Chapter 1

GENERAL INTRODUCTION
1 Aging and neurodegeneration

Aging can be defined as a multifactorial degenerative process resulting from the organism’s progressive loss of the ability to maintain homeostasis and less efficient ability to adapt to change. The impact of this age-related loss of adaptation is far more disruptive when integrative homeostatic systems are also affected. These systems include the autonomic nervous system and the neuroendocrine hypothalamic-pituitary-adrenal (HPA) axis. Their amine, peptide and steroid signals communicate specific information to ensure coordination of brain and body functions during daily and sleep-related events as well as in response to stressors. Such coordination involves the integration of emotions, cognitive processes and other central functions with energy metabolism, cardiovascular, inflammatory and immune regulations. In mammals and other vertebrates, aging in healthy individuals is therefore associated with a progressive decrease in the functionality of physiological, metabolic and reproductive systems compromising coping with environmental stressors and increasing vulnerability to stress-related disease. Thus, features like menopause or the decline in renal function intrinsically pertain to the physiology of aging, while others, like coronary artery disease, represent pathophysiological traits since they are age-related diseases not always present in aging individuals (Troen, 2003).

Many and different theories exist (around 300) investigating different aspects of the aging process. Nevertheless these theories can be roughly divided into two categories: those that answer the question “Why do organisms age?” and those that address the question “How do organisms age?” Only a few broad and overarching theories attempt to explain why nearly all living organisms age. These theories often compete with each other. Over time, some theories have fallen out of favor as others have become more widely accepted. As for the mechanisms, a number of hypotheses have been put forward to explain how we and other species age; likely, each of them, has elements of validity. Testing these hypotheses and investigating the mechanisms that underlie or affect the aging process is the current pursuit of most aging research.

Scope of the present research was to investigate the contribution of the p66Shc gene, and thus of oxidative stress, to behavioral and neuroendocrine regulations underlying the aging process.

1.1 Why do we age: an antagonistic pleiotropy theory of aging

Aging seems to be a paradox from an evolutionary perspective because it is a deleterious process. In the nineteenth century, Alfred Russel Wallace (1823-1913) proposed that aging and subsequent dying occurs to the benefit of the species namely because of the death of old individuals would free up resources for subsequent generations. This comforting scenario of aging as a natural virtue was later shown to be unsustainable by evolutionary biologists, since natural selection very rarely acts for the good of the species if it conflicts with selection on individuals. In fact, selection on individuals to produce more offspring is much more powerful than the selection on them to refrain from doing so for the good of their species. The idea that aging
evolved as a device to prevent old individuals from further reproduction so as to make way for the young is therefore theoretically unsound (Williams, 1957). In the 1940s J. B. S. Haldane studying Huntington disease (a genetic disease that causes severe mental illness and death) was puzzled by two unusual features: patients die later in life, usually in their 30s or 40s, and the mutation that causes the disease is dominant rather than recessive (Haldane, 1956; 1959). Thus, he first observed that dominant lethal mutations can be maintained in a population if their effects are delayed until after reproduction. Thus the evolutionary theory of aging started to take place around the idea that aging itself is the result of mutations that strike very late in life, at ages beyond the action of natural selection. These concepts were later formalized in the 1980s, in a mature evolutionary theory of aging. Two key theories had emerged: the mutation-accumulation theory and the antagonistic pleiotropy, or trade-off, theory (see BOX 1).

In light of these evolutionary theories, it can be stated that aging has evolved as a late-onset genetic disease that affects all organisms. The evolutionary theories of aging give a clear but stark picture of the biological function of aging: probably it is merely a non-adaptive epiphenomenon. Although many scientists believe the evolutionary theory of aging needs further refinement, most agree that this is currently the best explanation for why we and other organisms age.
Mutations that have deleterious effects on older organisms have a greater chance of escaping from natural selection. This leads to two theoretical predictions:

1) Mutation-accumulation theory

Mutations with a later age of onset will be able to reach a higher frequency in the population under mutation–selection balance. They will enter the population in accordance with their mutation rate, and selection will remove them with decreasing efficacy the later their age of onset. Aging can then occur as a result of the greater accumulation of mutations with deleterious effects later in life.

2) Pleiotropic antagonism or trade-off theory

Mutations that produce beneficial effects early in life, but that later increase the rate of aging can be incorporated into a population because natural selection will act more strongly on the early beneficial effect than on the later deleterious one. For example, a hypothetical mutation that promotes calcium deposition might accelerate bone growth and increase fitness early in life, but then might lead to hardening of the arteries later in life.

Both the mutation-accumulation and the trade-off theories for the evolution of aging have been subjected to extensive empirical testing, mainly in *Drosophila melanogaster*. These tests have all relied on either examining the properties of naturally existing genetic variation in fly populations, or the properties of new mutations as they occur.

The balance of experimental evidence is not strongly in support of the mutation accumulation as a significant mechanism for the evolution of aging. By contrast, pleiotropy/trade-offs and, in particular, a time-lagged cost of reproduction, is believed to be an important general mechanism for the kind of delayed genetic effect that will lead to the occurrence of aging (Gems et al., 2002).
1.2 How do we age: mechanisms of aging

In complex multicellular organisms, different causes of aging may rely on molecular, cellular and even systemic mechanisms. Thus, theories of aging may overlap at various levels of organization: alterations with aging of molecular events may lead to cellular alterations, and these, in turn, contribute to organ and systemic failure eventually with evolutionary implications for reproduction and survival. In this context, the study of interactions among intrinsic (genetic), extrinsic (environmental), and stochastic (random damage to vital molecules) causes provides a fruitful approach for a comprehensive and realistic understanding of the aging process.

In the following section for each group of theories (molecular - 1.2.1 -, cellular - 1.2.2 - and systemic - 1.2.3 -), the most representatives will be described and discussed. Section 1.2.4 provides an evolutionary/environmental explanation for a number of risk factors involved in the aging process.

1.2.1 Molecular theories: the gene regulation theory of aging

This theory proposes that senescence results from changes in gene expression (Kanungo, 1975). The evolutionary data imply that the same genes that enhance fitness by a pleiotropic mutation can cause a latent damage that takes time to emerge. This theory however does not specify the mechanisms generating the damage and the length of time between its first occurrence and the death of the organism. An approach to this problem points at identifying genetic modifiers of the rate of aging by generating mutants with increased lifespan. This approach during the past decade has allowed rapid progress in the field of aging research by isolating long-lived mutants in *Saccharomyces cerevisiae*, *Caenorhabditis elegans*, *Drosophila melanogaster* and mice (Bartke et al., 2001; Gems and Partridge, 2001; Kenyon, 2001). The longevity of the mutants has been found to be mostly mediated by genes, the products of which act in diverse ways, including modulating the stress response, sensing nutritional status, increasing metabolic capacity, and delaying reproductive age. In this context it is worth noticing that recent evidence points to genes involved in central nervous control of longevity pathways both in invertebrates and mammals (hypothalamic genes) (Bishop and Guarente, 2007). Interestingly, caloric restriction has been shown to reduce the rate of aging in many organisms (ranging from yeast to mammals) probably affecting similar or coincident pathways regulated by those genes that modulate longevity (for details on caloric restriction see paragraph 5.1.2).

1.2.2 Cellular theories: telomere shortening and oxidative stress (free radical)/mitochondrial DNA theories of aging

*Cellular senescence theory of aging*

This theory was formulated in 1965 when cell senescence was described as the process limiting the number of cell divisions that normal human cells can undergo in
culture (Hayflick, 1965). This “limit in replicative capacity” occurs after a characteristic number of cell divisions and results in terminally arrested cells with altered physiology (Campisi, 1996).

Replicative senescence is a specific type of cellular senescence that ultimately results from loss of telomeres (specialized structures composed of a repeating DNA sequence and located at the ends of each linear chromosome; see Blackburn, 2000). With each cell division, a small amount of DNA is necessarily lost at each chromosome end, resulting in ever-shorter telomeres, altered telomere structure, and eventual replicative senescence (Blackburn, 2000). Initial experiments with cells in culture showed a correlation between replicative potential and donor age, suggesting that cells from older individuals have a more limited capacity for further cell divisions. Similarly, organisms with short lifespan have cells that age more rapidly than organisms with longer lifespan. However, recent experiments have cast considerable doubt on these observations (Blackburn, 2000; Rubin, 2002; Wright and Shay, 2002). Experiments in rodents, have provided little support for this idea: gene targeting experiments have shown that telomerase-deficient mice do not age rapidly; in fact, overt phenotypes are not observed for several generations (Blasco et al., 1997; Lee et al., 1998).

An interesting model suggests that telomere shortening can promote tumorigenesis by enhancing genome instability: telomere-induced genome crisis leads to cell transformation, which is followed by telomerase activation to allow for unlimited cell proliferation (Maser and DePinho, 2002). Consistently, patients suffering by Dyskeratosis congenita (Mitchell et al., 1999) have an increased incidence of carcinomas, suggesting that telomere shortening may contribute to the development of cancer with aging (Wong and Collins, 2006).

**Oxidative stress (free radical)/mitochondrial DNA theory of aging**

Harman initially proposed that most aging changes are due to molecular damage caused by free radicals (Harman, 1956; 1981), which are atoms or molecules that contain an unpaired electron and are therefore highly reactive.

The free-radical or oxidative-stress hypothesis is one of the most accepted theories of aging. It states that oxidizing species are produced during aerobic metabolism, which consequently causes molecular damages and, over time, cell and tissue dysfunctions, ultimately increasing the risk of diseases. This theory is supported by a vast body of experimental evidence demonstrating that: aerobic organisms chronically generate powerful pro-oxidant species (reactive oxygen species; ROS); cells accumulate oxidative damage over time (oxidative stress); ROS induce cell senescence and apoptosis; and finally, ROS, senescence and apoptosis are mechanistically linked to enhanced vulnerability for aging-associated degenerative diseases (Harman, 1998). It is generally thought that ROS are generated accidentally as by-products of the aerobic metabolism and mitochondria are the major source of intracellular ROS. The accumulation of ROS in cells leads to various forms of reversible and irreversible
oxidative modifications of proteins (carbonylation or nitro-modifications), lipids (hydroperoxide lipid derivatives) and DNA (adducts and breaks) that eventually lead to loss of molecular functions. Cells can normally defend themselves against ROS damage through the use of specific enzymatic or non-enzymatic ROS reducing mechanisms. Thus, the levels of different ROS are buffered to regulate the intracellular redox balance at the same time avoiding the excessive oxidation of cellular components. A large body of experimental data indicates that intracellular oxidative stress increases during aging. This is in part due to the fact that intracellular ROS scavenging decreases progressively across life. However, it is still controversial whether ROS production increases in parallel during aging.

ROS contribute significantly also to the somatic accumulation of mitochondrial DNA (mtDNA) mutations, leading to the gradual loss of bioenergetic capacity and eventually resulting in aging and cell death. Mitochondrial DNA is subject to a much higher mutation rate than nuclear DNA. This is due, in large part, to the lack of histones and DNA-binding proteins to protect it, inefficient repair mechanisms and the proximity to the mitochondrial membrane where ROS are generated. Thus, oxidative modification and mutation of mtDNA occur with great ease, and the extent of such alterations increases exponentially with age.

Interestingly, defects in mitochondrial respiration with age are found not only in normal tissues (Trounce et al., 1989), but also in people with diseases that are increasingly manifest with age, such as Parkinson’s disease (Schapira et al., 1990a; Schapira et al., 1990b), Alzheimer’s disease (Hoyer, 1986), (Sims et al., 1987), Huntington’s chorea (Beal, 1994), and other movement disorders (Schulz and Beal, 1994).

1.2.3 System-based theories of aging: neuroendocrine and inflammaging theories of aging

In these theories, the aging process is related to the decline of the organ systems essential for: 1) the control and maintenance of other systems within an organism; and: 2) the ability of organisms to communicate and adapt to the environment in which they live. In humans, all systems may be considered indispensable for survival. However, the nervous, endocrine, and immune systems play a key role by their ubiquitous actions in coordinating all other systems and in their interactive and defensive responsiveness to external and internal stimuli.

Neuroendocrine theory of aging

An important component of this theory is the notion that the hypothalamic-pituitary-adrenal (HPA) axis, which is involved in the regulation of the stress responses, is the master regulator, the “pacemaker” that signals the onset and termination of each life stage. One of the major functions of the HPA axis is to master the physiological adjustments necessary for preservation and maintenance of the internal “homeostasis” (steady state) despite the continuing changes in the environment (Cannon, 1932). During lifespan, chronic exposure to severe stress from a multitude of physical, bio-
logical, or emotional stimuli may exhaust or weaken the capacity to adapt and lead to the so-called “diseases of adaptation” and death (Selye, 1936). Aging would then result from “a decreasing ability to survive stress”, one of the many definitions of aging that suggests a close relationship between stress and longevity.

**Inflammaging**

The ensemble of oxidative and inflammatory processes characterizing aging has been defined by Finch as a ‘gero-inflammatory manifold’, which may underlie Alzheimer’s disease and many other age-related neurodegenerative disorders (Finch, 2003; Finch and Longo, 2000; Finch et al., 2002).

A large part of the aging phenotype, including immunosenescence, can be explained by an imbalance between inflammatory and anti-inflammatory networks, which result in a low grade chronic pro-inflammatory status. Franceschi proposed to call it “inflammaging” (Franceschi, 2007). Inflammation is necessary to cope with damaging agents and is crucial for survival. However, chronic exposure to a variety of antigens, especially viruses, induces a chronic low-grade inflammatory status that contributes to age-associated morbidity and mortality. Within this perspective, healthy aging and longevity are likely the result not only of a lower propensity to mount an inflammatory response, due to a better control of neuroendocrine function, but also of efficient anti-inflammatory networks, which in normal aging fail to fully neutralize the inflammatory processes consequent to the lifelong antigenic burden and exposure to damaging agents. Centenarians are unique in that, despite high levels of pro-inflammatory markers, they also exhibit high levels of anti-inflammatory markers that may delay disease onset. Thus, the key to successful aging and longevity is to decrease chronic inflammation without compromising an acute response to pathogens. Franceschi and colleagues therefore proposed that “inflammaging” could be flanked by “anti-inflammaging” as major determinants not only of immunosenescence but eventually of global aging and longevity (Franceschi et al., 2007).

**1.2.4 The “thrifty hypothesis”**

Low birth weight has been strongly associated with higher risk of developing type 2 diabetes and related conditions, including dyslipidaemia, hypertension and cardiovascular disease, a whole of systemic risk factors to the aging process (Hales et al., 1997). This association has provided the ground for a particularly heated ‘nature-nurture’ debate on the etiology of diabetes: the thrifty genotype-phenotype hypotheses.

The thrifty gene hypothesis was proposed in 1962 by geneticist James V. Neel to explain the tendency of certain ethnic groups, such as Native Americans, towards diabetes (Neel, 1962). In the earliest formulation of the thrifty genotype hypothesis, Neel proposed that the exploding prevalence of diabetes amongst societies undergoing rapid Westernization resulted from selection for metabolically-thrifty genes. These may have enhanced survival during prehistory but can predispose to diabetes in the western society, given dietary abundance and a sedentary lifestyle. In 1992 Hales &
Barker proposed the thrifty phenotype hypothesis as an explanation for the association between early developmental factors and later disease (Hales and Barker, 1992). According to the original model, the organism adapts to poor nutrition in early life by programming its insulin metabolism to expect a similar environment subsequently. If the subject continues to be exposed to a relatively poor environment, the organism will be well adapted but if exposed to a richer diet, the programmed trait would become inappropriate. When it was proposed, the thrifty phenotype hypothesis made a significant contribution, by arguing that the pathway to certain adult diseases could be environmental rather than genetic. In their original paper, for example, Hales and Barker argued that the thrifty genotype hypothesis of Neel (1962) was less successful at explaining the incidence of type 2 diabetes in adult life. In a revised form, the thrifty genotype hypothesis proposes that insulin resistance may have been beneficial during periods of starvation due to its ability to maintain nutrition of the brain without degrading body protein stores, thus leading to natural selection of the underlying “diabetes genes” (Reaven, 1998).

Rather than being contradictory, the thrifty phenotype and thrifty genotype hypotheses are best seen as complementary. The thrifty genotype hypothesis can account for selection over many generations, and hence population differences in susceptibility to diabetes, whereas the thrifty phenotype hypothesis is relevant to adaptation within an individual’s lifespan (Lindsay and Bennett, 2001).

2 Oxidative stress

Oxidative stress is a common state characterizing biological systems in aerobic conditions derived from an imbalance between pro-oxidative and anti-oxidative molecules where the oxidants override defensive systems. Oxidative molecules, namely ROS, are produced primarily by the physiological metabolism of \( \text{O}_2 \) in cells (Kakkar and Singh, 2007). In addition, environmental stimuli such as cytokines, ultraviolet radiation, chemotherapeutic agents, hyperthermia and even growth factors generate high levels of ROS that can perturb the normal redox balance and shift cells into a state of oxidative stress (Finkel and Holbrook, 2000). ROS are highly reactive and may damage cellular structures, undermining or disrupting their biological functions and properties. A well known free-radical theory of aging originally implied that the targets of ROS were random, indiscriminate and cumulative. Oxidants may certainly function stochastically, however accumulating evidence implicates ROS as specific signaling molecules under both physiological and pathophysiological conditions. In this context the generation of ROS, within certain boundaries, is essential to maintain homeostasis. For example, ROS generation by phagocytic cells constitutes an essential host defense mechanism necessary to combat infections. Likewise, cytosolic ROS produced in response to stimulation by growth factors are involved in regulating proliferative responses (Finkel and Holbrook, 2000).

Regardless of how or where they are generated, a rise in intracellular oxidant levels has two potentially important effects: damage to various cellular components and
triggering the activation of specific signaling pathways. Mitochondrial respiration represents the major source of intracellular ROS and, under certain situations of metabolic stress, even mitochondrial-derived oxidants seem to function as signaling molecules (Nemoto et al., 2000; Nishikawa et al., 2000). Both of these effects can influence numerous cellular processes linked to aging and the development of age-related diseases (Finkel and Holbrook, 2000). Oxidative stress has been implicated in various pathological conditions such as cardiovascular disease, cancer, neurological disorders, diabetes, ischemia/reperfusion, to mention only a few (Dalle-Donne et al., 2006; Dhalla et al., 2000; Jenner, 2003; Sayre et al., 2001). Furthermore, ROS and oxidative stress have been found to be involved in the neuropathology of several neurodegenerative disorders associated with cognitive impairment (Finkel and Holbrook, 2000). Numerous reports have emphasized a clear association between enhanced neuroinflammatory processes, characterized by enhanced production of ROS, and neurodegenerative diseases (Finch and Marchalonis, 1996; Hensley et al., 1999; O’Banion and Finch, 1996; Zhu et al., 2001a; Zhu et al., 2001b). In particular, neuroinflammatory processes have been associated with the induction of the transcription of the inducible nitric oxide (iNOS) gene leading to the formation of a huge amount of nitric oxide that, together with its oxidation products (peroxynitrite and other species), is highly toxic to neurons (Floyd, 1999b). A series of observations made on rats showed that iNOS, nitrotyrosine, and apoptotic index were all increased in the brain regions examined in aged subjects compared to young ones (Ferrini et al., 2001; Kandeel et al., 2001; Vernet et al., 1998). Thus, it has been strongly suggested that enhanced neuroinflammatory processes and oxidative stress occur in the aging brain and that these processes exert dramatic effects on brain regions having important biological functions.

The mammalian brain is generally characterized by poor antioxidant defenses, high metabolic rate, and reduced capacity for cellular regeneration resulting particularly susceptible to oxidative stress insults (Floyd and Hensley, 2002). Floyd and Hensley extensively reviewed the main features that render the brain such a vulnerable organ: (a) the brain has a high content of easily peroxidizable unsaturated fatty acids; (b) it requires very high amounts of oxygen per unit weight (about 20% of the total amount used in humans); (c) it has a high content of both Fe and ascorbate (which are key elements in causing membrane lipid peroxidation); and (d) it is not highly enriched in antioxidant protective defenses and this then adds to its otherwise readily poised potential for oxidative damage (Floyd, 1999a).

The fact that the brain utilizes almost exclusively glucose as its energy source probably explains why it consumes very high levels of oxygen per unit weight. It is widely considered that the major portion (about 95–98%) of the total ROS produced during aerobic metabolism comes from by-products of the electron transport chain of mitochondria (Floyd and Hensley, 2002). Accurate studies of isolated mitochondria from brain and other organs showed that 2–5% of the total oxygen consumed in mitochondrial respiration yields ROS, mostly as H$_2$O$_2$ (Hensley et al., 1998; Papa and Skulachev, 1997; Perez-Campo et al., 1998).
A recent study from Giorgio and co-workers indicates one particular ROS - H$_2$O$_2$ - as a common mediator of aging signals (Giorgio et al., 2007). Not all ROS are equally implicated in signaling functions. For example, H$_2$O$_2$ is membrane permeable and diffusible, less reactive and longer-lived than OH or O$_2$ and, as such, it is best suited for intra- and even intercellular signaling (Stone and Yang, 2006). The physiological range of intracellular H$_2$O$_2$ concentrations appears to be remarkably conserved in different forms of life (Mueller, 2000). Notably, among ROS, H$_2$O$_2$ is the only species generated by several specific enzymes in the cell suggesting that its intracellular concentration is tightly regulated and may serve specific cellular functions. P66$^{\text{Shc}}$ is a peculiar protein acting specifically in the mitochondrion as a redox enzyme that generates H$_2$O$_2$ to trigger mitochondrial swelling and apoptosis (Giorgio et al., 2005). The H$_2$O$_2$ generated by p66$^{\text{Shc}}$ accounts for ~30% of the total pool of intracellular H$_2$O$_2$ and is biologically relevant, as shown by the finding that cells and tissues that are derived from p66$^{\text{Shc}}$-null mice accumulate significantly less oxidative stress, and because p66$^{\text{Shc}}$ can induce a mitochondrial permeability transition (a sudden increase in the permeability of the mitochondrial membrane) in vitro and in vivo (Giorgio et al., 2005). Notably, not only the main apoptotic response is strictly dependent on p66$^{\text{Shc}}$, but p66$^{\text{Shc}}$-mediated H$_2$O$_2$ production is also indispensable for cells to respond to the mitogenic effect of selected growth factors and for activated Ras to induce a p53-dependent checkpoint (Trinei et al., 2002).

Giorgio and co-workers further propose that genes that control H$_2$O$_2$ production are selected determinants of lifespan implying that aging should be considered as the expression of a selected genetic program that generates H$_2$O$_2$ as a signaling molecule.

Overexpression of H$_2$O$_2$ scavengers (catalase, glutathione peroxidase or superoxide dismutase) in some transgenic models of C. elegans and D. melanogaster, have been shown to increase oxidative-stress resistance and lifespan (Landis and Tower, 2005). Remarkably, transgenic mice that overexpress catalase in mitochondria show a specifically increased scavenging activity in mitochondria and a prolonged lifespan (Schriner et al., 2005). In addition, deletion of p66$^{\text{Shc}}$ gene in mice results in the decreased formation of mitochondrial H$_2$O$_2$ (Giorgio et al., 2005), which correlates with delayed aging (Francia et al., 2004; Menini et al., 2006), reduced incidence of aging-associated degenerative diseases (Francia et al., 2004; Menini et al., 2006; Napoli et al., 2003; Rota et al., 2006) and increased lifespan (Migliaccio et al., 1999) (see paragraph 5.2). Thus, genetic mammalian models of increased scavenging or decreased production of mitochondrial H$_2$O$_2$ directly implicate mitochondrial H$_2$O$_2$ in aging and lifespan determination.
Figure 1 Sources and cellular responses to ROS. Oxidants are generated by the organisms under physiological conditions by normal intracellular metabolism (mitochondria and peroxisomes, and cytosolic enzyme systems such as lipoxygenase, NADPH oxidase and Cytochrome P450) or may be the result of pathological conditions (cytokines, toxins, chemotherapeutics) as well as coming from exogenous environmental sources (ultra violet - UV - or ionizing - IR - radiations). Enzymatic and non-enzymatic antioxidant defenses include catalase (CAT), superoxide dismutase (SOD) and glutathione peroxidase (GPx) aimed to counteracts and regulate ROS levels in the range of homeostatic amount. Lowering ROS levels below the homeostatic set point may interrupt the physiological role of oxidants in cellular proliferation and host defenses. Similarly, increased ROS may also be detrimental and lead to the accumulation of random cellular damage as well as priming specific cell signaling pathways. Both these processes eventually end up to acceleration in aging and age-related diseases and cell death. *Figure and legend adapted from Finkel and Holbrook (2000).*
3 Stress and the HPA axis

Stress may be defined as any change of the internal or external milieu perturbing the maintenance of homeostasis of an organism; the disturbing factors are defined as “stressors”. In complex organisms, stress responses involve a coordinated set of intercellular signals and responses that result in removal of the organism from, or adaptation to, the stressful situation. Coping strategies, reflected in adaptive physiological changes, are therefore important components of the stress response. Thus, during stress, attention is enhanced and the brain focuses on the perceived threat. Cardiac output and respiration are accelerated, catabolism is increased and blood flow is redirected to provide the highest perfusion and fuel to the aroused brain, heart and muscles to allow an animal to “fright, fight or flight” (Chrousos and Gold, 1992). Pivotal to these allostatic/adaptive responses is the neuroendocrine system that employs neuropeptides and hormones as mediators. The HPA (which releases the glucocorticoids - GC - hormones) and sympathetic adrenomedullary systems (which releases catecholamines) coordinate the stress response in brain and periphery. They act as integrating units controlling the physiological and behavioral adaptive responses necessary for an organism to cope with stress (in a time-limited fashion) and their functioning is in turn affected by stress.

Under basal conditions the HPA axis coordinates daily and sleep related events through hourly secretory bursts of corticotrophin-releasing hormone (CRH), adrenocorticotropic hormone (ACTH) and glucocorticoids - see later - (Windle et al., 1998a; Windle et al., 1998b; Young et al., 2004). The amplitude of this ultradian adrenal GC rhythm is enhanced if the activity period starts, which is the night time in most rodents. Amplitude and frequency can change during disease and the aging process (Lightman et al., 2008). Activation of the HPA axis (by either psychological or physiological threats) can occur at any time and also results in the release from the hypothalamus of the neuropeptides CRH and arginine-vasopressin (AVP) into the hypophyseal portal blood system. The combined action of CRH and AVP stimulates the anterior pituitary gland to secrete peptides derived from the proopiomelanocortin transcript, which includes ACTH; ACTH, acting on the cortex of the adrenal glands eventually results in the synthesis and secretion of the main hormones of stress, GC (Engler et al., 1989; Rivier and Rivest, 1991). Glucocorticoids regulate the secretion of CRH, AVP and ACTH through negative feedback actions on the brain and anterior pituitary gland (Canny et al., 1989). In humans and many mammalian species, the predominant GC is cortisol, whereas in rodents and birds the key GC is corticosterone. It is of interest that the magnitude of the HPA response is larger when triggered during the ascending phase of the glucocorticoid pulses than during the descending phase.

Glucocorticoids exert action in the brain via high affinity Type I mineralocorticoid receptors (MR) and low affinity Type II glucocorticoid receptors (GR) (De Kloet et al., 1998). In the brain MR are located in the hippocampus - a region involved in memory formation and retention - and other regions of the limbic system such as the lateral septum and amygdala as well as hypothalamic sites, while GR have a more ex-
tensive distribution within the brain, being also present in the hippocampus, and most abundant in the hypothalamus and in pituitary corticotrophs. Both MR and GR belong to a large class of nuclear receptors that homodimerize and translocate from cytoplasm to the nucleus after ligand binding. There, both MR and GR bind to the consensus DNA sequence glucocorticoid-responsive-element, and modulate the transcriptional activity of downstream genes in a similar manner.

At basal levels, GC predominantly bind to MR and only slightly occupy GR; the affinity of the MR is high enough to retain circulating GC pulses in the nucleus (Conway-Campbell et al., 2007). In the early stage of a stressor, nuclear MR remains saturated, but the occupancy of the recently discovered membrane variant of MR (de Kloet et al., 2008; Joels et al., 2008; Karst et al., 2005; Olijslagers et al., 2008) and also of nuclear GR is increased. It is not until a sustained, major stressor that GR occupancy is saturated. Thus, MR is responsible for much of the effects of basal and low-stress levels of GC (i.e. the permissive effects) at the onset of the stressor, whereas GR largely mediates the effects of high-stress levels of GC, facilitating the re-establishment of homeostasis (negative feedback) when stress levels of GC prevail (De Kloet, 2004; De Kloet et al., 1998). This, combined with the fact that MR and GR signaling often have opposing outcomes, results in a U-shaped curve of GC action where intermediate (basal to low-stress) GC concentrations have the opposite effect of no GC or high-stress concentrations (Munck et al., 1984; Sapolsky et al., 2000).

Alterations in the effectiveness of negative feedback by GC have profound effects on the activity of the HPA axis and regulation of stress responses, such that aberrant action can increase the vulnerability of the individual to stress-induced disorders or diseases (De Kloet et al., 1998). There is also evidence that changes in the effectiveness of negative feedback by GC contribute to attenuated HPA responses to stress in some physiological states, thereby resulting in stress hyporesponsiveness.

Glucocorticoids are steroid hormones that exert potent and mainly catabolic effects on various peripheral tissues, prominent examples being modulation of energy metabolism in skeletal muscle, modulation of immune functions of lymphocytes (immunosuppressive effect), antireproductive and antigrowth effects temporarily beneficial rather than damaging. However, for the long-term welfare of the organism, it is important that neuroendocrine stress responses are turned off once the stressful situation is no longer present.

Chronicity of stress system activation leads to the syndromal state that in 1936 Selye described as the “general adaptation syndrome” (Selye, 1936). Thus GC have both protective and damaging effects on the body. In the short run they are essential for adaptation, maintenance of homeostasis, and survival (allostasis = maintaining stability through changes). Yet, over longer time intervals, they impose a cost (allostatic load) that can accelerate disease processes or participate to pathological changes ranging from immunosuppression to obesity, hypertension, and atherosclerosis (McEwen, 2000). During lifespan, chronic exposure to severe stress from a multitude of physical, social, or emotional stimuli may thus exhaust or weaken the capacity of an organism to adapt and cope with further stressors (Selye, 1936). Aging would then
result from “a decreasing ability to survive stress,” one of the many definitions of aging that suggests a close relationship between stress and longevity.

Gerontologists have long been interested in stress: whether aging impairs the ability to respond appropriately to stressful challenges, whether prolonged stress accelerates the aging process, and whether individual differences in coping with stress contribute to differences in “successful aging.” Many studies on brain aging have focused on altered neuroendocrine regulation and, in particular, on that of the HPA axis. Endogenous GC vary up to 50-fold over the diurnal variation set by the circadian rhythm and are further elevated in response to stress and disease. During aging negative feedback regulation of the HPA axis by GC has been found to be decreased, possibly due to decreased receptors (both MR and GR) in specific brain areas resulting in increased circulating levels of GC (Sapolsky et al., 1986). In addition, especially during challenges and in the presence of chronic diseases, aged individuals show difficulty in restoring GC levels to baseline (Sapolsky et al., 1986). Thus in humans, increased HPA axis activity during aging results in higher basal GC levels at the nadir of the circadian rhythm and in higher or prolonged GC levels after stress or in various disease states.

Based on initial studies in rodents and later in primates, a ‘glucocorticoid cascade hypothesis’ has been formulated whereby prolonged exposure to GC was damaging to the hippocampus resulting in loss of pyramidal neurons (Landfield et al., 2007; Landfield et al., 1978; Sapolsky et al., 1985). In addition, it was postulated that cumulative effects of stress and stress hormones exacerbated the effects of other insults and over the lifetime led to functional deficits, including cognitive decline. It has come to be recognized that GC excess can have adverse effects in the nervous system, particularly the hippocampus a brain area involved in memory formation and storage containing high levels of GR and MR, being an important site for the HPA axis negative feedback. These effects include disruption of synaptic plasticity, synaptic loss and atrophy of dendritic processes, compromising the ability of neurons to survive a variety of coincident insults and, at an extreme, overt neuron death (McEwen, 2000).

Glucocorticoids could endanger neurons by increasing voltage-gated calcium currents, contributing to the increase in cytosolic calcium seen in exacerbation of GC neurotoxicity (Joels and de Kloet, 1989; Kerr et al., 1989), or by decreasing the expression of neurotrophins and by inhibiting injury-induced sprouting (DeKosky et al., 1984; Smith et al., 1995). In addition, they can decrease glucose transport into hippocampal neurons resulting in a strong metabolic challenge for neuronal homeostasis. This is achieved most likely by decreasing the number of glucose transporters at the membrane and has been shown to be associated with a faster decline in mitochondrial potentials and ATP concentrations during insults (Horner et al., 1990; Kadekaro et al., 1988; Lawrence and Sapolsky, 1994; Tombaugh and Sapolsky, 1990). Such a metabolic disruption is believed to play an important role in neuronal death during critical energy demanding situations (such as seizures or hypoxia-ischemia). In fact, as a consequence of reduced energy, hippocampal neurons fail in the costly
task of maintaining synaptic glutamate concentrations in a safe range leading in turn to excessive mobilization of cytosolic calcium in the postsynaptic neuron. As a consequence, the costly task of carrying out calcium efflux is impaired. This leads to degenerative consequences, including cytoskeletal damage and, last but not least, oxygen radical generation and accumulation (Sapolsky, 1999).

4 Interaction between stress and oxidative stress

Aerobic cells are permanently challenged by oxidative stressors. Thus, cellular antioxidant defense mechanisms provided by enzymes (e.g., catalase, superoxide dismutase) and other compounds (e.g., vitamins, glutathione) have to maintain an equilibrium between ROS formation and detoxification in order to keep cells functional and alive (Sies, 1993). Oxidative stress has been implicated in the pathogenesis of various neurodegenerative disorders, including Parkinson’s disease, amyotrophic lateral sclerosis, and Alzheimer’s disease (Beal, 1995; Behl, 1997; Coyle and Puttfarcken, 1993; Ebadi et al., 1996; Markesbery, 1997; Olanow, 1993). Conditions characterized by persistent elevation in GC levels, such as chronic stress or aging (Delacourte et al., 2003) can result in neurotoxicity, atrophy and neuronal death through different mechanisms, including modifications in the cells’ energy metabolism. Disruption of neuronal metabolism is believed to play a crucial role in neuronal death especially during critical energy demanding situations under different stressful conditions (Madrigal et al., 2006; McEwen, 1992; Sapolsky, 1999). A number of clinical and experimental studies have described a close relation between the exposure to stressful events and the onset, evolution, and resolution of inflammation-related diseases in multiple body systems (cardiovascular, endocrine, digestive, immune, etc.) (Baum and Posluszny, 1999; Slimmer et al., 2001). In particular, neurological and neuropsychiatric diseases, including some related to stress exposure (neurodegenerative diseases, depression, posttraumatic stress disorder, and schizophrenia), have shown a strong neuroinflammatory component. In this context, animal models of chronic stress achieved by repeated and/or prolonged restraint stress exposure, have led to the identification of ROS, nitric oxide (NO) in particular, (Madrigal et al., 2006; McLeod et al., 2001) and of prostaglandins (PGs) (Madrigal et al., 2006; Oka et al., 2001) as endogenous key mediators of oxidative damage and of neuroinflammatory processes in the (rat) brain (Garcia-Bueno et al., 2008). Interestingly, inflammation may also behave as a stressful stimulus activating in turn the HPA axis with the consequent release of GC hormones (Black, 2002).

Evidence is mounting that stress and oxidative stress may act synergistically to induce or exacerbate neuronal insult, thus the question arises as to whether GC are able to directly affect the generation of ROS. Although little is known about interactions between GC and ROS, there is evidence that GC can affect both the generation of ROS and the cells’ protective antioxidant systems. Several studies reported that GC may alter antioxidant enzyme activity in peripheral tissues such as lung, kidney, liver, and epithelium (Asayama et al., 1992; Dougall and Nick, 1991; Frank et al., 1985;
Kawamura et al., 1991; Valentine and Nick, 1994; Yoshioka et al., 1994) and may directly induce Mn-superoxide dismutase (a ROS scavenger) in several of these tissues through a genomic mechanism (Valentine and Nick, 1994; Yoshioka et al., 1994). In addition, McIntosh and co-workers found that the in vivo long-term supplementation of GC in the rat brain was able to lower the antioxidant capacity in a region-specific manner although the deficit did not appear until the tissue was challenged with supernormal levels of oxidative products (McIntosh et al., 1998b). In a subsequent study, however, the authors found that the impairment in antioxidant enzyme defenses, following a treatment with kainic acid (an excitotoxin able to generate ROS in the brain, whose systemic administration produces neuronal patterns of damage similar to repeated seizures), did probably account for the GC-mediated neuroendangerment seen in the hippocampus of rats (McIntosh et al., 1998a). Overall, it appears that a main part of the neuronal insult resulting from a chronic stress conditions might be mediated via a direct or indirect effect of GC on ROS generation.

Different but much more intriguing would be the opposite question that is whether oxidative stress and ROS may affect the functioning of the HPA axis and GC-mediated effects. A number of transcription factors including the GR are regulated in a redox-dependent fashion. Upon hormone binding, the GR dissociates from hsp90 (molecular chaperones required both for the conformational competence of the steroid-binding pocket and to maintain the receptor in an inactive form in the absence of the hormone) and translocates into the nucleus to regulate the expression of target genes (Beato et al., 1995; Evans, 1988; Glass et al., 1997). Several studies presented evidence that the GC action in vivo is strictly controlled by cellular redox state (Makino et al., 1996; Tanaka et al., 1995). In addition Okamoto and co-workers have elucidated the molecular mechanism, showing that both ligand-dependent and -independent nuclear translocation of the GR is impaired under oxidative stress conditions in living cells and that Cys-481 is the residue involved in the redox regulation of the receptor. Thus, overall, redox-dependent regulation of GR nuclear translocation constitutes an important mechanism for modulation of GC-dependent signal transduction (Okamoto et al., 1999).

Inflammatory stress is usually associated with high plasma cytokine levels and increased generation of ROS that lead to a condition of oxidative stress. During immunogenic stressful conditions, such as acute inflammation and/or infection, up-regulation of the HPA axis is maintained in the face of sustained elevation of plasma GC hormones. Asaba and co-workers examined the effect of ROS on the GR-mediated negative feedback regulation of the HPA axis in vitro. The authors found that when the cells were treated with H$_2$O$_2$, GC suppression of the proopiomelanocortin (POMC) gene promoter was attenuated in a dose-dependent manner. In addition H$_2$O$_2$ inhibited the ligand-stimulated nuclear translocation of GR. Thus it has been suggested that increased ROS generation, occurring during inflammation-like conditions, may be able to attenuate the GC negative feedback system, at least in part, by interfering with the nuclear translocation of GR and consequently eliminating the repression on POMC gene expression (Asaba et al., 2004) see Fig. 2.
These data are particularly interesting especially if considered in a wider context such as that of aging both in terms of lifespan and in terms of “healthspan”, which can be defined as the period of life during which an individual is free of chronic illness and substantial functional decrements (Martin et al., 2007).

Life events (environment) and individual history (genetic background) largely contribute to shape the aging phenotype for each individual. Taking into account the recent evidence showing a direct interaction between ROS and HPA axis via the GR negative feedback, it is indeed intriguing to speculate that a different exposure to oxidants during lifespan might differently shape the neuroendocrine system thus resulting in different abilities to cope with stress. In fact it can be hypothesized that an organism constantly exposed to higher ROS levels would also be constantly exposed to high GC levels because of the inefficiency of GR-mediated negative feedback in conditions of oxidative stress. This would possibly result in a neuroendocrine phenotype resembling that generally characterizing aged individuals or that seen in depressed patients (Holsboer, 2000; Pariante and Miller, 2001). On the contrary, subjects that had experienced a permanent condition of lower levels of oxidative stress would have also experienced constantly lower circulating GC levels because of the more efficient negative feedback. This, in turn, would result in a healthier and more resilient neuroendocrine phenotype better equipped to cope with stress and less prone to develop the allostatic load exacerbating the aging process. In this scenario aging may be viewed as the final result of a fine tuning of the neuroendocrine system by the organismal redox milieu, ROS being one of the actors physically interfering on the GC negative feedback.

**Figure 2** Schematic representation of the mechanism hypothesized by Asaba and co-workers for the regulation of the HPA axis under conditions of oxidative stress. Under normal conditions GC feedback to GR in the corticotroph cells at the pituitary cells to down-regulate the POMC transcription. In condition of oxidative stress (inflammation/infection) ROS prevent GR from nuclear translocation leaving the HPA axis active in the face of high GC levels. *Figure adapted from Asaba et al., 2004*
5 The animal model

5.1 Genetic and environmental factors affecting lifespan: animal models

Animal models represent essential tools in studying the biological mechanism(s) underlying aging that allow to test the influence of genetic as well as environmental factors involved in the aging process. Most models in aging research rely on simple organisms such as the budding yeast or invertebrates. Two popular invertebrate models for aging study are the rounded worm *Caenorhabditis elegans* and the fruit fly *Drosophila melanogaster*. Their short life cycle and well defined physical or biological markers of aging make these two species well suited for studying the genetics of aging. Both species have yielded long-lived strains after genetic screens and selection immensely contributing to the understanding of mechanisms underlying aging (Guarente and Kenyon, 2000; Johnson et al., 2002; Lithgow, 1996; Partridge and Gems, 2002).

Mutations in genes affecting endocrine signaling, stress responses, metabolism, and telomeres can all increase the lifespan of model organisms. It seems likely that each known lifespan gene may act in one of several pathways that regulate aging, and that at least some of these pathways are highly conserved. For the remaining part of this paragraph some of the best known animal models of extended lifespan/“healthspan” will be described. Genetic models/factors in yeast, invertebrates and vertebrates as well as environmental/dietary factors affecting longevity will be analyzed eventually considering common features between these two main models of extended lifespan.

5.1.1 Genetic factors

Many mutations that extend lifespan perturb directly or indirectly endocrine signaling. The best understood of these signaling pathways is the insulin/insulin-like growth factor (IGF-1) pathway, which influences lifespan in worms, flies, and mammals (Tatar et al., 2003). This pathway was first linked to lifespan in the rounded worm *C. elegans*, where mutations in daf-2, a known regulatory gene encoding an insulin/IGF-1 receptor ortholog, were found to double the lifespan of the animal (Kenyon et al., 1993; Kimura et al., 1997). The lifespan extension caused by daf-2 mutations required the activity of daf-16 gene (Kenyon et al., 1993) encoding a transcription factor of the forkhead O (FOXO) family which participates in diverse physiologic processes: induction of cell-cycle arrest, stress resistance, differentiation, apoptosis, and metabolism (Lin et al., 1997; Ogg et al., 1997). In addition to DAF-16, HSF-1, the *C. elegans* heat-shock transcription factor, is also completely required for daf-2 mutations to extend lifespan (Hsu et al., 2003; Morley and Morimoto, 2004). The longevity of daf-2 mutants also requires the function of AAK-2, the catalytic subunit of the AMP-activated protein kinase (Apfeld et al., 2004). In mammals, AMP kinase regulates energy metabolism and food intake via phosphorylation of an array of substrates, including metabolic enzymes and transcription factors. Insulin/IGF-1 receptor mutations can also increase the lifespan of the fruit fly *D. melanogaster*, by
as much as 80% (Tatar et al., 2001). In addition, mutations in chico, a downstream insulin receptor substrate (IRS)-like signaling protein, increase lifespan by approximately 40% (Clancy et al., 2001; Tu et al., 2002). It seems likely that this lifespan extension is FOXO dependent (Giannakou and Partridge, 2004; Hwangbo et al., 2004; Junger et al., 2003; Lee et al., 2003).

Even the lifespan of yeast appears to be regulated by insulin/IGF-1 signaling components. Mutations in the yeast AKT ortholog Sch9 extend lifespan (Longo and Finch, 2003); Sch9 mutant yeast are much smaller than wild-type. Likewise, overexpression of the histone deacetylase Sir2, which is part of the insulin/IGF-1 pathway in *C. elegans*, extends the lifespan of both yeast and worms (Tissenbaum and Guarente, 2001).

Unlike worms and flies, which have a single insulin/IGF-1-like receptor, mice have separate receptors for insulin and IGF-1. IGF-1 receptor heterozygous knock-out mice live approximately 30% longer than wild-types, and males live roughly 16% longer (though the latter value was not statistically significant) (Holzenberger et al., 2003). Thus the ability of insulin/IGF-1 signaling to influence lifespan appears to have been distributed to both receptors during evolution. Mutations in upstream genes that regulate insulin and IGF-1 also extend lifespan by approximately 50%. For example, growth hormone (GH) stimulates IGF-1 production, and GH receptor mutants are long-lived (Coschigano et al., 2003). The Ames and Snell dwarf mice, which have pituitary defects and, consequently, low levels of GH and IGF-1, are also long-living (Brown-Borg et al., 1996; Flurkey et al., 2002). The small size of these mice is unlikely to be the cause of their lifespan extension because in worms, flies, and other mouse mutants, it is possible to change insulin/IGF-1 receptor activity in such a way that lifespan is increased with little or no changes in body size (Clancy et al., 2001; Garigan et al., 2002; Holzenberger et al., 2003; McCulloch and Gems, 2003). Whether FOXO proteins are responsible for lifespan extension in these mice is not known although these proteins function in mouse insulin and IGF-1 pathways that affect metabolism (Burgering and Kops, 2002). In addition, FOXO proteins have been implicated in the increased stress resistance of the long-living mice lacking p66Shc (Nemoto and Finkel, 2002), a gene whose product is an enzyme involved in the regulation of oxidative stress induced apoptosis (Giorgio et al., 2005, see paragraph 5.2).

Stress resistance is a general feature of insulin/IGF-1 pathway mutants, and in worms, this phenotype has been shown to be FOXO dependent (Clancy et al., 2001; Holzenberger et al., 2003; Larsen, 1993; Lin et al., 2001; Lithgow et al., 1995; Murakami and Johnson, 1996).

### 5.1.2 Environmental/dietary factors: caloric restriction (CR)

There are many questions about the determinants of biologic, genetic, evolutionary and social aspects of human longevity. Some of these may be answered by looking at the phenotype of extraordinary long-living humans such as centenarians. The most striking features of these individuals may be summarized as: efficient insulin re-
Before the development of genetically modified organisms, the only known mammalian model of retarded aging was caloric restriction (CR), i.e. reduced caloric intake without malnutrition (McCay et al., 1989). Up to day it is still the only known non-genetic intervention that robustly extends lifespan in every organism in which it has been tested, including yeast, worms, flies, rodents and in primates (Klass, 1977; Lin et al., 2002; Loeb and Northrop, 1917; Mattison et al., 2007; McCay et al., 1989).

Moderate CR in rodents is able to induce alterations in the physiology of many organ systems which have been characterized extensively, including a reduction of oxidative damage as well as reduced glucose and insulin levels (Lee and Yu, 1990; Masoro et al., 1983; Masoro et al., 1992). Even more significantly, CR has been shown to slow the progression of, or even prevent entirely, a range of age-dependent pathologies, including cardiovascular disease (Mattson and Wan, 2005), multiple types of cancer (Klebanov, 2007), several neurodegenerative disorders (Maswood et al., 2004; Wang et al., 2005) and diabetes (Anson et al., 2003) in mammalian animal models, and to reduce the risk of coronary disease and stroke in humans (Mattson and Wan, 2005).

Clearly, identification of the genetic mechanisms that underlie the protective effects of CR would have profound implications for the development of new medical interventions for diseases of aging. However, a clear understanding of which of these numerous changes are causally relevant to the increased longevity and improved health of CR animals is still lacking. To this purpose a number of genetically modified yeasts, worms, flies and mice have been developed. Most of these long-living mutants share common features, among each other, related to some or most characteristics of caloric restricted organisms, this implying that there might be some conserved mechanisms across taxa. One strategy to determine whether a given candidate gene functions in the same pathway as CR to regulate lifespan is to subject a mutant in the gene to CR and assess the normality of the response. Namely: non-additive effects of the mutated gene and CR (where the magnitude of the effect of the mutated gene depends upon whether the CR is also present) are taken to indicate that the treatments act in the same pathway or process in the determination of a phenotypic trait (Gems et al., 2002).

In metazoans many of the genes that act as key regulators of lifespan also have known roles in nutrient sensing or glucose transport, including homologues of yeast genes that are known to be involved in CR longevity, such as She9, Tor1 and Sir2. Intriguingly Bishop and Guarente (2007) suggest an overall neural basis of CR longevity in metazoans (Bishop and Guarente, 2007). Odor of food alone is sufficient to reduce the longevity response of calorie-restricted flies, suggesting that odor-sensitive neuronal pathways modulate CR longevity (Libert et al., 2007). ASI neurons are functional analogs of the mammalian hypothalamus that sense food in the environment and integrate this information with the intrinsic energy availability to modulate the hormonal signals in the head of C. elegans. Ablation of these neurons has been shown to prevent the CR-longevity response (Bishop and Guarente, 2007).
addition it has been shown that the smell of food is able to increase insulin in humans (Lindemann, 2001).

This overlap between genes that control metazoan lifespan and nutrient sensing suggests several candidate regulators of metazoan CR longevity, although few have been conclusively linked to it. Insulin signaling, TOR, AMPK and sirtuins (Olaharski et al., 2005; Wood et al., 2004) are all intriguing candidates for conserved regulators of CR induced-longevity in metazoans. None of them has been conclusively ruled out, although there is positive evidence for roles of AKT, TOR and sirtuins in multiple species.

CR also increases free plasma corticosterone. GC presents a paradox. The hormones are elevated and actually enhance the expression of trophic substances rather than suppress them as is the case during chronic uncontrollable stress (Patel and Finch, 2002). This study suggested that the neuroprotective effects of caloric restriction (which include decreased plasma glucose, attenuated free radical generation, alterations of the vasculature, increased expression of heat shock proteins and neurotrophic factors, and attenuation of age-related glial activation) outweigh the deleterious effects of glucocorticoids.

5.1.3 Common features for genetic and environmental extension of lifespan

Both genetic and environmental/dietary factors are able to affect the duration of life in different organisms. Many mutations that extend lifespan involve genes which have been found to be part of the longevity signal cascade mediated by CR. How could such common longevity pathways evolve?

Increased lifespan has been linked to increased expression of sirtuins, impaired function of insulin/IGF receptor homologs, and absence of the signaling protein p66Shc (Hajnoczky and Hoek, 2007). Several cell signaling pathways associated with these factors converge on the FOXO family of transcription factors, which regulate the expression of a battery of stress response proteins that affect antioxidant capacity, cell cycle arrest, DNA repair and apoptosis (Kenyon, 2005). There are many situations in biology in which low levels of stress trigger subsequent beneficial effects. This phenomenon, sometimes called “hormesis,” a term borrowed from toxicology, seems also to influence aging. A nice example of this phenomenon is the lifespan extension of flies and worms following a heat shock stress (Apfeld et al., 2004; Hercus et al., 2003; Lithgow et al., 1995). A general response to stressful stimuli - oxidative stress, stress caused by CR etc. - is that an increased stress defense program is initiated which involves the organism to be more ready to respond to a possible further stressful condition. In agreement with this, all long-lived mutants of *C. elegans* show an increased ability to respond to different stressors, including heat, UV, and ROS, irrespective of whether the genes providing the longevity phenotype are involved in the insulin/IGF-1 signaling pathway or not (Johnson et al., 2002). This seems logical, since metabolic and environmental stressors may inflict similar types of damage on cellular components (see conclusions of this paragraph). The same chaperones, antioxidants, and other proteins that protect against one type of stress could protect against an-
other. On the same line of evidence, the stress resistance of vertebrate cells in culture correlates with the lifespan of the species from which they were isolated (Kapahi et al., 1999).

It is worth noticing that several authors consider most of the beneficial effects observed in calorie restricted rodents to be mediated by a moderate increase in GC, the main stress hormones which are also involved in energy metabolism (Nelson et al., 1995; Sabatino et al., 1991; Sapolsky, 1995). See paradox above.

5.2 P66Shc knock-out mice as an animal model of extended longevity

5.2.1 The discovery

A Shc (Src homologous and collagen) proto-oncogene was first identified in 1992 while searching for containing Src homology 2 (SH2) domain sequences involved in growth factor signaling (Pelicci et al., 1992). In 1999 Migliaccio and co-workers (from the team of Pier Giuseppe Pelicci) described the serendipitous discovery of unexpectedly long-lived knock-out mice lacking the p66Shc gene (KO - p66Shc−/−). This study was the first of its kind to link a deletion in a single mammalian gene to longevity without reporting any apparent phenotypic abnormality (Purdom and Chen, 2003). Searching the pathway that links the lack of the p66Shc gene to longevity, the increased resistance to oxidative stress as shown by the mutants, appears to be crucial. In fact when KO subjects were challenged with a paraquat injection (an herbicide that induces a condition of massive oxidative stress), indeed they appeared to be better equipped to handle oxidative stress than their wild-type counterpart showing a 40% increase in survival time (Migliaccio et al., 1999).

At the cellular level, p66Shc has been shown to play a key role in the regulation of the oxidative stress response and apoptosis. Mouse embryonic fibroblasts (MEFs) derived from p66Shc−/− rodents have normal baseline levels of intracellular oxidative stress, however, unlike wild-type MEFs, p66Shc−/− cells are resistant to producing ROS in response to nutrient deprivation or serum starvation (Nemoto and Finkel, 2002). When subjected to oxidative stress through either H₂O₂ or ultra violet light (UV) treatment, KO MEFs are reluctant to undergo apoptosis compared to WT while expression of a plasmid containing p66Shc gene in KO cells is able to return the cells to levels of normal sensitivity (Migliaccio et al., 1999). In addition, overexpression of the p66Shc gene in normal MEFs results in increased sensitivity to apoptosis under the same conditions (Migliaccio et al., 1999). This evidence clearly indicates that p66Shc might participate in the regulation of the aging process in mammals through regulation of oxidant generation and (induction of) apoptosis (Migliaccio et al., 1999).

Thus, the main features of the p66Shc knock-out mice obtained in a 129Sv/Ev genetic background are: increased longevity (30% more than the wild-type counterpart under basal condition), increased resistance to oxidative stress, viability, with no reported developmental abnormality, and no increase in tumorigenesis although a source of apoptosis was lacking.
5.2.2 The structure

Three Shc genes have been found in the mammalian system: ShcA, ShcB (Sli) and ShcC (Rai) (Luzi et al., 2000). The ShcA gene encodes two mRNA species and three proteins: p66<sup>Shc</sup> and p46<sup>Shc</sup>/p52<sup>Shc</sup>. The p66<sup>Shc</sup> mRNA has an alternative transcription initiation site from that of the p46<sup>Shc</sup> and p52<sup>Shc</sup> isoforms (i.e., an alternative promoter) (Ventura et al., 2002). Each ShcA protein harbors three identical functional domains: an N-terminal phosphotyrosine-binding domain (PTB), which is slightly truncated in the p46<sup>Shc</sup> isoform, a central proline-rich domain (CH1), and a carboxy-terminal Src homology 2 (SH2) domain (Luzi et al., 2000). P66<sup>Shc</sup> differs from p46<sup>Shc</sup> or p52<sup>Shc</sup> by an additional N-terminal proline-rich domain (CH2) (Luzi et al., 2000). All three ShcA proteins (p46, p52 and p66) participate in mitogenic signaling and oncogenesis by regulating receptor tyrosine kinase signaling. The participation of p46<sup>Shc</sup> or p52<sup>Shc</sup> is thought to enhance certain weak signals from growth factor receptors or G-protein coupled receptors while no evidence indicates that p66<sup>Shc</sup> activates the Ras signaling pathway (Bonfini et al., 1996; Foschi et al., 2001; Migliaccio et al., 1997). P66<sup>Shc</sup> instead is thought to participate to this signal transduction pathway by competing with p46<sup>Shc</sup> or p52<sup>Shc</sup> for Grb2 binding, suggesting that it may act as a dominant negative regulator of p46<sup>Shc</sup> or p52<sup>Shc</sup>-mediated Ras signaling (Okada et al., 1997).

5.2.3 The function

The p66<sup>Shc</sup> protein contains a serine phosphorylation site, Ser36 in its CH2 domain, which is unique to this isoform. P66<sup>Shc</sup> becomes serine-phosphorylated in cells treated with UV or other inducers of oxidative stress, such as H<sub>2</sub>O<sub>2</sub>. On the contrary p66<sup>Shc-/-</sup> fibroblasts are resistant to UV- or H<sub>2</sub>O<sub>2</sub>-induced apoptosis, a finding mirrored by the increased UV- or H<sub>2</sub>O<sub>2</sub>-sensitivity conferred by overexpression of p66<sup>Shc</sup>. In p66<sup>Shc-/-</sup> cells a p66<sup>Shc-Ser36</sup> mutated transgene fails to restore apoptosis sensitivity, strongly indicating the importance of the Ser36 residue in regulating apoptosis (Migliaccio et al., 1999). Notably, insulin (presumably) also induces phosphorylation of this site suggesting a link between metabolism, oxidative stress and genetic pathways affecting lifespan (Kao et al., 1997). Important downstream target of Ser36-phosphorylated p66<sup>Shc</sup> are the FOXO proteins. Interestingly, this family of transcription factors regulates both the expression of genes crucial for the proliferative status of a cell as well as that of genes involved in programmed cell death (Burgering and Medema, 2003). At least in mammals FOXO factors can protect cells against oxidative stress damages by controlling the expression of antioxidant enzymes such as manganese superoxide dismutase (MnSOD) and catalase (Kops et al., 2002; Nemoto and Finkel, 2002) as well as by controlling the expression of proteins involved in the DNA repair mechanisms (Furukawa-Hibi et al., 2002; Tran et al., 2002). Nemoto and Finkel in 2002 showed that in p66<sup>Shc-/-</sup> cells the activity of the mammalian forkhead homolog FKHR-L1 was increased and redox-dependent forkhead inactivation was reduced. In addition, expression of FKHR-L1
resulted in an increase in both H$_2$O$_2$-scavenging and oxidative stress resistance (Nemoto and Finkel, 2002). These results suggest indeed that increased expression levels in scavenger genes may account, at least in part, for the lower levels of ROS seen in p66$^{Shc/-}$ mutant mice and for the consequent increased longevity (Kops et al., 2002; Trinei et al., 2002). However, the same authors recently argued that the increase in FKHR-L1 is modest thus it is unlikely that it can explain the much more significant decrease in basal and stress-induced ROS levels seen in p66$^{Shc/-}$ mice (Nemoto et al., 2006).

A great amount of work has been done by the team of P.G. Pelicci in order to characterize the cellular/molecular pathways underlying the increased resistance to oxidative stress seen in p66$^{Shc/-}$ mice.

The tumor suppressor p53 plays a central role in the regulation of oxidative stress-induced apoptosis (Vogelstein et al., 2000). In 2002 Trinei and co-workers showed that p66$^{Shc}$ is a downstream target of this tumor suppressor, indispensable for the ability of stress-activated p53 to induce elevation of intracellular oxidants, cytochrome c release and finally apoptosis (Trinei et al., 2002). Following UV and H$_2$O$_2$ treatment p53 becomes activated inducing p66$^{Shc}$ protein up-regulation by increasing its stability. Depending on the activating stimulus, p53 mediates apoptosis or cell cycle arrest (Vogelstein et al., 2000). P66$^{Shc}$ has been shown to be involved specifically in p53-dependent apoptosis, leaving unaffected other p53 functions. In particular p66$^{Shc}$ has been shown to act by regulating intracellular levels of ROS during p53-induced apoptosis. The proposed model is such that the p53-p66$^{Shc}$ pathway is able to act as a sensor of the levels of intracellular oxidative signals, regulating intracellular levels of oxidants and of oxidative damage. Thus, high intensity oxidative signals, characterizing cells exposed to acute oxidative stress, would result in high-level activation of the p53-p66$^{Shc}$ pathway and apoptosis. On the contrary, low intensity oxidative signals, as may occur in metabolically active cells, would result in chronic, low-level activation of the p53-p66$^{Shc}$ signaling pathway, allowing moderate ROS rises and accumulation of oxidative damage. The finding that p66$^{Shc/-}$ mice have no increased tumor formation and that the p53-p66$^{Shc}$ pathway is selectively involved in the propagation of pro-apoptotic oxidative signals suggest that p66$^{Shc}$ separates, genetically and biochemically, the aging and tumor suppressor activity of p53 (Trinei et al., 2002). Accumulation of ROS-damaged macromolecules with age has been hypothesized as the proximal causative agent of aging. Basal levels of ROS, and the extent of its oxidative damage to macromolecules, are generally considered to be the random consequences of physiological functions, such as cellular respiration (Finkel and Holbrook, 2000). An important implication of these findings is that, in mammals, these phenomena are genetically determined and controlled by a stress-induced signal transduction pathway, involving p53 and p66$^{Shc}$.

A further step in understanding the mechanism of action of p66$^{Shc}$ and its link to longevity has been taken by Nemoto and colleagues in 2006 (Nemoto et al., 2006). The authors found that a fraction of this protein localizes in the mitochondria and given that they represent the largest source of ROS generation within cells and that
oxidative stress represent an important determinant in the aging process, they sought to explore whether p66<sup>Shc</sup> might directly regulate ROS production by regulating mitochondrial metabolism. Indeed p66<sup>Shc</sup>-/- MEFs were found to have altered mitochondrial energy. In particular, the oxygen consumption of these mutant cells was lower than similarly maintained wild-type MEFs resulting in a reduction both in their resting and stress-induced levels of ROS. This was found to be achieved by a metabolic switch in the cellular respiration mode. That is, in the absence of p66<sup>Shc</sup> mitochondrial oxidative phosphorylation is reduced, whereas the dependence on glycolysis increased with the final result of a lower production of ROS. It can be concluded that p66<sup>Shc</sup> regulates mitochondrial oxidative capacity suggesting that it may extend lifespan by repartitioning metabolic energy conversion away from oxidative and towards glycolytic pathways (Nemoto et al., 2006).

Thus, the extended lifespan of mice lacking p66<sup>Shc</sup> has been correlated with decreased ROS production (Giorgio et al., 2005), as well as decreased mitochondrial metabolism (Nemoto et al., 2006). However, how these mitochondrial processes integrate with the upstream signaling events to control lifespan has remained enigmatic until recently. Pinton and co-workers in 2007 (Pinton et al., 2007) showed that p66<sup>Shc</sup> is required for early mitochondrial responses to an oxidative challenge (such as H<sub>2</sub>O<sub>2</sub>). Thus protein kinase C beta, activated by oxidative conditions in the cell, induces phosphorylation of p66<sup>Shc</sup> and triggers mitochondrial accumulation of the protein in the mitochondria (after it is recognized by the prolylisomerase Pin1). Once imported, the activated redox enzyme, p66<sup>Shc</sup>, oxidizes the reduced cytochrome <i>c</i> and catalyses the reduction of O<sub>2</sub> to H<sub>2</sub>O<sub>2</sub>. To do so, p66<sup>Shc</sup> uses electrons from the respiratory chain (complexes I–IV). H<sub>2</sub>O<sub>2</sub> then leads to opening of the permeability transition pore (PTP), mitochondrial swelling and finally apoptosis.

Thus, factors that set the stage for the p66<sup>Shc</sup> contribution to the aging process appear to act in a tightly regulated cascade of events finally leading to cellular death via apoptosis. Nevertheless, this does not exclude a physiological role for mitochondrial p66<sup>Shc</sup> in normal cell management of stress and damage repair. For example, a recent study from Pandolfi and co-workers reported that p66<sup>Shc</sup> is highly expressed in fibroblasts from centenarians (Pandolfi et al., 2005).

### 5.2.4 Site of expression, transcriptional and post-transcriptional modifications

Transcriptional and post-transcriptional modifications are important mechanisms regulating gene expression both spatially and temporally. P66<sup>Shc</sup> is a stress response protein that does not influence the mitochondrial function under steady state condition (inactive form), but is indispensable for mitochondrial-mediated apoptosis following a pro-apoptotic signal (active form) (Giorgio et al., 2005; Orsini et al., 2004; Trinei et al., 2002). Thus, a series of post-translation modifications that modulate its activity occur subsequent to treatment with pro-apoptotic signals, or upon stress stimuli. P66<sup>Shc</sup> is serine phosphorylated on Ser36 residual (Migliaccio et al., 1999); a small quantity of this protein translocates from the cytosol to mitochondria (Orsini et al., 2004); p53 induces an increase in p66<sup>Shc</sup> protein levels by increasing its stability.
(Trinei et al., 2002); lastly, under basal conditions, the mitochondrial p66{sup}Shc{sub} fraction is found within multimolecular complexes (including mtHsp70 and components of TIM-TOM import complex) becoming monomeric only following pro-apoptotic stimuli (Pinton et al., 2007).

Several lines of evidence suggest that, in addition to post-translational modifications, transcriptional regulation could also play a role in regulating p66{sup}Shc{sub} function. First of all, levels of p66{sup}Shc{sub} expression correlate with lifespan in mice, since heterozygous mice p66{sup}Shc{sub}+/− display an intermediate lifespan compared with wild-type and knock-out. Furthermore, p66{sup}Shc{sub} mRNA and protein are up-regulated in skeletal muscle, spinal cord and forebrain of aged rats and the extent of this upregulation increases with age (Jiang et al., 2003). The promoter of p66{sup}Shc{sub} gene contains relatively high guanine-cytosine content, although not enough to qualify as a CpG island. Apparently, cell lines with the least amounts of methylation of the cytosines in the promoter have the highest levels of p66{sup}Shc{sub} expression, and vice versa. Treatment of highly methylated cell lines with demethylating agents resulted in renewed transcription of p66{sup}Shc{sub} arguing for the importance of promoter methylation in silencing the p66{sup}Shc{sub} gene (Ventura et al., 2002).

In accordance with p66{sup}Shc{sub} promoter regulation by epigenetic modifications, p66{sup}Shc{sub} (mRNA and protein) is expressed at different levels in specific tissues, such as lung, spleen, liver, heart, and kidney, while it is absent in others (Trinei et al., 2002). Nevertheless, transcriptional levels of p66{sup}Shc{sub} have been identified in control cells, such as peripheral blood lymphocytes, mouse tymocytes and splenic T cells that acquire the capacity to express it in response to apoptogenic stimuli (Pacini et al., 2004).

Concerning the expression of p66{sup}Shc{sub} in the mammalian central nervous system, scarce data are available. ShcA adaptor proteins are known to function as initiators of Ras mitogenic signaling cascade in various non neuronal systems where they are considered to be expressed ubiquitously. Conti and co-workers in 1997 first investigated the role of the ShcA gene during neurogenesis in neuronal cells namely, whether and how it is involved in the proliferative and differentiative phases of the developing brain (Conti et al., 1997). Analyses of ShcA mRNA and protein in the rodent developing brain (both mice and rats; embryonic day 14-18 to early post-natal day 2) revealed progressive down-regulation of their expression during differentiation from neuroblasts to neurons suggesting that these proteins are lost from postmitotic neuronal cells. However, western blot analyses showed that p66{sup}Shc{sub} was the only isoform present at a low, although detectable, level in different adult brain regions (cortex, striatum, basal forebrain and hippocampus) (Conti et al., 1997). Data on the immunoreactivity of p66{sup}Shc{sub} in the mouse adult brain have been recently confirmed by our group for cortex, hippocampus, striatum, hypothalamus and pituitary gland (Berry et al., unpublished results, see Fig. 3). The role of p66{sup}Shc{sub} in differentiated tissue remains to be elucidated.
Although the involvement of p66Shc in cellular senescence may require further investigation, few recent reports show a correlation among high levels of p66Shc (mRNA and protein), senescence and increased ROS levels both in vitro (Favetta et al., 2004; Klein et al., 2005) and in vivo (Jiang et al., 2003). The finding that p66Shc deletion delays aging, and its implication in age-associated pathology is supported by numerous evidence: i) a high-fat diet has no effect on p66Shc-/- mice, whereas the same diet in wild-type results in signs of early atherogenesis (increased aortic cumulative early lesion, apoptosis in vascular cells and tissue oxidative stress) (Napoli et al., 2003); ii) p66Shc-/- mice do not show significant age-dependent decrease in ROS-mediated endothelial function, such as aortic relaxation that is impaired in old wild-type mice, but not in p66Shc-/- (Francia et al., 2004); iii) p66Shc-/- mice show decreased tissue damage and apoptosis induced by acute ischemia (Zaccagnini et al., 2004); iv) p66Shc deletion does not increase incidence of cancer.

In summary, insulin-like signaling, modulation of forkhead activity and p53 attenuation are among the mechanism that can increase lifespan, and now one interesting open question is how p66Shc mediated \( \text{H}_2\text{O}_2 \) production interacts with these pathways.

### 6 Summary rationale and objectives

#### 6.1 Current state of the art: aging, oxidative stress, the neuroendocrine system and p66Shc

Oxidative stress is a common condition suffered by aerobic organisms. The “free radical” theory of aging states that generated ROS may cause random damage to macromolecules (proteins, lipids and nucleic acids) as well as function as specific molecular signals activating pathways of cellular death, eventually enhancing vulnerability for aging-associated degenerative diseases. Gene mutations in invertebrates have been identified that extend lifespan and enhance resistance to environmental stressors, such as ultraviolet light or ROS, while in mammals such pathways are much more complex and overall still poorly understood. Nevertheless, a targeted mutation of the mouse gene p66Shc has been recently discovered to induce oxidative stress resistance and to prolong lifespan of the mutant subjects. Specifically, the p66Shc pro-
tein acts in mitochondria as a redox enzyme generating $\text{H}_2\text{O}_2$ in response to oxidative stress stimuli, to induce swelling and apoptosis. Thus, $p66^{\text{Shc}}$ has been pointed out as a critical component of the signal transduction pathway that regulates lifespan in mammals. Chronic pathophysiological conditions as well as physical or emotional stress may both induce an increase in ROS levels. Glucocorticoid adrenal hormones represent an essential tool for the organism to adapt and cope with stressful events. Upon stress, the HPA axis is activated, resulting in a prompt and temporary increase in GC. A prolonged exposure to GC is potentially harmful for the organism eventually resulting in neurotoxicity, atrophy and neuronal death. Thus, the secretion of these hormones is tightly and efficiently regulated via a mechanism of negative feedback mediated by GR in the pituitary and in the brain. A growing body of evidence has shown that a redox regulation exists of the function of several nuclear transcription factors, including the glucocorticoid receptor GR. In particular, recent evidence suggests that ROS may affect the HPA axis negative feedback by impairing the GC-mediated translocation of GR from cytoplasm to the nucleus. This would result in elevated levels of POMC mRNA, a result that could explain the chronic elevation in GC characterizing, for instance, inflammation-like states.

6.2 Statement of the problem

The $p66^{\text{Shc}}$ knock-out mouse is characterized by a lower susceptibility to oxidative stress, in addition to extended longevity. Thus, the main question addressed with this research was whether the complex interactions linking oxidative stress and the neuroendocrine system represent a major determinant for the aging process in $p66^{\text{Shc}^{-/-}}$ mice. In particular, the reduced levels of ROS, characteristic of these long-lived subjects, were expected to result in a more efficient regulation of the HPA axis - specifically under stressful conditions - and hence in an overall delayed aging process. To this aim the contribution of $p66^{\text{Shc}}$ to behavioral and neuroendocrine regulations was tested from early post-natal life to senescence.

6.3 Rationale of the studies

I. Investigate the contribution of the $p66^{\text{Shc}}$ gene to the reproductive success of the mutant mice and subsequent observation of maternal behavior to evaluate a possible effect of parental care in shaping the physiological/behavioral phenotype of the long-lived knock-out mice.

II. Study the neuroendocrine response to stress in mutant subjects by repressing or by stimulating HPA axis function. Psychological or systemic stress paradigms were also used which involve diverse oxidative stress components.

III. Study the effects of $p66^{\text{Shc}}$ on the behavioral phenotype of mutant subject at different ages. Specific behavioral tests evaluated if, in addition to increased lifespan, the reduced exposure of the $p66^{\text{Shc}}$ mutants to oxidative stress might account for a healthier phenotype because of a slower decay of brain functions underlying emotional arousal and cognitive performance.
IV. Study the role of the HPA axis and behavior on age-related differences in brain function.

7 Experimental approach and outline of the thesis

In Chapter 2 the effect of the lack of the p66Shc gene on the reproductive performance and maternal behavior of the 129Sv/Ev mouse strain is reported. Reproductive success was evaluated by taking into account: changes in fertility over time during 9 subsequent breeding cycles (performed on 40 WT and 40 KO breeding couples) considering the number of full term pregnancies. The body weight of virgin females, the number of pregnant subjects, number of delivered pups, number of litters with dead pups and number of litters in which cannibalistic episodes took place (over 8 breeding cycles of verging females) were also evaluated. In addition the effect of stress on the ability to carry-out full term pregnancies was assayed. Maternal behavior, (including cannibalistic behavior) was observed in primiparae female mice by instantaneous sampling. Finally the onset of puberty was also assessed by taking into account physical parameters such as vaginal opening (VO) and balanopreputial separation (BPS) respectively in female and male WT and KO mice.

Chapter 3 is focused on the regulation of the neuroendocrine HPA axis under conditions of oxidative stress. The studies were performed both by stimulating and by repressing the functionality of the HPA axis. Two different types of stressful challenges were compared for their ability to activate the HPA axis. Restraint stress (both acute and chronic) was chosen as a psychophysical stress and an LPS acute treatment as an immunogenic challenge able to induce an inflammation-like condition characterized by increased levels of reactive oxygen species (ROS). By contrast, the potent GR agonist Dexamethasone was used in combination with LPS, as a suppressor of the axis at the pituitary level. Functionality of the HPA axis was assessed by measuring the time course of CORT levels in the peripheral blood. Markers of oxidative stress and inflammation taken into account were respectively isoprostanes (15-F_{2α}-IsoP) and prostaglandine E₂ (PGE₂) in the hippocampus, a brain region involved in the GR-mediated negative feedback of GC. Levels of BDNF in the hippocampus, following the immunogenic challenge, were also evaluated as an index of neuronal protection.

In Chapters 4 and 5 the behavioral phenotype of WT and KO male mice at adulthood, middle-age and senescence is characterized. In particular, responses to stressful, painful (hot plate and tail flick tests) and novel/arousing stimuli (open field and elevated plus maze tests) were assessed together with spatial cognitive abilities (Morris water maze). Aim of this study was to characterize age-related changes especially in cognitive and emotional aspects and the neurobiological variables (such as Brain-derived Neurotrophic factor - BDNF -) associated to these brain functions.

All data are discussed in Chapter 6.
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