Apoptosis, Necrosis and Much More – Is Oxidative Stress Getting on Your Nerves?

Gavin Higgins[1], Philip Beart[2] and Phillip Nagley[1]*

[1]Department of Biochemistry and Molecular Biology, Monash University, Clayton, VIC 3800
[2]Florey Neuroscience Institutes, University of Melbourne, Parkville, VIC 3010
**Corresponding author: phillip.nagley@monash.edu*

Oxidative stress plays a central role in neuronal injury and cell death in acute and chronic pathological conditions. The cellular responses to oxidative stress embrace changes in mitochondria and other organelles, including endoplasmic reticulum. These responses can lead to a number of cell death paradigms, which cover a spectrum from apoptosis to necrosis and include autophagy. In some neuropathologies, protein aggregation provides further cellular stresses that can initiate or feed into the pathways to cell death engendered by oxidative stress. In this article, we first interrelate oxidative stress, mitochondrial dysfunction and programmed cell death due to the damaging effects of reactive oxygen species (ROS). Second, we outline studies at the cellular level, which not only define cell death outcomes but also allow elucidation of molecular and cellular pathways whereby neurones respond to oxidative stress and undergo injury and death.

Relationships Between ROS Generation and Neuronal Injury

Neurones are considered highly susceptible to oxidative stress, as they are intrinsically ill-equipped to defend against an increase in ROS, due to low levels of antioxidants relative to those in other mammalian cell types (1). Glial cells, including astrocytes, play a supplementary role in antioxidant defence of neurones. Oxidative stress occurs chronically in many neurodegenerative disorders, such as Parkinson's disease and Alzheimer's disease, and in more acute settings, such as during ischaemia-reperfusion injury following the onset of stroke (2).

In addition to ROS outlined elsewhere in this Showcase on Research, both nitric oxide (NO) and peroxynitrite (ONO_2^-) are encompassed within a larger collective known as RONS (for reactive oxygen/nitrogen species). At moderate or 'physiological' levels, RONS are critical players in normal cellular behaviour when tightly regulated. When the levels of RONS are excessive in terms of normal cellular requirements, molecular damage and cellular debilitation may result. Moreover, a paucity of RONS can cause cellular defects, particularly where individual RONS are involved in cellular signalling (3).

Sources of RONS have been recognised as intracellular, with mitochondria as one of the main sources. However, mitochondria are not the only organelles generating free radicals; endoplasmic reticulum (ER) enzymes such as P450 can also generate ROS. Cells may also be exposed to external sources of RONS. These may be from direct exposure to excessive oxygen, as in ischaemia-reperfusion injuries, or to excessive neurotransmitters or oxidation products thereof. Yet another source of external ROS under neuropathological conditions are phagocytes that are the main source of oxidants *in vivo*. In the central nervous system, the microglia account for the largest population of such cells involved in neuroinflammatory responses.

Mitochondrial Dysfunction, Oxidative Stress and Programmed Cell Death

Neuronal cell death induced by oxidative stress was for a long time noted to be dichotomous: either necrosis or apoptosis. In contrast to the unregulated necrosis seen under acute and intense oxidative stress, apoptosis was observed to occur under relatively mild conditions, such as in the penumbra regions of the brain infarction following a stroke (i.e., an episode of hypoxia-ischaemia involving severe oxidative stress) (4). The involvement of caspases has been central to defining into which each of these two broad cell death pathways a dying neurone can be categorised. In this classical view of cell death, apoptosis is a regulated programmed cell death (PCD) pathway defined by the activation of caspases, while necrosis is unregulated and independent of caspase activity. This basic (two-pathway) view of cell death has been challenged by the discovery of alternative forms of caspase-independent PCD (5), such as autophagy and programmed necrosis, considered in more detail below.

Two significant factors that might influence the patterns of recruitment of mitochondrial signalling to affect the resultant characteristics of PCD induced by oxidative stress are mitochondrial membrane potential ($\Delta\Psi_m$) and cellular calcium (6). Further, there is growing awareness that dysfunction of the ubiquitin proteasome system (UPS) and its ability to handle misfolded proteins might also exacerbate oxidative stress (7). These misfolded proteins that are pathogenic include Aβ in Alzheimer's disease, α-synuclein in Parkinson's disease, huntingtin in Huntington's disease, and SOD1 and other proteins in motor neurone disease. The extent to which modulation of $\Delta\Psi_m$ is critical in neuronal injury depends on the overall state of energisation of the neurone (for example, this can be severely affected in the excitotoxicity process that follows hyperstimulation by excess neurotransmitters in a stroke injury). Thus, $\Delta\Psi_m$ can be influenced by dynamic fluxes in various intracellular calcium pools (8). Moreover, $\Delta\Psi_m$ is also affected by transient variations in the physical interface between the inner and outer mitochondrial membranes. Such changes in mitochondrial organisation and energisation (9) affect the propensity of intermembrane space (IMS) proteins to be released through the outer mitochondrial membrane, after its

permeabilisation in death signalling. The resultant mix of IMS proteins can determine the relative contributions of caspase-dependent and caspase-independent PCD (6). Further, alterations in the H^+ gradient associated with the electron transport chain impacts upon $\Delta\Psi_m$ and the consequent mitochondrial permeability transition, with a likelihood of increasing the probability that a neurone will undergo PCD (6). There is also evidence that in some protein misfolding diseases (e.g., Alzheimer's disease), ER and mitochondria interact, perhaps via calcium signalling, to modulate the final route to PCD [summarised in (2)].

Diverse Modes of Cell Death Under Oxidative Stress

The above considerations set the stage for an overarching view of how cells (and neurones in particular) respond to oxidative stress with the possible death outcomes embracing apoptosis (PCD-type I) on the one hand and unregulated (accidental) necrosis on the other, with various other forms of cell death (e.g., autophagic death [PCD-type II] and programmed necrosis [PCD-type III]) as alternative forms of PCD (6). Our conceptualisation of the cellular processes is presented in **Fig. 1**. This schematic emphasises the main players in the cellular response to oxidative stress, once antioxidant defences are overcome. As mentioned, the source of RONS can be either external or internal, generated from mitochondria (as shown in the figure – red arrows indicate some targets). In chronic neurodegenerative disorders such as Alzheimer's disease, Parkinson's disease or motor neurone disease, toxic proteins contribute to, and synergise with, the effects of the oxidative stressors. Toxic proteins compromise the biology of ER, UPS and mitochondria. The net effect of various factors including ER stress, toxic proteins and UPS dysfunction on downstream pathways is represented by the wide purple arrow in **Fig. 1**. These pathways can lead to outcomes occurring as various forms of cell death (set out in lower half of this schematic), which include the diverse types of PCD (the main three are indicated here), as well as unregulated necrosis under conditions that overwhelm the potential death programs. Current research, some of which is expanded on below, is concerned with identifying the signalling molecules that lead the cell to execute one or other of the PCD pathways (downward-pointing black arrows), as well as elucidating the cross-talk that occurs between these downstream pathways (represented, in general, by the horizontal double-headed purple arrows).

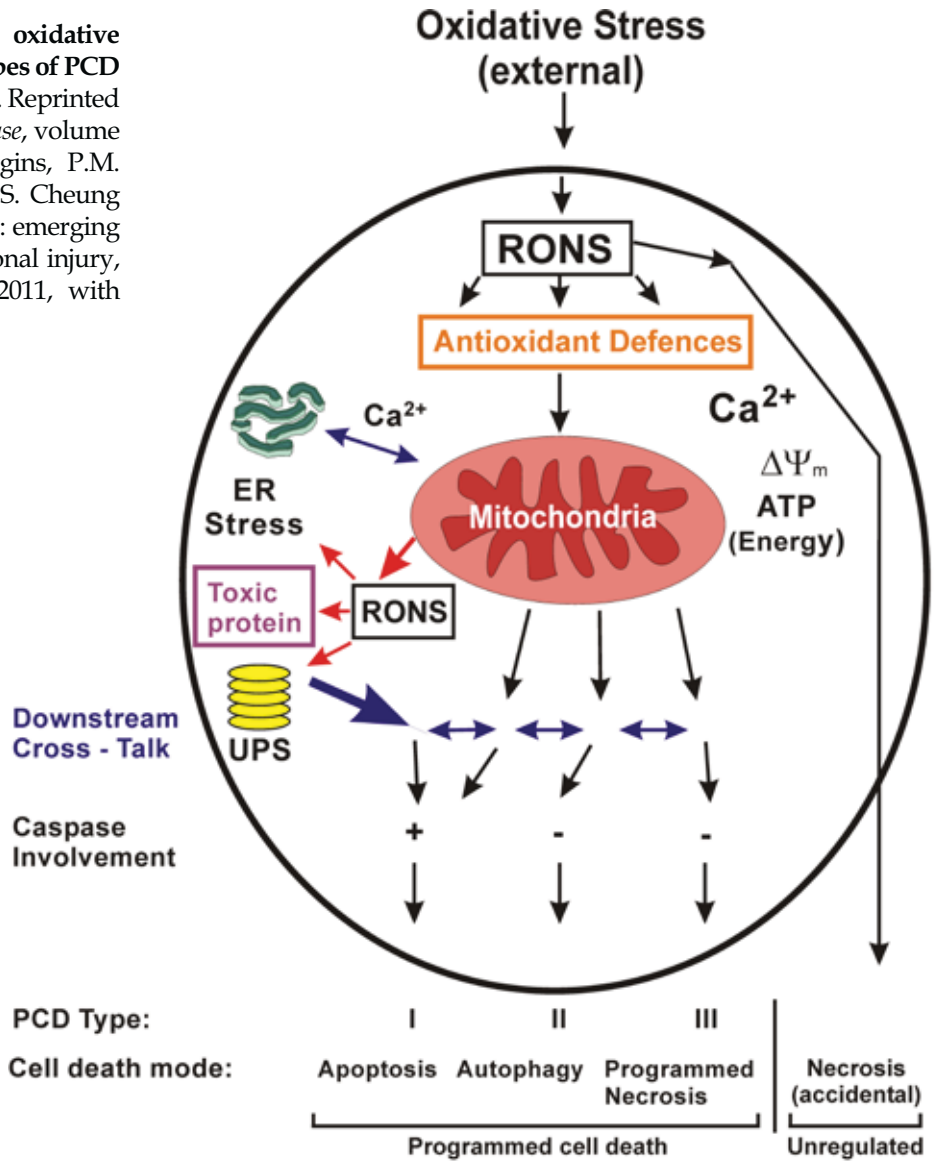

Fig. 1. Cellular responses to oxidative stress leading to the various types of PCD in neurones. See text for details. Reprinted from *Journal of Alzheimer's Disease*, volume 20 (Supplement 2), G.C. Higgins, P.M. Beart, Y.S. Shin, M.J. Chen, N.S. Cheung and P. Nagley, Oxidative stress: emerging themes and variations in neuronal injury, pages S453–S473, Copyright 2011, with permission from IOS Press.

Defining Alternative Death Modalities in Neurones Under Oxidative Stress

Recently, we undertook cell death studies using primary murine cortical neurones, which provide a powerful cellular neuronal model for studying death patterns in response to various oxidative stressors. We chose two distinct oxidative stress-inducing conditions to establish whether the cell death outcomes were unique or common to various forms of oxidative stress. Neurones were treated with either a bolus treatment of hydrogen peroxide (H_2O_2) (10,11) or a continuous source of superoxide ($O_2^{\bullet-}$) generated by xanthine plus xanthine oxidase in the presence of catalase to mop up H_2O_2 (G. Higgins, unpublished observations). The characterisation of caspase-independent PCD under oxidative stress in these neurones was clarified by using a reference inducer of apoptosis, namely staurosporine (STS). Thus, apoptosis involving the mitochondrial pathway can be invoked by STS in primary neurones (10); hallmarks are an early hyperpolarisation of mitochondria ($\Delta\Psi_m$ increases within 1 hour), followed by relatively late (about 24 hours) redistribution to cytosol of IMS proteins including cytochrome *c* (cyt c) and endonuclease G (Endo G). Downstream caspases, including caspase-9 associated with the intrinsic (mitochondrial) pathway of apoptosis and effector caspase-3, are activated in these neurones. The end point of apoptosis execution is determined by changes in nuclear morphology, including condensation and marked fragmentation, accompanied by generation of nuclear DNA breaks. Loss of plasma membrane integrity, measured by propidium iodide uptake into cells, is observed late (about 24 hours) after STS treatment, typical of apoptotic cell death (10).

The progression towards cell death initiated by H_2O_2 is in distinct contrast to that in STS-treated cells, both morphologically and mechanistically (10). Neurones treated with H_2O_2 undergo rapid loss of plasma membrane integrity (within 4 hours), with similar timing for nuclear condensation and DNA fragmentation. Changes in nuclear morphology generally display a marked condensation but with little fragmentation, in contrast to nuclei of STS-treated cells. At the mitochondrial level, there is a rapid loss of $\Delta\Psi_m$ (within 1 hour). This depolarisation is followed soon after by redistribution to cytosol of IMS proteins, including cyt c and Endo G. Neurones treated with H_2O_2 failed to activate caspase-9 or caspase-3 (10,11). Collectively, these cellular characteristics suggested a necrotic cell death. However, this turned out not to be a catastrophic unregulated necrosis but, in fact, has significant features of two sorts of programmed cell death, PCD-type II and PCD-type III (**Fig. 2**).

The definition of autophagic cell death (PCD-type II) requires not only the manifestation of autophagic events but also the demonstration that blockade of autophagy protects cells against the death outcome (12); see also (13) and other articles in *Australian Biochemist* Showcase on Autophagy (August 2011) for details of current autophagy research themes. In the cultured primary murine neurones, stimulation of autophagic activity occurred in the presence of both STS and H_2O_2 (11). Treated cells displayed increases in autophagic puncta (monitored by confocal microscopy using GFP linked to microtubule-associated protein light chain 3 [GFP-LC3]), as well as increased conversion of LC3-I to LC3-II (see (14) for autophagy methods). Whilst the autophagic inhibitor 3-methyladenine blocked these indicators of autophagy in both cases, autophagic cell death, as such, only occurred in H_2O_2-treated neurones. Thus, 3-methyladenine blocked cell death in H_2O_2-treated cells but not neurones treated with STS. More significantly, suppression of an essential autophagy gene *Atg7* using siRNA blocked cell death in H_2O_2-treated neurones (**Fig. 2F**) but not those treated with STS. These findings (11) provide clear indication of autophagic cell death (PCD-type II) in neurones subjected to oxidative stress.

The confirmation of programmed necrosis (PCD-type III) depends on identifying a regulated aspect of necrosis-like death outcomes to distinguish this from unregulated (or accidental) necrosis (5). In H_2O_2-treated neurones, suppression by siRNA of Endo G (a mitochondrial protein whose translocation to the nucleus engenders DNA breakdown independent of caspase activation) resulted in inhibition of cell death (**Fig. 2G**) monitored by propidium iodide uptake (10). By contrast, STS-treated neurones knocked down for EndoG still died, albeit less rapidly. Under H_2O_2, Endo G released from mitochondria acts in a caspase-independent manner to elicit changes in the nucleus that are associated with cell death (10), defined as programmed necrosis (PCD-type III).

The findings outlined above suggest the concurrent induction of both PCD-type II and PCD-type III in neurones exposed to H_2O_2. Interestingly, superoxide treatment of neurones superficially indicated concurrent PCD-type II and PCD-type III cell death responses devoid of caspase activity. Superoxide induced changes in mitochondria and nuclear morphology that were characteristic of necrosis, as with H_2O_2. Whilst indicators of both PCD-type II and PCD-type III in the cells were blocked (after 4 hours of exposure to superoxide) by suppression with siRNA of Atg7 and Endo G, respectively, cell death could not be stopped at later time points by those knockdown strategies. This evidence suggests that the continuous attack on the neurones by superoxide drives the cells ultimately to unregulated necrosis (G. Higgins, unpublished data) (**Fig. 2**).

Conclusions

These outcomes bring to light the importance of understanding the various forms of PCD invoked by oxidative stress, as well as the ultimate progression to unregulated necrosis under sustained stress. Yet the overall landscape of neuronal PCD remains an area for fruitful investigation. Other workers have reported on further sub-types of programmed necrosis, such as necroptosis (requiring RIP-1) (15) and parthanatos (requiring PARP-1) (16), which may fit within the cell death framework defined above. Further, the mechanisms that drive autophagic cell death and what regulates autophagic activity to switch from 'cell maintenance' to 'cell death' mode remain to be elucidated (13). Critical issues pertaining to the outcomes discussed here include why ROS initiate caspase-independent neuronal cell death over apoptosis, how cells 'choose' invocation of diverse modalities of cell death under ROS, what proteases are recruited, and

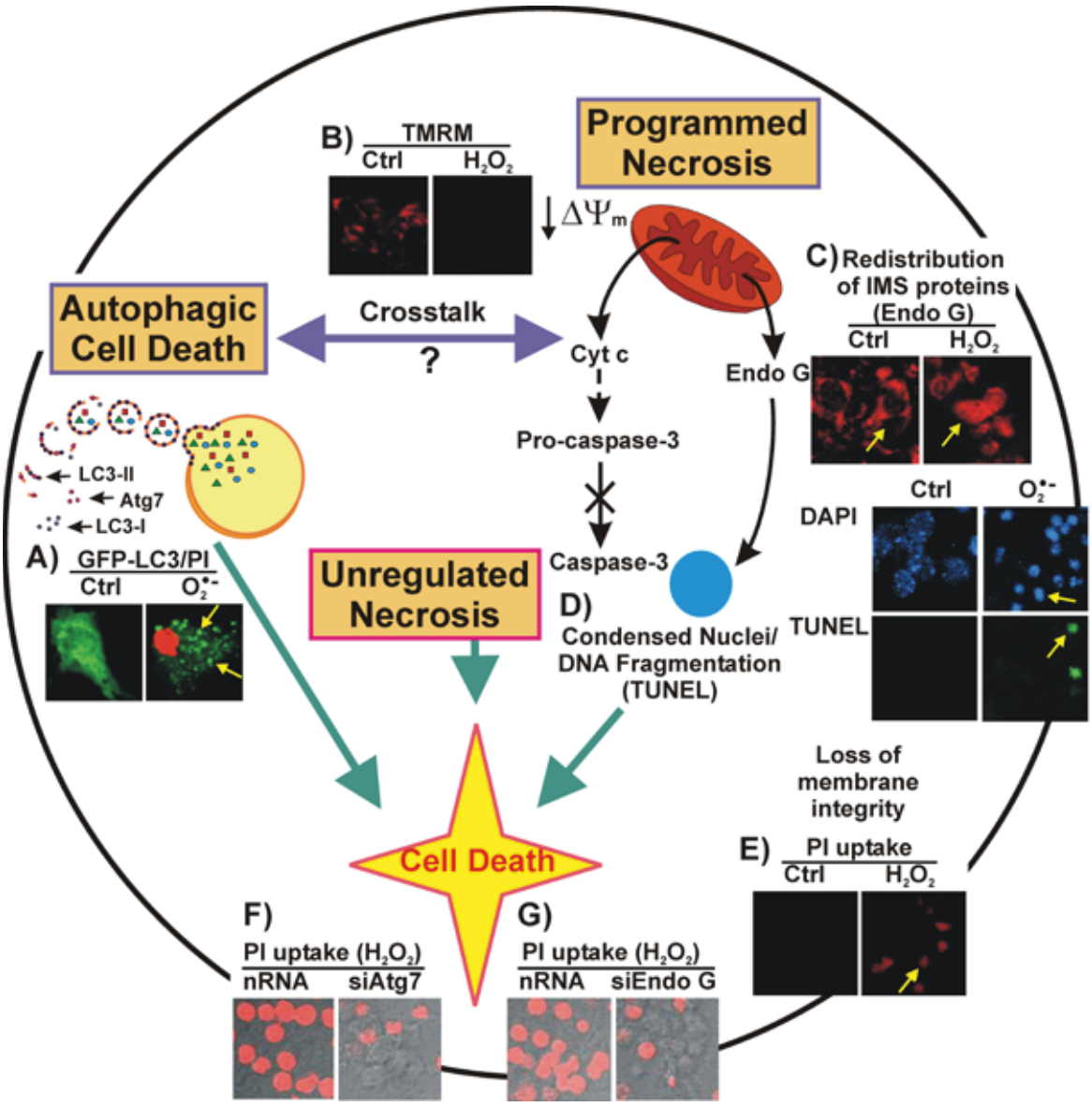

Fig. 2. Diverse neuronal cell death responses to oxidative stress with illustrative data. Autophagic cell death events: Upon oxidative stress insult, neurones undergo autophagy by initiating formation of autophagosomes that engulf cellular proteins and organelles within the cytosol. The formation of the isolation membrane of autophagosomes is dependent on Atg7 (pink dots). LC3-I (light purple dots) undergoes conformational modification to become LC3-II when bound to the isolation membrane. LC3-II remains attached to the autophagosome until fusion with the lysosome. **A)** LC3/PI: Confocal images show a viable neurone, control (Ctrl) displaying diffuse GFP-LC3 throughout the cytosol and no propidium iodide (PI) uptake. By contrast, the superoxide ($O_2^{\bullet-}$)-treated neurone displays puncta (indicated by yellow arrows; autophagosomes) formed by GFP-LC3 and shows PI uptake (red) into the nucleus, indicative of cell death. **Programmed necrosis events:** Neurones under oxidative stress undergo programmed necrosis via mitochondria. **B)** Mitochondria initially undergo depolarisation under oxidative stress, as reported by the membrane potential reporter, TMRM, which is no longer retained in mitochondria of H_2O_2-treated cells. **C)** Mitochondria redistribute intermembrane space (IMS) proteins including cytochrome *c* (cyt c) and endonuclease G (Endo G) (shown in confocal images). The yellow arrow in control image indicates a viable cell with Endo G present within mitochondria. The yellow arrow in H_2O_2-treated cells indicates a neurone with Endo G redistributed across the cell. **Common cell death features: D)** Nuclear changes under superoxide. Yellow arrows point to condensed nuclei (reported by DAPI, blue) and in some cases DNA fragmentation (TUNEL, green). **E)** Loss of plasma membrane integrity, as indicated by PI uptake. Yellow arrow indicates a PI-positive neurone. **Inhibition of cell death under oxidative stress: F)** Knockdown of Atg7 (siAtg7) and **G)** knockdown of Endo G (siEndo G) both block cell death relative to non-silencing control (nRNA), as measured by PI uptake. Note that all cells in these fields are visualised by differential interference contrast microscopy. In neurones under oxidative stress, caspases such as caspase-3 are not activated (illustrative data not shown here). Under prolonged oxidative stress achieved by continuous generation of superoxide, where both autophagic cell death and programmed necrosis mechanisms are overwhelmed, unregulated necrosis eventually ensues.

how these pathways interact (elucidating these issues in the framework of **Fig. 1** represents a current major challenge). For example, candidate 'higher-order regulators', such Beclin 1 (for apoptosis/autophagy) (17) and p62 (for autophagy/UPS) (18), are under current scrutiny in several laboratories internationally. Investigation of these issues raises the potential to arrest cell death induced by ROS in various neurodegenerative conditions. Hence, if oxidative stress can be minimised to an extent to prevent onset of unregulated necrosis and, further, to allow opportunity for the various forms of PCD to be modulated therapeutically, neurones and the functions they control may be better preserved in the face of neurodegenerative injury.

References

1. Cooper, A.J.L. (1997) in *The Molecular and Genetic Basis of Neurological Disease* (Rosenberg, R.N., Prusiner, S.B., Di Mauro, S., Barchi, R.L., and Kunk, L.M., eds), 2nd edition, Butterworth-Heinemann, UK
2. Higgins, G.C., Beart, P.M., Shin, Y.S., Chen, M.J., Cheung, N.S., and Nagley, P. (2010) *J. Alzheimers Dis.* **20 Suppl 2**, S453-473
3. Finkel, T., and Holbrook, N.J. (2000) Nature **408**, 239-247
4. Dirnagl, U., Iadecola, C., and Moskowitz, M.A. (1999) *Trends Neurosci.* **22**, 391-397
5. Kroemer, G., Galluzzi, L., Vandenabeele, P., Abrams, J., Alnemri, E.S., Baehrecke, E.H., Blagosklonny, M.V., El-Deiry, W.S., Golstein, P., Green, D.R., Hengartner, M., Knight, R.A., Kumar, S., Lipton, S.A., Malorni, W., Nunez, G., Peter, M.E., Tschopp, J., Yuan, J., Piacentini, M., Zhivotovsky, B., and Melino, G. (2009) *Cell Death Diff.* **16**, 3-11
6. Nagley, P., Higgins, G.C., Atkin, J.D., and Beart, P.M. (2010) *Biochim. Biophys. Acta* **1802**, 167-185
7. Hyun, D.H., Gray, D.A., Halliwell, B., and Jenner, P. (2004) *J. Neurochem.* **90**, 422-430
8. Kroemer, G., Galluzzi, L., and Brenner, C. (2007) *Physiol. Rev.* **87**, 99-163
9. Smith, D.J., Ng, H., Kluck, R.M., and Nagley, P. (2008) *IUBMB Life* **60**, 383-389
10. Higgins, G.C., Beart, P.M., and Nagley, P. (2009) *Cell. Mol. Life Sci.* **66**, 2773-2787
11. Higgins, G.C., Devenish, R.J., Beart, P.M., and Nagley, P. (2011) *Cell. Mol. Life Sci.* **68**, 3725-3740
12. Kroemer, G., and Levine, B. (2008) *Nat. Rev. Mol. Cell Biol.* **9**, 1004-1010
13. Xia, P. (2011) *Australian Biochemist* **42 (August)**, 14-16
14. Mizushima, N., Yoshimori, T., and Levine, B. (2010) *Cell* **140**, 313-326
15. Degterev, A., Huang, Z., Boyce, M., Li, Y., Jagtap, P., Mizushima, N., Cuny, G.D., Mitchison, T.J., Moskowitz, M.A., and Yuan, J. (2005) *Nat. Chem. Biol.* **1**, 112-119
16. Wang, Y., Dawson, V.L., and Dawson, T.M. (2009) *Exp. Neurol.* **218**, 193-202
17. Martin, S.J. (2011) *Autophagy* **7**, 922-923
18. Johansen, T., and Lamark, T. (2011) *Autophagy* **7**, 279-296