AN HYPOTHESIS CONCERNING CONTROL NETWORKS AND AGING IN DROSOPHILA MELANOGASTER AND CAENORHABDITIS ELEGANS.

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ABSTRACT

To explain trends emerging from the study of longevity mutants, a modification of the reactive oxygen species (ROS) model of aging is suggested.

ROS do not appear to be produced in greater quantities during cellular activity unless specific factors are also present. These include raised cytosolic calcium and sodium ions, nitric oxide (NO) or dopamine. Metabolically active cells that are repeatedly exposed to these factors, especially in combination, show the most ROS damage and may contribute most to aging. This explains the importance of neurons, which is highlighted by genetic studies, and points to which cells are the most aging-sensitive.

Control pathways disrupted in long-lived mutants are those which control one or more of these ROS promoting factors. The *daf-2/daf-16* pathway may interact with these factors in several ways. Changes in control networks may be propagated from a relatively small number of cells/junctions by neural connectivity and hormonal means.

INTRODUCTION

Control of longevity by the central nervous system in *Drosophila melanogaster* was suggested in 1976 (Miquel et alia) and has been supported by genetic analysis of aging in *D. melanogaster* and *Caenorhabditis elegans*. A large group of gerontogenes is involved in control networks, many of which are expressed in the central nervous system. In *C. elegans* this includes *unc-64* and *unc-31* (Ailion et alia 1999) and *AKT1* and *AKT2* (Paradis and Ruvkin, 1998). A key gerontogene *daf-2*, a homologue of the mammalian insulin/IGF receptor, is expressed in a number of tissues, but expression in only neurons is sufficient to control longevity (Wolkow et alia, 2000). In *Drosophila* the homologue of *daf-2*, *InR* is a gerontogene (Tatar et alia, 2001) and expressed at restricted numbers of neuronal and neuromuscular junctions (Gorczyca et alia, 1993). In addition a *SOD1* transgene targeted to the giant motor neuron produces increased longevity (Phillips et alia, 2000). What is so important about the neurons in control of longevity? What do the control networks do?

Furthermore, striking similarities exist between these two unrelated organisms. For example a longevity mutant common to both species is a hypomorphic version of the neuronal insulin/IGF receptor (Tatar et al., 2001). A second, equally striking observation concerns

diapause found in both species. Diapause essentially produces an extra larval stage used to extend the pre-adult life. In *C. elegans* this is known as the dauer phase. Diapause may have a role in permitting survival through tough times; for example, diapause will permit some species of *D. melanogaster* to over-winter. During diapause aging virtually ceases in both species. (Tatar and Yin, 2001).

What do these data tell us about the biology of aging?

The role of oxidative stress.

A theoretical model concerning the role of oxidative stress mediated by reactive oxygen species has evolved over the last half century, which posits that accrual of molecular oxidative damage, particularly to DNA, is the principal causal factor in the senescence-related loss of ability to maintain homeostasis. Under normal physiological conditions, the use of oxygen by cells of aerobic organisms generates potentially deleterious reactive oxygen metabolites. A chronic state of oxidative stress exists in cells because of an imbalance between prooxidants and antioxidants. The amount of oxidative damage increases as an organism ages and is postulated to be a major causal factor of senescence. Support for this hypothesis includes the following observations: (i) Overexpression of antioxidative enzymes retards the age-related accrual of oxidative damage and extends the maximum life span of transgenic *D. melanogaster*. (ii) Variations in longevity among different species inversely correlate with the rates of mitochondrial generation of the superoxide anion radical and hydrogen peroxide (Von Zglinicki et alia, 2001; Finkel and Holbrook 2000; Sohal et alia, 2000; Sohal and Weindruch, 1996). Furthermore, many authors (Wei et alia, 2001; Wallace 2001; Brewer 2000) point to mitochondria as a principal target of ROS damage because of the close proximity to the site of generation of ROS. According to this refinement of the model, the mitochondrial DNA progressively accumulates mutations that impair the ability to generate energy, maintain the membrane potential and maintain calcium homeostasis. Consequently the cells become functionally impaired and in addition, progressively more likely to undergo apoptosis.

Many findings in *D. melanogaster* long-lived strains are consistent with this model. For example the long-lived Methuselah strain of *D. melanogaster* produces less ROS (Lin et al., 1998). Polygenic strains that are long-lived are also resistant to oxidative stress, and produce less ROS (Arking et al., 1991) and have higher levels of anti-oxidants (Hari et al., 1998). A long-lived strain has been derived from the Arking strains, *La-mt*, in which only the cytoplasmic genome differs from that of the control. In this strain longevity is associated with

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reduced ROS production (Driver and Towadros, 2000), and reduced sensitivity to the ROS producer paraquat (Driver, unpublished data).

Similar observations are found in *C. elegans*. All the long-lived *C. elegans* mutants isolated to date are more resistant to oxidative and other stresses than control strains (Honda and Honda, 1999). The mutation *ctl-1* (a cytosolic catalase) shorten life span, and prevent the life span extension of *daf-2* mutants (Taub et al., 1999). *ctl-1* mutants accumulate the ROS derived product lipofuscin at a greatly accelerated rate compared to normal. Expression of *ctl-1* and *sod-3*, which encodes a Mn-superoxide dismutase, are increased in *age-1* worms. Conversely in *C. elegans*, the mitochondrial mutant *mev-1* differs in a succinic dehydrogenase sub-unit, resulting in impaired complex II function (Ishii et al., 1998). The mutants are short lived, oxygen sensitive, and produce increased ROS.

Antioxidant proteins

To control ROS damage a number of highly conserved proteins inactivate some of the key reactive intermediates. Such proteins include, for example catalase, superoxide dismutase (SOD) (Bai and Cederbaum 2001) and the thioredoxin and thioredoxin reductase pair (Nordberg and Arner 2001). These proteins can do much to protect the cell against ROS, but they cannot give 100% protection because there will always be the occasional molecule that will reach (and react with) important cellular molecules before they reach an anti-oxidant molecule. Thus in spite of the protecting molecules, the rate of ROS production remains a key to the amount of ROS damage.

The role of mitochondria

Since the principal site of ROS in most cells is the mitochondria, it could be predicted that mutations that alter mitochondrial function to reduce ROS production should be among the mutants that produce increased longevity. In the *D. melanogaster* mutant *Indy-1* reduced ROS production may occur because of reduced maximum respiration capacity, which may limit ROS production. The mutant *Indy-1* in *D. melanogaster* reduces function in a dicarboxylate transport protein (Rogina et al. 2000). By contrast, in La-mt strain described above, ROS production is reduced without changing respiration rate (Driver, unpublished data).

In *C. elegans*, the mutant *clk-1* (Miyadera et al., 2001) reduces synthesis of ubiquinone needed for mitochondrial respiration (Felkai et al., 1999). Braeckman et alia, (1999) show that in spite of a mild respiration reduction, ATP remains high in these mutants. Larsen and Clarke (2002) have produced a phenocopy of the *clk* mutants by showing that a diet with reduced ubiquinone produces an increased life span.

Dietary restriction, a common way to produce increased longevity in rodents has also been shown to increase life span in *D. melanogaster* and reduced the production of age pigment, a marker for ROS damage (Driver et al., 1986).

Although data has not presented for dietary restriction in *C. elegans*, *eat* mutants eat less. These mutants

may have an increased life span by up to 50%. This increase is additive with the effect of *daf-2*, but not with *clk-1*. This was taken to indicate that restriction of food intake and *clk-1* act at the same point, mitochondrial respiration, whereas *daf-2* has a different mode of action (Lakowski and Hekimi, 1998). Taub et al. (1999) show that catalase is required for the longevity increase induced both by the mitochondrial mutant *clk-1*, and the insulin/IGF receptor mutant *daf-2* indicating that both methods of control of aging act through ROS. Taken together these observations indicate that dietary restriction and the insulin/IGF receptor mediated pathway alter tissue ROS concentrations albeit in different ways.

The mitochondrial-ROS confusion: When is the most ROS produced by mitochondria?

In vitro most ROS production occurs during inactivity. Measurements on isolated mammalian mitochondria indicate that ROS production is much greater in state 4 where respiration has ceased because of depletion of ADP compared to state 3 where ADP is available. This was alleviated by uncouplers both in vitro and in vivo including a specialised uncoupling protein, leading some authors to suggest that most ROS is produced by mitochondria when energy demand is low (Brand 2000). This would make nonsense of the rate-of-living concept in aging and is in disagreement with a raft of observations discussed above, in mammals and other animals, indicating that in vivo ROS production increases with increasing respiration rate.

Some active tissues show little or no aging.

In both *D. melanogaster* and *C. elegans* diapause is observed. Diapause is a state of arrested development accompanied by physiological changes for somatic persistence. It is essentially another larval phase, and energy metabolism still proceeds. Better known in *C. elegans*, diapause also occurs in *D. melanogaster*. The endemic *D. triauraria* of Japan, and *D. littoralis* of Finland over-winter as adults in reproductive diapause. How *D. melanogaster* winters is poorly understood, but reproductive diapause can be cued by cool temperature. In the diapause phase aging effectively stops in both *D. melanogaster* and *C. elegans* (Tatar and Yin, 2001): extra time spent in the diapause state does not reduce the subsequent life span of the adult.

Since only a small number of cells are carried forward into the adult phase, a modest degree of aging in 'doomed' cells would be of little consequence. Therefore the above findings apply only to cells such as imaginal disc cells, which are precursors of adult cells. The special conditions that apply to these cells are not clear, but these results do indicate that metabolism is not obligatorily coupled to aging.

In vivo linkage with between activity and ROS production probably requires additional factors.

Calcium ions

In many tissues function is activated by a rise in the second messenger, calcium ions, producing coordinated activation of respiratory activity. For example in

pig muscle, ADP may remain constant when calcium activates respiration, producing a coordinated activation of ATP use, and ATP production by activation of calcium dependent dehydrogenases, and the mitochondrial F(0)/F(1)-ATPase (Territo et al., 2000). Calcium activation of muscle ROS production is also observed in humans (Gabbita et al., 2001). Calcium activation of respiration associated with increased ROS in human and animal neuronal cells (Carriedo et al., 2000; Gasso et al., 2001; Urushitani et al., 2001; Vergun et al., 2001).

Calcium activation of muscular and neuronal function has been shown in *C. elegans* (Kerr et al., 2000). In the fly, calcium activation of respiration in flight muscle has also been observed in the blowfly (Bygrave et al., 1975). Yan and Sohal (2000) showed that reduction of respiration by preventing flight, reduced respiration which also reduced ROS production and increased longevity.

Sodium ions

A rise in the concentration of sodium ions will also trigger a respiratory burst that generates ROS with isolated rat mitochondria. The ROS damage will produce functional impairment of electron transport complex 1 such as occurs in Parkinson's and Alzheimer's disease (Dykens 1994). Disturbances in sodium balance may be as important as disturbances in calcium balance during oxidative stress generated by hypoxia (Taylor et al., 1999).

Nitric oxide (NO)

NO is a key cellular communication molecule involved in an evolutionarily conserved manner in development, immune responses and memory in *D. melanogaster* (Schurmann, 2000; Nappi et al., 2000; Gibbs et al., 2001).

NO or its derivatives (reactive nitrogen species: RNS) have multiple actions in mammalian mitochondria which include direct reaction with superoxide to produce the highly reactive isomer of nitrate ions, peroxynitrite ions. It also will stimulate production of ROS and activate apoptosis or necrosis (Brown and Borutaite, 2001; Sastre et al., 2000). The signature modification of proteins, nitration of reactive tyrosine residues, may be observed to increase in proteins from some aged tissues (Schoneich et al., 1999). Nitration of tyrosine residues may contribute to peroxynitrite toxicity, as nitration precludes the phosphorylation or nucleotidylation of tyrosine residues and thereby seriously compromises one of the most important mechanisms of cellular regulation and signal transduction (Stadtman and Berlett, 1998). To date there has been little data NO concerning any role in aging processes in *D. melanogaster* or *C. elegans*, and it may be profitable to study NO further.

DOPA and dopamine

In some cells such as dopaminergic cells, mitochondria participate in the neurodegeneration in an apparently complex manner. In humans this produces Parkinson's

disease (PD), the most common movement pathology occurring in old age, severely afflicting dopaminergic neurons within the substantia nigra along with non-dopaminergic, extra-nigral projection bundles that control circuits for sensory, associative, premotor, and motor pathways. Factors unique to the substantia nigra may contribute to oxidative damage such the presence of neuromelanin, a high concentration of polyunsaturated fatty acids, and iron, and relatively low antioxidant complement with high metabolic rate of the neurons. However the chief factor probably relates to the neurotransmitter dopamine. Although the enzyme tyrosine hydroxylase DOPA and dopamine are potent sources of ROS (Haavik and Toska, 1998; Jones et al., 2000), the MAO metabolite of dopamine, 3,4-dihydroxyphenylacetaldehyde, is approximately 1000 more toxic to mitochondria and may be the principal cause of mitochondrial damage (Kidd 2000).

Other findings add weight to the concept of PD arising from mitochondrial poisoning. Exogenous agents such as the mitochondrial poison MPTP (1-methyl-4-phenyl-1, 2,3,6, -tetrahydropyridine) poisoning may produce PD (Kidd 2000) further adding weight to a mitochondrial role and implicating environmental agents in the development of PD.

Overexpression of bcl-2 suppresses ROS production induced by dopamine (Lud Cadet et al., 2000). The protein bcl-2 is a mitochondrial protein that functions to protect against apoptosis by blocking ROS production (see below). Oxidative phosphorylation abnormalities present in PD impair energetics in the substantia nigra mitochondria, also intensifying oxygen free radical generation. These pro-oxidative factors combine within the substantia nigra dopaminergic neurons to create extreme vulnerability to oxidative challenge (Kidd 2000).

There is a good case for the involvement of dopaminergic neurons in determination of longevity in *D. melanogaster*. Dopamine production falls during aging and the cell bodies producing dopamine show significant degeneration (Neckemeyer et al., 2000). The production of dopamine is vital and reduced levels of dopamine result in sharply reduced spontaneous activity and lethality (Neckemeyer, 1996). Furthermore dopaminergic pathways appear to be involved in feedback control of behavior by ovaries (Neckemeyer 1998a). Dopamine acts as a signaling molecule within the mushroom bodies to mediate a simple form of learning (Neckemeyer, 1998b) and so a dopamine deficit may contribute to the learning deficits observed in old *D. melanogaster* (Neckemeyer et al. 2000).

Neuronal cells

Neuronal cells are best known for their high cation fluxes and are therefore prime although not sole candidates for age-related functional loss. In mammalian cells, neurons that respond to glutamate via NMDA channels appear to be particularly sensitive to damage by reactive metabolites because in addition to the frequent rises of cytosolic sodium and calcium ions, synthesis of NO is activated (Urushitani et al., 2001;

Carrieodo et alia, 2000). Similar cells are apparently found in specific regions of *D. melanogaster* brains. (Schurmann, 2000). Among those cells there are those that are also exposed to dopamine and show substantial loss with aging (see above).

In *Drosophila*, patterns of activity can spread across most of the brain as indicated by seizures closely analogous to epilepsy, in which an activity 'storm' passes across the brain (Kuebler, 2001). Seizure can be blocked by a number of mutants including Shaker B (2)/Passover that blocks a specific electrical junction on the motor neuron, indicating that general activity can be controlled by very specific neurons/junctions. The curious observation that alterations in a number of different brain sites can have the same effect on longevity (Wolkow et alia, 2000; Ailion et alia 1999; Paradis and Ruvkin, 1998; Tatar et alia, 2001; Gorczyca et alia. 1993; Phillips et alia, 2000) may be a related effect. General brain activity may be modified by any of relatively small, presumably crucial, neuron or junction type. On the other hand a signal from a small number of cells may spread to the whole body via a hormone, for example Tatar et alia, 2001 indicate that the longevity of the insulin/IGF receptor mutant may be mediated in part by juvenile hormone.

Other tissues also show very high cation fluxes. For example, the indirect flight muscle depolarises at a rate several times faster than the afferent nerve (Koenig and Ikeda 1980) and loses function rapidly with age (Takahashi et alia 1970)

What do the gerontogenes do?

In view of the above considerations, gerontogenes would be expected to control the production of ROS by mitochondria (as above) or control the level of enzymes that protect against ROS, or control one or more of the above factors that induce ROS, and would be expected to be expressed in those cells that are most active in these factors.

The daf-16-related set of genes

The mutant *daf-16* is one of several longevity mutants in *C. elegans* that produces less ROS (Yasuda et alia 1999) and is more resistant to stresses such as hydrogen peroxide (Johnson et alia 2000). The gene *daf-16* is a point of convergence of several pathways. The lifespan of *C. elegans* is regulated by the insulin/insulin-like growth factor (IGF)-1 receptor homologue DAF-2, which signals through a conserved phosphatidylinositol 3-kinase (PI 3-kinase)/Akt pathway (Vanfleteren and Braeckman, 1999). Other pathways converging on *daf-16* include both sensory neurons and germline activities which regulate life span (Lin et al., 2001; Hsin and Kenyon 1999) and overexpressed SIR 2.1 (Tissenbaum and Guarente, 2001).

Multiple functions for daf-2 related genes

Although frequently envisaged as a linear pathway controlled by the insulin/IGF receptor, some of the genes on the *daf-2* pathway, Age-1 and *daf-16*, can be envisaged to have more than one function in control of

longevity.

Age-1, a potential cytosolic calcium regulator.

The age-1 protein (phosphatidylinositol 3-kinase (PI 3-kinase; Vanfleteren and Braeckman, 1999) may participate in a pathway that regulates cytosolic calcium via inositol 1,4,5-trisphosphate (IP3), releases Ca²⁺ from the endoplasmic reticulum to activate mitochondria metabolism (Brini and Carafoli 2000; Dutta 2000; Campbell 1997), thereby having a direct effect on the rate of calcium stimulated ROS production.

The key gerontogene daf-16 probably inhibits a bcl-2 homologue.

How do the control network gerontogenes reduce ROS production? If ROS production is reduced then presumably an anti ROS protein is up regulated or a pro-ROS protein is down regulated. There is one protein which is a good candidate for an up regulated anti ROS protein which also can act as a master protein to up regulate the antioxidant proteins catalase and superoxide dismutase.

Overexpression of *daf-16* increases life span suggesting that a principal action of the insulin-receptor pathway is to reduce *daf-16* function (Johnson et al. 2000). DAF-16 belongs to a homologous group of proteins called the Forkhead transcription factors, which includes the human proteins FKHR, FKHR-L1 and AFX (Lee et alia 2001). The Forkhead family of transcription factors may participate in the induction of death-related genes. One of these, FKHR inhibits the action of the anti-apoptotic protein bcl-2 with the protein Bim (Dijkers et alia, 2000 and 2002).

In *D. melanogaster*, insulin/IGF receptor related longevity mutants exist, implying the existence of a similar path. The next protein in the action of insulin, the insulin receptor protein substrate has also shown to be a gerontogene (Clancy et alia, 2001), Tatar et al. (2001) have argued that the increased longevity of the insulin receptor mutant of *Drosophila* is suppressed by added amounts of the morphogenesis controlling hormone, juvenile hormone (JH), and that the increased longevity may be mediated in part by lower levels of JH.

Bcl-2

The protein bcl-2 (and other members of the same family such as bcl-XL) has properties that make it a prime candidate for regulation by gerontogenes. At low ion fluxes bcl-2 promotes ROS production, associated with a high level of NAD(P)H and probably induces up-regulation of anti-oxidant proteins. In addition, at high ion fluxes, such as those initiated by apoptotic agents, it blocks a mitochondrial high flux channel and blocks ROS production (Reed 1997; Chao and Korsmeyer 1998, Reed et alia, 1999; Esposti et alia, 1999; Murphy et alia 2001; Kruman et alia, 1998).

The *C. elegans* protein that show sequence homology and probable functional similarity to bcl-2 is CED-9 (Xue and Horvitz, 1997). CED-9 is produced as a pro-protein that is converted to the bcl-2 homologue after hydrolysis by the cell-death protease CED-3. Presump-

tive evidence has been provided for a bcl-2 based system that controls apoptosis in *D melanogaster*, (Gaumer et alia, 2000) but the bcl-2 homologue or analog has not been identified at the time of writing.

Thus bcl-2 acts as an anti-oxidant protein, but more than this it acts under conditions where high amounts of ROS are likely to be generated, that is under high calcium fluxes. In addition the insulin receptor pathway probably controls it.

Bcl-2 is best known as an anti-apoptotic protein. If apoptosis precedes death of the individual when lethally stressed, the anti-apoptotic action may contribute to the greater stress resistance of long-lived *C elegans* strains (Barsyte et alia, 2001; Johnson et alia 2000). However it is likely that the production of ROS rises many times to a level which is not lethal, but which will over time produce substantial cellular damage. The known action of bcl-2 would protect the cell against this action as well.

The JNK/SAPK pathway and bcl-2

Seong et alia (2001) have introduced the probable involvement of the neural JNK/SAPK as another neural control network that modulates aging. Neural overexpression of *Drosophila* Plenty of SH3s (DPOSH) in adults produced a modest increase in life span in *D melanogaster*. *Puckered*, a target gene of the JNK/SAPK pathway was activated by DPOSH, and the increased longevity was suppressed by a mutation of *Drosophila* JNK (bsk) or JNKK (hep).

The JNK/SAPK pathway is a stress response pathway in which stress activated protein kinase (hence SAPK) controls bcl-2 (Yamamoto et alia, 1999). As with the above pathway, it is suggested that control of the bcl-2 homologue is probably the key to how this pathway produces increased longevity.

OLD-1

The gene OLD-1 is a key target for daf-16 action. It is a transmembrane protein tyrosine kinase with distant similarities to the Pdgfr, Fgfr, Ret, and Tie/Tek families (Rikke et alia, 2000) It is stress inducible, and is a positive regulator of longevity and stress resistance. The transcription of old-1 is upregulated in long-lived age-1 and daf-2 mutants and is upregulated in response to heat, UV light, and starvation. Expression is widespread throughout the body and dependent on daf-16. Finally, OLD-1 is required for the life extension of age-1 and daf-2 mutants. (Murakami S, Johnson, 2001). We await developments concerning the function of this protein.

CONCLUSION

One of the advantages of genetic analysis is that results obtained are less constrained by existing hypotheses and more likely to lead one into new fields. Nevertheless the mutants do not speak for themselves, and new hypothetical frameworks, such as the above need to be provided. This hypothesis would be a good one if it provides ideas for new experimental work and new

approaches to clinical management of age-related conditions.

Several new testable proposals arise from this argument.

A key role for calcium and sodium in the aging of many cells is proposed. The cells that are most subject to aging are those in which sodium and calcium fluxes are greatest and where NO and dopamine are also used.

A key gerontogene may be the homologue of bcl-2. Gerontogenes should alter brain electrical activity and hence behavior. The altered behavior should deteriorate with age, and more slowly in long-lived mutants.

A key question relates to the lack of aging in diapause. The hypothesis to be tested here is that the cells that are carried forward into adulthood produce little or no ROS. The hypothesis is not that the whole diapausing animal has little ROS, because many cells are destroyed during the metamorphosis, and most ROS damage to these would have little effect on the adults.

The new approach suggests some new methods of control of aging. In particular production of ROS may be reduced by pharmacological agents that block the factors that increase ROS production rather than interfering with energy metabolism.

This model is a simple one, probably too simple. Nevertheless it has explanatory power, and permits predictions concerning longevity in the experimental animals. The model must be extrapolated to humans with considerable caution. However it has a number of features that are probably general and are relevant clinically: production of damaging levels of ROS by mitochondria depends on additional factors such as raised cytosolic calcium and sodium ions, NO, and dopamine. From these suggestions arise the final and most interesting prediction: that a number of neurologically active drugs in clinical use at the moment may have a significant impact on aging.