Chapter 8
Stress-related Behavioural Responses, Immunity and Ageing in Animal Models

Carmen Vida and Mónica De la Fuente

8.1 Introduction

Health maintenance during the ageing process, which allows for increased longevity, is determined by the preservation of tissue homeostasis at all physiological levels. Healthy ageing depends both on the individual's genetic make-up and lifestyle factors (Kirkwood 2008). The age-related loss of homeostasis and capacity to react appropriately to stress, as a consequence of the deterioration of physiological systems, have been proposed to explain the increase of morbidity and mortality with ageing (De la Fuente 2008; Fig. 8.1). Although the effects of stressors on behaviour and immunity are very heterogeneous since they depend on type, frequency, duration, intensity, animal models, perception of subject and coping by the stressed animal (Costa-Pinto and Palermo-Nieto 2010), it has been suggested that immunosenescence is a significant consequence of chronic stress and the actions of stress hormones (Bauer 2008; Lord et al. 2009). Moreover, this age-related impairment of the immune system in turn appears to be involved in the increased oxidation and inflammation status that occurs in the ageing process, increasing its rate and representing a vicious cycle of decline (De la Fuente and Miquel 2009).

In the context of the neuro-endocrine–immune network, it is known that humans with psychological distress, anxiety or depression (Arranz et al. 2007, 2009a), and experimental animals (Viveros et al. 2007) with an inadequate response to stress situations show premature immunosenescence. Since immune function is a marker of health, alterations in immunity are accompanied by increased morbidity (Wikby et al. 2008). To study the effect of individual responses to stress in the ageing process and thus the involvement of immunosenescence in this process, models with a shorter longevity than humans are needed. Thus, we have proposed several murine models of premature immunosenescence. One of them, which is characterised by an altered stress-related behaviour response, shows accelerated immunosenescence, an oxidation–inflammation state and premature ageing in the nervous and endocrine

C. Vida (✉) · M. De la Fuente
Department of Physiology, Faculty of Biology, Complutense University of Madrid,
28040 Madrid, Spain
e-mail: mondelaf@bio.ucm.es

J. A. Bosch et al. (eds.), *Immunosenescence,*

DOI 10.1007/978-1-4614-4776-4_8, © Springer Science+Business Media New York 2013

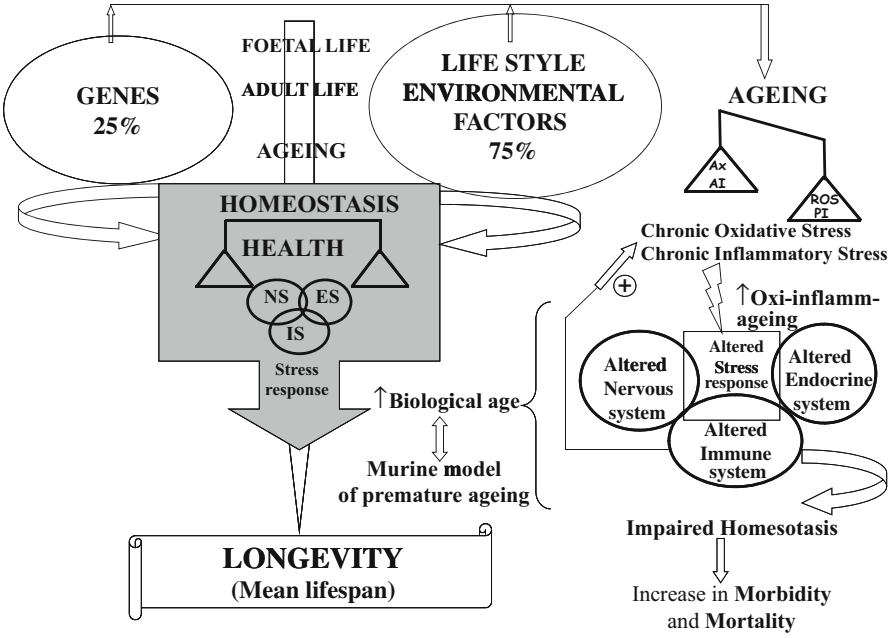

Fig. 8.1 A high longevity in each individual depends on health maintenance and homeostasis preservation, which is conditioned by genotype (approximately 25 % influence) and lifestyle and environmental factors (75 % influence). During the ageing process oxidative stress, affecting all cells and especially those of master regulatory systems, that is, the nervous, endocrine and immune system, and the communication among them, reduces the ability to maintain homeostasis. Moreover, the immune system could increase oxidative stress if it is not regulated thus accelerating the ageing process. This deterioration of homeostasis, which leads to the age-related increase in morbidity and mortality, is established at a different rate in each subject, and this rate is the result of individual epigenetic mechanisms acting on genes throughout the life of the subject. The use of animal models, specifically the prematurely ageing mouse (PAM) model, may be particularly useful to assess the relationships between a diminished ability to cope with stressful situations, ageing of homeostatic systems, accelerated biological ageing and a shorter longevity. *NS* Nervous System; *ES* Endocrine System; *IS* Immune System; *Ax* Antioxidant Compounds; *AI* Anti-Inflammatory Compounds; *ROS* Reactive Oxygen Species; *PI* Pro-Inflammatory Compounds

systems, as well as presenting a shorter life expectancy (De la Fuente 2010; Viveros et al. 2007). These theories of the ageing process and the model of prematurely ageing mice (PAM) are discussed in this chapter.

8.2 The Ageing Process and the Concepts of Longevity and Biological Age

The universal process of ageing may be defined as a progressive and general deterioration of the functions of the organism that leads to a lower ability to react to stress and preserve homeostasis. Although the accumulation of such adverse changes with

the passing of time should not be considered a disease, it strongly increases the risk of disease, and finally results in death. The ageing process is finished at the end of the maximum lifespan or maximum longevity, which is the maximum time that a subject belonging to a particular species can live. In human beings this is about 122 years, the record for human longevity held by Mme Jean-Louise Calment, whereas in mouse and rat strains it is approximately 3 and 4 years, respectively. It is very important to distinguish the maximum lifespan from the mean longevity or mean lifespan, which can be defined as the mean time that the members of a population that have been born on the same date live. The maximum longevity is fixed in each species, but the mean lifespan of individual organisms, even when they are of the same genotype and are raised in a common environment protected from extrinsic hazards, shows marked variability. Although presently it is impossible to increase the maximum longevity of a species, the mean lifespan can be increased by environmental factors that allow the maintenance of good health and thus, approach the maximum lifespan in good condition. Presently, since the mean longevity is very high in developed countries, about 75–83 years, and we start the ageing process at about 18 years old, we spend most of our life ageing. Therefore, it is very important to know which lifestyle factors can increase or decrease this longevity and how they do achieve this. A higher mean longevity is achieved by preservation of good health and this depends approximately 25 % on genetic factors and 75 % on lifestyle and environmental factors (Fig. 8.1).

The ageing process is highly heterogeneous, and thus, there are different rates of physiological changes in the various systems of the organism and in the diverse members of a population of the same chronological age. This justifies the introduction of the concept of "biological ageing ", which determines the level of ageing experienced by each individual and therefore, his/her life expectancy. The biological age is related to mean longevity, subjects of a population with a higher rate of ageing show an older biological age and have a shorter lifespan. Since chronological age fails to provide an accurate indicator of the rate of ageing, it is necessary to select parameters useful as biomarkers of ageing to find out this rate and therefore, the probable longevity of each subject (Bae et al. 2008; Borkan and Norris 1980).

Although almost 400 single-cause theories have been proposed to explain the ageing process, we have recently published an integrated theory of ageing (De la Fuente and Miquel 2009). To answer the question of "how" ageing happens, a chronic oxidative stress condition (increase of oxidant compounds and decrease of antioxidant defences), which is linked to many age-related changes, has been proposed. In addition, emerging evidence shows the close link between oxidation and inflammation, and with ageing the pro-inflammatory compound levels are higher than those of the anti-inflammatory (termed inflammageing; Franceschi et al. 2007), leading to inflammatory stress. Therefore, an oxidative and an inflammatory state have been suggested as the cause of the loss of function that appears with senescence. Thus, after adult age the establishment of what has been called "oxi-inflamm-ageing" occurs (De la Fuente and Miquel 2009).

8.3 Neuro-endocrine–immune Communication in Ageing: The Immune System as a Marker of Biological Age and Predictor of Longevity

It is known that the three master regulatory systems, namely the nervous, endocrine and immune systems, are intimately linked and interdependent. Thus, there is a neuro-endocrine–immune axis that allows the preservation of homeostasis and health. The communication between these three systems has permitted the understanding of why situations of depression, emotional stress and anxiety are accompanied by a greater vulnerability to infections, cancers and autoimmune diseases (Ader et al. 2001; Besedovsky and Del Rey 2007; Irwin and Miller 2007). In fact, many neurotransmitters and hormones modulate immune cell functions and consequently behaviours, emotional states and stressful life experiences significantly affect the immune response (Ader et al. 2001). With ageing there is an adjustment in these physiological regulatory systems and in their communication, with the loss of homeostatic capacity and the resulting increase of morbidity and mortality that appears with the passage of time (De la Fuente 2008; Fabris 1990; Gemma 2010). For example, the nervous system changes with ageing showing a decrease of synaptic density (Hedden and Gabrieli 2004). The age-related decrease of neurogenesis, which clearly affects the hippocampus, explains the learning and cognitive impairment in aged subjects (Couillard-Depres et al. 2011), the hippocampus neurogenesis and plasticity being also altered by stress (Kim et al. 2006). In the endocrine system several changes accompany healthy ageing, these include, for example, the decrease of the growth hormone/insulin-like factor-1 axis (somatopause) and of the sexual hormones, namely estradiol (menopause), testosterone (andropause) and dehydroepiandrosterone (adrenopause) (Makrantonaki et al. 2010). Moreover, the age-related disturbances of the hypothalamic–pituitary–adrenal (HPA) axis are responsible for decreasing stress adaptability in old subjects, this being, at least in part, the cause of their health impairment (Aguilera 2011) and peripheral immunodepression (Woiciechowsky et al. 1999). Thus, the inadequate response to stress that occurs with ageing can be understood in the context of compromised neuro-endocrine–immune communication, this altered response being one of the conditions leading to an acceleration of ageing accompanied by the poor functioning of the immune system (Bauer 2008; Gouin et al. 2008). In addition, chronic stressful conditions modify immune functions and their interaction with the nervous system, causing detrimental effects on memory, neural plasticity and neurogenesis (Yirmiya and Goshen 2011).

With age, there is an increase in susceptibility to infections and increased risk of cancer, which indicates an inadequate immune response and influences age-related morbidity and mortality. The profound impact of ageing on immunity is widely accepted (see Chaps. 1 and 2), with age-related changes of the immune system that may include not only diminished but also enhanced functions such as pro-inflammatory cytokine production in unstimulated cells. Thus, the components of the immune system undergo striking age-associated re-structuring, termed immunosenescence (Weiskopf et al. 2009). Some of the key and most marked changes are a pronounced

age-related decrease in T-cell functions (Haynes and Maue 2009), especially in the CD4 T-helper cell, which affects cell mediated and humoral immunity and causes an impaired B-cell function (Eaton et al. 2004; Frasca and Blomberg 2009). Phagocytic cells, including neutrophils and macrophages, show functions that decrease with ageing and the antitumoral activity of natural killer (NK) cells, in most of the work published, shows an age-related decrease (reviewed in Shaw et al. (2010)). The network of cytokines produced in response to immune challenge has also shown changes with ageing, notably a shift towards Th2 (effecting humoral antibody mediated immunity) responses and reduced anti-inflammatory cytokine production (Arranz et al. 2010a, 2010b).

Importantly, it has been demonstrated that the competence of the immune system is an excellent marker of health and several age-related changes in immune functions, have been established as markers of biological age and therefore as predictors of longevity and have been termed the immune risk profile or phenotype (Wikby et al. 2008). In order to identify the above parameters as markers of biological age, it has been necessary to confirm that the levels shown in particular subjects reveal their real health and senescent conditions. This has been achieved in the following two ways: (a) ascertaining that the individuals with those parameters indicative of a greater biological age, die before their counterparts. This can be confirmed only in longitudinal studies; (b) finding that the subjects reaching a very advanced age for their species, preserve these immune functions at levels similar to those of adults. For example, this can be tested in extremely long-lived subjects, such as centenarians or the extremely long-lived mice used in our own studies. Whilst biologically older individuals showing the immune competence levels characteristic of chronologically older subjects have been found to die prematurely, centenarians (Alonso-Fernandez et al. 2008) and long-lived mice (Arranz et al. 2010a, 2010b) exhibit a high degree of preservation of several immune functions, which may be related to their ability to reach a very advanced age in a healthy condition. All the above results confirm that the immune system is a good marker of biological age and a predictor of longevity (Wikby et al. 2008).

8.4 The Oxidation–Inflamamtion Theory of Immunosenescence and Ageing

According to our recently proposed theory of oxidation–inflammation in ageing (De la Fuente et al. 2005; De la Fuente and Miquel 2009), the age-related changes in the organism are linked to a chronic oxidative and inflammatory stress, which leads to the damage of cell components, including proteins, lipids and DNA. This affects all cells and especially those of the regulatory systems, including the immune system, which partially explains their impaired function. Moreover, the immune system, due to its capacity of producing oxidant and inflammatory compounds in order to eliminate foreign agents, could, if it is not well regulated, increase the general oxidative and inflammatory stress, and thus increase the rate of ageing.

Thus, immunosenescence could be involved in oxi-inflamm-ageing and affect the functions of other regulatory systems, resulting in age-related homeostatic decline and the consequent increase in morbidity and mortality. In this context, a relationship has been found between the redox and inflammatory state of the immune cells, their functional capacity and the life span of a subject. Thus, when subject shows a high oxidative stress in its immune cells, these cells have an impaired function and that animal shows a decreased longevity (Viveros et al. 2007). In contrast, subjects who achieve greater longevity, such as human centenarians and extremely long-lived mice, show a preserved redox state and immune functions (Arranz et al. 2010a, 2010b; Alonso-Fernandez and De la Fuente 2011). In summary, aged individuals that maintain a good regulation of the leukocyte redox state and consequently, a good function of their immune cells, with levels similar to those of healthy adults, have the greatest chance of achieving very high longevity.

8.5 Murine Models of Premature Neuro-endocrine–immune Ageing

Support for the role of the immune system in oxi-inflamm-ageing, in the context of neuroimmunomodulation, may be obtained by the study of animal models in which, individuals showing premature immunosenescence and a high oxidative and inflammatory stress in their immune cells (and in other cells), as well as a premature alteration of the nervous system (shown principally by behavioural tests) show decreased longevity in relation to other members of the group of the same chronological age. This has been studied using several murine models investigated and developed during the last few years (De la Fuente and Gimenez-Llort 2010; De la Fuente 2010) and summarised below.

8.5.1 Menopausal Models

Menopausal women as well as ovariectomised rats and mice (a model for human menopause) constitute a model for assessing premature ageing, since they show premature immunosenescence, with decreased leukocyte chemotaxis, lymphocyte proliferation and NK cell activity (Baeza et al. 2010a, 2011; De la Fuente et al. 2004), and a higher oxidative stress condition with higher GSSG/GSH ratio (Baeza et al. 2010b, 2011) as well as a decrease in anti-inflammatory cytokines such as IL-10 (Baeza et al. 2011). Moreover, ovariectomy causes the premature ageing of several behavioural responses such as sensorimotor abilities (loss of muscular vigour, impaired equilibrium and traction capacities) and reduction of exploratory activity (Baeza et al. 2010a). In addition, ovariectomised female rats and mice show a redox state and function in leucocytes similar to those in males (Baeza et al. 2011; De la Fuente et al. 2004). In mammalian species, males have a higher oxidative state and

a lower function in their immune cells than those of females and also have a lower mean life span than the latter (Baeza et al. 2011; De la Fuente et al. 2004; Guayerbas and De la Fuente 2003).

8.5.2 Obesity Models

Obese subjects show a higher incidence of infections and some types of cancer, suggesting an impaired immune function (Lamas et al. 2002a). In general, the few studies of immunity in obese compared to non-obese subjects of the same chronological age, show a worse immune function, which has been observed in both genetically and diet-induced obese rats (De Castro et al. 2009; Lamas et al. 2002a, 2002b). Moreover, obesity is associated with an inflammatory state (Ye 2011), and immune cells from obese rats show premature immunosenescence, with a decreased proliferation and NK activity with respect to non-obese animals of the same chronological age (De Castro et al. 2009; De la Fuente an De Castro 2012; Lamas et al. 2002a, 2002b) as well as an oxidative stress situation (De Castro et al. 2010).

8.5.3 Alzheimer's Disease Model

The age-related changes in the neuro-endocrine–immune network influence both the progress of ageing and its related diseases such as neurodegenerative disorders. Alzheimer's disease (AD) is the most common of these disorders, with the main pathological hallmarks being the aberrant protein aggregates, amyloid plaques, comprising the amyloid β peptide and neurofibrillary tangles that consist of hyperphosphorylated tau protein. Synaptic and cholinergic deficits, reactive gliosis, an inflammatory profile and an oxidative stress situation as well as psychological symptoms of dementia and the impairment of cognition and behaviour are other neurodegenerative changes. The triple-transgenic mice for AD (3 x Tg-AD) harbouring $PS1_{M146V}$, APP_{Swe} and tau_{P301L} transgenes, represent a unique animal model, which mimics both amyloid and tau AD neuropathologies, besides presenilin overexpression, in an age-dependent manner and in disease-relevant brain regions (Oddo et al. 2003). In this model, the key role of the neuro-immuno-endocrine network in the etiopathogenesis of AD has been shown. Thus, 3 x TgAD mice suffer an age-related impairment at the level of behaviour (lower ability to cope with stressors such as novelty, increased emotionality and anxiety-like behaviours, neophobia and reduced exploratory capacity), endocrine (higher plasma corticosterone levels) and immune parameters (decreased proliferation of lymphocytes and reduced NK cell activity), with males being more affected than females and showing higher mortality rates (Gimenez-Llort et al. 2008, 2012).

8.6 Models of Poor Response to Stress, Anxiety and Depression

It is accepted that an inadequate response to stress is one of the conditions leading to an acceleration of ageing, accompanied by an impaired immune system and other physiological systems. Moreover, the changes in cellular trafficking as well as cell-mediated immunity observed in ageing are similarly found following stress or chronic glucocorticoid exposure (Bauer 2005). Thus, it has been shown that mice with chronic hyper-reactivity to stress and anxiety show a premature ageing of the immune and nervous systems, a higher oxidative stress and a shorter life span (Viveros et al. 2007). These animals show premature ageing, and this model will be explained in more detail later. It has also been observed recently that mice exposed to the stressful condition of isolation have behavioural responses that reveal a certain degree of depression and a more evident immunosenescence than control animals of the same age housed in groups (Arranz et al. 2009b). These animal models show a significant premature immunosenescence and oxidative stress similar to those in human subjects suffering chronic anxiety or depression (Arranz et al. 2007, 2009a).

8.7 A Model of Premature Ageing in Mice Based on an Altered Stress-related Behavioural Response

The lifespan of rodent strains appears to be inversely related to the intensity of their behavioural and neuro-endocrine responses to stressful stimuli in an exploration test (Dellu et al. 1994), and reduced longevity could be caused by an accelerated age-dependent neurodegeneration (Gilad and Gilad 2000). In this context, several studies from our laboratory have shown that inter-individual differences among members of outbred Swiss and inbred BALC/c mouse populations, both male and female, may be related to their behaviour in a simple T-maze test. Moreover, animals which exhibit immobility or "freezing behaviour" (high levels of anxiety) when placed in a new environment, for example the T-maze, fail the test and show a worse immune function than those mice that performed the test correctly (De la Fuente et al. 1998; Guayerbas et al. 2000; 2002a, 2002b, 2002c; Viveros et al. 2001). These animals with premature immunosenescence also have a shorter life span (Guayerbas et al. 2002a, 2002c; Guayerbas and De la Fuente 2003). Thus, a model of premature ageing in mice based on an altered stress-related behavioural response in an exploratory test and a premature immunosenescence was established (Viveros et al. 2007). The studies carried out to characterise this model are discussed below.

8.7.1 Simple T-maze Exploration Test

Mice of the same strain, sex and chronological age are tested individually in a simple T-maze test (Guayerbas et al. 2000). The performance of the spontaneous exploratory

behaviour of each mouse (marked for individual monitoring) is evaluated measuring the time elapsed until the animal crosses the intersection of the three arms with both hind legs. The test has to be performed once a week, for 4 weeks, in order to sort out the non-prematurely ageing mice (NPAM), which complete the exploration of the "vertical" arm of the maze four times in 10 s or less, from the PAM, which required over 10 s. Those animals showing an intermediate response in the T-maze are removed from the study.

8.7.2 Behavioural Characterisation

Behavioural tests have been major components of batteries designed to assess biological ageing in animal and human populations. Moreover, the performance in certain behavioural tests is considered as a marker of neurological ageing (Dellu et al. 1994), which is related to individual longevity (Gilad and Gilad 2000). Different behavioural tests have been carried out with chronologically adult–mature Swiss and BALC/c PAM and NPAM (both sexes) (Guayerbas et al. 2000, 2002a, 2005b; Viveros et al. 2001). In addition, certain behaviour characteristics of PAM, have been also investigated in young mice (Pérez-Álvarez et al. 2005). Our battery of tests provides relevant information about the strength-coordination (tightrope test) and diverse aspects of the adaptive response to stress, emotionality and anxiety (the hole board, the open field and the plus-maze test). PAM have shown an impaired neuromuscular vigour and coordination, a decreased locomotor activity and adaptive response to stressful situations, as well as an increased emotional reactivity and anxiety when compared to NPAM of the same age (the results have been summarised in Table 8.1).

The tightrope test, a method for evaluation of neuromuscular coordination and vigour (Miquel and Blasco 1978), is positively correlated to lifespan in rodents (Ingram and Reynolds 1986). Cross-sectional studies in adult Swiss and BALC/c mice (both sexes), reveal that PAM spend more time in performing this task than NPAM, which shows a decreased neuromuscular coordination and vigour in PAM (Guayerbas et al. 2000; Pérez-Álvarez et al. 2005). Moreover, this neuromuscular capacity decreased with age in both groups of mice, but more markedly in PAM (Guayerbas et al. 2002a). In the hole board and in the elevated plus-maze test, Swiss PAM (both sexes) show an increased grooming frequency, a decreased internal-central ambulation and a lower percentage of entries and time in open-arms in comparison to NPAM, which show an increased emotionality and anxiety. Interestingly, when the animals are submitted to a higher stress situation in the open field (bright white light), PAM show lower motor activity than NPAM, being the PAM females the most affected for external and internal ambulation, and showing the highest grooming frequency. Regarding to the gender differences, female mice (NPAM and PAM) show higher levels of emotionality than males, being more marked in the case of PAM.

Ageing is associated with alterations in neuro-endocrine responses to stress, such as an altered function of the hypothalamus–pituitary–adrenal axis (Orentreich et al. 1984), which is crucial for the regulation of stress and anxiety-related responses.

Table 8.1 Behavioural characterization of adult prematurely and non-prematurely ageing male and female Swiss mice. PAM, prematurely ageing mice; NPAM, non-prematurely ageing mice. Symbols represent statistical differences between the two categories of mice (PAM vs. NPAM), in both (♂) males and (♀) females mice: decreased (↓) $P < 0.05$; (↓↓) $P < 0.01$; increased (↑) $P < 0.05$; or (=) not change. (−) behavioral test not analyzed

Adult Swiss mice	PAM vs. NPAM	
A. Sensorimotor abilities	♂	♀
Thigtrope test (60 s trial)		
Muscular vigour (% mice falling off, latency)	↓↓	↓↓
Motor coordination	↓	↓↓
Traction	↓	↓
B. Exploratory and anxiety-like behaviors	♂	♀
Holeboard test		
External ambulation	=	=
Internal ambulation	↓↓	↓
Total ambulation	↓↓	↓
Central ambulation	↓↓	↓
Grooming	↑	↑
Open field test		
External ambulation (squares entered)	=	↓
Internal ambulation (squares entered)	=	↓↓
Total ambulation	↓↓	↓↓
Central ambulation	=	↓↓
Grooming	↑	↑
Plus-maze test		
% time in open arms	↓	↓
% open-arm entries	↓	↓
Closed-arms entries	↑	↑
Porsolt test		
Immobility	–	↓↓

Interestingly, PAM exhibited an increased baseline corticosterone levels and a blunted stress response when compared to NPAM (Pérez-Álvarez et al. 2005).

All these findings demonstrate that the PAM resemble those behavioural characteristics found in chronologically aged mice and confirm that the T-maze test could provide a simple and fast approach for the determination of murine biological age (Viveros et al. 2001).

8.7.3 Characterization of Monoaminergic Systems

There is an evidence indicating that ageing is accompanied by some alterations in the neurotransmission systems. In aged rodents, many studies have shown reductions of the levels of neurotransmitters and of the activities of the enzymes involved in their synthesis, as well as an age-related behavioural impairment, which is the result of dysfunction in the neurotransmission, but not the loss of neurons of the Central nervous system (CNS) (Magnone et al. 2000). Thus, with the aim to provide a neurochemical characterization of PAM, a comparative study with NPAM regarding their noradrenergic, serotonergic and dopaminergic systems were carried out in discrete brain regions, which are relevant for the behavioural responses, such as hypothalamus, hippocampus, striatum, frontal cortex and midbrain. For this purpose, the

levels of noradrenaline (NA), serotonin (5-HT), dopamine (DA) and their respective metabolites (3-methoxy-4-hydroxyphenyl glycol (MHPG), 5-hydroxyindol-3-acetic acid (5-HIAA) and 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA), respectively) were analysed in the brains of the same animals (males and females) used in the previous behavioural study (Viveros et al. 2001).

The results (De la Fuente et al. 2003) showed that PAM of both sexes show decreased NA levels in hippocampus, frontal cortex and midbrain in comparison to NPAM. Although the data on the sensitivity of noradrenergic systems to the ageing process are very heterogeneous, a decrease in the NA content of at least some brain regions, mainly in hippocampus has been proposed. PAM of both sexes also show decreased levels of 5-HT in hippocampus, striatum, midbrain and hypothalamus in comparison to NPAM, as well as an increase in the turnover rate (5-HIAA/5-HT) in hypothalamus and in hippocampus. The influence of age on the levels 5-HT in different strains of rodents have shown different results, but several studies show decreased levels of 5-HT and an increased 5-HIAA/5-HT ratio in several brain regions (Lee et al. 2001). Since there is abundant evidence about the involvement of the serotonergic system in the modulation of anxiety, the altered emotional responses in PAM, which present higher levels of emotionality and anxiety, could be related with these altered serotonergic indices. With respect to the dopaminergic system, the majority of the studies have shown a decrease of DA levels and its metabolites (DOPAC and HVA), as well as an increase of the DA turnover rate (DOPAC/DA and HVA/DA) with ageing. These alterations are in accordance with the majority of the changes found in the dopaminergic system of PAM, which (in both sexes) show a marked reduction in the DA content in most brain regions analysed (hypothalamus, hippocampus, striatum and frontal cortex), as well as a decrease in the levels of HVA in the hypothalamus and striatum, whereas in the hippocampus decreased levels of DOPAC and HVA are only observed in female PAM. The turnover rates (DOPAC/DA and HVA/DA) of PAM (both sexes) are either increased (hippocampus and frontal cortex) or unchanged with respect to NPAM, depending on the brain region analysed (De la Fuente et al. 2003). An age-related decrease in the levels of DA and its metabolites in striatum, as well as a correlation between this fact and diminished motor function in aged rodents have been observed. Thus, it is likely that the altered dopaminergic indices in PAM, particularly in striatum, are related to the impaired motor function, such as neuromuscular vigour and coordination in the tightrope test, and the decreased locomotor activity in three standard behavioural tests (Viveros et al. 2001) observed in these animals when compared to NPAM.

In spite of the sex differences in the age-related changes in the noradrenergic, serotonergic and dopaminergic systems, which depend on the brain region analysed, in most cases the differences between PAM and NPAM involve both sexes, with the exception of the hypothalamus, a typically sexual dimorphic area, where some differences only affect the male mice. In conclusion, the neuro-chemical modifications found in the monoaminergic systems in brain regions of PAM, which involve both sexes, clearly resemble some of the alterations reported for ageing animals, and this brain neurochemistry characteristic of older animals seems to be related to the

Table 8.2 Comparative changes in the level of monoamines and their respective metabolites from noradrenergic, serotonergic and dopaminergic systems in different brain regions (hippocampus, hypothalamus, striatum, frontal cortex and midbrain) of non-prematurely and prematurely ageing adult Swiss mice, both males and females. PAM, prematurely ageing mice; NPAM, non-prematurely ageing mice; NA, noradrenaline; MHPG, 3-methoxy-4-hydroxyphenyl; 5-HT, serotonine; DA, dopamine; DOPAC, 3,4-dihydroxyphenylacetic acid; HVA, homovanillic acid; 5-HIAA, 5-hydroxyindol-3-acetic acid. Symbols represent statistical differences between the two categories of mice (PAM vs. NPAM), in both (♂) males and (♀) females mice: decreased (↓) $P < 0.05$; (↓↓) $P < 0.01$; (↓↓↓) $P < 0.001$; increased (↑) $P < 0.05$; (↑↑) $P < 0.01$; (↑↑↑) $P < 0.001$; (=) or no change

Adult Swiss mice	PAM vs. NPAM	
	♂	♀
Hippocampus		
NA	↓	↓
MHPG	=	=
5-HT	↓↓↓	↓↓↓
HIAA/5-HT	↑↑↑	↑↑↑
DA	↓↓↓	↓↓↓
DOPAC	=	↓
HVA	=	↓
HVA/DA	↑↑	↑↑
DOPAC/DA	↑	=
Hypothalamus		
NA	↓↓	↓↓
5-HT	↓	↓
DA	↓	↓
Striatum		
NA	=	=
5-HT	↓↓↓	↓↓↓
DA	↓	↓
Frontal cortex		
NA	↓	↓
5-HT	=	=
DA	↓↓↓	↓↓↓
Midbrain		
NA	↓	↓
5-HT	↓	↓
DA	=	=

behavioural features found in PAM (De la Fuente et al. 2003). All these results on the monoaminergic systems have been summarised in Table 8.2.

8.7.4 Characterization of Immune Functions

Ageing has been associated with immunological changes, which presently are considered as good markers of health, biological age and longevity (De la Fuente and

Miquel 2009; Wikby et al. 2008). Moreover, the immunological changes observed in healthy ageing may be closely related to psychological stress, which may lead to an earlier onset of ageing-related diseases and premature ageing. Indeed, there is an evidence suggesting a link between chronic psychological stress and impaired immune function (Bauer 2008). In this context, preliminary studies showed a relation between the T-maze performance and the functions of immune cells from old and adult Swiss mice, animals with slow performance showing a less competent immune system (De la Fuente et al. 1998; Correa et al. 1999). This immunosenescence of PAM was confirmed after performing several investigations on a wide range of functions in immune cells from peritoneum, thymus, spleen and axillary nodes from PAM and NPAM of different strains (Swiss and BALB/c), chronological ages (young, adult, mature and old), both sex as well as in cross-sectional and longitudinal studies (Table 8.3). In fact, the results show, in general, an increased macrophage and lymphocyte adherence in PAM compared with NPAM, which suggests an impaired capacity of these cells to move to the infectious focus, this function increasing with ageing. Moreover, the spontaneous mobility and chemotaxis capacity of macrophages and lymphocytes, the phagocytic capacity and bacteriocidal activity of macrophages, the lymphoproliferative response to mitogens (Con A and LPS), the IL-1β and IL-2 release as well as the NK activity, which are the functions that decrease with ageing, are lower in PAM in comparison to NPAM (Alvarado et al. 2005, 2006b; Alvarez et al. 2006; De la Fuente 2010; Guayerbas et al. 2002a, 2002b, 2002c; 2005a; Guayerbas and De la Fuente 2003; Puerto et al. 2002; Viveros et al. 2001). Moreover, young PAM are more susceptible than NPAM to the effect of an acute stress (which includes forced swim) on the mitogen-induced lymphoproliferative responses, decreasing this activity (Pérez-Álvarez et al. 2005). The results on the immune functions in PAM versus NPAM are shown in Table 8.3.

Considering the relevance of optimal immune functions for successful ageing, the present data justify the view that PAM have worst preserved immune functions compared to NPAM, and they show values more similar to those of older animals. Since the immune functions studied have been proposed as markers of health and biological age, the impairment in these functions observed in PAM could play a central role in the shorter life span of these animals. The above-mentioned facts demonstrate the premature immunosenescence of PAM and support the increasing evidence on the key role played by the immune system in premature ageing (De la Fuente 2008; De la Fuente and Miquel 2009).

8.7.5 Inflammatory and Oxidative Stress

Progressive dysregulation of immune responses associated with ageing may be a result of increased oxidative stress (De la Fuente et al. 2005). In view of the link between oxidative and inflammatory stress and the ageing process, several oxidant and pro-inflammatory compounds, as well as antioxidant defences, have been evaluated in the model of premature ageing in mice, in order to characterize redox state

Table 8.3 Changes in immune functions in (A) peritoneal leukocytes (macrophages, lymphocytes and natural killer cells) from chronologically young, adult and old Swiss female mice and in (B) axillary nodes, spleen and thymus leukocytes from adult and old female Swiss and BALC/c mice, both in prematurely and non-prematurely ageing mice. PAM, prematurely ageing mice; NPAM, non-prematurely ageing mice. Symbols represent statistical differences between the two categories of mice (PAM vs. NPAM), in young, adult and old Swiss and BALC/c mice: decreased (↓) $P < 0.05$; (↓↓) $P < 0.01$; (↓↓↓) $P < 0.001$; increased (↑) $P < 0.05$; (↑↑) $P < 0.01$; or (=) no hange. (*) decreased (↓) $P < 0.05$ in BALC/c mice; (**) decreased (↓↓) $P < 0.01$ in BALC/c mice. (−) not analyzed

A) Peritoneal leukocytes	PAM vs. NPAM		
Chronological age	Young	Adult	Old
Macrophage functions			
Adherence capacity	–	↑↑	↑↑
Chemotaxis	↓↓↓	↓↓↓	↓↓
Phagocytosis capacity	=/↓*	↓↓	=/↓↓*
Intracellular O_2 anion levels	↓	↓↓↓	↓
Intracellular ROS levels	↓↓	↓	↓
IL-1ß release	–	↓	↓
Lymphocyte functions			
Adherence capacity	–	↑	↑
Chemotaxis	↓↓↓	↓↓↓	↓↓
Proliferation to Con A (%)	↓↓	↓↓	↓
IL-2 release	↓↓	↓	=
Natural Killer (NK) functions			
NK activity (lysis %)	↓↓↓	↓	↓

B) Leukocytes	PAM vs. NPAM					
Chronological age	Axillary nodes		Spleen		Thymus	
	Adult	Old	Adult	Old	Adult	Old
Swiss mice						
Chemotaxis	↓↓	↓	=	↓	=	=
Basal proliferation (cpm)	–	=	–	↓↓	–	↓↓
Proliferation to Con A (%)	↓↓	↓	↓↓	↓↓↓	↓↓	↓↓↓
NK activity (lysis %)	↓↓	=	↓	=	=	↓
IL-2 release	↓↓↓	↓	–	–	–	–
BALC/c mice						
Chemotaxis	=	↓↓	↓↓	↓↓	↓↓	=
Basal proliferation (cpm)	=	=	–	=	–	=
Proliferation to Con A (%)	↓↓	↓↓	↓↓	↓↓	↓	↓
NK activity (lysis %)	↓	↓	↓	↓↓↓	↓↓	↓↓↓
IL-2 release	↓↓	↓	–	–	–	–

Table 8.4 Comparative changes in several oxidative stress parameters in peritoneal leukocytes from prematurely versus non-prematurely ageing female Swiss mice in young and adult mice. PAM, prematurely ageing mice; NPAM, non-prematurely ageing mice. Symbols represent statistical differences between the two categories of mice (PAM vs. NPAM): decreased (↓) $P < 0.05$; (↓↓) $P < 0.01$; (↓↓↓) $P < 0.001$; increased (↑) $P < 0.05$; (↑↑) $P < 0.01$; (↑↑↑) $P < 0.001$; or (=) no change. (*) increased (↑) $P < 0.05$ in old Swiss mice; (**) increased (↑↑↑) $P < 0.001$ in BALC/c adult mice; (***) increased (↑) $P < 0.001$ in old Swiss mice; (–) not analyzed.

Peritoneal leukocytes	PAM vs. NPAM	
	Young	Adult
Oxidant/Pro-inflammatory compounds		
Extracellular superoxide anion levels	↑↑	↑/↑*↑/↑↑↑**
Nitric oxide (NO) levels	↑↑	↑
Oxidized glutathione (GSSH)	↑	↑↑
Oxidized/reduced glutathione (GSSH/GSH)	↑↑↑	↑↑↑
Prostaglandin E2 (PGE2)	↑	↑↑
Xanthine oxidase (XO) activity	–	↑↑
Tumor necrosis factor alpha (TNF-α) levels	↑	↑/↑***
Antiaoxidant Defenses		
Reduced glutathione (GSH) levels	↓↓	↓↓
Glutathion peroxidase (GPx) activity	=	↓↓
Glutathion reductase (GR) activity	↓↓↓	–
Superoxide Dismutase (SOD) activity	↓↓↓	↓
Catalase (CAT) activity	↓	↓
Oxidative Damage		
Malondialdehyde (MDA) levels	↑↑	↑
8oxo-7,8dihydro-2deoxiguanosine (8oxodG)	–	↑↑

of PAM in comparison to NPAM. These parameters of the oxidative and pro-inflammatory stress status have been studied in leukocytes (from peritoneum, axillary nodes, spleen and thymus), as well as in other tissues of Swiss and BALC/c PAM and NPAM of different chronological ages (Alvarado et al. 2006a, 2006b; Guayerbas et al. 2002b; Viveros et al. 2007). In general, PAM show higher levels of oxidant and pro-inflammatory compounds as well as decreased levels of antioxidant defences in comparison to NPAM (results summarised in Table 8.4).

Regarding pro-inflammatory cytokine release, such as tumor necrosis factor-α (TNF-α) and prostaglandin E2 (PGE2), in Swiss female PAM increase more than in NPAM. Oxidants such as extracellular superoxide anion, nitric oxide (NO), oxidized glutathione (GSSG) and the GSSG/GSH ratio (a marker of oxidative stress situation) in young and middle-aged peritoneal leukocytes of female Swiss PAM, show higher levels than in those of NPAM (Alvarado et al. 2006a, 2006b). In addition, these leukocytes from PAM also show, in comparison to NPAM, a decrease in reduced glutathione levels (GSH), a key non-enzymatic antioxidant, as well as in the enzymatic antioxidant defences, namely superoxide dismutase, catalase, glutathione peroxidase and reductase activities which are the main antioxidant enzymes that eliminate the excess of reactive oxygen species (ROS). Therefore, PAM show oxidative stress in their leukocytes, which induces oxidative damage of biomolecules

Table 8.5 Xanthine oxidase (XO) activity (mU XO/mg protein) in different tissues (cerebral cortex, liver, spleen and kidney) from chronologically mature prematurely (PAM) and non-prematurely (NPAM) ageing female BALC/c mice. Data are mean and standard deviation for eight animals. Statistical differences between the groups were analyzed by the *Student's t* test. * $P < 0.05$; ** $P < 0.01$ with respect to the corresponding values in NPAM

Xanthine Oxidase (XO) activity (mU XO/mg protein)		
Tissue	BALC/c mature mice	
	NPAM	PAM
Cerebral cortex	29.83 ± 8.24	34.20 ± 6.39*
Spleen	4.78 ± 1.67	7.25 ± 3,58
Liver	54.36 ± 9.60	65.69 ± 10.9**
Kidney	9.53 ± 1.59	11.74 ± 1,69*

such as lipids and DNA, a characteristic of ageing, and contributes to the inappropriate function of these cells (De la Fuente and Miquel 2009). In fact, the oxidative stress damage to lipids and nuclear DNA measured by malondialdehyde (MDA) and 8-oxo,7,8-dyhidro-2′-deoxyguanosine (8-oxodG) levels, respectively) in peritoneal leukocytes from adult and young Swiss PAM are higher than those found in cells from NPAM (Alvarado et al. 2006a, 2006b). Moreover, an imbalance between oxidant and antioxidants, leading to an oxidative stress situation, has been observed in several tissues such as brain, liver, heart and kidney in Swiss and BALB/c PAM with respect to those in NPAM (Viveros et al. 2007).

All these findings demonstrate that the PAM suffer a situation of oxidative and inflammatory stress in their leukocytes and tissues that are characteristic of mice of an older chronological age. Because oxidative stress may lead to loss of homeostasis in immune cells, all these findings could explain the immunosenescence exhibited by PAM. Moreover, as mentioned above, it has been demonstrated that the PAM have a shorter life span than NPAM (Guayerbas et al. 2002a, 2002c; Guayerbas and De la Fuente 2003). Therefore, all these facts confirm that PAM are biologically older, at the same chronological age, than NPAM (Viveros et al. 2007).

8.7.6 Xanthine Oxidase in PAM and NPAM

Xanthine oxidase (XO), an enzyme characterized by its generation of ROS, such as superoxide anion and hydrogen peroxide, which increase in tissues and leukocytes of older mice (Arranz et al. 2010a; Vida et al. 2009, 2011), shows higher activity in cerebral cortex, liver and kidney of mature BALC/c PAM than in NPAM (Table 8.5). This increase in XO activity was also found in peritoneal leukocytes of adult PAM (in Swiss and BALC/c mice) than of NPAM (Table 8.4), being more evident in phagocytes than in lymphocytes (unpublished data). This adds support to the suggestion that phagocytes are the immune cells most involved in the ageing rate of organisms (De la Fuente and Miquel 2009).

8.8 Conclusion

The use of animal models, especially the PAM model, may be particularly useful to assess the relationships between a diminished ability of coping with stressful situation, immunosenescence, a chronic oxidative stress, an accelerated biological age and a shorter longevity.

Acknowledgments The authors would like to thank Dr. Miquel for his critical revision of the article. This work was supported by grants of the MICINN (BFU2011-30336), Research Group of UCM (910379ENEROINN) and RETICEF (RD06/0013/0003) (ISCIII).

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