to MPTP. We and others found that although complex I-linked ADP-stimulated respiration from isolated *harlequin* brain mitochondria was normal, O₂ consumption was more sensitive to rotenone or MPP⁺ inhibition (Chinta et al., 2009, Perier et al., 2010). The level of complex I inhibition in wild type and *harlequin* mice treated with MPTP was not examined. Therefore, it is possible that *harlequin* mice are more sensitive to MPTP simply because for a given MPTP concentration they display greater complex I inhibition.

How complex I inhibition causes or exacerbates Parkinsonian neurodegeneration remains an open question, especially in light of the NDUFS4 knockout findings. In further support of the hypothesis that complex I deficiency has a sensitizing effect on Parkinsonian pathology, partial inhibition of complex I activity (30%) similar to that found in Parkinson's disease was sufficient to significantly increase ROS in isolated synaptic nerve terminals (Sipos et al., 2003) and sensitize *in situ* synaptosomal mitochondria to H₂O₂-induced depolarization (Chinopoulos and Adam-Vizi, 2001). However, in support of a prominent causative role of impaired complex I in rotenone and MPTP neurotoxicity, introduction of rotenone- and MPP⁺-insensitive yeast alternative NADH-quinone oxidoreductase, Ndi1 provides potent neuroprotection (Richardson et al., 2007, Seo et al., 2006b, Barber-Singh et al., 2009, Marella et al., 2008). Ndi1 maintains high redox potential in the presence of complex I inhibitor and abrogates ROS production by rotenone (Marella et al., 2007, Seo et al., 2006a).

5. Loss of mitochondrial AIF vs. gain of nuclear AIF—which is the real culprit in acute neurodegeneration?

Although *harlequin* mice with a deficiency in AIF were sensitized to *in vivo* MPTP intoxication (Perier et al., 2010), a differentiated midbrain-derived dopaminergic MN9D cell line with stable AIF knockdown was resistant to MPP⁺ toxicity *in vitro* compared to control-transfected cells (Chu et al., 2005). This resistance was suggested to be due to interruption of an intricate caspase-independent pro-death pathway requiring AIF (Chu et al., 2005, Delavallee et al., 2011). As with caspase-dependent apoptosis, the initiating event in AIF-mediated death is permeabilization of the mitochondrial outer membrane (Moubarak et al., 2007). Calcium-dependent calpain protease then cleaves AIF, detaching the protein from the outer face of the inner membrane (Polster et al., 2005, Moubarak et al., 2007, Cao et al., 2007). Truncated AIF translocates to the nucleus, forms a complex with cyclophilin A and H2AX, and initiates large-scale DNA fragmentation (Artus et al., 2010). It is possible

that in cultured cells with low energy demand this pro-death pathway mediated by AIF determines the sensitivity to MPP⁺ while *in vivo*, the effect of AIF deficiency on complex I levels determines the outcome of MPP⁺-mediated injury.

What is the evidence that nuclear AIF is actually the primary cause of neuronal demise in caspase-independent cell death? Yu et al. first implicated AIF as a primary mediator of neuronal death caused by overstimulation of calcium permeable N-methyl D-aspartate (NMDA) glutamate receptors (Yu et al., 2002). Neurons could be rescued from excitotoxic cell death by delivering a neutralizing AIF antibody using a protein transfection reagent (Wang et al., 2004). Because an acute neutralization rather than a knockdown approach was used, this study argues that a gain-of-function following AIF release rather than a loss of normal mitochondrial function underlies the role of AIF in excitotoxicity. However, a major unanswered question is how neuronal cells can survive mitochondrial permeabilization, increased ROS production, and intracellular calcium deregulation despite attenuation of the nuclear function of AIF. In the AIF antibody neutralization experiment, cell death was scored morphologically by counting condensed nuclei (Wang et al., 2004). Therefore it is possible that changes in nuclear morphology were blocked by AIF inactivation without a long-term rescue of neuronal death. Alternatively, it is possible that interfering with AIF activity counteracts some of the other deleterious consequences of NMDA receptor overactivation such as mitochondrial compromise, calcium deregulation, and increased ROS production. AIF was shown to localize to the nucleus prior to cytochrome *c* release from mitochondria, consistent with the possibility of an early role in excitotoxicity before mitochondrial compromise is complete (Yu et al., 2002, Wang et al., 2004, Diwakarla et al., 2009). A third possibility is that neurons have the ability to recover from mitochondrial permeabilization if nuclear integrity is preserved. Several years ago it was shown that sympathetic neurons could recover from mitochondrial cytochrome *c* release following nerve growth factor withdrawal if caspase proteases were inhibited and nerve growth factor was subsequently re-added (Martinou et al., 1999). Mitochondria regained normal morphology and cytochrome *c* content through a process requiring protein synthesis. Real-time quantitative measurements of mitochondrial membrane potential changes, intracellular calcium deregulation, and ROS production in AIF-depleted neurons compared to wild type cells would help answer the mystery of how AIF-neutralized neurons survive NMDA-mediated toxicity.

To truly distinguish the relative contributions of loss of a pro-survival mitochondrial AIF function vs. gain of a pro-death nuclear function to neuronal cell death, it would be necessary to prevent mitochondrial AIF loss or restrict AIF nuclear entry without influencing other events in the cell. Cheung et al. designed a clever set of experiments to accomplish just that. To assess how loss of mitochondrial AIF influences DNA damage-induced cell death, they overexpressed membrane-anchored AIF mutants in wild type neurons and treated them with the DNA damaging agent camptothecin (Cheung et al., 2006). Translocation of wild type AIF to the nucleus was preserved, however high AIF levels in the mitochondria were maintained due to the retention of mutant AIF lacking the calpain cleavage site necessary for release. Preventing the depletion of mitochondrial AIF preserved mitochondrial membrane potential, inhibited cytochrome *c* release, and increased survival at 12 and 24 hours after camptothecin treatment despite the presence of wild type AIF (Cheung et al., 2006). A significant rescue of cellular oxygen consumption and total ATP was also observed. However, at 36 hours the survival of cells overexpressing mutant AIF was the same as wild type, indicating that the protection afforded by membrane-anchored AIF was not sustainable over the long-term.

To address whether nuclear AIF also contributes to DNA damage-induced death, the same group first cultured neurons from telencephalon-specific AIF knockout animals that were

also knockout out for the caspase-activating protein Apaf-1 (Cheung et al., 2006). As expected, these neurons were resistant to camptothecin because they lacked the machinery for both caspase-dependent (Apaf-1) and caspase-independent (AIF) programmed cell death. Transduction of wild type AIF restored cell death in response to the DNA damaging agent. In contrast, an AIF mutant engineered to contain a nuclear export sequence failed to increase death relative to control-transfected cells. This was likely because AIF with the nuclear export sequence failed to accumulate in the nucleus and initiate chromatin condensation and DNA fragmentation. However, cell death was scored by nuclear morphology, raising the possibility that nuclear AIF is indeed required for chromatin condensation and DNA fragmentation but is not required for the ultimate death of the cell.

Adding another chapter to the story of mitochondrial vs. nuclear AIF in shaping cell death, Öxler and colleagues recently provided evidence that protection mediated by AIF depletion is due to a preconditioning-like effect (Oxler et al., 2012). They examined the impact of siRNA-mediated AIF knockdown on oxidative stress induced by the treatment of immortalized HT-22 hippocampal cells with glutamate. Glutamate induces oxidative stress in these cells by inhibiting cystine transport, which causes depletion of the intracellular antioxidant glutathione due to impaired synthesis (Aminova et al., 2005). In contrast to AIF-deficient *harlequin* cerebellar granule neurons which were sensitized to glutamate-induced, oxidative stress-mediated cell death (Klein et al., 2002), HT-22 cells depleted of AIF were resistant to glutamate-induced injury (Oxler et al., 2012). Strikingly, AIF-depleted cells were less sensitive to glutamate-induced lipid peroxide accumulation, mitochondrial fission, mitochondrial depolarization, and ATP degradation (Oxler et al., 2012). The complex I subunit NDUFA 8 was greatly diminished by AIF knockdown. NDUFA 8 reduction could be mimicked in control HT-22 cells by an 18 hour incubation with a low dose of rotenone (20 nM) that was insufficient to cause ATP depletion or cell death. Like AIF knockdown, low-dose rotenone pre-incubation protected HT-22 cells from glutamate-induced cell death and the associated lipid peroxide accumulation, mitochondrial fission, mitochondrial depolarization, and loss of ATP. Consequently, it was suggested that moderate inhibition of complex I as a result of either AIF depletion or low-dose rotenone treatment pre-conditions cells upstream of mitochondrial damage to withstand oxidative stress-induced injury.

One caveat with this scenario is that the 18 hour low-dose rotenone treatment also led to a reduction in AIF protein levels (Oxler et al., 2012). The extent of AIF translocation to the nucleus was not quantified in AIF knockdown cells or rotenone pre-conditioned cells treated with glutamate. Therefore, the possibility that a decrease in nuclear AIF plays some role in the protection from injury cannot be entirely excluded. However, because AIF knockdown or rotenone pre-conditioning protected against the early stages of injury upstream of mitochondrial compromise, a scenario where AIF deficiency induces protective mitochondrial alterations is most likely. The reduction in lipid peroxides implicates improved handling of oxidative stress, which would be consistent with some but not all studies of AIF-deficient cells. Although there is ample evidence from the literature that AIF can cause apoptotic nuclear changes, the lesson from the Öxler study is that one should interpret results from AIF knockdown studies cautiously until cellular and mitochondrial changes secondary to AIF loss are fully characterized. Proteomics should help in this regard.

6. So what can we conclude about the role of AIF in mitochondrial ROS generation?

At this juncture, perhaps the only conclusion is that there is no real conclusion. Similar experiments by different groups have yielded vastly different results in different cells. We propose that a critical factor determining how AIF- and complex I-deficiency impact ROS production is cellular energy utilization. Interestingly, studies using the same model of

glutamate-induced oxidative stress yielded different conclusions in cerebellar granule neurons vs. immortalized HT-22 cells. Primary neurons are excitable cells which often experience high energy demand whereas immortalized cells in culture typically rely heavily on glycolysis for energy production and have substantial reserve respiratory capacity. It was in fact shown that cerebellar granule neurons use all of their spare respiratory capacity when exposed to glutamate due to the activation of ionotropic glutamate receptors and the consequent energy demand (Yadava and Nicholls, 2007). In the presence of glutamate, even mild complex I inhibition by rotenone potentiated cell injury (Yadava and Nicholls, 2007), similar to the potentiation due to combined AIF and complex I deficiency (Klein et al., 2002). This potentiation by rotenone was due to the bioenergetic consequences of complex I inhibition rather than oxidative stress (Yadava and Nicholls, 2007). We speculate that glutamate-treated HT-22 cells are able to meet cellular energy requirements using primarily glycolysis, therefore allowing the protective effects of AIF and complex I depletion to dominate rather than the negative consequences of decreased respiratory capacity. The protective effects may include an attenuation of mitochondrial ROS generation, although the jury is still out on how that could occur.

The only situation where AIF deficiency was found to have a dramatic effect on ROS emission by isolated mitochondria was when ROS from *harlequin* brain mitochondria oxidizing succinate was measured (Chinta et al., 2009). In that case, complex I-derived ROS was reduced to half of the wild type rate and residual ROS emission was inhibited by rotenone, as was the case for wild type mitochondria. Although the succinate concentration is normally very low in the brain, succinate increases dramatically following brain ischemia, with a concomitant decrease in complex I-linked substrates (Folbergrova et al., 1974, Benzi et al., 1979). Niatetskaya et al. recently demonstrated that the complex I inhibitor pyridaben is protective in a neonatal hypoxia-ischemia model (Niatetskaya et al., 2012). The investigators suggested that protection was due to inhibition of reverse electron transport-mediated ROS production by complex I. *Harlequin* mice with reduced complex I were also protected from neonatal hypoxia-ischemia (Zhu et al., 2007). However, the free radical scavenger edaravone protected the *harlequin* brain further and had a greater effect in the *harlequin* animals than in wild type mice (Zhu et al., 2007). This finding suggests but does not prove that protection due to AIF deficiency was unrelated to attenuation of ROS production. Evidence that reverse electron transfer-mediated ROS production can occur in living cells is scarce (Shabalina and Nedergaard, 2011), although a few *in vivo* studies using complex I inhibitors have supported this idea (Ambrosio et al., 1993, Hirata et al., 2011, Piantadosi and Zhang, 1996, Niatetskaya et al., 2012). Attenuation of complex I-mediated ROS production could potentially explain protection by either AIF knockdown or low-dose rotenone treatment against glutamate-mediated HT-22 cell toxicity.

In summary, further work is clearly needed to dissect out if/how AIF and complex I modulate mitochondrial ROS levels under normal conditions and following the onset of acute or chronic neurodegeneration. Although complex I deficiency has been thought by many to cause Parkinson's disease, evidence from the complex I- and AIF-depleted *harlequin* mouse, as well as from NDUFS4 knockout dopaminergic neurons lacking functional complex I, suggests that this may not be the case. Nevertheless, complex I deficiency sensitizes neurons and animals to Parkinsonian toxins and it is likely that impaired bioenergetics and oxidative stress both play a role. How AIF deficiency leads to a decrease in assembled complex I remains a mystery. Elucidation of the mitochondrial function of AIF should continue to garner much interest, especially following the recent discovery of encephalopathy caused by the first identified AIF mutation in humans (Ghezzi et al., 2010). Finally, in light of Öxler et al.'s recent findings of a preconditioning-like effect of AIF knockdown, the role of AIF nuclear translocation in acute neurodegeneration should also be re-visited. In particular, it will be interesting to explore if and how mitochondria are

spared when the nuclear function of AIF is blocked. Available AIF mutants of the calpain cleavage site, the FAD binding domain, or the DNA binding domain should ultimately help illuminate the multiple functions of this mysterious protein.

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Abbreviations

AIF	apoptosis inducing factor
DCFH-DA	dichlorodihydrofluorescein
MPP+	1-methyl-4-phenylpyridinium
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
NMDA	N-methyl D-aspartate
ROS	reactive oxygen species

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