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BEHAVIORAL CHANGES AND MECHANISMS – AN EXPERIMENTAL STUDY ON AGING IN RODENTS

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Front cover:
Individual exploration trajectories and rears in dark and light arena (300 s). Representative individual trajectories showing the change in exploratory activity across adult life span. Brown lines indicate locomotion (horizontal movements) while blue indicates rears (vertical movements). Exploratory activity declines with age and more time is spent in the light arena. Aged mice maintained on DR explore more than ad libitum fed age-matched controls. In accord with younger mice, aged DR mice explore the full arena including all corners. *Fahlström A, Zeberg H, Ulfhake B. Changes in behaviors of male C57BL/6J mice across adult life span and effects of dietary restriction. AGE.* Epub 2011/10/12.

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ABSTRACT

In paper I, we studied alterations in behavior with advancing age in female C57BL/6 mice (of Jackson origin). In parallel, growth and survival data were collected. In a protected environment the median survival age was 32 months. Our behavioral data show that aging modulates certain aspects of basic behavior in a continuous manner. However, behavioral aging differentially affects genetically closely related individuals housed under strictly standardized conditions. Thus, subtle environmental factors and epigenetic modifications may be important modulators of aging.

In paper II, we analyzed behavioral changes during aging in male C57BL/6J mice. A group of aged males maintained on dietary restriction (DR) was included. The most conspicuous alteration was the decline in exploration activity with advancing age. Comparison with results from paper I revealed that alterations in aged males and females are similar. Moreover, behavioral indices in 22-month-old males could predict remaining life span; exploratory activity and motor skills accounted for up to 65% of the variance in survival. Consistent with a high level of exploratory activity and preserved motor capacity indicated a long post-test survival, aged mice maintained on DR were more successful in such tests than ad libitum fed age-matched males.

In paper III, we studied changes in DNA methylation during aging in the central nervous system in a Sprague-Dawley rat model. Using the LUminometric Methylation Assay (LUMA), which assays methylation status in CCGG sequences across the entire genome, and 5-methyl cytosine labelling in neurons, we report a decline in global DNA methylation in several but not all regions examined. In females there was a gradual drop in DNA methylation in the spinal cord, while both cerebellum and striatum were unaffected. The unexpected observation of a decrease in DNA methylation confined to the middle-ages when reproduction ceases suggested that gonadal steroids influence DNA methylation. To test this, ovariectomy was performed in fertile females, resulting in lowered DNA methylation in frontal cortex to levels observed in middle-aged and aged females. In males, DNA methylation was unchanged in the frontal cortex and the decline in DNA methylation in hippocampus occurred at more advanced age than in females. Another conspicuous difference between sexes was the demethylation of DNA in the aged male striatum.

In paper IV, we compared age-related loss of muscle mass (sarcopenia) in C57BL/6J mice (wild type; WT) and a transgenic model with accelerated aging: the mtDNA mutator mice; in an effort to asses if an increased load of mtDNA mutations is sufficient to phenocopy sarcopenia as it occurs in normal aging. We found that both WTs and mutator mice lose muscle mass during aging but that this process is more advanced in normal aging. A distinct difference between WTs and mutator mice, was the dramatic re-expression of γ-subunit of the nicotinic acetylcholine receptor (nAChR-γ) caused by failure of muscle innervations in normal aging. In mtDNA mutator mice increased levels of nAChR-γ were infrequent and importantly seen in both young adults and aged; with only a small increase across adult life-span.

LIST OF PUBLICATIONS

I. **Andreas Fahlström**, Qian Yu and Brun Ulfhake. Behavioral changes in aging female C57BL/6 mice. Neurobiology of Aging. 2011 Oct;32(10):1868-1880. Epub 2009/12/14.

II. **Andreas Fahlström**, Hugo Zeberg and Brun Ulfhake. Changes in behaviors of male C57BL/6J mice across adult-life span and effects of dietary restriction. Age. Epub 2011/10/12.

III. **Andreas Fahlström,** Mikael Altun, Mohsen Karimi, David Gomez-Cabrero Lopez, Louise Sjöholm, Tomas J. Ekström and Brun Ulfhake. Changes in DNA methylation in the aging rat brain and spinal cord is region specific and gender dependent. *Under revision.*

IV. **Andreas Fahlström**, Mikael Altun, Hossein Parnow, Takashi Yamada, Ingrid Liljefors, Nils-Göran Larsson, Håkan Westerblad and Brun Ulfhake. Sarcopenia in aging C57BL/6J and mtDNA mutator mice. *Manuscript.*

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LIST OF ABBREVIATIONS

ANOVA	ANalysis Of VAriance
BB	Beam Balance
BW	Body Weight
CA	Closed Arm
DNA	DeoxyriboNucleic Acid
DR	Dietary Restriction
EDL	Extensor Digitorum Longus
EPM	Elevated Plus Maze
EMG	ElectroMyoGraphic
Gpx1	Glutathione peroxidase 1
IR	ImmunoReactive
LUMA	LUminometric Methylation Assay
MRF	Myogenic Regulatory Factor
mRNA	messenger RiboNucleic Acid
nAChR	nicotinic Acetyl Choline Receptor
NeuN	Neuronal-specific Nuclear protein
OA	Open Arm
OF	Open Field arena
OR	Object Recognition
OVX	OVariectomized
PCR	Polymerase Chain Reaction
Pgc-1a	Peroxisome proliferative activated receptor, gamma, coactivator 1 alpha
PPAR-g	Peroxisome Proliferator Activated Receptor gamma
RNA	RiboNucleic Acid
ROS	Reactive Oxygen Species
RR	RotaRod
RT	Reverse Transcription
TFAM	Transcription Factor A, Mitochondrial
WT	Wild Type

1 INTRODUCTION

1.1 AGING

The consequences of an aging population is today one of the greatest challenges in developed countries (Buttler et al., 2008, Kirkwood, 2010). It is well documented that people above 85 years of age is the most rapidly growing segment of the population in these countries. This group is also the most susceptible to disease and disability. Somewhat surprisingly, life expectancy is still lengthening almost linearly in most of the developed world. If this trend continues through the 21st century most babies born since 2000 will celebrate their 100^{th} birthday in these countries. (Cristensen et al., 2009).

Aging can be seen as a process with an increasing risk of disease and death. It is accompanied by a progressive decline of functional capacities and an increased morbidity; impairments that represent major threats to our independence and intensify our need for health care, often a tragedy for the individual and a burden for relatives and society. Similar to life expectancy the pace and extent of aging-related impairments are species specific. It is clear that aging affects individuals within a species differently; a difference distinguished with the terms biological- and chronological age. Aging affects different organs and tissues differently. Signs of aging are more prominent in tissue built up by postmitotic cells as the brain and skeletal muscles in contrast to tissue built up by rapidly replicating cells, such as the intestinal mucosa. (Harman, 2001; Holliday, 2006; Hyflick, 2007; Kirkwood, 2005).

1.2 THEORIES ON AGING

Over the years a number of theories on why we age have been launched. It is outside the scope of this thesis to present or discuss the pros and cons of these theories. But I will briefly illustrate the complexity of this field by presenting the very first question a theory on aging has to answer: *is aging programmed or not?* At one end of the spectrum we have the theory of programmed aging (Lamberts et al., 1997; Weisman, 1889) claiming that aging is only the final chapter in the genetically governed developmental process of an organism. At the other end we have the theories claiming that aging it not programmed, arguing that there has been no natural selection during evolution for growing old per se. From this standpoint aging only occurs in rare niches where a species is highly favored or able to control the environment like humans and our domestic animals. Normally, the competition for limited resources in nature terminates life before any signs of aging has occurred. (Holliday, 2006; Hyflick, 2007; Kirkwood, 2005, Medawar, 1952).

Having said that, it is relevant to discuss what mechanism(s) underpin aging. Below, a brief account of the current concepts and mechanism(s) in the processe(s) of aging follows, focusing on mammals.

1.3 MECHANISMS OF AGING

1.3.1 Genetic make-up

The genome is the entirety of an organism's hereditary information and holds the blueprint for the survival machineries on cellular as well as organism level. During the past decade a number of genes effecting life span have been identified. Discoveries were mainly done through systematic mutagenesis of simpler organisms like Caenorhabditis elegans, Saccharomyces cerevisiae and Drosophila melanogaster (e.g. Hekimi, 2006; Hekimi and Guarente, 2003; Kenyon, 2005; Liang et al., 2003; Mackay, 2002; Pletcher et al., 2005). The identified genes largely regulate cell metabolism, cell repair and stress responses, they are conserved through evolution as is the case of the sirtuin class of enzymes (Guarente and Picard, 2005; Johnson et al., 1999). It is possible that these genes are conserved through evolution due to their fundamental function in cell maintenance and adaptation to environmental cues and pressures (*idem*). Most probably, the number of identified genes that modulate life span will expand during coming years.

A striking difference between long- and short-lived species is the amount of investment in cellular repair and maintenance machineries. Based on comparison of genome it appears that long-lived species have done a greater investment in these machineries. (Kirkwood, 2002; Mackay, 2002; Sanz et al., 2006). Finally, variations in mutations of these and other genes may, in part, explain inter-individual variation of life span within species (*idem*).

1.3.2 Replicative senescence

The genome in dividing cells is controlled by the telomeres and associated enzymes, a mechanism connected to the number of possible cell divisions (Blackburn, 2000). A telomere is a region of repetitive DNA sequences at the end of a chromosome protecting genes near the ends of chromosomes from the degradation during cell division, when shortening of chromosome ends occur. Telomeres can be maintained or elongated by the enzyme telomerase, but the transcription of telomerase ceases in most cells already during embryogenesis (Ahmed and Tollefsbol, 2001). If the telomere shortening goes too far, cell division can no longer occur and the cell enters a state of replicative senescence; a condition associated with the appearance of aging stigmata. The significance of this regulatory machinery and its possible role in the aging of organisms is still an open question (Ben-Porath and Weinberg, 2005; Blasco, 2007; Chai et al., 2005; Cristofalo et al., 2004; Monaghan and Haussmann, 2006; Sarin et al., 2005). Although available evidence suggests that this may be true for cellular senescence in replicating cells, it has not been proven for determining the life span of an organism. Transgenic animals deficient of telomerase show no decrease in life span until the sixth generation (Rudolph et al., 1999), making it safe to assume that the individual organism will die long before replicative senescence will take its toll on cell division. Furthermore, the major hallmarks of aging in complex organisms, such as mammals, stem from changes in postmitotic cells (myocytes and neurons) which with

few exceptions do not replicate and where no telomere shortening can be detected (Renault et al., 2002).

1.3.3 Cell metabolism and the accumulation of non-degradable molecules

The cell is far from perfect. Proteins and lipids are constantly damaged due to errors of metabolism, exogenous chemicals, somatic mutations and cell stress. The results of these events are molecules that are difficult for the cell to degrade or recycle because of carbonylation, oxidative modifications, protein unfolding and misfolding. (Bokov et al., 2004; Halliwell, 2006). The damaged molecules will accumulate over time and appear as intracellular deposits, or inclusion bodies; a situation commonly seen in aging postmitotic cells since the damaged molecules are not diluted by cell divisions (Cuervo et al., 2005; Terman and Brunk, 2005; Terman et al., 2007). Lipofuscin (age-pigment) is perhaps the most abundant among non-degradable waste material. Its accumulation has been associated with metabolic rate and redox stress (Kurz et al., 2007; Terman et al., 2004). In this sense, aging is similar to several degenerative diseases and is often considered a co-factor in the natural progression of late-onset neurodegenerative diseases (Bokov et al., 2004; Korolainen et al., 2006).

1.3.4 Cell respiration and aging

Cellular damage caused by free radicals will accumulate during an organism´s life-time according to the "free radical theory of aging" (Harman, 1981; Harman, 1994; Harman 2009). Ever since symbiotic bacteria developed the function of mitochondria within eukaryotic cells, oxygen has been necessary for cellular respiration (Martin and Russell 2003; Searcy 2003). Mitochondria convert dietary calories to energy through oxidative phosphorylation releasing reactive oxygen species (ROS), as a by-product. While mitochondria produce most of the cellular ROS, free radicals are also generated by several other enzymatic reactions, and may themselves serve important functions. For example, in inflammatory reactions, immune cells use targeted release of ROS to attack invading microorganisms and in cellular signaling pathways (Beckman and Ames, 1998).

Since ROS might damage vital cell functions through oxidative reactions with DNA, RNA, lipids and proteins several scavenging systems have evolved converting ROS to less reactive molecules (Bokov et al., 2004; Sanz et al., 2006; Wallace, 2005). The defense system for ROS includes: superoxide dismutase, catalase and glutathione peroxidase (Squier, 2001). Glutathione is probably the most important system (Cooper and Kristal, 1997). It is directly involved in redox cycling reactions, in which the reduced form is converted to its oxidized counterpart, glutathione disulfide. A number of studies have found an age-related shift towards the oxidized form of glutathione together with an accumulation of oxidative damage to DNA in aged individuals (Barja and Herrero, 2000; de la Asuncion et al., 1996; Soong et al., 1992).

Neurons, postmitotic cells with a high metabolic rate are particularly vulnerable to oxidative damage during aging. If the ROS production in these cells exceeds the

scavenging capacity, they are under oxidative stress (Harman, 1981; Harman, 1994; Halliwell, 1992; Keller et al., 1998). Studies have shown that the level of oxidative damage appears to correlate with life span as well as age-related impairments, including decline in cognitive function and motor skills and a loss of muscle mass (Forster et al., 1996).

Oxidative stress is considered a major contributor to DNA damage and posttranslational modifications of proteins during aging in general and could be instrumental in both cytoskeletal and cell membrane changes seen in aging. But it is important to stress that even if increased production of ROS and oxidative damage are heavily implicated in aging the impact of this type of damage in aging is still much debated. Furthermore, there is little direct evidence to support the free radical theory of aging (Bokow et al. 2004; Park and Larsson, 2011; Salmon et al., 2010; Larsson, 2010).

1.3.5 DNA methylation and aging

Epigenetics influence gene function without affecting the DNA nucleotide sequence (Bird, 2007). DNA methylation, together with post-transcriptional histone modifications, are the best characterized epigenetic modifications. DNA methylation refers to the addition of a methyl group to one of the four bases that constitute DNA. In eukaryotes, DNA methylation occurs only on the 5th position of the cytosine bases in the genome. Methyl cytosine is found in the genomic DNA of many eukaryotic organisms, ranging from fungi and plants to invertebrate and vertebrate animals. Between 2% and 8% of cytosines in mammalian DNA are methylated; 5-meC (Zhu, 2009) and DNA methylation influence a range of cellular functions such as genome stability, imprinting, X chromosome inactivation, tissue-specific gene regulation, carcinogenesis, and aging (Feinberg and Vogelstein, 1983; Wilson, 1987; reviewed in Zhu, 2009; Liu et al., 2008). DNA methylation in differentiated cells is heritable through cell division with a tissue/cell-type specific signature (Razin and Riggs, 1980; Riggs, 1975, Yoder et al., 1997; Eckhardt et al., 2006). More recently, this concept was challenged by studies showing increased divergence in DNA methylation in human monozygotic twins over life span and, moreover, aging-related changes possibly associated with disease development (Fraga et al., 2005; Karminsky et al., 2009; Mastroeni et al., 2009;). Evidence suggests that DNA methylation can be dynamically altered by methylation and demethylation processes (reviewed in Zhu, 2009) in response to a range of stimuli (Ma et al., 2009; Kim et al., 2009; Weaver et al., 2005). In the brain, changes in DNA methylation have been associated with mnemonic functions and synaptic plasticity (Levenson et al., 2006; Miller et al., 2007; reviewed in Liu et al 2008), sex differences (reviewed in McCarthy et al., 2009) as well as development of neurodegenerative diseases, like Alzheimer diseases (Mastroeni et al., 2009; reviewed in Liu 2008). In humans and other mammals alike, aging is associated with alterations in DNA methylation and may result in genome instability and cell dysresponsiveness (Fraga et al., 2005; Wilson, 1987; Bjornsson et al., 2008; Siegmund et al., 2007; Hernandez et al., 2011; Bolatti et al., 2009; Thompson et al., 2010; see however Eckhardt et al., 2006).

1.4 IMPACT OF AGING

1.4.1 Behavioral changes and aging

Rodents are used as models for human diseases. During the 20th century a number of mice strains were introduced (Fox 1965; Irwin et al. 1968), many of which are still in use (Bucan and Abel 2002; Chia et al. 2005; Taft et al. 2006; Mekada et al. 2009). Early-on efforts were made to characterize the behavior of rodent models by systematic testing. Results from this work stressed inter strain behavioral differences (Thompson, 1953; Southwick and Clark, 1968). The introduction of techniques to engineer targeted gene modifications led to a demand on a batch-like approach to speedily characterize phenotypic changes (caused by a genetic modification), including organism behavior. This impetus generated today widely accepted behavioral test-batteries (e.g. SHIRPA and EMPReSS, http://empress.har.mrc.ac.uk/; (Crawley and Paylor 1997; Rogers et al. 1997; Paigen and Eppig 2000; Moldin et al. 2001; Karl et al. 2003) which essentially capitalized on already existing behavioral testing protocols, arranged them into comprehensive batteries followed by validation across laboratories (*idem*). Because targeted gene modifications have been easier to do in the mouse, this is now the dominating rodent model of human diseases. Over the past decade the accumulation of behavioral data generated for, in particular C57BL/6 substrains, has rapidly accelerated which among other things has re-emphasized the importance of using genetically well-defined mouse models (Rogers et al. 1999; Crabbe et al. 1999; Bothe et al. 2004; Bryant et al. 2008; Crusio et al. 2009; Crawley et al. 1997; Matsuo et al. 2010). However, most of the data generated so far concerns adult mice and developmental aspects, while behavioral characterization of aging in mice is scarcer (Sprott and Eleftheriou 1974; Goodrick 1967, 1973, 1975; Dean et al. 1981; Ingram and Reynolds 1986; Ingram et al. 1982; Ingram et al. 1981; Lau et al. 2008) see also (Collier and Coleman 1991; Ingram 1988; Ingram and Jucker 1999). Rodent models have proven to be very important in aging research and, combined with work on simpler organism models, our insights have greatly expanded over the past decades concerning mechanisms governing life span, aging related impairments and the biology of normal aging. Previous studies have shown that aging-related changes in body composition and sensorimotor behaviors in rodents appear similar to those seen in humans (reviewed in (Alliot et al., 2002; Cowen et al., 2005; Gutman and Hanzlikova, 1972; Ulfhake et al., 2000, 2002)). It has also been shown that aged mice are impaired in several sensorimotor-dependent behaviors and that non-prompted physical activity decrease with age (Benice et al., 2006; Dean et al., 1981; Forster et al., 1996; Ingram, 1988; Lau et al., 2008). Furthermore, deficits in sensorimotor-dependent behaviors and body weight were found useful to predict remaining survival of old mice (Ingram et al., 1982; Wax and Goodrick, 1978). Several groups have reported on aging-related impairment of cognitive functions in C57BL/6 mice (Benice et al., 2006; Dean et al., 1981; Forster et al., 1996; Frick et al., 2000; Frick and Gresack, 2003).

Nevertheless, the aging mouse is not as comprehensively characterized as the rat. An animal model of aging must be characterized thoroughly including survival data and should allow for a read-out on the organism level in behavioral tests.

1.4.2 Sarcopenia – senile muscle atrophy

A hallmark of human aging is a progressive loss of skeletal muscle mass, a process referred to as sarcopenia (Rosenberg 1997). On average, aging individuals lose about one-third of the skeletal muscle mass or more precisely, at a rate of 1–2% per year past the age of 50 (Marcell, 2003). This age-related loss of muscle mass is accompanied by a loss of muscle strength that exceeds what would be expected from the reduced muscle mass (Frontera et al. 2000). During aging, this quantitative and qualitative loss of skeletal muscles have significant effects on physical function and quality of life, it is also associated with increased morbidity (Evans 1995; Jette and Jeete 1997).

The mechanisms underlying this profound atrophy remain enigmatic but must include an imbalance in the continuous build-up and break-down of skeletal muscle tissues. In this context it is interesting to note that sarcopenic skeletal muscle shows many alterations typical of a regeneration, including increased expression of myogenic regulatory factors (MRFs, Musaro, Cusella De Angelis et al. 1995; Dedkov, Kostrominova et al. 2003; Edstrom and Ulfhake 2005), essential for the development and regeneration of skeletal muscle, increased precursor cell proliferation (Dedkov, Kostrominova et al. 2003), and a high content of the embryonic myosin heavy chain isoform (Edstrom and Ulfhake 2005). Despite the regenerative activity, loss of skeletal muscle progresses, suggesting that the regenerative machinery fails in senescence (reviewed in (Edstrom, Altun et al. 2007)).

Among the candidate mechanisms underpinning this progression is mitochondrial failure due to an accumulation of DNA damages caused by a defective repair of altered DNA and/or cellular damage by reactive oxygen species (ROS).

Impaired innervations (Gutman and Hanzlikova 1972) (Larsson and Ansved 1995; Valdez, Tapia et al. 2010)(Leksell, 1995) caused by an aging-related loss of motor neurons is also a major candidate mechanism of sarcopenia. However, pending unbiased quantifications, available data suggest that motoneuron loss is too small (<15%) to account for the massive loss of skeletal muscle seen in sarcopenia (reviewed in Ulfhake, Bergman et al. 2000; Edstrom, Altun et al. 2007). Disturbed innervation, however, can be a peripheral process primarily affecting the motor axon's capacity to maintain myofiber innervation or to innervate regenerating myofibers (See Cowen et al., in Dyck and Thomas 2005; Valdez, Tapia et al. 2010). Signs of skeletal muscle denervation are typically found in sarcopenic skeletal muscle. Electromyographic analyses indicate an age-related decrease in the number of active motor units (Campbell, McComas et al. 1973; Brooks and Faulkner 1988), while low-threshold motor units increase in size (Sperling 1980). This is also supported by data based on glycogen depletion techniques, finding increased innervation ratios, and larger motor unit territories (reviewed in Larsson 1995). Based on EMG amplitudes and fiber densities, about a fourth of the motoneurons have been estimated nonfunctional (Stalberg and Fawcett 1982). An important event in the transition of new, or regenerating, fibers to a mature functional phenotype is to establish neuromuscular connectivity. Newly formed myotubes and regenerating myofibers not yet innervated, express an alternative fifth member of the nicotinic acetyl choline receptor (nAChR), referred to as the gamma subunit. Upon innervation this subunit is replaced by the adult

epsilon isoform to produce the a_2bde-pentamer found at mature neuromuscular junctions (reviewed in Millar 2003).

2 AIMS

The general aim of this thesis was to investigate processes that may be significant to aging on a behavioural as well as organ specific level. The focus has been on the nervous system and skeletal muscles in rodents.

The first part of this project addresses the usefulness of C57BL/6 mice as a model of human aging on a behaviour level.

> A behavioral characterization of aging female C57BL/6 mice (of Jackson origin).
>
> A behavioral characterization of aging male C57BL/6J mice and the effect of dietary restriction on behaviour.

The second part of this project addresses two processes in aging: epigenetic DNA modifications by methylation in the nervous system and the progressive loss of skeletal muscle mass.

> Study alterations in DNA methylation of discrete regions of the aging nervous system in the Sprague-Dawley rat.
>
> Investigate if skeletal muscles in aged C57BL/6J mice show signs of an insufficient neuromuscular connectivity.
>
> Evaluate if the mtDNA mutator mouse show changes in skeletal muscles in accord with the alterations seen in WT C57BL/6J mice at corresponding biological age.

3 MATERIAL AND METHODS

3.1 ANIMAL MODELS

All animals used in paper I were off-springs to pregnant C57BL/6 mice (of Jackson origin) delivered from Charles-River, Germany. In paper II all animals used were off-springs to pregnant C57BL/6J mice delivered from Charles-River, Germany. In paper III all animals used were CR-CD rats (of Sprague-Dawley origin) from Charles River, Germany. The C57BL/6J mice used in paper IV were of the same origin as the animal in paper I and II. The mtDNA mutator mice (Trifunovic et al., 2004) were a kind gift from Dr Nils-Göran Larsson. Only homozygous mtDNA mutator mice, properly backcrossed for six to ten generations to C57BL/6J mice, were used in the study.

All animals were purchased as SPF (specified pathogen free) animals and kept under standardized conditions in the animal facility at the Department of Neuroscience, Karolinska Institutet. Health monitoring, according to the FELASA recommendations (http://www.felasa.org/ recommendations.htm), showed that the animals were free of pathogens with the exceptions of *Helicobacter spp*. species.

Food and water were served ad libitum (AL), and changed once a week at about 10 AM. All animals were fed commercially available food-pellets (Lactamin R34, Lantmannen, Sweden). Animals were weighed at regular intervals (every other week or once a month). Animals maintained on a dietary restriction (DR) in paper II received 70% of the intake recorded for AL fed animals. Animals on DR were drawn from the same colony as those fed AL and maintained on this regime from the age of 3 months.
All experiments were conducted under the ethical permits: N122/03, N122/06, N253/08, N120/09, N394/09.

3.2 BEHAVIORAL ANALYSIS

3.2.1 Elevated plus maze (EPM)

EPM relies on the animal's preference for dark and enclosed spaces over bright, exposed spaces and involves a conflict between the desire to explore and the anxiety of exposure and height (Lau et al. 2008). The EPM has a centrally placed open platform (height above floor: 50 cm) from which four 30 cm long arms extend, two open (i.e. without walls) and two closed (i.e. with 30 cm high walls) (Lister 1987; Montgomery 1958; Pellow et al. 1985; Walf and Frye 2007; Fahlstrom et al. 2011a). The following behaviors were recorded during 300 seconds: time spent in closed arms (CA) and open arms (OA), respectively; ambulation into the CA and OA.

3.2.2 Open field arena (OF)

Explorative behavior was examined in a circular arena with walls (diameter 50 cm; height 30 cm) in gray colored plastic (Altun et al., 2007; Dorce and Palermo-Neto, 1994; Drago et al., 1996; Peng et al., 1994). The animal was placed in the arena with the head towards the wall and allowed to freely explore the field for 180 s. During this period (a) ambulation frequency, (b) rearing frequency and (c) time spent in the inner zone of the arena (diameter 39 cm) were evaluated.

3.2.3 Open field (Actimot detection system, TSE, Germany)

This apparatus was used to assess behavior including horizontal locomotor activity and rears (vertical movements), enabling examination of exploration (exploration track) as well as habituation (Diaz Heijtz et al. 2004). In paper I activity over 90 minutes were recorded, enabling examination of exploration as well as habituation. In paper II The first 3 minutes were used to assess the behavior in a novel open field (OF) while the extended recording (60 min) was used to assess locomotion and pattern of habituation.

3.2.4 Dark and light arena transition test (TSE, Germany)

The same detection system was used as above but the arena consisted of two plexi-glass chambers connected by a small opening (2x2 inch). One part of the arena was dark (<10 Lux, stray light from the illuminated arena), while the light arena was illuminated by 400 Lux. The mice were placed in the light arena and the following indices were recorded during 300 sec: latency to enter the dark arena, exploration track, and locomotor activity in respective arena.

3.2.5 Object recognition (OR)

Two related versions of OR were used, one designed to evaluate working memory (OR1) (Ennaceur and Delacour 1988) and one to evaluate memory consolidation (OR2) (Fernandez et al. 2008). Following habituation and pretrial (see (Fahlstrom et al. 2011a)), the time and number of visits to the two objects were recorded, and the preference for the novel object was calculated as (N-F/(F+N)). Latency between pretrial and probe trial was 1 minute. In this OR test, following habituation and pretrial ((Fahlstrom et al. 2011a)), the animal was allowed to explore the objects until 10 seconds had been spent at the objects. In the OR2 test, the latency between pretrial and probe trial was 24h. The cut-off time was set at 15 minutes. Object preference was derived directly from the fraction of the 10 seconds spent at each object. In paper I different animal groups were used for OR1 and OR2. In paper II the animals were first tested with OR1 and one week later with the OR2.

3.2.6 Rotarod

Rotarod (LE8200, LSI Letica, Scientific Intsruments, Spain). In the Rotarod experiments we followed the protocol available at http://empress.har.mrc.ac.uk/ with the exception that routinely a 4th test round was included (Carter et al., 2001). Briefly, in the training sequence the animal was placed on the rod for one minute while it was not revolving; after a pause the animal was placed on the rod again, now revolving at slow speed (4 rpm) and this procedure was repeated once. In the trial sequence, 3 of the 5 Rotarod slots were used, i.e. one slot in-between was left vacant, and the animals were placed on the rod starting at a revolution speed of 4 rpm which successively increased up to 40 rpm during 300 sec. Time to fall-off and speed at fall-off were recorded. Animals were immediately returned to their home-cage after fall-off. If an animal clung to the rod and made a full revolution it was considered as a fall-off. The test was repeated four times.

3.2.7 Beam balance (BB)

Beam balance was used to assess motor coordination and balance as described in (Altun et al., 2007; Clifton et al., 1991). In brief, a 50cm long and 2.3 cm wide rod was suspended between a vertical plastic wall and the open home-cage with litter-mates, at a height of 50 cm above a thick layer of soft bedding material (to reduce impact, should the animal fall-off). The animal was placed on the beam at the plastic wall-end and given 3 trials to cover the distance to the home-cage. Speed and number of limb misplacements (slips) were recorded. In addition, the performance (Beam balance score) on the beam was ranked according to the following scale [adopted from: Clifton et al., 1991]:

1. Balances with steady posture; paws on top of the beam;
2. Balances with steady posture; uses the full-width of the beam;
3. Grasps sides of beam and/or has shaky movement;
4. Attempts to balance on beam but falls;
5. Drapes over beam and/or hangs on beam and falls off;

All animals were given a score regardless of if they failed or not in the beam walk test.

3.2.8 Walking track analysis

Walking track analysis was used to assess gait ((Altun et al., 2007). The following records were made from the walking tracks: (a) stride length (distance between fore paw-fore paw and hind paw-hind paw); (b) gait width (distance between left and right hind paws), (c) placement of hind paw relative to fore paw (distance between hind paw-fore paw in each step cycle).

3.2.9 Grip strength

Grip strength (body suspension test) as described at http://empress.har.mrc.ac.uk/ was used to assess fore-limb muscle strength (Metz and Schwab 2004). A 1 mm thick steel rod was suspended at a height of 50 cm above a thick layer of soft bedding material (to reduce impact should the animal fall-off). The animal was made to grab the rod with both fore-paws and then released in a suspended position. Time to fall-off was recorded and a cut-off at 60 seconds was used.

3.2.10 Geotaxis and ladder climbing

Geotaxis and *ladder climbing* (see http://empress.har. mrc.ac.uk/ and (Metz and Schwab, 2004)) was combined into one test where the animal was placed on a vertically arranged ladder with the head pointing towards the floor. Time to turnaround (geotaxis) and successfully climbing the ladder were recorded. Time cut-off was set to 60 s.

3.2.11 Hot plate test

Hot plate test was used to assess nociceptive threshold (Altun et al. 2007; Espejo and Mir 1993; Langerman et al. 1995). The animal was placed on the heated surface (52^{o}C) until it licked paws, stamped, jumped or vocalized. The time lapse between placement and reaction was recorded as response latency. The cut-off time was set at 30 sec to avoid tissue damage.

3.2.12 Visual placement

Visual placement (see http://empress.har.mrc.ac.uk/ and (Metz and Schwab 2004). Animals were held by the tail while they were gently moved towards a table edge in order to assess visual placement reaction. A score of two was given if the animal vigorously stretched its fore-limbs towards the table surface well in advance of being able to reach it with any part of its body. A score of 1 was given if the stretching was less marked and/or slow but the animal still correctly placed the fore-paws on the table. A score 0 was given when the first encounter of the table was not with the fore-paws.

3.3 SURGICAL PROCEDURES AND TISSUE PREPARATION

In paper III, 20 female rats were ovariectomized under deep general anesthesia according to standard surgical procedure. After surgery, the animals received analgesics (buprenorphine 0.025 mg/kg, s.c.; Schering-Plough, Brussels, Belgium) and returned to their home cages until euthanized. Ten of the ovariectomized females received estradiol benzoate (in sesame oil; 5 mg in 0.05 ml) given s.c. every third day until euthanized after one month; while another ten females received vehicle only. At sacrifice, sera

were collected and shipped for analysis of estradiol content at the Clinical Chemistry Laboratory, Government's Agricultural University, Uppsala, Sweden.

All rats collected for tissue analysis in paper III were decapitated under deep anesthesia and the brain and spinal cord were then dissected out, snap frozen and kept at -80^{o}C until further use.

In paper IV, the following hind limb muscles were dissected out under deep general anesthesia: the tibialis anterior, extensor digitorum longus, gastrocnemius and soleus. The muscles were then weighed, snap frozen and kept at -80^{o}C until further use.

3.4 DNA ANALYSIS

3.4.1 Isolation of DNA

To isolate pure genomic DNA from the different regions of the nervous system, Sigma's *GenElute Mammalian Genomic DNA Miniprep Kit* (G1N350) was used according to the manufacturer's instructions with the exception that a chloroform step was added to the protocol to increase the yield of DNA.

3.4.2 LUminometric Methylation Assay (LUMA)

The LUMA is based on DNA cleavage by methylation sensitive or insensitive restriction enzymes followed by a luminometric polymerase extension assay to quantify the extent of restriction cleavage. The CpG methylation sensitive restriction enzyme *Hpa*II (recognition sequence CCGG) and its methylation insensitive isoschizomer *Msp*I were used in parallel reactions. *EcoR*I (recognition sequence GAATTC) was included in all reactions as an internal reference. *Msp*I and *Hpa*II both leave 5-CG overhangs after DNA cleavage, whereas *Eco*RI produces 5-AATT overhangs, which are then filled in a polymerase extension assay during the stepwise dispensation of dNTPs on the PyrosequencingTM platform. DNA methylation was defined as the *Hpa*II/*Msp*I ratio. If the DNA is completely unmethylated, the *Hpa*II/*Msp*I ratio would be 1.0, and if the DNA is 100% methylated, the *Hpa*II/*Msp*I ratio would approach zero. LUMA analysis of genomic DNA was performed as previously described (Karimi et al. 2006).

3.4.3 Methodological considerations

LUMA is a quantitative DNA methylation assay and a suitable method in DNA methylation studies with large numbers of samples since LUMA is performed in a 96-well plate format and easily scaled up and automated. The method is highly sensitive and versatile, does not require base modifications and permits accurate studies of DNA methylation in 6 hours. In addition, the assay requires only 200–500 ng of genomic DNA. LUMA is also unique in having an internal control for DNA input. Normalization of genomic DNA input has been recognized as a challenging problem in most DNA methylation assays based on restriction enzyme cleavage. LUMA

circumvent this problem by including *Eco*RI as an internal indicator for DNA input and digestion. The exact determination of DNA concentration is unnecessary when *Eco*RI is used as an internal control. Nevertheless, it is important to note that by using the *HpaII*/*MSPI* isoschizomers, all CpG sites in a genome are not covered.

3.5 IMMUNOHISTOCHEMISTRY

3.5.1 Tissue preparation

In paper III frozen forebrains and lumbar spinal cord segments were cut into 14 μm coronal sections. The forebrain sections corresponded to stereotaxic levels -0.7 to -0.2 mm from Bregma (George Paxinos & Charles Watson: The rat brain in stereotaxic coordinates, Academic press 1982 Sidney). In paper IV extensor digitorum longus and solus muscles were cut into 10 μm transverse sections.

3.5.2 Immunohistochemistry

5-methylcytosine and neuronal-specific nuclear protein immunohistochemistry. After air-drying (30 min, 22°C), sections were fixed (10 min, 22°C) in paraformaldehyde (4%), washed with 50% ethanol (30 min, 22°C), blocked in 10% normal donkey serum in PBS containing 0.2% Triton X-100 (2 h, 22°C), and immersed in 2N HCl (2 h, 37°C)(Brown et al. 2008). Sections were then incubated with anti-5-methylcytosine mouse (1:8000, Calbiochem, Merck, Darmstadt, Germany) antibody followed by DyLight 549-conjugated donkey anti-mouse IgG (1:100; Jackson Immuno Research Laboratories Inc., West Grove, PA, USA) antibody. After this, vacant binding sites on the primary and secondary antibodies were blocked with 10% normal mouse serum followed by an excess of unconjugated Fab fragment donky anti-mouse IgG (Lewis et al. 1993). The purpose of the normal mouse serum is to saturate open binding sites on the first secondary antibody with IgG so that they cannot capture the second primary antibody. The purpose of the Fab antibody is to block the mice IgG so that the second secondary antibody will not bind. After rinsing, slides were incubated with neuronal-specific nuclear protein (NeuN; 1:100; a kind gift from Dr. Jonas Frisén, Karolinska Institutet, Stockholm, Sweden) mouse antibody, followed by rhodamine-conjugated donkey anti-mouse IgG (1:100); rinsed, and finally mounted in DPX (Fluka Chemie AG, Buchs, Switzerland). Single labeling and omission of primary antibody were used as controls.

3.5.3 Microscopy and imaging

All images were captured on an Olympus FV1000 confocal microscope using a X60/NA 1.2 water immersion objective, with a lateral point-spread function of 0.15 mm, and image pixilation set to 1600x1600 (pixel size=0.06mm) to avoid aliasing. Image size corresponded to 96 mm in the specimen (zoom x1.8). All images were collected from a z-plane half way through the sections to avoid tissue-surface aberrations. Multichannel image recordings were done sequentially as follows: Ch0

with El=470nm was used to detect autofluorescense; Ch1 with laser El=561nm was used to record 5meC-IR signals; and Ch2 with El=630nm was used to detect NeuN-IR. In the calibration of the channels we followed a previously published protocol (Ulfhake et al. 1994). The lack of bleed-through between Ch1 and Ch2 was assessed by single labeled specimens and combined with the sequential recording strategy, cross-talk between Ch1 and Ch2 should be minimal. The Olympus FV1000 allows the recorded signal intensities to be binned in 4096 levels and to optimize the recording dynamics, a range of primary anti-5meC-ab dilutions were tested (from 1:500 to 1:16000) and the 1:8000 dilution was selected for the intensity recordings. Finally, the laser power and PMT voltage in Ch0 was adjusted to allow autofluorescence to be subtracted from Ch1 prior to intensity measurements of 5meC-IR (Fig. S2). Images used as illustrations had their intensities adjusted using linear tools in ImageJ 1.44i (http://rsb.info.nih.gov/ij/); while all measurements and image subtractions were performed on raw image data.

From each animal (n=4 in each age-group) a systematically sampled row of images was collected from M2 frontal cortex from layer I-II down to layers V-VI. From these raw images and following subtraction of autofleurescence (Ch0) *all* 5meC-IR nuclei (Ch1) also IR to NeuN (Ch2) had their 5meC-IR signal measured using ImageJ 1.44i. Typically, one row of images yielded between 100-150 neuronal nuclei. In order to normalize for any slide-to-slide variation, 5meC intensity was expressed as a ratio over nearby background (unspecific labeling). The ratios were used in the statistical analysis. Corresponding images of the laminae of the spinal cord dorsal and ventral horn were systematically sampled from the L4-L5 lumbar segments and used for 5meC intensity measurements in neuronal profiles as described above.

3.5.4 Methodological considerations

The use of immunohistochemistry has become an extremely common and important tool in biological sciences. But it is important to stress that the reliability of the techniques depends on the antibodies that are used (Saper, 2005). Antibodies used in paper III and IV are well characterized and have been evaluated with complementary techniques. All secondary antibodies used were also incubated on tissue without the presence of primary antibody in order to exclude unspecific binding from the secondary antibody.

3.6 RNA ANALYSIS

Analysis of relative mRNA levels in gastrocnemius muscles was performed using reverse transcription and real-time PCR.

3.6.1 Preparation of RNA

Total RNA was isolated from gastrocnemius muscles according to the TRIzol® protocol (Invitrogen, CA, USA). The samples were DNAse treated (DNA-free™, Ambion Inc., TX, USA) according to the manufacturers protocol to minimize protein

and DNA contamination. RNA amount and purity was measured in a spectrophotometer (Thermo Scientific NanoDrop™ 1000, DE, USA) resulting in OD 260/280 values above 1,9.

3.6.2 Reverse Transcription

Reverse transcription was typically conducted in a reaction volume of 40 µl containing 600 ng of total RNA, 100 units MuLV reverse transcriptase (Perkin Elmer, Applied Biosystems, CA, USA), 2,5 µM Oligo d(T)16, 40 units RNase inhibitor (Perkin Elmer), 1 mM of each dNTP (dATP, dCTP, dGTP, dTTP), 5mM $MgCl_2$ and 1xPCR Buffer II (Perkin Elmer). The RT-reaction mixture was incubated for 10 minutes at 25°C, brought to 42°C in a PC960G PCR thermal cycler (Corbett Research, Mortlake, Australia) for 15 minutes and finally terminated by 5 minutes incubation at 99°C.

3.6.3 Real-time PCR

Real-time PCR was carried out on cDNA transcribed from 30 ng RNA, with Platinum® SYBR® Green qPCR SuperMix-UDG (Invitrogen, CA, USA) and the appropriate primer pairs (paper IV, table 1) in a 7500 Fast Real-Time PCR System (Applied Biosystems, CA, USA). Analysis of results relied on the use of GPX1 as internal controls. Correct melting temperature and size of the amplified products were confirmed using melting curves and agarose gel electrophoresis respectively.

Real-time analysis of SYBER® Green chemistry allows relative quantification of template (cDNA) amount through comparison of number of cycles needed to reach defined signal level, typically the detection threshold. Thus, data for comparison of expression levels are given in numbers of cycles at detection threshold. These cycle numbers represent the exponential growth from cycle to cycle and should be treated as log-values. Normalization to internal controls is consequently carried out through subtraction of their values from target template cycles at detection threshold.

3.6.4 RNA analysis – methodological considerations

Measurements of transcriptional changes are performed to provide information about the regulatory state of a cell or tissue. In this thesis work measurements of mRNA have been used. After homogenization, total mRNA is easily extracted from tissues, and available for further analysis. However, homogenization of the tissue presupposes loss of proportionality to the cellular level; results from comparison of mRNA levels in different tissue specimens should not be inferred to represent regulatory events occurring in single cells. But, to understand the changes in individual cells is usually the motivation behind the analysis of mRNA-levels. Messenger RNAs are blueprints for the synthesis of proteins, but the relationship between changes in levels of mRNA and output of functional proteins is not always straightforward. Pre-translational factors that will affect the proportionality between mRNA and protein levels include the efficiency of mRNA processing, splicing, turnover rate and availability for translation.

These factors are generally not controlled for. Finally, a general issue of relevance to all comparisons of lysates, is the problem of normalization. To ensure correct comparisons of samples they should somehow be adjusted to ensure comparability. Thus, preparation and analysis of samples is standardized in different ways: equal tissue amounts or equal amounts of mRNA are compared and in many instances this is accompanied by the use of "house keeping genes", genes which are supposed to be unresponsive to changes affecting the target genes. House keeping genes serve as internal controls and data are adjusted relative to their levels. But, a growing body of evidence suggests that there are no true housekeeping genes (reviewed in Bustin 2002). If housekeeping genes are regulated in relation to the experimental situation, normalization will introduce a bias. In this thesis a number of housekeeping genes have been tested and discarded in relation to aging and sarcopenia. Among the ones tested, DPX1 has been shown to be among those least affected by aging and sarcopenia. To ensure the use of equal RNA amounts, RNA quality and integrity was carefully characterized (see Bustin 2002).

3.7 STATISTICS

Statistics were performed using Statistica 6.1 (Statsoft, Tulsa, USA) and Mathematica 8.0 (Wolfram Research,Champaign, IL, USA). Comparisons of experimental groups were carried out with parametric or nonparametric testing depending of the data type. Statistical significance levels were set to: *=$\mathbf{p}<0.05$; **=$\mathbf{p}<0.01$; ***=$\mathbf{p}<0.001$.

In paper I, comparisons of experimental groups were mainly carried out with analysis of variance (ANOVA) and Bonferroni's post hoc test (multiple comparison of all groups; two-tailed test). Comparison of two dependent samples was accomplished using Wilcoxon matched-pairs test. Record series of repeated measurement were analyzed by ANOVA for repeated measurements and matched-pairs test. Kaplan–Meier plots were used to analyze survival records and two-sided log-rank test was used to compare samples (Lee and Go, 1997). Correlation of two parameters (interval scale) was accomplished using least square linear regression and calculation of the fraction of explained variance ($\mathbf{r}2$) or by Spearman rank order correlation (ordinal scale). In addition clustering of data was analyzed with **K** —means clustering.

In paper II, comparisons of experimental groups were carried out with analysis of variance (ANOVA) and Bonferroni**'**s post hoc test (multiple comparison of all groups; two tailed test). Record series of repeated measurement were analyzed for differences between groups by ANOVA for repeated measurements. Changes within a group across trials were analyzed using Friedman**'**s ANOVA and Kendall**'**s concordance. Correlation of two parameters (interval scale) was accomplished using least square linear regression and calculation of the fraction of explained variance ($\mathbf{r}2$) or by Spearman rank-order correlation (ordinal scale and interval scale).

The capacity to predict remaining life span of behavioral indices recorded at 22 months was analyzed by repeated linear regression and calculation of the adjusted coefficient of determination ($\mathbf{R}2$) with $\boldsymbol{\alpha}\leq 0.01$ as threshold (for further details, see Supplemented

materials and paper II, fig. S3). In the latter analysis, we used case-wise censoring if a data entry was missing in the behavioral analysis.

In Paper III, comparisons of the LUMA assay results from experimental groups were carried out with Kruskal-Wallis analysis of variance and Wilcoxon test because some of the data sets did not meet the prerequisites of parametric tests such as full factorial ANOVA (Siegel. S., Nonparametric statistics, McGraw-Hill, 1956). Because of the repeated testing, associated with an increased risk of type I errors, the obtained p values were adjusted using Bonferronis algoritm. If the Kruskal-Wallis test yielded a significant difference (adjusted $p<0,05$), pair-wise testing was performed with Wilcoxon test. Analysis of nuclear 5meC-ir was carried out with ANOVA and Tukey's post hoc test.

In paper IV comparisons of groups were carried out with parametric testing using either Student's t-test or ANOVA (analysis of variance), and when significant differences were found, Bonferroni's post hoc test was used for pair-wise comparisons.

4 RESULTS AND DISCUSSIONS

4.1 PAPER I

In this study we have characterized aging-related modulation of the behavioral phenotype in the inbred mouse strain C57BL/6. All animals derive from an in-house colony. Off-springs allocated to this study were arranged in age-cohorts to simplify data processing and comparisons across the expected median life-span (paper 1, table 1). A cross-sectional design is commonly used in aging studies and, as discussed in related work on the laboratory rat (Altun et al., 2007), there is good agreement between results of behavioral analysis obtained with multiple age-groups and those obtained with a longitudinal study-design.

Morbidity increases with advancing age. Although the animals used were under the supervision of a veterinarian and animals with clinical signs of disease considered terminal were excluded (paper 1, section 2), it is important to be aware of the prevalent pathologies of the species and strain used. For a comprehensive account on common diseases in C57BL/6 we refer to standard textbooks and information on http://jaxmice.jax.org/strain/. The perhaps most common pathology in C57BL/6 is lymphoproliferative disease while cardiac failure and renal diseases are less common (*idem*). The 75% and 50% survival ages were 25 and 32 months, respectively (paper 1, fig. 1). Our results are very close to and well within the 95% confidence interval of corresponding data available at The Jackson Laboratory database (http:// phenome.jax.org/pub-cgi/phenome/mpdcgi?rtn=docs/home). This is about 15% longer than that reported for the closely related C57BL/6NNia (Turturro et al., 2002; and references therein for further discussion on life-span variations) but in line with other publications (Ingram et al., 1982; Perez et al., 2009; Ran et al., 2007). This indicates that there may be differences in survival among C57BL/6 sub-strains stressing the importance of well-characterized animal models in aging research. In this context it should be noted that an expected median life-span in excess of 30 months is about 3 times longer than that expected for wild-life mice (Phelan and Austad, 1989) and underscores the impact of reducing environmental hazards in prolonging mammalian life-span (Kirkwood, 2005).

The data presented here on body weight gain and its fluctuation through-out the expected median life span agrees closely with those at The Jackson Laboratory (http:// research.jax.org/resources/index.html) as far as the comparison can be made; i.e. up to 18 months of age, as well as with the complete life-span data described by Turturro (Turturro et al., 1999, 2002) (paper 1, fig. 2). The drop in body weight observed here in advanced age is similar to that reported to occur in several strains of laboratory rats. Since a similar trend is also seen at advanced age in humans, weight reduction might be a trait common to mammalian aging. Importantly, the decrease in gross body mass in senescence is associated with an apparently involuntary change in body mass composition (Altun et al., 2007 and references therein).

The accumulated results of the behavioral tests indicate that certain domains of mouse behavior are modulated through-out life while others only change at an advanced age (paper 1, fig. 4-12). For example, changes in rearings and beam walk are gradually altered during aging whereas performance of other behaviors, like beam balance, are maintained until old age. The pattern of gradual versus more sudden alterations in behavior is consistent with earlier reports on age-related alterations and similar to that observed in the aging rat suggesting that it is not a feature particular for the C57BL/6 (Altun et al., 2007; Dean et al., 1981; Forster et al., 1996; Ingram, 1988). Consistent with this and data in the literature (see above) a number of the aging-related behavioral modifications showed a significant correlation with age (paper 1, table 3). The main rationale for the selection of tests applied here was that they commonly are included in general recommendations for phenotyping- and toxicology screening (http://empress.har.mrc.ac.uk/; Karl et al., 2003). However, some of these tests will favor a small body size over a large, like the beam balance test and the wire-test. The poorer performance in 8- and 15-month-old mice compared with the 3-month-old can certainly be, at least in part, explained by the continued body growth up to 15–20 months. However the drop in suspension time in the wire-test between 15- and 28-month-old mice occurs when gross body weight decreases and, therefore, probably reflects decreased muscle power. Another example of impairment fairly specific to old age is the very poor ability to acquire new motor skills, evident in both the beam walk test (paper I, fig. 8) and the Rotarod test (paper I, fig. 9), suggesting an impairment of motor learning in senescence.

A striking observation was the gradual loss of explorative activity with advancing age (see also Dean et al., 1981; Forster et al., 1996; Ingram, 1988). Both the 3- and 8-month-old age-groups (covering 2–10 months of age, paper I, table 1) of C57BL/6 mice were eager explorers (paper I, figs. 4–6) but this “drive” gradually dissipates as evident in 14–16-month-old mice and manifested among the 27–29-month-old. In fact, many of the 28-month-old mice made very little effort to explore at all (paper I, fig. 12). There has probably been a strong selection for explorers in wild-life given the significance of this behavior in the search of nutrients and a safe environment. Still we know very little, if anything, about the cellular and biochemical events underlying explorative drive and activity. This is likely to be quite an important issue, since a decrease in explorative drive (as seen during aging) probably influences, on several levels, the performance in many cognitive tests (see below). Interestingly, when challenged with explorative tasks wild mice show an even broader repertoire of behaviors than do laboratory mice (Holmes et al., 2000).

There are fewer data on memory deficits and changes in learning ability during aging of mice (Benice et al., 2006; Dean et al., 1981; Forster et al., 1996; Frick et al., 2000; Frick and Gresack, 2003) as compared to corresponding data available for the rat (e.g. Gerrard et al., 2008; Lewis et al., 2008; Scali et al., 1997; and references therein). We used the object recognition task to test working memory and memory consolidation (Bevins and Besheer, 2006). While elderly mice were able to discriminate a novel object from a familiar one within the working memory time window (paper I, fig. 10), they were impaired when the delay was extended to 24 h (paper I, fig. 11). This is consistent with the data of Dean et al. (1981) in their one-trial passive avoidance test, showing an intact performance of working memory but an impairment of memory

retention at longer delays in 24-month-old C57BL/6J mice of both sexes. However, it should be noted that the OR1 and OR2 tests were conducted on different animal groups, and therefore the conclusion of an intact working memory and an impaired memory consolidation in 28-month-old female C57BL/6 is by inference. Previous studies on female C57BL/6 (J or Nia) indicate that memory impairment may occur at an earlier age in female than male mice (Benice et al., 2006; Frick et al., 2002; Frick and Gresack, 2003). This would be in agreement with our 15-month-old female mice that scored less well than young adults in the object memory consolidation test, although the difference was not statistically significant (Paper I, fig. 11).

Although brain structures of critical importance for an intact working memory and also for memory consolidation are still being debated (Dere et al., 2007) it seems as if working memory relies on prefrontal cortex regions whereas memory consolidation also depends on an intact hippocampus function. Our data on old mice indicate an impairment of both 'object' memory consolidation and ability to learn new motor skills suggesting that this model may be useful in dissecting the underlying cellular mechanisms of importance in these behaviors.

Object recognition tests of memory rely on a drive to explore, as do many other cognitive tests. The drastic reduction in explorative behavior observed in aged mice led us to re-examine the explorative behavior-dependent data sets (paper I, fig. 12). This exercise confirmed that aged C57BL/6 mice do not represent the homogenous population expected from their close genetic relationship and the highly standardized conditions of upbringing and housing. This is consistent with earlier observations on age-related behavioral alterations showing that aging affects individuals differently when subjects are grouped according to chronological age (Collier and Coleman, 1991; Forster et al., 1996; Ingram, 1988). The existence of subgroups of aged-matched mice with intact and impaired explorative behavior, respectively, could be verified by cluster analysis among 28-month-old mice (Paper I, fig. 12). This finding is relevant to the ongoing debate on the relative contribution made by the genetic make-up, the environment and the epigenetic arrangement of the DNA (Fraga and Esteller, 2007; Lathe, 2004; Mathers, 2006; Wong et al., 2005). It seems clear that subtle environmental factors may play a more significant role than hitherto recognized (Ingram, 1988; Lathe, 2004); however, the earlier notion that what is not in the genes should be searched for in the environment, is now being challenged by a view that nuclear DNA is rearranged epigenetically through-out life, by DNA-methylation and histone modifications. Recent data on DNA-methylation suggest that this may be a highly dynamic mechanism far beyond the embryological imprinting and the 3–5% DNA-methylation rearrangements occurring at somatic cell divisions (Liu et al., 2007; Mathers, 2006; Nguyen et al., 2007; Siegmund et al., 2007; Wong et al., 2005). Furthermore, a body of data indicates that global changes in methylation associates with tumor progression and cell replicative senescence, suggesting alterations in DNA-methylation as one important mechanism in the aging process of neurons that certainly deserves further attention (Liu et al., 2007).

4.2 PAPER II

In this study we have analyzed behaviors in 4-, 22- and 28-month-old male C57BL/6J mice with a broad range of tests. For comparison, a group of 28-month-old males maintained on dietary restriction (DR) was included.

Decline in exploratory activity marks the aging C57BL/6J male mouse
Male C57BL/6J mice are active explorers in early adulthood and our results on adult male mice are in line with data published by Crabbe et al. (Crabbe et al. 1999; Wahlsten et al. 2003) and Matsuo et al. (Matsuo *et al.* 2010). The most conspicuous alteration in behavior during aging is the decline in exploratory activity (paper II, figs. 1-5), a change noted by several investigators (Goodrick 1967; Goodrick 1973; Sprott & Eleftheriou 1974; Goodrick 1975; Dean *et al.* 1981; Ingram *et al.* 1981; Ingram *et al.* 1982; Ingram & Reynolds 1986; Forster *et al.* 1996; Lau *et al.* 2008). The drive to explore is a fundamental behavior that likely impact other behaviors when affected (see below and (paper I)) and the decline is a gradual process clearly evident at 22 months that deteriorates further in advanced age. Although the functional domain of the brain responsible for decisions underlying exploration behavior has yet not been revealed, experimental work suggests that in mammals these processes reside in the prefrontal cortex and that (projections to the prefrontal cortex of) the ascending locus coeruleus–norepinephrine system modulates these processes (reviewed in (Aston-Jones and Cohen 2005)). Just recently, this concept gained further support from work in *c. elegance* ((Bendesky et al. 2011)) showing that the decision to explore (in a foraging choice situation) is governed by catecholamine signaling. This is of considerable interest since the central aminergic systems (at least) in rodents are vulnerable to aging with overt signs of axon terminal loss and neuroaxonal dystrophy (Johnson et al. 1993; Cowen et al. 2005; van Luijtelaar et al. 1988). As discussed below high exploratory activity also correlates positively with long post-testing survival expectancy.

With advancing age also other behaviors are affected. Skilled motor performance assessed in the beam and rotarod tests declines as does grip strength (paper II, figs. 6 and 7)(Dean III et al. 1981; Ingram 1988). However, some behaviors are well preserved in advanced age as the placement reaction and hot plate latency, suggesting that sensorimotor mechanisms underlying these behaviors are intact. Furthermore, both 22- and 28 months old males managed as well as young adults in the object recognition test of working memory (paper II, fig. 8). The sparing of non-spatial working memory also in advanced age confirms and extends Benice and coworkers (Benice et al. 2006) observation that memory function is intact in 18-20 month-old male and female C57BL/6J mice (Dean III et al. 1981). In contrast, object memory consolidation was impaired in both 22- and 28 month-old male mice examined here (paper II, fig. 8). The impairment of a delayed memory recall in aged C57BL/6J is in line with several reports using different tests to probe memory recall function (Dean III et al. 1981; Benice et al. 2006; Frick et al. 2000; Forster et al. 1996).

Dietary restriction modulates behavioral alterations seen in aging ad libitum fed mice
Reducing calorie intake by manipulation of diet formulas or food restriction (DR) is the most universal regime to challenge the process of aging ((McCay et al. 1989; Stunkard 1983) see recent reviews by (Anderson and Weindruch 2010; Baur et al. 2010)). The

detailed mechanism(s) by which food restriction modulates aging remains largely unknown but it increases health span and life span by curbing morbidity and by modulating basal metabolism reviewed in (Guarente and Picard 2005). Food restriction impacts organism behavior not only by preserving adult behaviors into advanced age (Ingram and Reynolds 1983, 1987; Idrobo et al. 1987) but apparently also by "setting" the level of basal physical activity already in young adulthood (Weed et al. 1997; McCarter et al. 1997; Holloszy and Schechtman 1991; Chen et al. 2005). We included a group of 28 month old male mice maintained on a modest DR regime (30% reduction of ad libitum consumption) for comparison. In related work we have shown that a 30% food restriction increases the (median) expected life span by about 20-25% (Altun et al. 2007); Fahlstrom et al., unpublished results), for C57BL/6 from about 30 to 36 months. The 28 months old DR mice were markedly different from the AL fed aged matched controls in all tests with an exploratory component (paper II, figs. 1-4) and in locomotor activity as well as adaptive motor performance (paper II, figs. 5 and 7). In several aspects they were indistinguishable from young adult male mice. However, on closer inspection there were qualitative differences between young adult and aged DR males behaviors; for example in the dark and light arena transition test aged DR males covered a larger distance and used more time to explore the light compartment (paper II, figs. 1-2) and in the locomotor test (paper II, figs. 4 and S1-S2) DR animals were more active in the central field, thus did not show the same preference for the peripheral field as did young adults and aged AL-fed mice. Finally, aged males on DR did not show the same degree of habituation over time in the locomotion activity test (paper II, fig. 5). In contrast to these distinct effects, the DR regime used here did not impede the devastating effects of aging on grip-strength, beam balance or delayed object memory recollection (OR2) (paper II, figs. 6B-D and 8). The beneficial effects of DR on memory-dependent behaviors remains somewhat controversial ((Burger et al. 2010; Minor et al. 2008; Fontan-Lozano et al. 2007; Mattson 2010)) and it cannot be excluded that particularities of different DR regimes alone or interacting with strain (genetic) differences account for these differences.

Correlations among tests within and across age groups

When using a range of tests to behaviorally characterize mice, cross-correlation analysis of the results is helpful to assess if tests are complementary or if they *measure* the same underlying properties of an organism's behavior (Ingram 1996, 1988; Wahlsten et al. 2003; Fahlstrom et al. 2011a). Within age group cross-correlation analysis of the mice studied here revealed that results from sensorimotor and rotarod tests did not correlate consistently with the outcome of the exploratory tests in young adult males (EPM, OF, dark-light transition or OR). In contrast, among tests with an exploratory component, consistent cross-correlations were observed such as males that were active in the EPM and made multiple entries into the OA also were very active, including exploration of the central field, in the OF and locomotor tests (paper II, supplementary table S1-S2). Mice spending more time in the CA and made fewer entries to the OA of the EPM also avoided the central field of the OF arena and showed a preference for the dark compartment in the dark and light transition test. Although the level of exploratory activity was significantly reduced in 22 month-old males (paper II, figs. 1, 3 and S2), the correlations among exploratory indices were similar as in young adult mice (paper II, table S2). However some differences were noted, for example the capacity to manage the rotarod in the first trial correlated positively with activity level

in the EPM. This incipient change in cross-correlation pattern became overt in the 28 month-old AL fed mice. In this age-group only few variables of the tests with an exploratory component cross-correlated, while fairly strong cross-correlations became evident between indices of the sensorimotor test and different exploratory indices (paper II, table S2). In contrast, among aged males maintained on DR, cross-correlations among test variables were in close agreement with those seen in young adult males.

The EPM, light and dark arena transition test, and OF test, all share that they involve a conflict between the desire to explore and the anxiety of exposure or height. Among the young adult mice there were individuals with a "daring" or an "anxious" behavioral profile as judged by the correlations among tests with explorative and anxiety components, a pattern that was not as evident in the 28 month-old mice. A comparison between the 4 month-old and 28 month-old mice across tests done here with an anxiety component is not conclusive because the results varied from test-to-test. For example, the EPM read-outs suggest an increased level of anxiety among the 28 month-old mice c.f. 4 month-old (paper II, fig. 1), however, in the light and dark arena transition test the latency to escape into the dark compartment tended to be longer in aged mice and, furthermore, aged mice spent more of the total time in the light compartment (paper II, fig. 3). Earlier studies on aging-related behavioral changes in rodents, using the number of deposits and/or urinations as indicators of stress/anxiety level during behavioral testing, showed that anxiety level did not change with advancing age (Edstrom and Ulfhake 2005; Altun et al. 2007).

Comparisons of behavioral changes during aging in female and male C57BL/6
Behavioral analysis of adult C57BL/6J have mainly considered male mice, however, a few studies included both genders and reported no effect of sex in the tests used for behavioral phenotyping (Wahlsten et al. 2003; Lau et al. 2008). Corresponding data on possible gender differences during aging is even scarcer, Wax and Goodrick (Wax and Goodrick 1978) studied wheel running in males and females of two different mouse strains and concluded that although there were differences between strains they saw no effect of gender within strains. Also in the study by Dean and collaborators no differences were noted between sexes (Dean III et al. 1981). Comparison of the results obtained in male C57BL/6J here with recently published behavioral assessments of female C57BL/6 (Paper I) reveal that alterations evident in 28 month-old male and female mice are very similar. In general, exploration activities decline progressively during aging and are conspicuous at 28 months in both sexes (paper I, figs. 4-5). Many capacities assessed by the sensorimotor tests are reduced to a similar extent with advancing age in both genders (c.f. Paper II, figs.6-7 with paper I, figs. 7-9)) or not affected by aging in either sex (thermal nociceptive threshold). Thus, changes of several behavioral indices correlate with age in both male and female C57BL/6 (paper II, table 4). Males and females showed no impairment of working memory at 28 months (paper II, OR1; c.f. Paper II, fig. 8 with paper I, fig. 10) while at this age both genders failed in the object memory consolidation test (paper II, OR2; c.f. paper II, fig. 8 and paper I, fig. 11). The lack of a gender difference in object recognition tests appears to be at variance with the accumulating body of evidence suggesting a gender difference in cognitive decline and memory function in early senescence (22-24 month-old), considered to be caused by the cessation of reproduction and decreased systemic

levels of gonadal hormones in females (reviewed in (Frick 2009)). The current study and the previous one (paper I) were not designed specifically to address the issue of a gender difference in early aging and at advanced age studies agree that both sexes are affected. However, there are some gender differences among the animals used here and in paper I, evident in results of the EPM (c.f. paper II, fig. 1 and paper I, fig. 4). While total number of arm entries was similar in young adults of both genders, females showed a strong preference for the closed arms (~80%) not evident in males (~50%). The decline in arm entries with advancing age had the same magnitude in both genders (-60% to -70%) but affected entries into the OA the most in males. Thus, in the EPM, aged males are more similar to aged females than young adult males to aged-matched females. This observation and earlier notification of differences in aggressive behavior between genders in adulthood (Van Loo et al. 2003) deserves further attention and suggests that a test capturing secondary manifestations of sexual differentiation should be included in a primary behavioral screen of genetically modified mice.

Biological and chronological age and prediction of remaining life span from behavioral indices in early senescence

The pace of aging and the expected life-span vary among individuals (Ulfhake et al. 2002; Collier and Coleman 1991) also in inbred mice strains such as C57BL/6J ((Ingram 1988)). Individual trajectories of aging are shaped by the genetic makeup, the environment and interactions on the epigenetic level and as a consequence biological age is in part dissociated from chronological age of an organism. The search for markers of *biological age* in chronologically age-matched populations has attracted considerable interest in research on human but also in rodent aging ((Swindell et al. 2008) and references therein). Behavioral changes/impairments can be considered a high-end read-out of aging impacts on the organism; and a few studies have analyzed the capacity of behavioral indices to predict (remaining) life-span (Wax and Goodrick 1978; Ingram et al. 1982; Ingram and Reynolds 1987). Ingram and Reynolds ((Ingram and Reynolds 1987)) assessed predictive value of behavioral indices in aging C57BL/6J males and found linear relations between life-span and several of the test indices (exploratory and locomotor activity, and grip strength) which combined, using multiple regression analysis, accounted for about 40% of the differences in observed life-span. Consistent with this we found that tests holding an exploration component were somewhat more powerful to predict remaining life-span (EPM>OF) than motor skills and muscle strength (RR>grip strength) (paper II, table 3). The linear correlations obtained here were stronger than those seen among the mice analyzed by Ingram and Reynolds ((Ingram and Reynolds 1987)) and another difference between studies was that RR (here executed according to the protocol of EMPReSS; but with 4 trials instead of 3) in the present study showed a fairly high linear correlation with remaining life-span. It cannot be excluded that this discrepancy was caused by differences in RR platform and protocol used in the two studies; this is in fact plausible since the two studies agree on that other indices of high locomotor activity correlate positively with life-span. In the present study, the best single indices could explain 60-65% of the observed variances in life-span, combining two indices (multivariate approach; see Supplemented materials in Paper II) generated linear models that could account for 70-80% and models with three indices: 92-93% of the variances in remaining life-span (paper II, table 3). An important observation made here was that the best models (based on one, two or three indices) all held at least one exploratory component, suggesting

that alterations in exploration activity reflect important underlying modulations caused by biological aging (see also above).

In this study whole body weight (paper II, table 1) at 22 months did not correlate with remaining life-span (r^2=0.108; p=0.388)(cf. Ingram, 1988; Ingram and Reynolds, 1987). However, whole body weight was a useful measure to predict remaining life span when combined with behavioral indices (see paper II results and table 3) in this age-cohort. As evident in paper II, tables S1 and S2, whole body weight did not correlate significantly with any of the recorded behavioural indices *within* age groups. The impact of whole body weight on behavioural indices such as grip strength and RR was limited and could not account for the pattern of changes observed across age-groups (cf paper II figs. 6B and S5, 7 and S6, respectively). Since whole body weight is a compounded measure it may not be suited to use in this context. A measure which reflects changes in body composition across life span, such as lean body mass, muscle mass-to-whole body mass or MRI/DXA; is probably better (see also, (Edstrom and Ulfhake 2005; Altun et al. 2007)). Although the latter methods are more laborious or expensive, we strongly recommend such measures to be included in future studies.

Consistent with that individuals with a high level of spontaneous exploratory activity and relatively preserved capacity to manage motor tests such as RR and grip strength test were the most long-lived among the 22 month-old male C57BL/6J, 28 month-old males maintained on dietary restriction which challenge normal aging and extend expected survival (see above) performed much better than ad libitum fed aged-matched males in these tests and, moreover, were almost indistinguishable from young adults in the EPM, locomotor test and rotarod (paper II, figs. 1, 5, 7 and S2). The only exemption was that 28 month-old males maintained on DR were not successful in the grip strength test (paper II, fig. 6).

4.3 PAPER III

In this study we have used a well characterized Sprague-Dawley rat model (paper III, fig. 1) (Altun *et al.* 2007), to assess changes in global DNA methylation in the central nervous system during aging. During the progress of this work, it became evident that both nervous system region and gender had to be considered. Using the LUMA assay for genome-wide screen of DNA methylation at CCGG sequences (Karimi *et al.* 2006), and cellular labeling for 5meC immunoreactivity (IR; see paper III, supplemented materials; (Lewis Carl *et al.* 1993; Brown *et al.* 2008), we observed a region specific decrease in DNA methylation during aging in several but not all regions studied and that some of these changes showed a gender-specific pattern. Importantly, in females gonadal functions seem to affect DNA methylation in hippocampus, frontal cortex and the spinal cord but not in striatum.

LUMA assay of frontal cortex, hippocampus, striatum, cerebellum and the lumbar spinal cord revealed between 40% and 60% DNA methylation at CCGG sites in young adult (male and female) rats (paper III, fig. 2A-B) with no significant difference between regions within genders. Across adult-life span global DNA methylation showed either no change, or decreases between -15% to -60% which were region- and

gender specific (Kruskal-Wallis, effect of age: $p<0.001$; in both males and females)(paper III, fig. 2A-B). In females, DNA methylation decreased in both frontal cortex and hippocampus from young adulthood to one year of age and a further drop in hippocampus during the second year of life (young adult vs. 20 month-old; $p<0.01$), while no further change was observed in advanced age (paper III, fig. 2A). In sharp contrast, the cerebellum and the striatum showed no significant change in DNA methylation across adult life-span (paper III, fig. 2A). The most dramatic drop in DNA methylation (in aging females) was in the lumbar spinal cord (-60%; $p<0.001$) (paper III, fig. 2A).

The intriguing observation that DNA methylation in frontal cortex and hippocampus decreased when female fertility declines (Markowska 1999) with no further change in advanced age led us to examine if the ovaries influence DNA methylation in adulthood. Two month-old females were ovariectomized (ovx) and sacrificed for analysis 1 month later. Ovx lowered circulating estrogen to, or below, detection level (≤7pmol/l) and in these animals, DNA methylation in the frontal cortex decreased to levels seen in intact 12- and 20 month-old females ($p<0.05$; paper III, fig. 2C cf fig. 2A). DNA-methylation also declined in the hippocampus and spinal cord following ovx but the changes did not reach statistical significance. Interestingly, ovx did not influence DNA methylation in the striatum (paper III, fig. 2C). Blunt estrogen substitution raised circulating levels of estrogen above the linear range of the test used (>180 pmol/L) but could not impede the decline in DNA methylation significantly (paper III, fig. 2C).

In contrast to females, males showed no significant change in DNA methylation in the frontal cortex across life-span and the decline in 5meC observed in the hippocampus ($p<0.01$) occurred at more advanced age than in females. Another difference between sexes was the significant ($p<0.01$) decline in DNA methylation in the striatum of males. In contrast to these gender differences, the decrease in DNA-methylation across adult-life span in the spinal cord was similar in males and females (paper III, fig. 2A, B). It is also noted that the different trajectories of DNA methylation changes observed between regions during aging result in an increased difference between regions that in several cases reached statistical significance (paper III, fig. 2A-B).

Since neurons represent only one of several cell populations in the central nervous system, it was important to determine if the observed changes in DNA-methylation also affected neurons. We used immunohistochemistry to determine levels of immunoreactive (IR) 5meC in neuron nuclei in the layers of the frontal cortex and the laminae of the lumbar spinal cord (paper III, fig. 3). In the frontal cortex of aged female rats, there was a decrease ($p<0.001$) in nuclear 5meC-IR (in neurons) of all layers (paper III, fig. 3G). Although the cortical neurons of all analyzed aged females, on average, had lower levels of 5meC than the young adults, there were also differences among the aged animals that reached statistical significance ($p<0.01$). Such large differences were not observed among the young adults, suggesting that DNA hypomethylation during aging varies among individuals. Analysis of the spinal cord revealed an overall decrease in neuronal 5meC-IR ($p<0.001$) which was marked in the dorsal horn (lamina I-IV; $p<0.001$) but more diminutive in the ventral horn (laminae V-IX; $p<0.05$) (paper III, fig. 3H).

DNA hypomethylation in the brain during aging was first described by Wilson and Jones (Wilson & Jones 1983) and the current study extends these findings by showing that loss of DNA methylation is both region and gender specific, and importantly affects neurons. Global loss of DNA methylation in aging seems to be common in several tissues and is a conspicuous feature in cellular aging and tumorigenesis (Feinberg & Vogelstein 1983; Esteller 2003). In these conditions, DNA hypomethylation may play a role in loss of cell phenotype fidelity and changes in DNA methylation have been associated with several neurodegenerative diseases, many of which are late-onset conditions where aging may be a significant cofactor.

Since the results of several of the LUMA-based experiments were not suitable for parametric statistical analysis, nonparametric statistics were used. To protect towards type I errors associated with multiple testing conditions, Benferooni's correction factor of the p values (adjusted p values) was used. This is quite a conservative method providing a good protection towards type I errors but has been criticized because it tends to generate type II errors, i.e. false negative results. This should be kept in mind and is the rational why we bring up some non-significant tendencies in this report.

An intriguing observation made here is the decline in DNA-methylation in the frontal cortex and the hippocampus occurring when reproductive capacity ceases in females. At the age of one year when female rats normally retire as breeders, and both reproduction and gonadal cycling ceases in the vast majority of female rodents during the 2nd year of life (Markowska 1999). Thus, there seems to be a close concordance between DNA hypomethylation in frontal cortex and hippocampus, on the one hand, and cessation of reproduction and gonadal cyclicity, on the other. Furthermore, a significant cognitive decline is evident in one-year-old female but not male rodents, accentuated during the 2nd year of life, and correlating with loss of estrous cycling (Markowska 1999; Frick *et al.* 2000); reviewed in (Frick 2009). Our finding that ovx in young adult females induced a DNA hypomethylation similar to that seen in 12- and 20-month old females suggests a direct link between gonadal steroids and DNA-methylation in brain regions relevant to cognitive and memory processes (Squire 2004; Dere *et al.* 2007) and dysmethylation as a possible mechanism in female post-fertile cognitive decline.

The failure to rescue DNA methylation by estradiol-benzoate supplementation suggests a more complex interaction and that progesterone also plays a role in DNA methylation in this brain region. However, the non-physiological supplementation of estradiol used here must be acknowledged (see (Frick 2009; Foy *et al.* 2010) and references therein). Importantly, estrogen supplementation seems to improve cognitive capacity in ovariectomized and acyclic adult and aged female rodents (reviewed in (Frick 2009)). This notion is consistent with a recent observation that estrogen may enhance object memory in rodents through *de novo* DNA methylation and histone acetylation (Zhao *et al.* 2010).

In contrast to female rats and consistent with the gender difference in aging-related cognitive decline (*idem*), males showed no significant decline in global DNA methylation in the frontal cortex across adult life-span while not until more advanced age DNA methylation declines in the male hippocampus. The marked aging-related

drop in DNA methylation in the lumbar spinal cord was similar in males and females, and indicates that DNA hypomethylation may play a role in the development of the severe hind limb sensorimotor disturbances that is equally evident in both sexes (Altun *et al.* 2007).

4.4 PAPER IV

In this study we have compared sarcopenia in C57BL/6J WT mice and the mtDNA mutator mice in an effort to address if mitochondrial impairment caused by an accumulation of mtDNA damage is sufficient to generate the inevitable loss of muscle mass that occurs during normal aging.

Our survival data of female and male mtDNA and WT mice confirm that the median expected life span is 10-11 month in mutator mice while maximum life-span in the cohorts studied was 14.4 months (paper IV, fig 1A) (c.f. Trifunovic, 2004). WT from the same breeding colony had a median expected survival of 29-30 months and a maximum survival of 35 months. We could not detect any gender difference in either group of mice. The WT data was further compared with ad libitum and DR restricted C57BL/6J cohorts from other in-house colonies (paper IV, fig. 1C) and we could conclude the WTs of the polgA breeding colony did not deviate in life-span characteristics from other C57BL/6 colonies (paper I). Based on these survival curves we decided to compare biological aging using mutator mice at 10-11 months of age and WT at 28-30 months of age. Young adults (3 to 5 months old) were used as controls for respective genotype.

Body weight (BW) increases rapidly the first 8 weeks of post-natal life in both mutator and WT mice (paper IV, fig. 1B). In WT, BW reaches a plateau towards the end of the 2nd year followed by a drop in advanced age ((Turturro et al. 2002; Nadon 2004; paper I) A similar but in time much compressed pattern of BW changes was evident in the mutator mice. These mice peak at BWs comparable to those recorded for WT mice maintained on dietary restriction (DR) (paper IV, fig. 1D).

In line with previous observations (paper I), C57BL/6J mice show upon inspection overt signs of aging including kyphosis, graying and balding of the fur, and a stiffening of the tail. Our observations on the mtDNA mutator mice were consistent with previous observations including kyphosis and balding of the fur (Trifunovic et al. 2004). Graying of the fur and stiffening of the tail seen in the aged C57BL/6J mice were not seen in the mtDNA mutator mice.

Skeletal muscles
We have used two muscles to analyze changes across life-span, extensor digitorum longus (EDL) as a representative for fast muscles, completely dominated by type II fibers (IIb and IId), and m. soleus, a component of the triceps surae muscle, which is composed of both fast and slow twitch fibers but dominated by the latter (~70-80%) and characterized by slow contraction and long duty-cycles (Hennig & Lomo 1985). The second component of this muscle, the gastrocnemius, was used to extract protein and mRNA for expression analysis.

EDL wet-weight decreases significantly across life span in both WTs and mutator mice (paper IV, fig. 2A). In considering adaptation to changes in gross body weight, the ratio: muscle weight to whole body weight was computed and the drop in ratio at advanced age was more marked than observed for muscle wet-weight (paper IV, fig. 2B). This latter analysis revealed also that the drop of EDL muscle mass is more pronounced in WTs than mutator mice ($p<0.05$). EDL force-output (isolated EDL) per unit muscle was significantly reduced in the mutator mice but not significantly affected in the aged WT EDL (paper IV, fig. 3A-B). We could not detect any change in distribution of adult MyHC IIb and IId isoforms during aging of WT or mutator mice (paper IV, fig. 4A) suggesting that no major fiber-type shift occurs during aging of EDL in mice.

Consistent with the observations in EDL, the soleus muscle wet-weight decreased during aging (paper IV, fig. 2C). Due to the large variability and rather small number of observations among mutator mice this decline did not, as in WT mice, reach statistical significance. The drop in muscle mass is even more conspicuous when set in relation to whole body weight (paper IV, fig. 2D; see also above under EDL). As with EDL the drop is somewhat more pronounced in WTs than mutator mice, however, the size-order is similar. In contrast to EDL, soleus force-output per unit muscle did not change significantly during aging of WTs or mutator mice (paper IV, fig. 3C-D). In accord with the observation in EDL, we could not detect any significant shift in distribution of adult MyHCs (I, IIa, IId or IId) (paper IV, fig. 4B).

Regulation of biomarkers associated with mitochondrial biogenesis

We examined if standard biomarkers of mitochondrial biogenesis were induced in normal aging or in the mutator mice. Neither TFAM-1 nor pgc-1a or PPAR-g, a potent activator of pgc-1a, is induced in normal aging (paper IV, fig. 5). Surprisingly, of these molecules only PPAR-g was up-regulated in aged mutator mice (paper IV, fig. 5E). These observations suggest that the feedback signaling to increase mitochondrial production is not activated in normal aging and, perhaps more unexpected, only PPAR-g was induced in the mutator mice. There are several reports providing support for the notion that mitochondrial dysfunction increases with advancing age and this process is markedly accelerated in the mutator mice; still the accumulation of impaired mitochondria/shortage of intact mitochondria do not appear to trigger an increased mitochondrial biogenesis, at least not along so far established signaling pathways.

Expression of nicotinic Acetylcholine receptor subunit -γ (nAChR-γ)

During normal aging there is a dramatic up-regulation of nAChR-γ mRNA (paper IV, fig. 6A), the magnitude of this induction is similar to that found in nerve injuries (Grönholdt-Klein, Edström et al., in preparation) suggesting that a rather large number of aged myofibers lack appropriate innervation (see also (Valdez, 2010; Edstrom, 2007). Immunoblotting of muscle extracts revealed that this transcriptional induction is translated to increased levels of nAChR-γ protein (paper IV, fig. 6B). In mutator mice, the expression of nAChR-γ mRNA was more modestly increased, occurring already in young adults (paper IV, fig. 6A) with no further increase across adult life-span. Immunoblotting revealed a small increase compared to young adult WTs (~25%) which increased somewhat across life-span (~75%). Thus, while the induction of

nAChR-γ is a progressive process in hind limb skeletal muscles among aging WTs, it appears more like a stochastic event among mutator mice, affecting also some young individuals and not very dramatically accentuated by aging.

5 CONCLUDING REMARKS

In paper I and II alterations in behavior with advancing age in C57BL/6 mice were studied. Our behavioral data show that aging is a continuous process modulating certain aspects of the basic behavior in a continuous manner. Advanced age is associated with an accelerated decay in behavioral performance evident in most of the tests used. The observed changes in the aging mice are very similar to alterations seen in the senescent human. This is of course fascinating from a biological perspective that changes that take decades to develop in humans are evident in the mouse in less than 20-25 months. But more importantly, it makes the mouse with its short life span an ideal model for human aging, not only for practical but also for a number of scientific reasons. The possibility to isolate a problem or a specific question is rather simple in a mice model living in an environment under the scientist´s control. A good example is the effect of dietary restriction on behavior and the short life span of mice allow for relatively rapid answers to the addressed question and in the end advances in understanding of aging. An even more important reason for using the mice is the advances in genetic engineering and as a matter of fact the C57BL/6 mice is the golden standard on which most genetically engineered mice are back-crossed. The mtDNA mutator mice used in paper IV is a good example on how we addressed a specific question with the help of a transgenic mouse model: if mitochondrial impairment, caused by an accumulation of mtDNA damage, is sufficient to cause changes in skeletal muscles in accord with the alterations seen in WT C57BL/6J mice at corresponding biological age. A question that would not be possible to answer in such a direct way with out the advance in the field of genetic engineering.

In paper III we have used rats instead of mice. The reason was practical: it was too difficult to extract enough DNA from the discrete brain regions used in the mice since the mice central nervous system is much smaller than the rats.

Finally, I would like to comment on the skepticism concerning the use of rodents as a model for human biology, a skepticism that is more common among my clinical colleagues than one would expect. This is very difficult to understand or defend from a scientific point of view. The reason why I bring this up is because I think mice are absolutely crucial for fast and reliable advances in medical research now and in the future. It is important to understand the value of a good animal model since translational medicine will always go through the mice.

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