Mitochondria and Ageing

Jose Viña and Consuelo Borras

Abstract Mitochondria are the major sites of oxygen utilisation for energy production in cells. Indeed, all the reactions of the Krebs' Cycle take place in mitochondria and they produce NADH and succinate, which are then oxidised in the respiratory chain. Experiments dating back to the early part of the twentieth century seemed to indicate that at a high rate of oxygen consumption (referred to gram of body weight) was normally associated with a low maximum lifespan. Thus, it was thought that it was the rate of oxygen utilisation that was related to "the rate of living". However, more recent data pointed out that birds are unique because they combine high rates of oxygen consumption with a high maximum lifespan. It would later be pointed out that the maximal lifespan is more correlated with the rate of free radical production by mitochondria rather than the rate of oxygen utilisation. These experiments were performed under the general scheme of the free radical theory of ageing. Still, more than 300 theories have been postulated to explain ageing and this can indicate that none of them is completely satisfactory to explain a complex phenomenon such as ageing. We postulate in this chapter that the free radical theory of ageing could be revisited and that it is the age-associated derangement of the free radical signalling network that is central to understand ageing.

Keywords Free radicals · Oxidants · Longevity · Antioxidants · Frailty

1 The Free Radical Theory of Ageing. The Mitochondrial Free Radical Theory of Ageing as Proposed by Miquel

Mitochondria are the major sites of oxygen utilisation for energy production in cells. Indeed, all the reactions of the Krebs' Cycle take place in mitochondria and they produce NADH and succinate, which are then oxidised via complexes 1 and 2 of the

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respiratory chain. Experiments dating back to the early part of the twentieth century seemed to indicate that at a high rate of oxygen consumption (referred to gram of body weight) was normally associated with a low maximum lifespan. Thus, it was thought that it was the rate of oxygen utilisation that was related to "the rate of living" (Pearl 1928). However, Barja and co-workers (Barja de Quiroga 1999) pointed out that birds are unique because they combine high rates of oxygen consumption with a high maximum lifespan. It would later be pointed out that the maximal lifespan is more correlated with the rate of free radical production by mitochondria rather than the rate of oxygen utilisation. These experiments were performed under the general scheme of the free radical theory of ageing.

This theory was first postulated by Denham Harman who proposed that "ageing and the degenerative diseases associated with it could be attributed basically to the deleterious side attacks of free radicals on cell constituents and on the connective tissues" (Harman 1956). An important antecedent of this critical paper was that of Gershman and co-workers who proposed that oxygen poisoning and X irradiation have a mechanism in common (Gershman et al. 1954). They are both caused by deleterious effects of free radicals. Therefore, back in the 1950s, the essential bases for the free radical theory of ageing were proposed.

Importantly, Harman himself already mentioned that mitochondria are essential to his free radical theory of ageing (Harman 1972). However, it was Jaime Miquel in 1980 who clearly formulated the mitochondrial free radical theory of ageing (Miquel et al. 1980). What Miquel stated was that mitochondria are the origins of a substantial part of the free radical production by cells and that because the close vicinity of mitochondrial DNA and the sites of radical productions, mitochondrial DNA should be considered as an essential target of the free-radical-induced damage to cells.

It is outside the scope of this review to dwell on the number of theories that have tried to explain ageing; the reader is referred to a review on this topic by the authors of this paper in IUBMB Life (Vina et al. 2007). Suffice it to say that more than 300 theories have been postulated to explain ageing and this can indicate that none of them is completely satisfactory to explain a complex phenomenon such as ageing. We postulated that the free radical theory of ageing could be revisited and that it is the age-associated derangement of the free radical signalling network that is central to understand ageing (Vina et al. 2013). However, mitochondria are central to most of theories proposed for ageing that have high explanatory power.

2 Mitochondrial Disruption of Cell Signalling in Ageing

Mitochondria were originally thought of as organelles that produce energy and they were also considered as a source of free radicals that would literally cause damage to cells. Around the turn of the century, however, the idea that free radicals had a physiological function and were not just there to damage cells began to take shape. A critical paper by Wolf Droge published in physiological reviews in 2002 summarised the knowledge in this field at the time (Droge 2002). What Droge pointed out was that

"at moderate concentrations" nitric oxide superoxide anion and other reactive oxygen species were not causative of damage, but would play an important role as regulatory mediators in signalling processes. This author went on to say that "many of the ROS mediated responses actually protect cells against oxidative stress and re-establish redox homeostasis" (Droge 2002). Therefore, the concept of oxidative stress as first pointed out by Helmut Sies was then extended to the concept of redox homeostasis. Another paper published in 2004 by Enrique Cadenas had the title "Mitochondrial function in ageing: coordination with signalling and transcriptional pathways" (Yin et al. 2004). The title itself underpinned the importance of mitochondria in generating signals that are free radicals in chemical nature, but that are essential for normal cell functioning. It is not the aim of this chapter to review the role of free radicals produced by mitochondria as signalling molecules. But what we want to point out is that in ageing, the whole network of the free radical based cell signalling pathways is deranged.

In the last 15 years, the very concept of the free radical theory of ageing as postulated by Harman has been challenged. On some occasions, ageing has been associated not with an increased oxidative damage, but with a reductive damage. In our opinion, many of the criticisms of the free radical theory of ageing are quite correct in that if one considers the strict concept of free radicals "causing damage" to cells. We have proposed the cell signalling disruption theory of ageing. The major postulate of this theory is that, as stated before, reactive oxygen species generated by mitochondria are responsible for an altered cell signalling network. It is not just molecular damage to structures like double bonds that is responsible for the altered cell function in ageing tissues, but the altered signalling causing a cascade of event leading to disrupted function in old animals and even persons (Vina et al. 2013).

3 Mitochondrial DNA Is More Susceptible to Damage than Nuclear DNA

One critical aspect of the free radical-associated damage was put forward by Britton Chance and his colleagues who proposed the idea that because radicals are so reactive they would do much of the damage at sites near their production (Boveris and Chance 1973). It was therefore important to try and show which the major sites of free radical production in ageing were.

DNA damage has been observed in a large number of cell lines from mammals exposed to oxidative stress (Halliwell and Auroma 1991). This damage includes double and single chain breaks, deletions, base changes, oxidative damage, and even chromosome aberrations. The main molecular mechanisms involved are the direct reaction of hydroxyl radicals and carbonyl compounds with DNA and the activation of nucleases (Halliwell and Auroma 1991). The superoxide anion and H_2O_2 do not react with DNA unless there are transition metal ions that allow the formation of hydroxyl radicals. Hydroxyl radicals are capable of attacking the deoxyribose, purines and pyrimidines, generating numerous products, such as 8-hydroxydesoxyguanosine (8-oxodG), thymidine glycols and 8-hydroxyadenosine (Halliwell and Auroma 1991).

Bruce Ames and co-workers calculated that reactive oxygen species modify approximately 10,000 bases of DNA per cell (Ames et al. 1993). DNA repairing enzymes are able to repair the vast majority of these lesions, but not all. Therefore, DNA lesions that go un-repaired, such as 8-oxo-dG accumulate with age.

As stated before, according to the mitochondrial theory of ageing, mitochondria are the main source of free radical production. This implies that they are also the main target of free radicals, as they are very unstable molecules (Miquel et al. 1992). Therefore, it is well known that mitochondrial DNA is much more oxidised with age than nuclear DNA (Richter et al. 1988). Our group, in 1996, showed that oxidative damage to mitochondrial DNA correlates with oxidation of mitochondrial glutathione (García de la Asunción et al. 1996).

Moreover, mitochondrial DNA (mtDNA) is especially susceptible to oxidative damage and mutations because it lacks protective histones (Johns 1995). Thus, the formation of 8-oxoDG in mitochondrial DNA increases as the rate of hydroperoxide production increases by mitochondria (Giulivi et al. 1995). Suter and Richter have reported that oxidized bases are present in moderate amounts in 16.3 kb mitochondrial DNA molecules but are found in large numbers in mitochondrial DNA fragments (Suter and Richter 1999). These results, together with the discovery of endonucleases related to mitochondrial oxidative damage, demonstrate the existence of a mitochondrial DNA repair system (Suter and Richter 1999; Shen et al. 1995; Croteau et al. 1999).

According to the mitochondrial aging theory, Barja and Herrero found that the oxidative damage associated with mtDNA is inversely related to the maximal survival of mammals, while oxidative damage to nuclear DNA is not (Barja and Herrero 2000). In addition, several studies have reported that levels of oxidative damage to mtDNA are several times higher than those produced in nuclear DNA, and that mutations in mtDNA are also more frequent than in nuclear DNA (Richter et al. 1988; Shigenaga et al. 1994; Suter and Richter 1999; Barja and Herrero 2000). However, Anson et al. pointed out that when oxidative damage to mtDNA purified from isolated mitochondria is measured, it is observed that it has been over-estimated (Anson et al. 2000). In fact, they observed similar levels of 8-oxodG in nuclear and mitochondrial DNA. Our group published a method of isolation of mtDNA that does not require previous isolation of mitochondria (Asunción et al. 1996). Using this method we obtained levels of 8-oxodG three to nine times higher in mtDNA than nuclear DNA in the eight species studied (Sastre et al. 1998). Nevertheless, these results should be confirmed using different methods of isolation of mtDNA.

Oxidative lesions to mtDNA accumulate with age in human and rodent tissues. (Halliwell and Auroma 1991; Asunción et al. 1996; Ames et al. 1993).

Two characteristics were thought to occur that supported this conclusion. The first is that it was believed that mitochondrial DNA is much less protected against free radical attack than nuclear DNA because of a lower number of histones and other DNA-associated proteins. This is still thought to be the case. On the other hand, it was also thought that mitochondrial DNA does not have repair mechanisms and therefore the damage could be less reversible than nuclear DNA. This is not thought to be the case anymore. We know now that mitochondrial DNA contains repair mechanisms to counteract oxidative damage. In any case, it still holds true that oxidative damage to

mitochondrial DNA is (at least as determined by measuring 8-hydroxy-2-deoxy-guanosine) ten times higher than nuclear DNA damage.

The mtDNA repair system is unable to counteract the amount of ROS generated in mitochondria throughout life. Point mutations and deletions in mtDNA are produced in the tissues of old animals (Gadaleta et al. 1992; Lezza et al. 1994; Lee et al. 1997). In humans the deletions in mtDNA increase more than 10,000 times with age (Lezza et al. 1999). According to the Miquel's hypothesis, the highest percentage of mtDNA deletion is observed at the end of life in postmitotic tissues such as brain, heart and muscle (Lezza et al. 1999). Point mutations and aberrant forms of mtDNA from postmitotic cells are also related to degenerative diseases associated with aging.

Age-associated mtDNA deletions display mosaic distribution. This supposes a localized distribution of the deletions even in the same tissue, so that some cells possess a greater percentage of deletions than others. Thus, a difference of two or three orders of magnitude for the deletion at the 4977 bp level—the most common deletion—is observed in different regions of the brain (Corral-Debrinski et al. 1992; Cortopassi et al. 1992).

Damage to mtDNA may affect mitochondrial gene transcription (Kristal et al. 1994). In fact, it has been reported that there is a decline associated with age of mitochondrial transcript levels in some rat tissues and in Drosophila (Gadaleta et al. 1990; Calleja et al. 1993). In addition, since mtDNA lacks introns, any mutation will affect DNA coding sequences (Johns 1995). Thus, Lezza et al. observed a correlation between the decrease in oxidative phosphorylation capacity and the increase in the percentage of current deletions in mtDNA during aging (Lezza et al. 1994). Therefore, it has been suggested that mutations in mitochondrial DNA may contribute significantly to the aging process and to the development of neurodevelopmental diseases.

Furthermore, we have more recently proposed that there is also a relationship between mitochondrial and nuclear DNA damage. We found that mtDNA fragments are inserted into nuclear DNA contributing to aging and related diseases by alterations in the nucleus (Caro et al. 2010). Consequently, mitochondria can be a major trigger of aging but the final target could also be the nucleus.

4 Mitochondria Are Damaged Inside Cells

We provided the first evidence that mitochondria are damaged inside cells (see Fig. 1) (Sastre et al. 1996). The question in the early '90s was whether mitochondria were damaged when isolated from old tissues because they were more fragile and therefore they were damaged in the isolation procedures or whether they were already damaged inside cells. By using metabolic as well as flow cytometry studies we demonstrated that mitochondria were damaged inside cells and not during isolation. The metabolic approach consisted of checking that those metabolic pathways that involved mitochondria were more affected in aged tissues than those that involved only extra mitochondrial compartments. For instance, the rate of gluconeogenesis from lactate

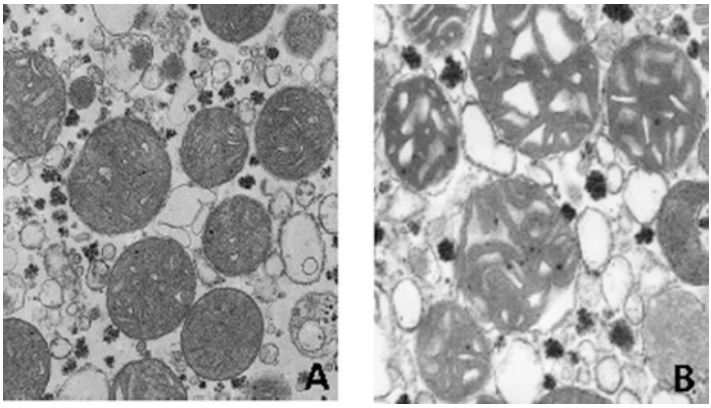

Fig. 1 Electron microscopy images of young (**a**) and old (**b**) mitochondria

and pyruvate was much more affected than that from glycerol, the first involving mitochondria and the second not (Sastre et al. 1996).

Our findings were independently confirmed almost simultaneously by the group of Bruce Ames who showed the mitochondrial decay in ageing and postulated that this was majr characteristic of the ageing process (Ames et al. 1995, Hagen et al. 1997).

Once it was clear that free radicals produced by mitochondria were responsible for much of the damage that was associated with ageing, a number of laboratories set out to test with newer techniques the old idea that the rate of respiration was responsible for "the rate of ageing". Prominent among these laboratories was that of Gustavo Barja in Madrid. Barja et al. compared the rate of oxygen consumption in tissues, for instance muscle from pigeons and rats, two species whose individuals are approximately the same body weight (Barja 1998; Barja de Quiroga 1999). There was a very significant difference between the longevity of the maximal longevity in these two species: rats live up to 3 or 4 years whereas pigeons can live more than 25. What Barja and his colleagues observed was that longevity did not correlate with the oxygen consumption by mitochondria but with the rate of free radical production by these organelles (Barja et al. 1994). These results have strong correlative evidence in favour of the free radical theory of ageing. These experiments have been confirmed in many laboratories and some of the criticisms of the free radical theory of ageing are based on assumptions that are not strictly based on the idea that the rate of production of radicals is what determines the rate of ageing.

Damage to DNA as well as to other critical components of the mitochondria like lipids, results in two important pathophysiological facts. The first is that since mitochondria are damaged, this may explain in a large part the energy collapse that occurs with ageing. The second conclusion is that oxidant production by mitochondria from old animals will be increased when compared with that of young ones. The old idea that "2% of all oxygen consumption in organisms is converted to free radicals rather than to water and energy" is not true. It may serve as an "average value", but the fact is that when electrons flow through the respiratory chain and

active oxidative phosphorylation takes place, the rate of radical production in mitochondria can be as low as 10% that of damaged mitochondria when energy production is far less. This idea which is based on clear chemical considerations has far-reaching biological conclusions. For instance, the old idea that in exercised muscle, more free radicals are produced because the cells consume more oxygen is wrong. When an active respiratory chain and oxidative phosphorylation takes place, fewer radicals are produced. The inverse can be said of ageing mitochondria. When mitochondria from old animals use oxygen less efficiently, more radicals are produced. Therefore, the "inefficient work" of mitochondria from old animals explains both the energy collapse that occurs in aged cells and the increased production of oxygen radicals that not only alter the cell signalling network but also cause damage to susceptible molecules like the double bonds in unsaturated fatty acids or the deoxyguanosine residues in mitochondrial DNA.

5 Sex Differences in Free Radical Production and Its Relationship with Longevity

Differences in longevity between sexes offer interesting possibilities to understand ageing. Animals with a very similar genetic background may differ in their average life span for a much as 10%. Of paramount importance is the fact that these differences is also occur in humans. However, in order to find proof that this is not due to sociological changes or peculiarities between societies (i.e. whether women smoke more of less than men etc.) one must understand the differences in longevity in animal species.

We and others tried to explain why Wistar rat females live longer than males, the maximal life span being approximately 10% (Borras et al. 2003). In a similar fashion, using Fisher 344 rats, i.e. the same species but a different strain, in which longevity is also higher in females than in males, the group of Leeuwenburgh observed that males produce more reactive oxygen species than females (Jang et al. 2004). However, the higher longevity of females as compared with males is not a universal phenomenon. In other species of rodents such as mice, some strains, for instance the C57BL6, show a higher longevity of males when compared with females (Ali et al. 2006). Moreover, a variation of this strain which was used by Leeuwenburgh's group, i.e. C57B16J mice show no differences in longevity between sexes (Sanz et al. 2007). In contrast, the Swiss albino mouse shows an increased longevity in females when compared with males (Navarro et al. 2004). So not only do we observe different sex specific longevity in different species, but also in different strains of the same species. This offers a unique opportunity to study comparative ageing, i.e. whether there are hormonal differences, different sensitivity or reactivity to hormones or different fundamental molecular mechanisms of ageing. Since we are dealing with the same species, only different sexes, we are faced with optimal animal models to understand fundamental gerontological aspects as well as the hormonal regulation of ageing (Borras et al. 2003; Ali et al. 2006; Sanz et al. 2007)

In many species in which females live longer than males, the former produce approximately half the amount of mitochondrial peroxide than the latter (Borras et al. 2003).

In early 2000s, we measured peroxide production by mitochondria from female and from male Wistar rats and found that females produce approximately half the amount of peroxides than males. This was completely reversed when rats were ovariectomised, thus tracing the differential gender effect on radical production to ovarian hormones. We then measured the free radical production of mitochondria from ovariectomised females which had been treated with estrogens. In this case, estradiol was able to reverse the effect of ovariectomy and the rate of peroxide production was similar to that of females. The decrease in radical production resulted in a significantly lower damage to mitochondrial DNA in females than in males. In fact, the level of 8-oxo-deoxyguanosine was up to four-fold higher in males than in females (Borras et al. 2003).

However, Ali et al. showed that in those strains of mice like the C57BL6 they were using in which males live longer than females it is males that produce fewer oxidants than females (Ali et al. 2006). The authors measured not only oxidant production by determining dihydroethidium oxidation in brain but they also measured the EPR signals in brain mitochondria of their mice. Thus, in this strain of mice in which males live longer than females it is males that produce fewer radicals. Far from contradicting results fromour laboratory, these results nicely confirm our results. The claim by all of us is that the sex that lives longer produces fewer radicals independently of whether it is males or females who live longer. The critical test will be to see why estrogens promote the expression of antioxidant genes in a given species and do not promote that expression in other species or strains. A third confirmation of this hypothesis came from the laboratory of Christiaan Leeuwenburgh (Sanz et al. 2007). These researchers studied a particular strain derived from the C57B6J in which males and females live the same. Not surprisingly, these authors did not observe changes in either oxygen consumption, complex 1 and complex 3 oxidant production, protein carbonyls, oxidised DNA or other indicators of oxidative stress. Thus, in those strains in which females live the same as males, there is no difference in oxidant production or in oxidative stress associated with sex. The overall conclusion is that estrogens promote a lower rate of production of radicals in those species or strains in which females live longer than males. On the other hand, in those animals in which males live longer than females it is males that produce fewer radicals and those in which longevity is the same in both sexes, radical production is also similar.

6 Mitochondrial Diseases

It has long been recognised that mitochondria are essential, and therefore when altered, can cause diseases many of which are very severe. These are known as mitochondrial diseases. It is not within the scope of this chapter which is aimed more towards understanding the role of mitochondria in ageing, to describe in detail these mitochondrial diseases. Tissues most susceptible to mitochondrial-driven disease

states are those with higher metabolic demand, i.e. brain, eye, liver, heart, and skeletal muscle. Mitochondrial disease states include:

1. The mitochondrial myopathies, a group of neuromuscular diseases that includes Kearns-Sayre syndrome, mitocondrial encephalopathy lactic acidosis and strokes, myoclonic epilepsy with ragged red fibers, and mitocondrial neuro-gastrointestinal encephalomyopathy that have genetic origins (Schapira 2006).
2. Disorders of mitochondrial electron transport chain that affect its assembly and/or stability and function. They involve both genetic factors and cofactor deficiencies (coenzyme Q10) that can lead to decreased ATP production and increased free-radical production. This free radical production leads to neurodegenerative diseases such as Alzheimer's disease, Parkinson disease, Huntington's disease, and amyotrophic lateral sclerosis (Johri and Beal 2012).

Leber hereditary optic neuropathy, which involves visual failure caused by the degeneration of retinal ganglion cells, is the most common disease with mtDNA mutations with a prevalence of approximately 12 cases per 100,000 in the population (Schapira 2006).

7 Toxicological Aspects: The Treatment of AIDS with Zidovudine Causes Mitochondrial Pathology that Explains Muscle Damage Associated with AIDS Treatment

Moreover, mitochondria are involved in not only genetic diseases but also pharmacological and toxicological diseases. For instance, patients who suffer from HIV infection, who are successfully treated with a cocktail of drugs including Zidovudine (AZT) frequently suffer from muscle myopathies. These were attributed to free radical damage associated with increased production of mitochondrial reactive oxygen species. We were interested in this pathology because first of all HIV patients can be considered as an accelerated model of ageing and secondly because mitochondria were clearly involved in this myopathy which is, it is important to underline, associated with the treatment and not with the primary disease. In any case, we observed that patients who suffer from HIV infection have a decreased activity of the cystathionase activity, an important pathway in the biosynthesis of thiols from methionine (de la Asuncion et al. 1998). Cystathionase itself is lower in patients suffering from HIV infection (Martin et al. 2001) and this renders patients more susceptible to oxidative insults. When these susceptible patients are treated with Zidovudine, mitochondrial damage clearly occurs, and this can be seen from both biochemical and molecular analysis. Histological evidence can also be observed. These alterations can, we observed, be successfully treated when patients (or experimental animals) who are treated with Zidovudine, receive high doses of vitamins C and E. We mention these toxicological aspects of the mitochondrial function because it is now becoming a trend to think that vitamin supplementation is never useful. It can be so if the patients are under specific oxidative damage, especially associated with mitochondria as is the case in Zidovudine treatment.

8 Alzheimer's Disease

On a different note, one of the most prevalent and devastating age-associated diseases is Alzheimer's. Early work from George Perry and Mark Smith showed that many of the manifestations of Alzheimer's disease can be traced to free radical damage (Perry et al. 1998). What Smith and Perry did was analysing the brains of Alzheimer's patients and seeing that in the areas more damaged in the disease, one could observe oxidative damage even by histochemical analysis.

Our understanding of the pathophysiology of Alzheimer's changed dramatically when it was appreciated that amyloid beta caused damage from inside the cells (Selkoe 1991) and not only from the plaques as was Alzheimer's original observation. In fact, some researchers believe that amyloid beta when it is in the plaques is in quite an inert form and it is the soluble Abeta what causes damage. Pioneer work by Catarina Oliveira at the University of Coimbra in Portugal showed that mitochondria were affected by amyloid beta and that in fact, cells that were artificially depleted of mitochondria were much more resistant to damage associated with amyloid beta than those containing normally functioning mitochondria (Pereira et al. 1998). The work of Oliveira prompted us to study the rate of radical production by mitochondria in the presence and absence of amyloid beta. We observed that the toxic peptide causes an increase in the rate of oxygen production by mitochondria and that this could be prevented when mitochondria were co-incubated with amyloid beta and heme (Lloret et al. 2008). The protective effect of heme was based on the observation by Atamna and co-workers that amyloid beta strongly binds to iron in heme and that this can explain the lower rate of respiration in mitochondria in the presence of Alzheimer's peptide (Atamna and Frey 2004). As stated before, a lower rate of oxygen consumption by mitochondria is usually associated with an increased production of radicals. This was the case and therefore the increased rate of oxygen production could contribute to damage to the mitochondria and to other organelles and explain the low energy production in areas of brain affected by Alzheimer's disease.

The altered free radical production in mitochondria of neurons in the presence of amyloid beta, prompted us to study the role of this increased free radical production in the cell signalling associated with Alzheimer's. For instance, we observed that RCAN 1 which is an adaptive enzyme that is upregulated in the presence of chronic oxidative stress (Davies et al. 2007) and that is an inhibitor of calcineurin could contribute to understanding the relationship between amyloid beta and Tau, originally thought of as two distinct and independent hallmarks of Alzheimer's disease (Lloret et al. 2015). Indeed, an upregulation of RCAN 1, will inhibit the dephosphorylation of phospho-Tau and lead to increase its levels of phosphor-tau (see Fig. 2). Altered radical production by mitochondria not only in normal ageing but also age-associated disease such as Alzheimer's can lead to pathophysiological hallmarks of the disease and of the ageing process itself.

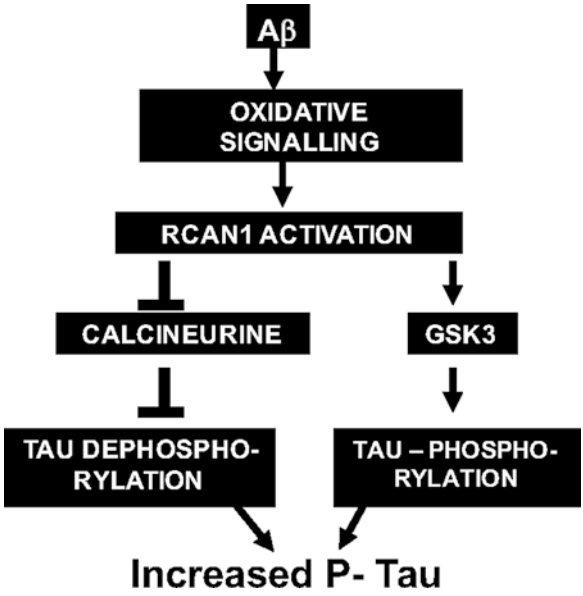

Fig. 2 Abeta and p-Tau interaction model

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