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Hormones in Ageing and Longevity

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Hormones in Ageing and Longevity

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Preface

Hormones are natural chemical signals synthesized from specialized group of cells to influence bodily functions. They are the excellent system of communication from one tissue/cell to others within an organism, and are involved in the dynamic control of biochemical and physiological functions. Hormonal imbalance creates an upset in the regulatory mechanisms thereby disturbing the homeodynamic balance. Besides the level of hormones, their cognate receptors and post-receptor events also have a large influence on the final response to a particular hormone. There also exists a cross-talk in such signaling with an added advantage to the organisms. Hormones also coordinate a wide range of processes in biological systems including neuroendocrine and immunological controls. However, the competence of body's homeodynamic adjustments tends to decline as one ages. A scholarly collection of updates on various hormonal signaling in ageing, health and longevity is of great importance to the readers working in the area of hormone signaling in general and in the field of ageing research in particular.

This multi-chapter review book presents the present state of knowledge on the role of hormones in health, ageing and longevity. The book is divided into four major parts: Part I embodies history and conflux on more than 100 years of hormone science and its challenges; Part II presents varied chapters on hormones involved in growth, stress and metabolism; Part III encompasses chapters on neuroendocrine axis and rhythms during ageing process; and Part IV has chapters on hormones affecting the brain, immunity and lifespan, including a chapter on plant-based cytokinin hormones for the modulation of ageing and longevity.

Engrained with the up-to-date information about role of hormones in health, ageing and longevity, this collection is a valuable addition to the book series "Healthy Ageing and Longevity", and provides a reliable source of information and knowledge useful for understanding and developing potential hormone-based interventions for modulating ageing and longevity.

Aarhus, Denmark — Suresh Rattan, Ph.D., Dr. Scient.
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Part I
History and Conflux

Chapter 1
How Hormones, as Ancient Signalling Molecules, Regulate Diverse Biological Processes Through Evolution

Jamshed R. Tata

Abstract Hormones are chemical messengers that regulate and coordinate all major metabolic, growth and developmental activities of different populations of cells of an organism. Since most hormones of higher organisms can be detected in primitive organisms, it follows that hormones arose during evolution well before many of the functions they regulate in higher organisms. Two examples to illustrate this principle are the protein hormone prolactin and the iodothyronine thyroid hormone. The first regulates such diverse activities as lactation in mammals, crop sac development in birds and migration in fish; the second hormone controls metabolic rate in homeotherms and different functions in different tissues of the same developing individual as during amphibian metamorphosis, such as restructuring of the digestive system, gene switching for new blood proteins, new cell development during limb formation and programmed cell death in unwanted tissues in the tail and intestine. The acquisition of hormonal function during evolution is likely to have coincided with the appearance of hormonal receptors, which are the key to understanding the mechanism or specificity of action of a given hormone. The hormone-receptor complex for protein hormones can be considered to have co-evolved as a unit. Most animal hormone receptors can be divided into two classes according to their localization in the hormone's target cell: (a) those located in the cell membrane and (b) in those in the nucleus. Work based on the exploitation of recombinant DNA and cell transfection has established a high degree of homology between oncogenes and both membrane and nuclear receptors. An early consequence of the formation of hormone-membrane receptor complex is at the modulation of processes such as phosphorylation of proteins, as exemplified by the control of levels of cyclic AMP. Nuclear receptors control the chromatin structure of their target genes through interaction with or structural modification of chromatin. The key role of receptors in explaining hormone action has to be considered in the context of evolution of a system of molecular linguistics in intercellular communication.

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1.1 Introduction

Although it is only 110 years ago that the word **Hormone** was coined by Starling (1905) to mean *I stimulate*, the principles of Endocrinology had already emerged many decades earlier. In the 19th century, Claude Bernard's studies in Paris on the secretions of the dog pancreas and sugar metabolism had already established the presence of regulatory constituents in this secretory organ. Similarly, the work of Brown-Séquard, also in Paris and Adolphe Berthold in Germany, on testicular extracts established the presence of secretions that promoted and regulated sexual functions and cellular longevity in male accessory tissues (see Turner and Bagnara 1971; Baulieu and Kelly 1990). In the same century, several physicians described successful treatment of patients with certain disorders by administering extracts of animal endocrine tissues, such as the thyroid, adrenals and pancreas; they subsequently showed that these disorders were due to hormonal deficiencies. More recently, the study of hormones has led to enormous benefits to human health, social and economic progress, such as contraception, in vitro fertilization (IVF) and the availability of recombinant human hormones (Edwards et al. 1984; Pincus et al. 1958; Sharpe and Skakkebaeck 1993). At the same time, the subject of hormones and hormonally regulated development, has also found much interest among public health experts and the larger public, in view of the emerging evidence of various man-made chemicals that can act as hormone disruptors to cause a wide range of diseases and disorders in man and wildlife.

Starling's proposal of a new term sparked off enormous interest in determining the nature of endocrine secretions and soon led to the discovery of how these substances exerted their actions. In the first half of the 20th century, researchers thus concentrated their efforts on identifying the source of these internal messengers, with the result that many hormones have been named after the gland or organ from which they were secreted, e.g. thyroid, adrenal, etc. This system of nomenclature was not always perfect, since quite distinct hormones are now known to be secreted by the same gland, as, for example, the pituitary and pancreas. Scientists soon succeeded in deciphering the chemical nature of hormones. For example, Kendall purified and determined the structures of cortisone (a steroid) and thyroxine (an iodoamino acid), while Charles Harington first chemically synthesized a hormone: thyroxine. This breakthrough work was soon followed by the characterization of the nature and activity of the pancreatic hormone insulin—a protein—by Banting and Best, while in the 1920s and 1930s, Budenandt, Reichstein and Doisy discovered and characterized various steroid hormones, including oestrogen, testosterone and progesterone. The growing knowledge of the physiological actions also led to many hormones being named according to their actions, such as growth hormone and prolactin. However, this nomenclature can still be unsatisfactory when a hormone exerts different actions in different target tissues or organisms at different developmental stages.

1.2 Evolution: Hormones Are Messengers but Do not Carry Any Information

It is obvious from studies on comparative endocrinology of the effects of endocrine organ extracts in heterologous tissues that many hormones have been conserved across species during evolution. This fact is central to the question of which came first in evolution: the hormone or its actions? One of the most striking illustrations of this generalization is provided by the hormone prolactin. As its name suggests, prolactin was named after its action in regulating lactation. But it also emerged that this protein hormone made in mammalian pituitary is also known to have potent action in regulating important physiological functions in other non-mammalian vertebrates. As depicted in Fig. 1.1, the major functions of this hormone in mammals are the regulation of lactation and luteotropic activity. Prolactin also regulates major functions in non-mammalian species (Gorbman and Bern 1962; Tata 1993, 1998). It stimulates crop-sac development in birds, induces "water drive" in terrestrial urodeles and regulates salt adaptation and melanogenesis in fish. Prolactins from lower vertebrates generally do not exert their function in higher forms, whereas mammalian prolactin is quite effective in fish and amphibia. Since the amino acid sequence of prolactins is known to vary in different species, this latter fact reflects a concomittant evolutionary variation in the structure of protein hormone receptors as well. It is at once obvious from Fig. 1.1 that the molecule of prolactin does not carry the information for such a wide variety of physiological activities that it regulates in different organisms, as, for example, salinity detection in fish, water drive in neotenic amphibia, metamorphosis in amphibians, crop sac development and function in birds, and lactation and female reproductive functions in mammals. The "information" content is the way in which the hormonal target tissue interprets the signal.

Not only is the nature of response determined by the targets in different species but no two tissues within the same individual organism may respond to the hormone in the same manner. Amphibian metamorphosis is a good example to illustrate this point. The discovery by Gudernatsch in 1912 that extracts of horse thyroid tissue can induce the complete metamorphosis of frog tadpoles. The availability of synthetic thyroid hormone further established the extraordinary multiplicity of responses to this hormone, as can be discerned from Table 1.1. Thyroid hormone, which is present in primitive organisms, such as amphioxus, fish, birds, amphibians and mammals, regulates very different physiological processes, such as moulting in birds, metamorphosis in amphibia and basal metabolic rate in mammals.

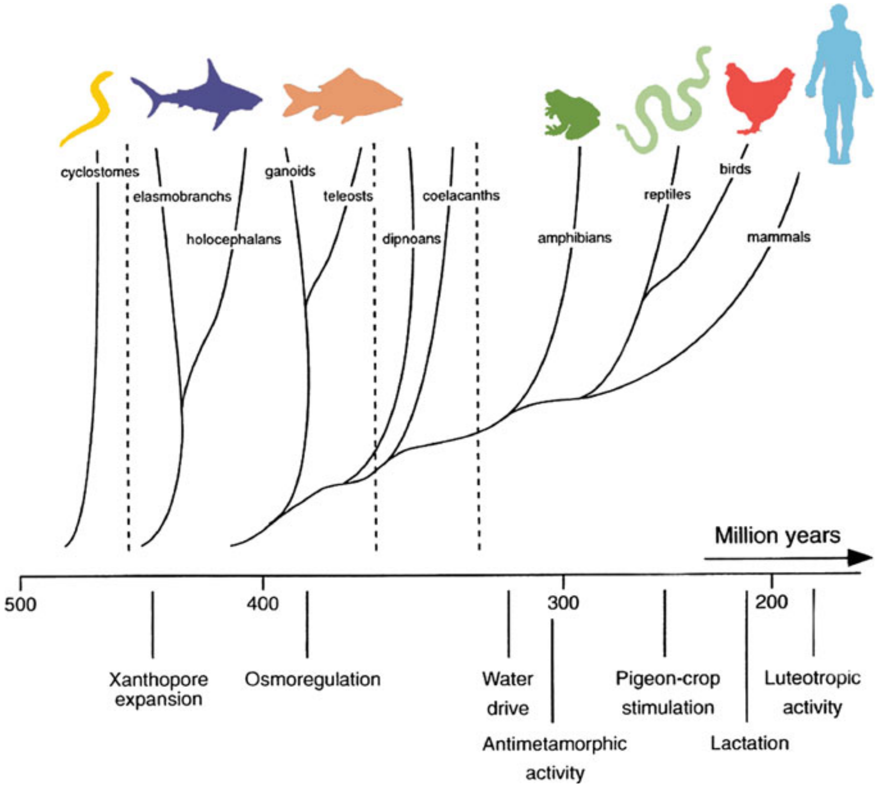


Fig. 1.1 Acquisition by a hormone of different regulatory physiological functions in different species during evolution, as exemplified here for the hormone prolactin. At the *bottom* of the figure is the evolutionary time scale, below which are given the important regulatory functions of prolactin. See Tata (1998) for further details

Table 1.1 Multiplicity of physiological and biochemical actions of thyroid hormone

Growth and developmental actions	Metabolic actions
Rate of postnatal growth of many mammalian species	Regulation of basal metabolic rate and avian tissues
Functional and biochemical maturation of foetal brain and bone	Movement of water and Na^+ ions across cell membranes
	Calcium and phosphorous metabolism
Morphogenesis, gene switching and cell death in amphibian metamorphosis	Regulation of metabolism of cholesterol and other lipids
Control of molting in birds	Nitrogen (urea, creatine) metabolism
Regulation of synthesis of mitochondrial respiratory enzymes and membranes	Control of oxidative phosphorylation and energy metabolism

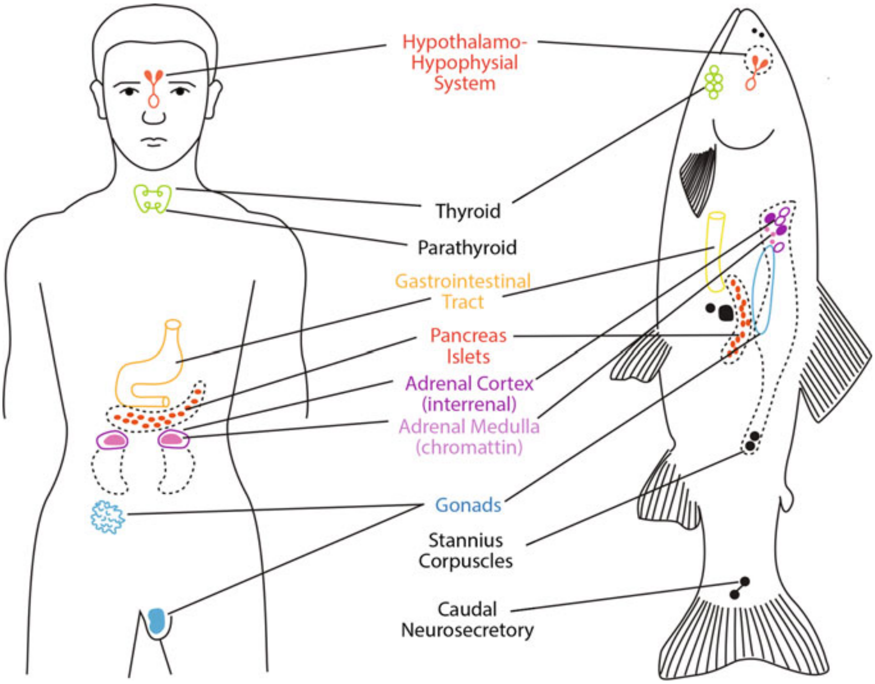
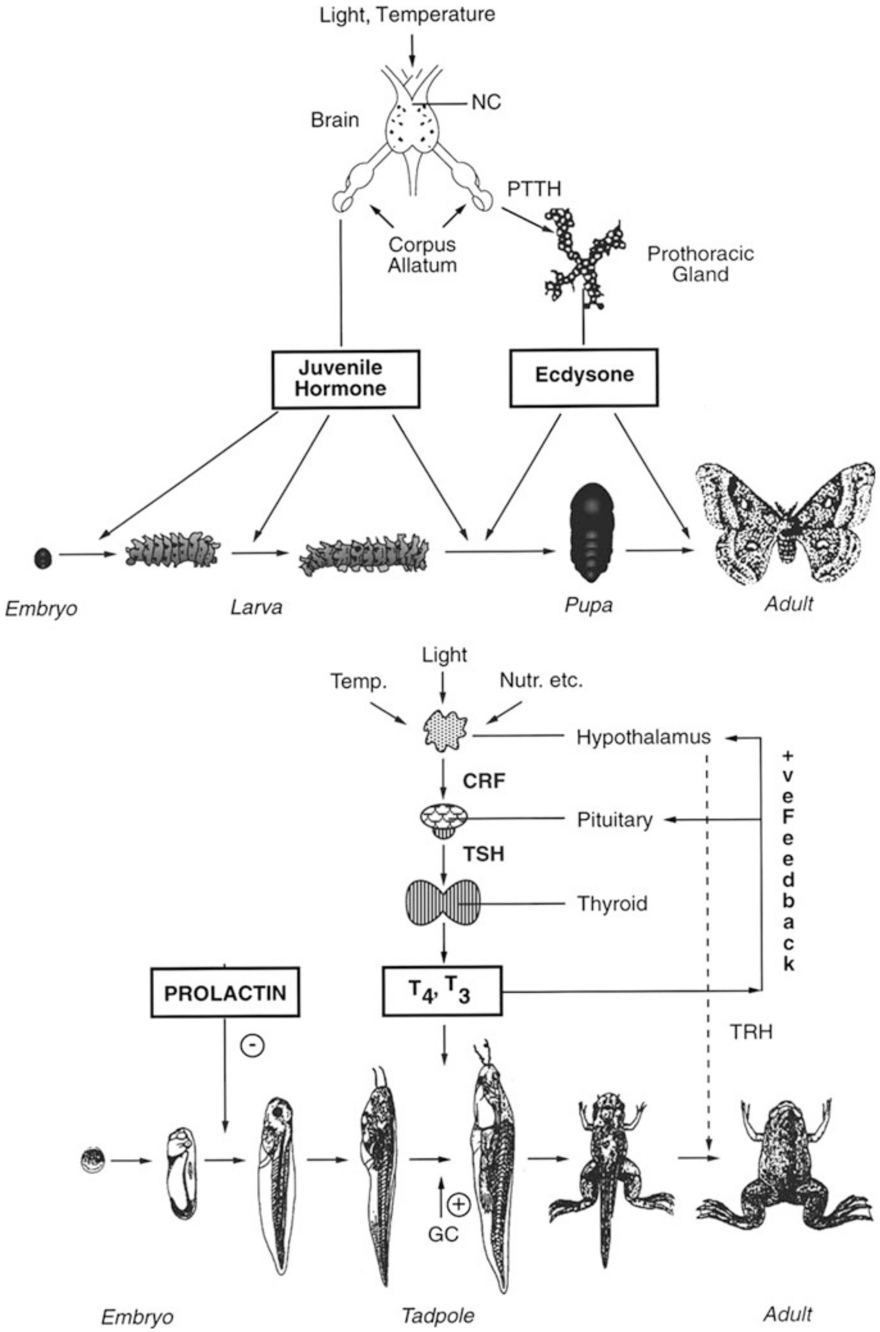


Fig. 1.2 Conservation of endocrine glands in two evolutionarily distant vertebrates, producing similar hormones but whose actions may vary according to the species and target tissue (Gorbman and Bern 1962)

Furthermore, even in the same individual organism, a given hormone may exert different, if not opposing actions, as illustrated by thyroid hormone in the initiation of cell death in the frog tadpole tail and gills, while simultaneously promoting differentiation and growth of limbs in the immature limb buds. Another example to illustrate this point of conservation of both the hormonal signal and its receptor through evolution is the anti-metamorphic action of human prolactin in thyroid hormone-induced amphibian metamorphosis in the tadpole of the amphibian *Xenopus*, both in vivo and in organ cultures (Tata 1993, 1996). It is also an important feature of endocrinology that both the internal secretions and the organs producing them were conserved across species (see Fig. 1.2).

1.3 Environmental Cues and Endocrine Cascades

A major principle of endocrinology involves the interplay and feedback between the central nervous and endocrine systems. Most of the external physical signals, such as photoperiodicity, temperature, etc., are transmitted to the CNS, where neurotransmitters act on specific target neurons, which in turn produce neurohormones, and set


Light, Temperature
Brain
NC
PTTH
Corpus Allatum
Prothoracic Gland
Juvenile Hormone
Ecdysone
Embryo
Larva
Pupa
Adult
Light
Temp.
Nutr. etc.
Hypothalamus
CRF
Pituitary
TSH
Thyroid
+ve Feedback
PROLACTIN
T_4, T_3
TRH
⊖
⊕
GC
Embryo
Tadpole
Adult

◂**Fig. 1.3** Schemes depicting the similar patterns of hormonal regulation of metamorphosis in **a** insect and **b** amphibian. In response to environmental cues the brain of the moth larva stimulates specialized cells to secrete Juvenile Hormone and the hormone ecdysone. The balance between these two hormones determines the initiation of formation of the adult insect (here depicted for the moth). Similarly, in the frog tadpole, environmental factors trigger the sequential release hormones in the brain which stimulate the thyroid gland to secrete thyroid hormone (T3, T4). Although not fully understood, the hormone Prolactin prevents or slows down the action thyroid hormone. Other hormones also exert a modulatory effect. At the tissue and cellular levels the same hormone regulates new tissue formation and programmed cell death. Other abbreviations and details to be found in Tata (1998)

up a cascade of endocrine secretions Hormones can thus be considered as intermediaries in the transfer of information from the environment to the organism. Their overall purpose is to coordinate and integrate the activities of metabolic and developmental processes in diverse target cells in response to environmental signals. By the 1950s, important associations between the neural and hormonal signalling pathways were established in vertebrates for a variety of hypothalamic “releasing” hormones or factors. It is an interesting fact that some neurotransmitters, such as serotonin, are also signalling molecules in plants, while many animal hormones are found in primitive organisms and obviously have been put to different uses through evolution (Gorbman and Bern 1962; Barrington 1964).

Figure 1.3 shows the extraordinary similarity of how information originating as changes in the environment (such as temperature, photoperiodicity, nutritional elements, etc.) is transmitted via the central nervous system, or other sensory apparatus, and then to the target organs via the synthesis and secretion of specific hormones. In insects, certain neurosecretory cells in the larval or pupal brain act on the *corpus allatum* to produce a hormone called prothoracicotropic hormone (PTTH) which acts on specialized cells of the prothoracic gland. The latter when stimulated by PTTH will, in turn, secrete a group of steroid hormones termed ecdysteroids (the principal member being ecdysone) which induce metamorphosis. At well-defined periods of the developmental progression leading to metamorphosis, different cells in the invertebrate larval and pupal brain stimulate the *corpus allatum* to produce and secrete a group of terpenoid compounds collectively termed juvenile hormone or JH. It is significant that JH will counteract many biochemical actions of ecdysteroids at the cellular level.

In amphibians (and other metamorphosing vertebrates) it has been known since the discovery by Gudernatsch in 1912 of the precocious induction of metamorphosis in frog tadpoles that the process is under hormonal control (Gorbman and Bern 1962; Tata 1993). With the recognition of the central role played by the hypothalamus-pituitary-thyroid axis in vertebrates, the link between environmental signals and the initiation of metamorphosis could also be traced to the central nervous system through the intermediary of hypothalamic hormones TRH (thyrotropin releasing hormone), CRF (corticotropin releasing factor) and TSH (thyrotropic hormone) made in the pituitary.

1.4 Receptors: Key to the Understanding of the Mechanism of Hormone Action

Early studies on the mechanism of hormone action sought to uncover a unique mode of action for all hormones and vitamins, which often involved adding a given hormone or active principle to isolated tissues, cell homogenates or sub-cellular preparations. The availability of purified enzymes in the 1930s and 1940s allowed studying direct hormone-enzyme interactions. For a few years, it was thought that hormones induced allosteric or conformational changes in proteins, as, for example, the effects of insulin on hexokinase. These studies were eventually vitiated by the very high concentrations of hormones needed to elicit a direct effect on a given enzyme, nor was this approach compatible with the high degree of tissue specificity exhibited by hormones. By the end of the 1950s there was little enthusiasm for the idea of direct hormone-enzyme interactions as the basis for a common mode of action (Tata 1986, 1998).

1.5 Membrane Receptors

Hormone receptors have been commonly classified into two major groups: cell membrane and nuclear receptors. Receptors for protein hormones and growth factors, such as insulin, epidermal growth factor, growth hormone and prolactin, as well as many neurotransmitters, are all located in the target cell membrane; most of them are products of the oncogenes *v-erb*B, *v-ros* and *v-mpl*. Many membrane receptors are closely linked to adenylyl cyclase and G-proteins, and, through these, to cytoplasmic protein phophokinases, which transfer extracellular signals to the intracellular regulatory machinery (Parker 1996).

In the early 1940s, Levine had proposed that insulin controlled sugar metabolism by regulating its transport into the target cell, a proposal which led several years later to the concept that proteins and smaller peptide signals interact with the cell membrane. The discovery of cyclic AMP by Sutherland in 1956 as a "second messenger" of adrenaline and glucagon, followed by the discovery that adenylyl cyclase, the enzyme synthesizing cyclic AMP, was located in the plasma membrane, further consolidated the view that the cell membrane was a major site of action for many hormones (Sutherland 1972; Beavo and Brunton 2002). With the discovery of other secondary signalling molecules, such as inositol trisphosphate, G-proteins, oncogenes and the advent of gene cloning and sequencing technologies, it soon became possible to identify and characterize several membrane hormone receptors. Binding of the ligand to these receptors initiates a cascade of protein phosphorylation and de-phosphorylations in the cytoplasm, eventually leading to the physiological action of the hormone (Parker 1996; Hunter 1997, Fig. 1.4).

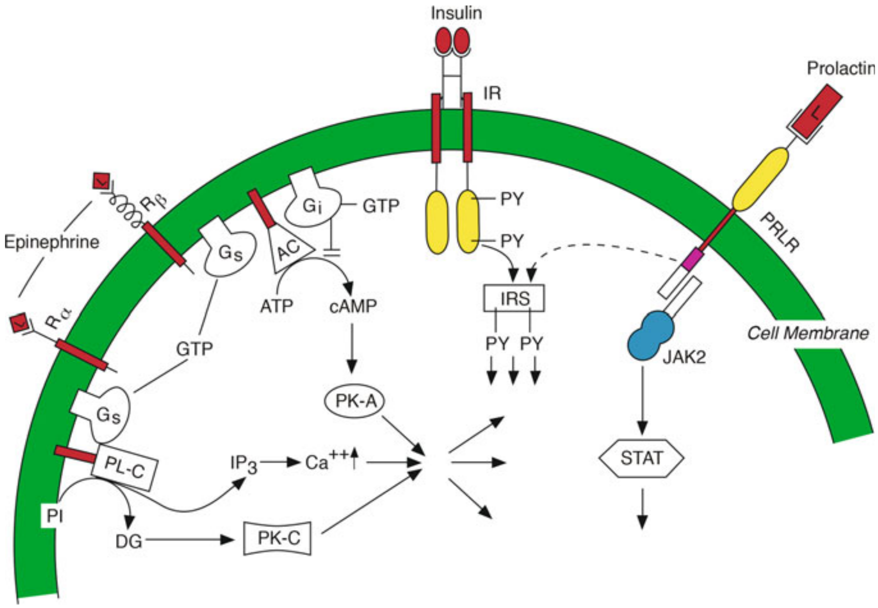


Fig. 1.4 A simplified diagram to illustrate "second messenger" and protein phosphorylation pathways associated with target cell membrane receptors for three hormones: epinephrine (adrenaline), insulin and prolactin. *R£∨Rß, IR* and *PRLR* denote receptors for epinephrine, insulin and prolactin, respectively; *Gs* and *Gi* are stimulatory and inhibitory G proteins; *PL-C*, phospholipase C; *AC*, adenylyl cyclase; *PI*, phophatidyl inositide; *IP3*, inositide trisphosphate; *DG*, diacylglycerol; *PK-A* and *PK-C*, phosphokinases A and C; *cAMP*, cyclic AMP; *PY*, phosphotyrosine; *IRS*, insulin receptor substrate protein; *JAK*, Janus kinase; *STAT*, signal transduction and transcription factor. See Tata (2005) for details

1.6 Nuclear Receptors

At almost the same time as cyclic AMP was discovered, Knox demonstrated that glucocorticoids regulate hepatic metabolism by selectively enhancing the synthesis of the enzyme tyrosine aminotransferase (Knox et al. 1956). New methods to study cell-free protein synthesis, and the availability of specific transcription inhibitors, allowed a more precise analysis of how growth and developmental hormones influenced protein synthesis in their respective target cells. The resulting observations that all steroid and thyroid hormones, administered in vivo affect the protein synthesizing machinery in vitro soon shifted the attention to transcriptional control (Tata 1986). That hormonal signals regulate transcription rather than translation, first became evident in the early 1960s from work demonstrating that puffing of polytenic chromosomes in larval salivary glands of insects is regulated by the steroid moulting hormone ecdysone (Ashburner 1974). Kinetics of labelling of nuclear RNA further revealed that all steroid and thyroid hormones strongly influence the formation and turnover of messenger RNA. In the mid-1960s several investigators were able to reproduce the transcriptional effects of steroid and thyroid hormones in cell-free transcription systems using isolated nuclei and nuclear extracts from target tissues (Tata 1986, 1998). In the 1970s and 1980s, the laboratories of Chambon and O'Malley described in detail how oestrogen activates tissue-specifically and selectively the genes for egg white protein (ovalbumin, conalbumin, ovomucoid) and yolk proteins (O'Malley 1978; LeMeur et al. 1981; Tata 1998; Chambon 2004).

Although we know much about the details of the transcriptional machinery, it would be impossible to understand how hormones or other signals regulate gene expression without a reasonable knowledge of the structure and function of their receptors. First evidence for the existence of nuclear receptors came in 1961 from the work of Jensen and colleagues in which they tracked radioactively-labelled oestradiol-17ß in female sexual tissues, and found that it forms a complex in the nucleus with a protein, which fulfilled the criteria for a receptor (Jensen 2004). The cloning of receptors for oestrogen, glucocorticoids and thyroid hormone in the 1980s in the laboratories of Chambon, Evans and Vennström, showed that all nuclear receptors are cellular homologues of the oncogene *v-erb*A and function as ligand-activated zinc-finger transcription factors. Today, more than 30 nuclear receptors encoded by this gene superfamily have been cloned, sequenced and many obtained as pure recombinant proteins (Mangelsdorf et al. 1995; Benoit et al. 2004; Chambon 2004), including several 'orphan' receptors whose ligands have not yet been identified. What is most remarkable for these nuclear hormone receptors is their high degree of target gene specificity, which is achieved by a precise spacing of nucleotide repeats in the target gene promoter's hormone response element (HRE) that interacts with the DNA-binding domain (DBD) of the receptor.

As depicted in Fig. 1.5, the central DNA-binding domain a nuclear hormone receptor is the most conserved region, while the 3′-terminal ligand—binding domain is the most variable (not shown in Fig. 1.5). Nuclear hormone receptors can be sub-divided into two groups, according to whether they form cytoplasmic complexes with hsp90 (heat-shock proteins of around 90 kDa molecular weight), and can be active as monomer, homodimer or heterodimers. All vertebrate steroid hormone receptors, belong to the first category, while the liganded receptors for retinoic acid, thyroid hormone (TR), vitamin D_3 (VDR) and peroxisome prolifetor (PPAR) function as heterodimers with RXR, the 9-cis-retinoic acid receptor (RXR does not have to be liganded to function as heterodimer). At first, only the second group of receptors were found to exist as multiple isoforms, the multiplicity residing in the N-terminus of the receptor, but more recent work has shown that the same holds true for other steroid receptors.

An interesting question arises as to how the high degree of target gene specificity for a given hormone and its receptor is achieved. The answer lies in the highly precise spacing of nucleotide repeats in the hormone response element (HRE) of the promoter of the target gene and the DNA-binding domain (DBD) of the receptor which recognizes it. A consensus hexanucleotide sequence, usually present as a pair, is the most common feature of all the known HREs but the sequence of each hexad, and the relative position of the two hexads, exhibit considerable variability

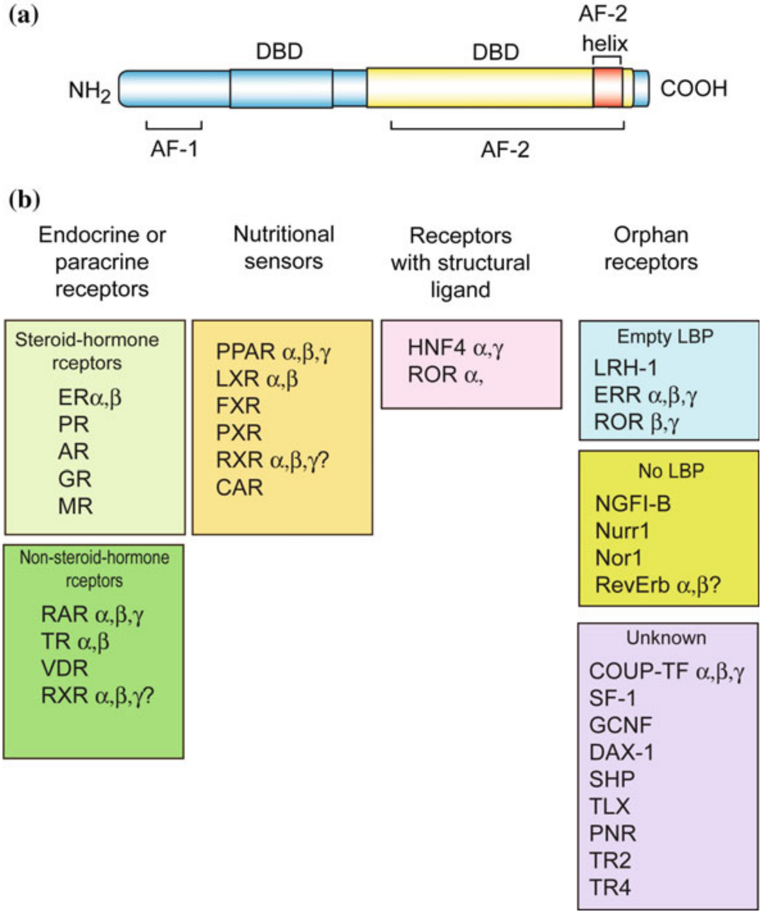


Fig. 1.5 **a** Representation of the common structural features of all members of the supergene family as ligand-activated transcription factors. *DBD* DNA-binding domain; *AF* transcription activating factors. The ligand-binding domain (LBD) is located at the COOH terminus. **b** Different categories of nuclear receptors divided by Benoit et al. (2004) into four sub-groups according to the nature of the signalling molecules. Only the two groups on the *left in green boxes* refer to hormonal signals and only these will be considered in this article. ER, PR, AR, GR and MR in the *light green box* are receptors for oestrogen, progesterone, androgen, glucocorticoids and mineralocorticoids, respectively. RAR, TR, VDR and RXR in the *dark green box* are receptors for retinoic acid, thyroid hormone, vitamin D_3 and 9-cis retinoic acid, respectively. For all other receptors and abbreviations refer to Benoit et al. (2004)

to generate the high degree of specificity of interaction between the receptor and its target gene. The HREs recognized by the receptors of steroid hormones oestrogen, progesterone, glucocorticoid, androgen and mineralocorticoid (ER, PR, GR, AR, MR) in the top green box in Fig. 1.5 are characterized by a 15-nucleotide motif consisting of two hexads in a palindromic configuration separated by 3 nucleotides.

The hexad sequences show a variability within this group of receptors. On the other hand, and more interestingly, the HREs of the non-steroid receptors shown in the bottom green box in Fig. 1.5 (TR, RAR, RXR, VDR, PPAR) all share the same AGGTCA hexad of ERE but are organized as direct repeats (DRs) separated by one to five nucleotides. This arrangement of HREs explains the fine discrimination of target genes by the heterodimers formed by each of these receptors with RXR and which confers an extraordinary hormonal specificity. It is a most remarkable example of selective transcriptional regulation (Mangelsdorf et al. 1995; Tata 1998). Dramatic confirmation of the above biochemical findings regarding the interaction between DBD of nuclear receptors and their cognate HREs has been provided in the last decade by NMR spectroscopy and x-ray crystal structure analysis (Chakravarti et al. 1996; Evans 2004).

What is most remarkable for these nuclear hormone receptors is their high degree of target gene specificity, which is achieved by a precise spacing of nucleotide repeats in the target gene promoter's hormone response element (HRE) that interacts with the DNA-binding domain (DBD) of the receptor. As shown in Fig. 1.6, large proteins termed CBP (CREB binding protein) and p300 are thought to form bridges between nuclear hormone receptors and other transcription factors. Other important elements of this complex are the p160 nuclear receptor coactivator and the 270 kDa nuclear receptor co-repressor (N-CoR) (McKenna and O'Malley 2002). The CBP/p300 complex serves to integrate multiple signalling pathways in the cell nucleus and many more such modulators will most likely be discovered in the very near future, forming even more complex structures with nuclear receptors.

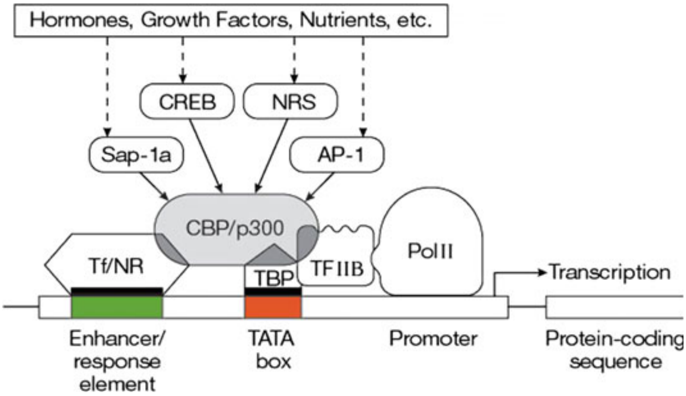


Fig. 1.6 How nuclear hormone receptors are thought to form complexes with other factors to regulate transcription. A bridging protein such as CBP/p300 would be in close contact with nuclear receptors and transcription factors (*Tf*) that recognize specific DNA sequences, TATA box binding protein (*TBP*) and transcription factor IIB (*TFIIB*), which would form a complex with RNA polymerase II. CBP/p300 is thought to form complexes with other transcription factors without involving DNA, such as CREB, AP-1 and Sap-1a. The activities of many of these components are modified by phosphorylation. See Tata (1998) and Evans (2004) for further details

1.7 Chromatin Structure and Hormonal Regulation

The higher order of organization of genes within the nucleus and the growing number of cross-interactions among regulatory factors have focused attention on the important role of chromatin structure in hormonal regulation of gene expression. Studies from Chambon's laboratory in the mid-1980s on the DNase I-hypersensitive regions of the chicken ovalbumin gene promoter led to the first understanding of how oestrogen reversibly modifies the chromatin structure of this target gene in a tissue-specific manner (see Chambon 2004). Later studies provided evidence that the function of glucocorticoid receptor in regulating the target gene promoter is determined by the manner in which it itself is organized within the chromatin structure (Beato 1996). Recent work on how steroid and thyroid hormones modify the chromatin structure of their target genes has provided evidence that the HREs of the glucocorticoid receptor are highly organized in phased nucleosomes. The binding of the hormone to its nuclear receptor causes an alteration in the chromatin structure, such that it induces the incorporation of other transcription factors into chromatin, thus facilitating the transcription of the hormone-regulated gene promoter (Beato 1996; Wolffe 2000; Evans 2004). So far, most conclusions drawn from chromatin experiments are based on indirect observations in vitro. The recent development of chromatin immunoprecipitation (ChIP; Sachs and Shi 2000) is therefore a new promising technique. Workers in Gannon's laboratory have used it to show that the oestrogen receptor activates its target gene in a cyclical manner, in that the receptor-ligand complex is continuously removed by a protease to be replaced by a new receptor (Métivier et al. 2003; Reid et al. 2003).

According to a simple model proposed by Wolffe (2000), the HREs are highly organised in phased nucleosomes and the binding of the hormone to its nuclear receptor causes an alteration in the chromatin structure such that it will induce the binding of non-receptor transcription factors, such as NGF-1 and OTF-1, and thus allow the transcription of the hormone-regulated gene promoter. Similarly, as depicted in Fig. 1.7, Wolffe's group has suggested that both the silencing and activation of the Xenopus thyroid hormone TRß gene is determined by processes controlling nucleosome assembly (see Wolffe 2000). As pointed out earlier (see Fig. 1.3), thyroid hormone is obligatory hormonal signal for all tissues of the amphibian larva to develop to the adult organism. The conclusions of such chromatin studies have largely been inferred indirectly from techniques such as cross-linking to determine points of contact between DNA and protein. More direct information of the spatial organization and mobility of receptors is needed before we can draw precise conclusions as to the role played by chromatin rearrangements within the nucleus in vivo.

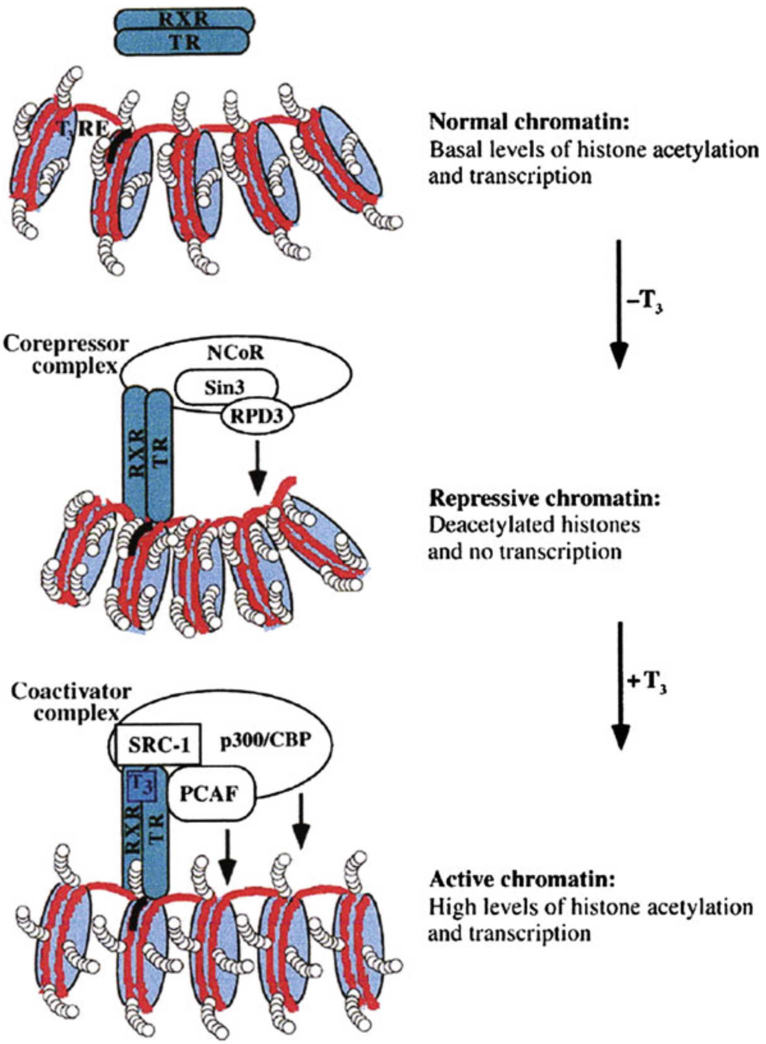


Fig. 1.7 How a ligand-activated nuclear receptor could modify the higher-order structure of chromatin. The packaging of DNA into chromatin is visualized in three transcriptionally active states: normal, repressive and active. In this example, the region of chromatin chosen contains the thyroid hormone receptor (TR)/RXR heterodimer, with or without its ligand triiodothyronine (T_3), bound to the thyroid responsive element (TRE) in the target gene. In normal chromatin, histone acetylation is at its basal level and so is the transcriptional activity. In the absence of T_3 (as during early stages of development), chromatin exists in its condensed and transcriptionally repressive form whereby the histones are in a largely deacetylated state with no transcription of the TR's target gene. In the presence of T_3 the chromatin is now active with elevated levels of histone acetylation and transcription. The other components are proteins that form 'corepressor' and 'coactivator' complexes with complexes with the TR/RXR receptor heterodimer. For more details see Wolffe (2000)

1.8 Integration of the Membrane- and Nuclear Receptor-Linked Hormonal Pathways

The functions of many transcription factors and co-regulators of nuclear receptors are regulated by protein phosphorylation (Wu et al. 2004). Since the processes of protein modifications are often linked to membrane components, this phenomenon is a reflection of the existence of a wider network that links membrane and nuclear receptor into complex signal transduction pathways. In this context, and as illustrated in Fig. 1.8, work, from Darnell's laboratory has highlighted the importance

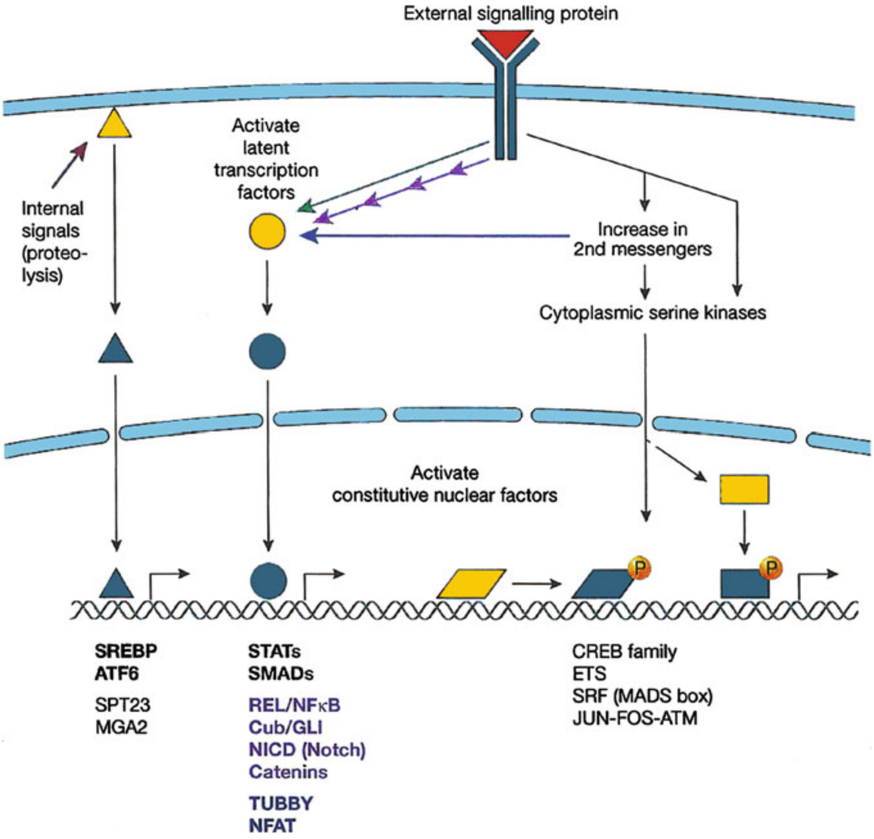


Fig. 1.8 According to this model, proposed by Brivanlou and Darnell (2002), latent intracellular regulatory factors, which include nuclear receptors, transcription factors and other modulators of nuclear functions, are activated or inhibited by post-translational modifications such as phosphorylation, proteolysis or through 'second messenger'-dependent functions. The latter would be regulated by signals impinging on the cell membrane. Thus, for example, the activities of nuclear proteins, receptors and transcription factors can be modified by phosphorylation by a convergence of functions operating at the levels of cell membrane and nucleus. For definitions of the abbreviations and other details see Brivanlou and Darnell (2002)

of protein phosphorylation in the control of gene expression, for example, as seen with the JAK/STAT pathway and that of nuclear factors, such as CREB (Brivanlou and Darnell 2002). The importance of the JAK/STAT pathway and the control of gene expression in general by transcription factors is a good example of how such complex interactions bring together nuclear and extra-nuclear regulatory processes and thus allow the setting up of a network linking membrane and nuclear receptor signal transduction pathways.

It is clear from what has been said above that hormone receptors now occupy a central role in our current concepts of signalling mechanisms. One should add that equally important is the complex of the target cell's signalling component that is linked to the receptor. Over the past 25 year, the application of gene cloning, cell transfection, transgenic and gene knock-out techniques, x-ray crystallography and NMR analysis of DNA-protein and protein-protein interactions have significantly advanced our understanding of the structure and function of receptors beyond all expectations. It therefore follows that our further understanding of the mechanism of hormone action will be a function of our changing perception of the different branches of the science of genomics, cell structure and cell signalling processes.

1.9 Hormones and Their Actions Through Evolution

While considering the progress resulting from the above advances in the future, the question of whether or not the hormone molecule itself carries the information for how the target cell has to respond needs to be kept in mind. The answer to this question is most convincingly answered by the example, depicted in Fig. 1.1, of the wide variety of actions that have been ascribed to the hormone prolactin through evolution—from a marine worm to man. Several similar examples of species-wide multiplicity of action can be found for many other hormones (see Gorbman and Bern 1962; Barrington 1964; Tata 1986). Not only is this multiplicity of action seen in different species of organisms, but is easily discerned in different tissues of the same organism, or even in the same tissue at different developmental stages of same tissue. The importance of this latter point concerning hormone action is most obvious from the example given in Table 1.1 on the induction and regulation of metamorphosis by thyroid hormone in amphibians. Thus the genes whose expression is regulated by this hormone in the larval and adult frog liver and intestine are nowhere similar. At the whole tissue level, the same hormone activates cell death in the larval muscle, skin and nerve cells of the tail and gills, while at the same time promoting the growth and development of the same cells in limb buds and lungs. The chemical structure of both prolactin and thyroid hormone is the same in the most primitive organism in which they are made as in man. These examples serve quite categorically that the hormone molecule in itself does not carry any information for its target cells. It serves as a trigger or signal for the responding cell to initiate the first step in the complex between the hormone receptor and its key partner that initiates the chain of downstream responses and events that lead to the physiological action of the hormone. Thus, the focus has to shift to the complex of the hormone receptor and the immediate cellular regulatory element in order to explain how the actions of an ancient hormone molecule have varied through evolution. This conclusion lies at the heart of the current research on various facets of the mechanism of hormone action. At the same time, this research will continue to reflect our knowledge of cellular regulatory mechanisms at any one given time. Conversely, many important advances in biochemistry, cell and molecular biology are also the result of work on hormones.

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Chapter 2
Somatotropic Axis' Role in Ageing and Longevity Could Depend on Life-History Strategies of Species

Éric Le Bourg

Abstract It is often argued that food restriction and modulation of the somatotropic axis could increase lifespan in all species, and particularly in human beings. However, this rationale does not take into account the life-history strategies of species and the way they adapt to environmental challenges, particularly to food restriction. It is argued that, for short-lived species of a small size, the best strategy to survive starvation is staying at the same place and increasing lifespan, because they cannot migrate to discover new food sources, because of a high predatory load and/or an inability to cross long distances. Emigration is an appropriate strategy for long-lived species of a large size less at risk of predation. Because humans tend to emigrate when facing unfavourable conditions rather than staying at home, food restriction is not expected to increase lifespan in humans. As an outcome, modulating the somatotropic axis would probably not increase human lifespan, because increased lifespan has not been selected as a strategy: how a genetic pathway could modulate lifespan in the absence of any selective pressure?

Keywords Lifespan · Ageing · Food restriction · Somatotropic axis · Human beings · Model organisms · Life-history strategies

2.1 Introduction

It has been said that "molecular, cellular, and developmental biologists ask "how" questions about mechanisms", while "evolutionary biologists ask "why" questions" (Masel and Promislow 2016). The role of the somatotropic axis in health, ageing and longevity cannot be considered only as a "how" question, i.e. wondering "how" the information needed to manage energy supply is best used by the organism, via hormones, cellular receptors, transcription factors and so on. This is also a "why"

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question linked to evolutionary biology, and this article wonders whether, depending on their life-history strategies, the somatotropic axis modulates the ageing process and the longevity of species. This chapter does not recapitulate the information provided in the second part of this book and some views could be at variance with those expressed elsewhere in this book.

On the one hand, it is often possible to increase lifespan in various species by relying on environmental means. For instance, decreasing the environmental temperature increases lifespan of *Drosophila melanogaster* flies (e.g. David 1988), food restriction increases lifespan of the nematode *Caenorhabditis elegans* (e.g. Johnson et al. 1984; Houthoofd et al. 2005) and various chemicals increase lifespan in these species (e.g. Frankowski et al. 2013; Brack et al. 1997). In mammals, Turkish hamsters *Mesocricetus brandti* kept at 22 °C and transferred 5.5 months a year at 5 °C lived longer if they hibernated for 12–33% of their life (Lyman et al. 1981). Food-restricted mice and rats often live longer than ad libitum-fed ones (Speakman et al. 2016, but see Liao et al. 2010 and Harper et al. 2006).

On the other hand, the search for long-lived mutants was disappointing up to the seminal article of Klass (1983) who isolated 8 long-lived mutants among 8000 *C. elegans* clones. Two of these mutants spontaneously entered the Dauer larval stage, as wild-type nematodes do when starved (Klass and Hirsh 1976), and the remaining 6 mutants had a reduced food intake. Later on, Friedman and Johnson (1988) or Kenyon et al. (1993) isolated other long-lived *C. elegans* mutants and mutations increasing lifespan were also discovered in *D. melanogaster* (e.g. Clancy et al. 2001) and mice (review in e.g. Bartke 2005). As emphasised by Klass (1983), "there were no mutants that specifically altered only life span", and these mutations in these species were all linked to the somatotropic axis (Bartke et al. 2013) and to the homologue of the insulin-like growth factor 1 (IGF-1) signalling pathway in invertebrates (Kenyon 2010). In other words, these mutations disturb the normal way of using energy like food restriction does.

Relying on results showing that lifespan can be increased either by mutating the somatotropic axis or by food restriction, some authors have concluded that similar results could be observed in human beings. For instance, Kenyon (2010) wrote that "it seems reasonable to think that mutations in our future evolution could give us longer lifespans and that, if they do, then drugs that mimic their effects should too". Similarly, Weindruch (2006) wrote that "it would be surprising if appropriately applied, chronic dietary restriction would not significantly increase the average lifespan of people".

Recently, Bartke (2016) summarised the whole idea: "both average and maximal longevity can be increased by various environmental factors as well as by dietary, genetic, and pharmacological interventions in species ranging from yeast and worms to insects and mammals. There is also increasing appreciation of the fact that many of the fundamental mechanisms of aging are evolutionarily conserved … and shared by most if not all living organisms … and that interventions that extend life in experimental animals can be expected to have similar effects in our own species." However, this rationale could be wrong because it does not take into account the contrasted life-history strategies of species and the way they adapt to environmental challenges, particularly to food restriction, and thus it is not sure "that interventions that extend life in experimental animals can be expected to have similar effects in our own species".

In 1973, Theodosius Dobzhansky wrote his famous article "Nothing in biology makes sense except in the light of evolution". This title has become a motto, because it grasps in a single sentence a fundamental idea now common to nearly all biologists. Paraphrasing Dobzhansky (1973), and turning our eyes towards the biology of ageing, one could add that "nothing in biology of ageing makes sense except in the light of life-history strategies".

2.2 Short- and Long-Lived Species Have Different Life-History Strategies

Because all species do not face the same challenges, not all life-history strategies—the way to manage survival and reproduction—are probable to the same extent for each species. For instance, African elephants *Loxodonta africana* living for only 20 years could simply not exist as a species because they would not have a sufficient time to reproduce, because of a long time to maturity (ca 10 years), a 2-year gestation time (only one singleton), a 4–5-year inter-birth interval, and a long parental care. Thus, elephants *must* live long to survive as a species and one can easily understand why the various life-history traits of species (fecundity, lifespan, gestation time, and so on) are highly correlated. A low fecundity is correlated with a long lifespan because species giving birth to only one offspring at a time must repeat reproductive episodes, which often implies to wait for the next year or more, and thus to live long. By contrast, mice reproducing quickly and giving birth to many offspring in a single reproductive season can afford to live only for a few months in the wild (Phelan and Austad 1989).

This comparison of elephants and mice shows that different species have different life-history strategies (Pianka 1970). In mammals, there is a continuum (Stearns 1983). Short-lived species with a small body size mature quickly after a short gestation time and give birth at short intervals to many offspring, as mice and rats do. These species are opposed to large ones that live long and need long gestation time and parental care to reach adulthood, and give birth to a few offspring during successive years, like elephants or primates do. It is however not to say that all mammals perfectly fit to these life-history because some of them can be as small as mice and live much longer (e.g. bats, Austad and Fischer 1991).

In the wild, because of a high predatory load, short-lived species of a small body size may reproduce only once or, if they are lucky, several times in a single season. By contrast, larger and longer-lived species are more spared from predation because of their size that makes them not so easy prey. Indeed, hunting a mice or an elephant is not the same challenge. However, because of the contrasting life-history strategies of elephants and mice, hunting adult elephants makes them soon an endangered species but mousetraps do not bear such a risk for mice.

Because of their life-history strategies, some species can quickly exploit a new environment if resources are plentiful and produce many offspring to do so ("opportunistic species": Demetrius 2005), while other ones are usually unable to so quickly thrive in a favourable environment ("equilibrium species"). In mammals, opportunistic species are short-lived and of a small body size, and equilibrium species are usually large and long-lived ones.

Could these contrasting life-history strategies imply that means to cope with ecological constraints in the wild, and particularly food shortage, vary among species?

2.3 Different Life-History Strategies Imply Different Means to Cope with Food Shortage

There are mainly two strategies to cope with food shortage: looking for food elsewhere, i.e. emigration, and waiting at the same place for better times, which can imply to increase lifespan. At a first sight, one could argue that emigration is the best strategy, because it can be implemented immediately and one can bet there is a higher chance to eat soon if trying to discover food, rather than staying at the same place. To make an analogy with human behaviour, if the supermarket is closed, it seems better to shop in another one than waiting for the next opening hours. However, emigration is not possible for all species.

2.3.1 Short-Lived Species

Nematode worms *C. elegans* live for less than 2 days in the soil (Van Voorhies et al. 2005) and ca 3 weeks in the laboratory. If food is lacking, they enter the Dauer larval stage for up to 2 months before resuming the normal life cycle, which thus increases longevity (Klass and Hirsh 1976). This strategy—waiting at the same place for better times and increasing lifespan—is surely better than emigration, which seems simply impossible for a worm living in soil for a very few days.

The same could be said regarding the spider *Frontinella pyramitela*. These sedentary spiders live for ca one week in the wild and several weeks in the laboratory. In the laboratory, their lifespan increases and their fecundity decreases when food supply decreases (1, 3 or 5 *D. melanogaster* flies per week, Austad 1989): in the wild, this strategy would be more optimal than emigration, because spiders catch prey on the web.

Small rodents could attempt to emigrate when facing famine, but as body size is positively correlated with the maximal distance covered by mammal species

(r^2 = 0.50 to ca 0.70: ca one km in the meadow vole *Microtus pennsylvanicus* and 300 km in the wolf *Canis lupus*, see Bowman et al. 2002), they would have a low chance to discover a better environment. In addition, as small rodents are subjected to a high predatory load, emigration is a very risky decision. Taking into account the low covered distance and the high predation risk, it is not so surprising that food-restricted mice often live longer than ad libitum-fed ones, possibly up to the next reproductive season, i.e. to the next year, before resuming reproduction (see Holliday 1989; de Grey 2005), because it is maybe the only possible strategy available to them.

Therefore, it can be concluded that, for some species, increasing lifespan in the event of starvation has been selected as an appropriate response, and thus that some species have a very plastic lifespan. In other words, species unable to emigrate are expected to live longer when food-restricted.

2.3.2 Long-Lived Species

Large and long-lived species reproduce in successive years and have a lower predation risk than small species. In such conditions, migration seems to be a wise strategy in the event of a too low food supply, and this is observed every year in migratory birds. However, migratory species can become resident ones if migration is no longer needed. For instance, the long-lived white storks *Ciconia ciconia* (39–48 years: Wasser and Sherman 2010) are now becoming resident birds, because landfills in Portugal provide them with food along the year, and thus they do not longer need to winter in sub-Saharan Africa (Gilbert et al. 2016). Humans is another example of a species well able to emigrate in the event of famine (or of other disasters): the history of mankind is replete with people walking across countries or even continents to find a better place to live. The difference between migration of various species and emigration of humans is that the latter is not linked to the season and is often a one-way process.

Unfortunately, only a very few studies on the effect of food restriction on lifespan have been carried out in these long-lived species, even if many species are known to migrate (ungulates, elephants, and so on). However, the effect of food restriction on lifespan of Rhesus macaques *Macaca mulatta* has been studied and is still in progress.

On the one hand, Colman et al. (2009, 2014) have reported that "age-related mortality", not linked to mere accidents, and overall mortality of animals food-restricted from the age of 7–14 years were lower than those of control ones and that food-restricted animals were less affected by diseases (Colman et al. 2009). On the other hand, no decreased age-related and overall mortalities were observed in animals food-restricted from the ages of 16–23 or 1–14 years (Mattison et al. 2012, see Le Bourg 2016 for other details).

In both studies, food-restricted monkeys weighed ca 25% less than control ones, but animals had a lower weight in Mattison et al. (2012). In Colman et al. (2009, 2014), monkeys were fed with a “typical Western modern diet rich in refined and processed foods” (Cava and Fontana 2013) and control monkeys were ad libitum fed. Animals in Mattison et al. (2012) ate a diet “more similar to the traditional Mediterranean or Japanese diet” (Cava and Fontana 2013), the food of control monkeys being portioned to prevent obesity, and thus control monkeys were not really ad libitum-fed. Thus, control monkeys of Colman et al. (2009, 2014) ate ad libitum a diet known to be detrimental while those of Mattison et al. (2012) had a better diet in conditions preventing obesity. Could it explain that, both sexes pooled, control monkeys lived for ca 26 years and food-restricted ones for 29 years in Colman et al. (2014), while females and males lived longer, respectively ca 28 and 35 years, in Mattison et al. (2012)? If it were the case, showing that restricting a “typical Western modern diet” can increase longevity would not mean that food restriction increases longevity, but more probably that this diet is hazardous. For the time being, one can conclude that food restriction does not increase lifespan in *M. mulatta*, provided the control group is not offered a bad diet.

Food-restricted dogs of the labrador breed were reported to live longer than control ones (median lifespans of 13 vs. 11 years, Kealy et al. 2002) but, as the authors made use of a breed prone to severe obesity (fat mass was 40% of weight in control dogs at 12 years of age and 23% in food-restricted ones: 13 vs. 6 kg, lean weight being ca 20 kg for both groups), it is probable that food-restriction simply lowered the deleterious effect of obesity on lifespan (other details on this study in Le Bourg 2010). However, because the domestic dog *C. lupus familiaris* is a very peculiar sub-species of *C. lupus*, it is necessary to wonder what could happen in food-restricted dogs.

Dogs are long-lived and probably well able to migrate, like the wolf *C. lupus* or the coyote *C. latrans* (Bowman et al. 2002) and, at first sight, they should be an equilibrium species, but this is probably no longer the case. The wolf *C. lupus* has a seasonal reproduction (with the same father among seasons), a rather small litter size (around 6 pups), a long parental care with an active social life in the pack, and offspring do not reproduce before the third year of life (Mech 1974): all these features do not favour an explosive reproduction. Thus, *C. lupus* can be considered as an equilibrium species (Demetrius 2005). In sharp contrast, dogs have more pups, reproduce twice a year and at any time (with different fathers) as soon as at 6 months of age, parental care is short, and pups are soon independent because there is no pack. In such conditions, dogs cannot be considered as an equilibrium sub-species but rather as an opportunistic one (Demetrius 2005) able to quickly thrive in a favourable environment. Thus, the original species *C. lupus* and one of its sub-species, *C. lupus familiaris*, display highly different life-history strategies. The domestication process can explain the evolution of a different strategy in dogs (Lord et al. 2013). Dogs live with or near humans and have no need to hunt for food, which is provided by owners or, e.g., in landfills for free dogs. In such conditions, fecundity can be not seasonal and more important than in wolves but, as emphasised by Lord et al. (2013) “the increase in fecundity has the consequence of an increased juvenile mortality when population levels reach carrying capacity”, exactly as it happens in opportunistic species. However, because dogs were originally an equilibrium species and food restriction is not a threat for most of the dogs for millennia, one can hypothesise that food restriction would not increase lifespan in this sub-species, contrarily to what happens in opportunistic species such as mice, because there is no selective pressure for an increased lifespan in starvation conditions. Thus, it seems probable that, if food restriction would be a threat, dogs would emigrate, like the original species does, rather than staying at the same place and living longer like mice do.

2.3.3 Conclusions

Strategies of species in case of food restriction are dependent on their life-history strategies. Obviously, a species like the nematode cannot leave its environment, i.e. the soil. For mice, emigrating under the sight of predators is probably a suicidal strategy and they cannot cover a long distance (Bowman et al. 2002). As an outcome, for these species, the best strategy is maybe to wait at the same place until the threat is over: short-lived species reproducing in a single season have a better chance to survive if staying at the same place and increasing their lifespan, because they cannot rely on migration.

Long-lived species, which reproduce in successive years and can thus delay reproduction to the next year or even later, can afford to migrate because they are less exposed to predation and can cover long distances (Bowman et al. 2002). There is maybe no selective pressure for a genetic pathway favouring increased lifespan when food is scarce in these long-lived species. Therefore, one could expect that food restriction does not increase lifespan in these species. It seems that, for the time being, no data contradict this conclusion.

Most et al. (2016) argued that "a forced 20% CR (calorie restriction) without malnutrition" during World War II made human mortality to decrease in Norway. "Circulatory diseases" strongly decreased in a strict parallel with consumption of fat, before both rose again in post-war times (Fig. 4 in Strøm and Jensen 1951). However, as tobacco consumption of men was cut by half at the same time (Lund et al. 2009), one may bet that this decrease explains a part of the mortality fall. Indeed, while the positive effect of food restriction on human lifespan is a hypothesis, the deleterious effect of tobacco is not.

2.4 Can a Genetic Pathway Modulate Lifespan in the Absence of Any Selective Pressure?

On the one hand, it thus seems that, depending on their life-history strategies, food restriction either increases lifespan of species or has no effect. On the other hand, because the somatotropic axis is evolutionary conserved, it has been argued by many authors that its modulation (e.g. by mutations) could make lifespan to increase, including in human beings (e.g. Milman et al. 2016). These two previous conclusions seem to be at variance: if the somatotropic axis regulates the use of nutrients by increasing lifespan when they are scarce, how explaining that food restriction does not increase lifespan in some species? This contradiction can be overcome by postulating that the size of the effect of the somatotropic axis on lifespan is similar to that of food restriction: if food restriction makes lifespan to strongly increase in a given species, one could expect that some mutations of the somatotropic axis in this species could strongly increase lifespan; if food restriction does not increase lifespan in another species, no mutation of this axis is expected to increase lifespan in this species.

This rationale could be falsified (Popper 1935) if there were a strong discrepancy between the effect of food restriction and that of the modulation of the somatotropic axis in a species. Let us examine what happens in *C. elegans*, *D. melanogaster,* mice, and humans.

Food restriction strongly increases lifespan in *C. elegans* (e.g. Johnson et al. 1984) like do mutations of IGF-1 (e.g. Fontana et al. 2010), as expected.

Mutations of IGF-1 also increase lifespan in *D. melanogaster* (e.g. Clancy et al. 2001; Martins et al. 2016). However, the effect of food restriction on lifespan has been debated, some authors claiming to observe such an effect while other ones did not report a positive effect (review in Le Bourg 2010). As flies can fly, it could be hypothesised that, in sharp contrast with nematodes, they could emigrate, and thus that increasing lifespan when facing starvation is not mandatory. It could also be argued as well that they cannot cover long distances and that an increased lifespan could be a valuable strategy. However, food restriction did not increase lifespan in other fly species (*Ceratitis capitata*, *Musca domestica*, *Anastrepha ludens*, *Bactrocera tryoni*, review in Le Bourg 2010). Therefore, it is not clear whether results of the effect of food restriction and of mutations of IGF-1 in *D. melanogaster* are in accordance in this species.

Food restriction increases lifespan in mice (but see Harper et al. 2006; Liao et al. 2010) and most of the results on the positive effect of the genetic modulation of the somatotropic axis have been obtained in mice (Bartke 2016). Therefore, results on the effect of food restriction and on this modulation seem to point in the same direction.

There is no result showing that food restriction can increase lifespan in human beings (reviews in Le Bourg 2010, 2012, 2016) and genetic modulation of the somatotropic axis does not seem to strongly modulate human lifespan. The few dwarf human mutants do not seem to live longer than their contemporaries (review in Le Bourg 2016) and, even if some studies have reported that lifespan can be linked to the *FOXO3A* gene polymorphism in some cohorts (e.g. Flachsbart et al. 2009), other ones have shown that the results could be dependent on the studied birth cohort (Nygaard et al. 2014). A high plasmatic IGF-1 level has been linked to a low remaining lifespan in nonagenarian women with a history of cancer, but not in all men and in women without a history of cancer (Milman et al. 2014). By contrast, a high growth hormone (GH) level at fasting was linked with a high all-cause mortality in ca 50-year-old men at baseline followed for ca 15–20 years (Maison et al. 1998; Hallengren et al. 2014), but not in women whose plasmatic GH level is nevertheless 10-fold higher than that of men (Hallengren et al. 2014).

On the whole, it has not been shown that food restriction can increase human lifespan, and results on a link between IGF-1 and GH, on one hand, and lifespan on the other hand can be opposite in men and women, when such links do exist (Kaplan et al. 2012). Obviously, studies of the effect of, e.g., IGF-1 on lifespan and ageing of very old people, such as nonagenarians (e.g. Van der Spoel et al. 2015), do not explain ageing and lifespan of the numerous people dying before this very old age. Milman et al. (2016) reviewed the results on the links between the somatotropic axis and human ageing and lifespan. They reported either positive, negative, or no effects of e.g. IGF-1 level and concluded that "much of the knowledge about the effects of the GH/IGF-1 axis on age-associated diseases and aging in humans remains inconsistent".

For the time being, it seems possible to conclude that no mutation (or allelic variants) of the somatotropic axis can increase human lifespan and that food restriction does not increase human lifespan: because humans can emigrate in the event of food starvation, there is probably no need to rely on the somatotropic pathway to increase lifespan in starved humans.

On the whole, it thus seems possible to strongly increase lifespan by modulating the signalling pathway regulating energy use in species whose lifespan increases when facing starvation, but not in species whose lifespan does not increase. However, it is maybe not an all-or-none answer and one could imagine a more graduated response. Food restriction and the modulation of IGF-1 could have no effect on lifespan in some species at one extremum, while huge effects could be observed at the other extremum, say 100% lifespan increase, with some species showing less important effects, say 10 or 20% increases.

2.5 Conclusions

Model organisms used in the laboratory to study ageing are usually opportunistic species (Demetrius 2005) with a short lifespan, such as the nematode *C. elegans* (ca 3 weeks at 20 °C), the fly *D. melanogaster* (ca 2 months at 25 °C), or the mouse (ca 2–3 years), and it is possible to increase their lifespan in the laboratory by modulating their environment, and particularly the availability of nutrients (but see the debate above on the effect in flies).

It is often accepted that these results could be extended to equilibrium species (Demetrius 2005) such as humans (e.g. Bartke 2016), but one can argue that experiments showing lifespan increases in short-lived model organisms will be never replicated in long-lived species such as humans, because life-history strategies of these species strongly differ. In contrast to opportunistic species, equilibrium species do not rely on lifespan increase to survive environmental threats in the wild: human beings facing famine and staying at the same place die while people emigrating get a chance to survive. Because a lifespan increase has not been selected as a strategy during evolution, there is no ground to expect that food restriction could increase human lifespan, as argued for instance by Yu (2006).

If food restriction does not increase human lifespan there is no reason to expect that modulating the human somatotropic axis could increase it. One can thus hypothesise that the modulation of e.g. IGF-1 and GH levels (e.g. by food restriction mimetics, see de Cabo et al. 2014) or food restriction would have no beneficial effect on ageing and lifespan in people without any pathology linked to this axis. Obviously, this conclusion does not apply to people suffering from acromegaly, obesity and other ailments linked to the somatotropic axis. To give a clear example, young men with a body mass index (BMI) of 21 kg/m^2 and eating a well-balanced diet, such as the Mediterranean one, without calories excess, should not expect to live longer if restricting their food. By contrast, overweight (BMI $\geq$ 25), obese (BMI $\geq$ 30), and severely obese people (BMI $\geq$ 35) eating a too caloric junk food, like in the 2004 movie *Super Size me*, could maybe live longer if choosing a more appropriate food. This result would be more probably due to the avoidance of life-shortening diseases rather than to a true life-extending effect of food restriction.

One could argue that there is a caveat in the fact that some favourable effects of food restriction on health indicators have been observed in the USA (e.g. Omodei and Fontana 2011). However, 30% of US people are expected to be overweight in 2020, 45% of them being obese (Stewart et al. 2009). Because the percentage of proteins in the US diet has regularly decreased in favour of fat and carbohydrates from at least 1971 to 2004, before a slight increase and a plateau from 2005 to 2010 (Ford and Dietz 2013), or since 1960 according to Simpson and Raubenheimer (2005), having a well-balanced diet for life is a daily challenge, if not an unreachable goal. At the same time, and maybe as a consequence of a decreased proportion of proteins in the diet (Simpson and Raubenheimer 2005), the mean food intake strongly increased in the USA (Ford and Dietz 2013). In such conditions, one can bet that adopting a better diet and decreasing the caloric intake would have favourable effects in most of US subjects, even if they are not (still) overweight. One can bet, too, that such a result would be less observed in European countries less affected by the obesity epidemics than the USA.

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Part II
Growth, Stress and Metabolism

Chapter 3
Glucocorticoid Hormones in Aging

Banteiskhem Kharwanlang and Ramesh Sharma

Abstract The discovery of glucocorticoids is a hallmark achievement in clinical science, as they have profound effects and consequences on various physiological and pathological conditions. Glucocorticoids (GCs) regulate a cohort of physiological functions like intermediary metabolism, immune system and influence development, growth and aging. Aging which is a physiological process characterized by reduced response to stress and imbalance homeostasis share similar physiognomies with impaired glucocorticoids functions like diabetes, cardiovascular system and bone disorders. Glucocorticoid signaling impairment with aging like heightened GCs level, disproportionate hydroxyl steroid dehydrogenase (HSD) enzymes and reduced glucocorticoid receptor level could have implications in the age-related disorders and may be ultimately death. Positive interventions by anti-aging nutraceuticals, dietary restriction and plausibly selective glucocorticoid receptor modulators having good effects sans side effects could be beneficial in managing pathological conditions during aging, possibly add life to years for healthy aging and better health management with reference to the recent increase in greying populations.

Keyword Aging · Glucocorticoids · Glucocorticoid receptors · Nutraceuticals · Dietary restriction

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3.1 Introduction

In 1950 the Nobel Prize in Physiology or Medicine was bestowed to Edward Calvin Kendall, Tadeus Reichstein and Philip Showalter Hench for their discoveries and inquisition on “the hormones of the adrenal cortex, their structure and biological effects” (Szabo et al. 2012). Preliminary groundwork in glucocorticoids research dates back to the 1920s. In 1929, Philip Hench found the improvement of an arthritic patient who becomes jaundiced. From the observation he predicated a hypothesis that an endogenous substance *x* is responsible for the remission of the symptoms in which the origination of its physiological justification is related to the adrenals. Towards the middle part of the 20th Century the substances were isolated from the adrenal gland and given the cognizance preferably as steroids. Compound Fa, which was known by Reichstein, was implicated to be the substance *x* by Kendall and Hench which later came to be known as dehydrocorticosterone (Lloyd 2002). The term glucocorticoid was coined by Hans Selye and designated alternatively by different names such as glucocorticosteroids or corticosteroids. In human, the glucocorticoid is cortisol whereas in rodent it is corticosterone (Szabo et al. 2012). The era on glucocorticoids study and research, which is almost touching a century in less than a few years from now, wield an extensive continuum on the glucocorticoids physiological processes, nonetheless the underlying molecular mechanism of GCs action is insubstantially elucidated. An extensive but intensive research is required to fully elucidate the already emerging mechanisms and as well other unknown domains. Such an approach will have profound effects on diseases and the science of aging (Kharwanlang and Sharma 2013).

Glucocorticoids regulate blood pressure, salt and water balance regulation, cardiovascular system and central nervous system. GCs also regulate cellular processes like the intermediary metabolism, immune-inflammatory reactions and metabolic processes like regulating the flux of glucose, amino acids and fatty acids into oxidative metabolism (Fig. 3.1) (Sharma 1988; Kharwanlang and Sharma 2011; Szabo et al. 2012). The above consequences co-ordinately metamorphose energy allocation, physiology and behaviour which indirectly manipulate key life-history traits like age-specific transitions, reproduction and survival (Crespi et al. 2013). The wide spectrum of GCs biological functions stem from the stupendous and mammoth gene expression activity induced by GCs. For example in mouse liver, GCs result in the differential expression of 1300 genes in which 53 of the genes expressive profile is dependent on ligand-bound glucocorticoid receptor. GCs-regulated genes control growth and apoptosis, signal transduction, inflammation, metabolism and other unknown functions (Wang et al. 2004; Le et al. 2005). Diverse molecular mechanistic action of the GCs signaling pathway can be elucidated by probing the various feats of variant glucocorticoid receptor isoforms, variant DNA regulatory sequences and differential mode of hormone-receptor complex (HR-complex) interaction with the DNA regulatory sequence, GR transcriptional complexes and post-transcriptional regulation of GR-mediated gene expression. Some of these GCs mediated mechanistic actions are found to be age related and play an important role in the physiology of aging and age-related diseases (Ranhotra and Sharma 2001; Dutta and Sharma 2004; Kharwanlang and Sharma 2011). Aging is characterized by the decline in the ability to respond to stress and impasse homeostatic balance thereby leading to incidence of pathological conditions and ultimately death. The underlying mechanism in aging process manifests striking parallelism with the unregulated GCs signaling pathway specifically with respect to stress response and homeostasis imbalance. Hence, dissecting on the GR signaling pathway molecular mechanism during aging could unfold interventions that could be helpful in tackling geriatric therapy and as well as maintaining a healthy life during aging (Weinert and Timiras 2003).

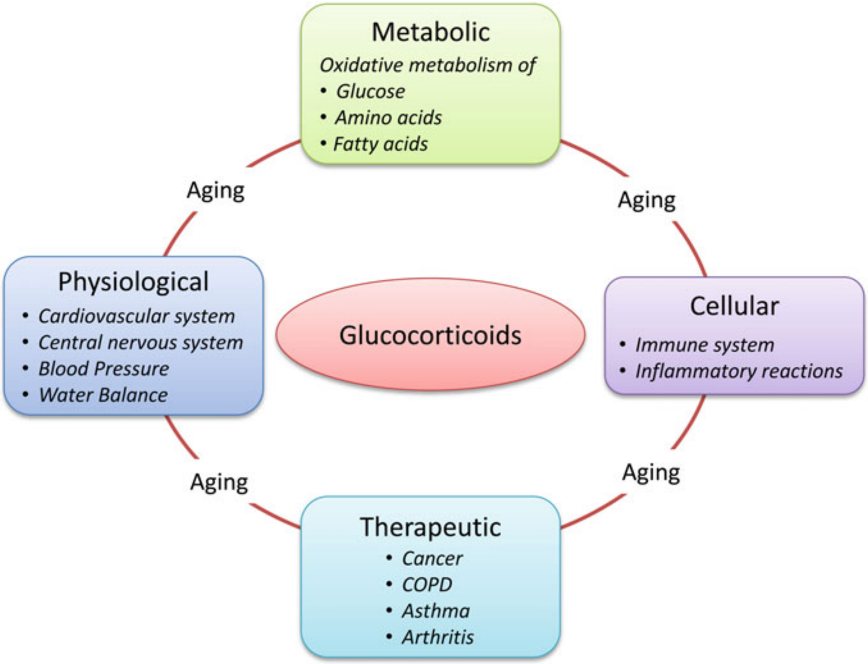

Fig. 3.1 Glucocorticoids (GCs) display physiological, metabolic, cellular and therapeutic activities. During the lifespan of an organism the diverse biological functions are controlled by GCs which are also age–regulated. The cellular, physiological and metabolic functions seem to be impaired with age and whose underlying mechanism is highly correlated to dysregulation of GCs signaling in aging. COPD, Chronic Obstructive Pulmonary Disease

3.2 Glucocorticoid Cascade Hypothesis

The glucocorticoid cascade hypothesis in hippocampal aging put forward by Sapolsky states that stress and reiterate bout of high GCs in the systemic circulation lead to downregulation of glucocorticoid receptors, which in turn result in further glucocorticoid hypersecretion and eventually hippocampal neuronal loss. The underlying mechanism is that the excess glucocorticoids participate in a feed-forward cascade on the brain and body culminating in progressive glucocorticoid-induced damage to the hippocampus which further promotes progressive elevation of adrenal GCs through dysregulation of the hypothalamus-pituitary-adrenal (HPA) axis (Sapolsky et al. 1986; Mc Ewen 1999; Young and Korzsun 2010).

3.2.1 Glucocorticoids in Health

The role of glucocorticoids in orchestrating the organism's behaviour, physiological and anatomical transition from developmental stages to aging is crucial in maintaining the integrality of the health and fitness of the organism. When GCs deviate from their normal physiological roles due to various known and unknown causes it leads to various physiological and pathological conditions. Cushing's syndrome, a disease of excess GCs leads to morbidity and mortality. In contrast, insufficient GCs lead to weight loss, lethargy and postural hypotension. GCs signaling is relevant in age-related diseases such as coronary heart disease, arteriosclerosis and leukemia. Obesity, osteoporosis, hypertension and hyperglycaemia are observed when GCs level is beyond normal (Sharma and Timiras 1987; Macfarlane et al. 2008; Zannas et al. 2015).

3.2.2 Glucocorticoids in Clinical Science

Glucocorticoids have profound clinical effects and are prescribed in various formulations which include oral, topical, eye solutions, eye ointments, oral inhalers, nasal formulations, parenteral and rectal preparations (Dhaou et al. 2012). Synthetic glucocorticoids, which include prednisolone, methylprednisolone, dexamethasone, betamethasone and triamcinolone, will remain the therapeutic cornerstone in management of various pathological conditions like cancer, asthma, arthritis, chronic obstructive pulmonary disease, allergy, multiple sclerosis and physiological conditions like adrenal insufficiency (Barnes 2006; Chapman et al. 2016; Merkulov et al. 2016). Antenatal GC therapy reduces the incidence of respiratory distress syndrome in preterm infants and a reduction in neonatal mortality (Bonanno and Wapner 2012). For the elderly population, having complications like systemic diseases, GCs like corticosteroids are routinely prescribed procedural drugs and manifest imperative positive outcome in a variety of malady (Dhaou et al. 2012).

3.2.3 Glucocorticoids Side Effects

Prolonged and repeated usage of exogenous glucocorticoids is associated with severe and detrimental side effects on the endocrine system such as adrenal suppression and insufficiency, Cushing's syndrome, hyperglycemia, central obesity, glucose intolerance, cardiovascular disease, osteoporosis, dyslipidemia, dermatologic, ocular and gastrointestinal complications (Lansang and Hustak 2011; Vienberg and Bjornholm 2014; Oray et al. 2016). GCs induce impaired peripheral glucose uptake, insulin resistance and gluconeogenesis that is correlated to the hyperglycemic action (Macfarlane et al. 2008). Glucocorticoids in antenatal therapy are associated with low birth weight and linked with undesirable side effects such as coronary heart disease, stroke, hypertension, anxiety-related behaviour and type 2 diabetes later in adulthood. Intake of GCs as therapeutic application also leads to psychological, cognitive and behavioural complications. Prolonged GCs therapy in geriatrics, which is exacerbated by physiological and neuroendocrinological changes during aging, is associated with many side effects like infection, iatrogenic diabetes, arterial hypertension, osteoporosis, depression and cataract (Ogueh and Johnson 2000; Cottrel and Seckl 2009; Asztalos 2012; Dhaou et al. 2012; Judd et al. 2014).

3.3 Glucocorticoids in Aging

Glucocorticoids physiological and pathological activities in aging as well as their non-therapeutic side effects are correlated to various molecular mechanistic actions of GCs signaling ranging from GCs secretion regulated by HPA axis, its bioavailability depending on HSD enzymes, glucocorticoid receptor (GR) signaling pathway which is further complicated by its isoforms, downstream genomic and non-genomic effects of GR.

3.3.1 Glucocorticoids, HPA Axis and Aging

The stress response mechanism by glucocorticoids is articulated tightly to the hypothalamus- pituitary-adrenal (HPA) axis (Fig. 3.2). In response to stress, HPA axis initiates a cascade of neuroendocrinological events culminating in the release of GCs from the adrenals to maintain homeostasis. The hypothalamus releases corticotropin releasing hormone (CRH) which activates the anterior pituitary to secrete adrenocorticotropic hormone (ACTH) into the systemic circulation. On reaching the vicinity of the adrenals, ACTH stimulates the adrenal gland which results in the synthesis of GCs from the zona fasciculata of the adrenal cortex. Physiological level of GCs in the systemic circulation is regulated by a negative feedback action by GCs-mediated inhibition of the CRH gene in the hypothalamus (Smith and Vale 2006; Kageyama and Suda 2009; Sriram et al. 2012). The systemic circulation of GCs follow a circadian rhythm in which during the 24 h cycle in human it shows a low level at sleep onset, increases abruptly in the middle of the night reaching maximum level by morning and again decreases through the day up to the phase of repetition of a new daily cycle. However, during the circadian rhythm, the GCs systemic circulation shows discrete pulses that last for an hour which are referred to as the ultradian rhythm. GCs circadian and ultradian rhythms stimulate accurate GCs response resulting in specific hormonal action at a particular instant but a differential physiological outcome during the 24 h cycle

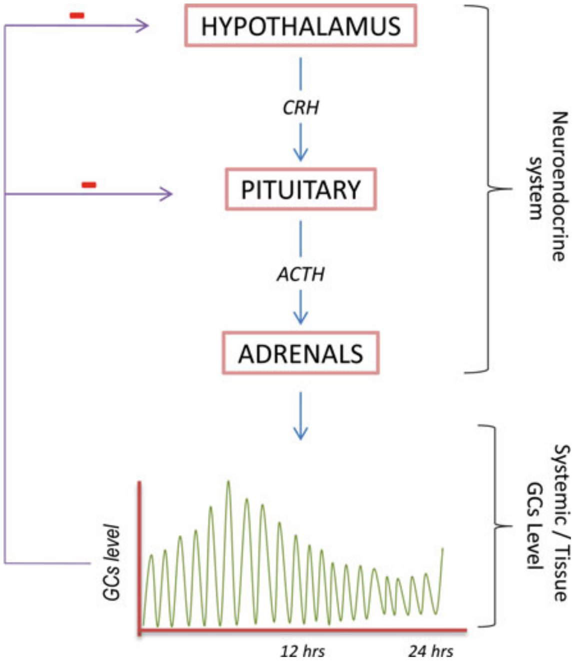

Fig. 3.2 Regulation of systemic glucocorticoids level by hypothalamus-pituitary-adrenal (HPA) axis. The feed forward cascade and feedback inhibition on the HPA axis regulates the circadian and ultradian glucocorticoid secretion. Stress activates the HPA axis resulting in GCs secretion by the adrenal glands (Feed forward cascade). Excess systemic GCs negatively regulate the HPA axis, by down regulating the secretion of corticotropin releasing hormone (CRH) by hypothalamus and adrenocorticotropic hormone (ACTH) by pituitary gland, resulting in decreased basal level of GCs (Feedback inhibition)

(Merkulov et al. 2016). In addition to the hypothalamus, the hippocampus also regulates the glucocorticoid systemic circulation (Jacobson and Sapolsky 1991).

Glucocorticoids level plays an important role in physiology of aging. Increased glucocorticoid activity has also been associated with greater hippocampal atrophy, memory impairment and poor cognitive function in the elderly. Rapid progression of Alzheimer's disease is associated with higher GCs. In transgenic Alzheimer's disease mice, glucocorticoids or stress induced-GCs were shown to potentiate memory impairment, hippocampal damage, β-amyloid formation and *Tau* accumulation (Green et al. 2006; Lee and Thomas 2007; Caroll and Zhang 2011; Yao et al. 2011; Batalha et al. 2016). Aberrant GCs systemic ultradian and circadian circulation in aging could lead to various pathological disorders associated with age like cardiovascular diseases (Vogelzangs et al. 2010).

3.3.2 Glucocorticoids Extra- and Intra-cellular Bioavailability with Age

The GCs bioavailability of various tissues is dependent on the GCs carrier protein called corticosteroid binding globulin (CBG) which transports GCs in blood circulation and as well regulates entry of GCs into the cells. Intracellular GCs availability is also regulated by a set of enzymes called 11β-hydroxysteroid dehydrogenases (11β-HSDs) which catalyze the inter-conversion between active GCs and the inactive keto forms. Thus, GCs bioavailability is regulated by the circadian, ultradian rhythms, CBG carrier protein and HSD enzymes (Stewart et al. 1993; Sandeep and Walker 2001; Petersen et al. 2006). In relation to age, there seems to be no change in CBG level (Table 3.1) (Veldhius et al. 2013) but HSDs

Table 3.1 The molecular components of glucocorticoids signaling pathway during aging, pathological conditions and their plausible intervention

GCs molecular mechanistic action	Aging	Pathological condition	Plausible intervention
GCs level	Increased	Improper cognitive function Memory impairment	Nutraceuticals like resveratrol, ginsenoside
HPA axis	Dysregulated	Decreased stress response	Resveratrol
HSDs level	Increased HSD1 Decreased HSD2	Hallmarks of age-related disorders	HSD1 Inhibitor Nutraceuticals
GR signaling pathway	Unregulated	Age-related disorders	Curcumin, Capsaicin, SGRMs
GR level	Decreased	Homeostasis imbalance	Dietary restriction

levels show an age-related pattern. The level of 11β-HSD1, converting inactive GCs into active GCs, increase with age in brain, adipose tissue, ovary, bone and immune system. In contrast, 11β-HSD2 seems to show an inverse relationship with age which increases the bioavailability of intracellular GCs. This implies a relationship of unregulated GCs bioavailability in aged tissues and hallmarks of age-related disorders like metabolic diseases, cognitive decline and cardiovascular risk (Chapman et al. 2013).

3.3.3 Glucocorticoids Genomic Signaling as a Function of Age

Intracellular glucocorticoids mediate their biological functions via the glucocorticoid receptor (GR) signaling pathway in vertebrates including human, rat and mice (Fig. 3.3). GCs bind to the glucocorticoid receptor, belonging to the class of nuclear receptors, to elicit physiological functions. Prior to GCs binding, the unbound GR is retained in the cytoplasm in association with the inhibitory protein complexes comprising of hsp90, immunophillins and various other modulatory proteins. In the absent of GCs, the inhibitory complex halts the GR nuclear-cytoplasmic trafficking and creates an environment which keeps GR in the appropriate folded state so that it could bind to GCs with high affinity on its intracellular emergence. When the GR binds to GC the inhibitory complex dissociates resulting in nuclear translocation of the hormone receptor complex (HR-complex). In the nucleus, the HR-complex binds to DNA regulatory sequences of a plethora of genes (Sanchez et al. 1987; Picard et al. 1990; Tai et al. 1992; Truss and Beato 1993, Zannas et el. 2015). From the binding of ligand with the receptor and to the interaction of the HR-complex with the DNA regulatory sequences constitute the GR signaling pathway. GR signaling pathway induces a subset of genes expression programing human growth and maturation in a time-dependent phase (infancy, childhood, puberty and adulthood) and in a tissue-dependent manner. Glucocorticoid receptor is strongly associated with development-related pathways and networks like NOTCH, VEGF, TGFB and WNT signaling showing tissue- and time-dependent events (Stevens et al. 2013). GR is also involved in aging of thymocytes which may have probable implication for the thymic involution process (Lustig et al. 2007). In aged rats there is a decrease in hsp90 and hsc70 in both cytoplasmic and nuclear fractions suggesting an age-dependent role of inhibitory complex in the GR signaling pathway (Murphy et al. 2002). In aged mice, the level of GR in liver and kidney decreases as compared to adult mice. However, the binding affinity of glucocorticoid for the receptor does not change with age (Ranhotra and Sharma 2001; Dutta and Sharma 2004). Ligand binding affinity, GR level, hsp90 and hsc70 differential expressions in development and aging could manipulate GCs physiological responses. The complete elucidation on their relation with age could pave a way on understanding and management of GCs in health and aging.

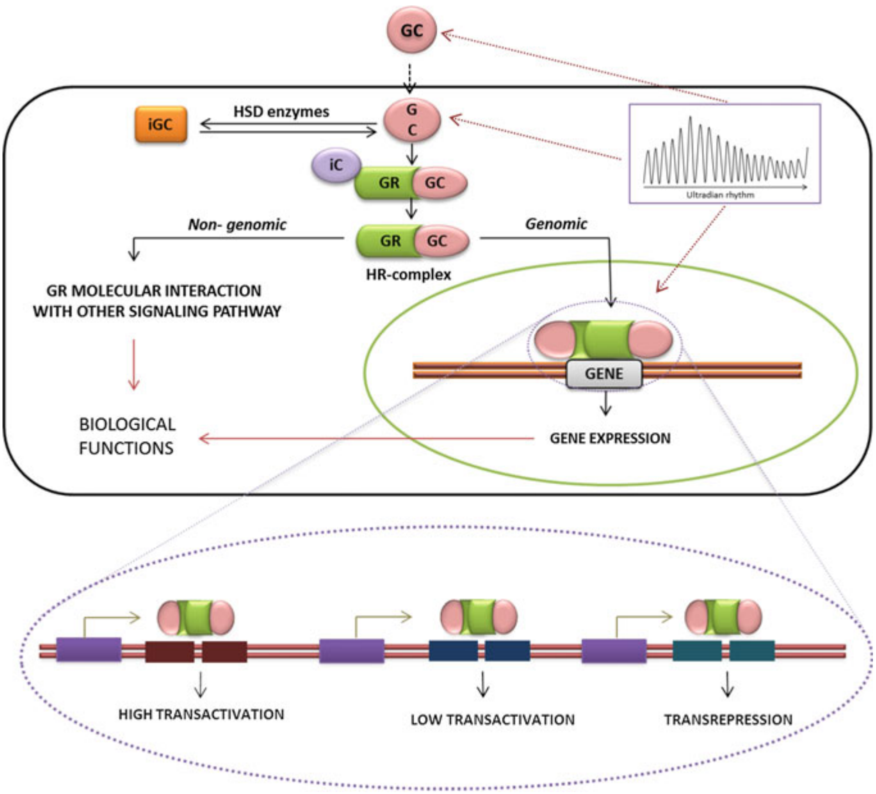

Fig. 3.3 GCs-mediated gene expression by glucocorticoid receptor (GR) signaling pathway. The pulsatile rhythm of extracellular, intracellular GCs and hormone receptor (HR)-complex interaction with the glucocorticoid response element (GRE) is maintained in GCs signaling resulting in normal physiology. GCs exert their effect via genomic and non-genomic actions. In genomic action, the level of GCs-mediated gene expression is highly regulated at the level of GRE and also by regulatory sequence (*purple box*) shown upstream of the GRE. The nature of the flanking nucleotides at either end of the palindromes of the GRE further fine tune GCs-mediated transactivation/transrepression. The highly transactivating GRE (*brown box*) is flanked by A/T nucleotides; the low transactivating GRE (*indigo box*) is flanked by G/C nucleotides; the nGRE is represented by *green box*. Non-genomic action of GCs is via interaction of GR with other signaling molecular players. *iGC* inactive GC; *iC* inhibitory complex

The meagre binding of the HR-complex to the DNA regulatory sequences simply does not account for the description on the comprehensive physiological action of GCs. Heterogeneous molecular mechanistic action of the GR signaling pathway can be explained by diverse feat like the variant GR isoforms, variant DNA regulatory sequences positioned in the vicinity or sequestered from the related gene, differential mode of HR-complex interaction with the response element and HR-complex chaperoning with various accompanying transcription regulatory proteins, post-transcriptional regulation, post- translational modification of

GR-mediated proteins. These mechanisms are further regulated at the cellular and tissue level in an age-dependent fashion (Kharwanlang 2015).

Identification and primary structural determination of a handful variant isoforms of GR substantially explained the plethora of GCs-mediated physiological functions. The main functionally active isoform having universal presence across various species is GRα. Another variant called GRβ is transcriptionally inactive. A new variant GRγ is also identified (Rivers et al. 1994). Alternative splicing of GR gene (NR3C1) and alternative translation of such transcripts generate around 16 isoforms of GR (Lu and Cidlowski 2006). Differential GR isoform expression in neonatal and adulthood was found in human cortical development (Sinclair et al. 2011). Interleukin-1β regulation on the expression of glucocorticoid receptor isoforms in nasal polyps were studied in patients of 41–46 years of age (Wang et al. 2015). Absence of GRβ mRNA in adult mouse tissues was reported in one study (Otto et al. 1997). Hence, GR isoforms show age associated pattern which could mediate age specific physiological changes.

The interaction of homodimer or heterodimer GR with the glucocorticoid response element (GRE) which is palindromic in nature mediates gene expression resulting in either induction or repression which depends on the nature of the sequence (Fig. 3.3). Sequences inducing gene expression are associated with genes like lipocortin 1, IkB, gluconeogenic genes like PEPCK and metallothionein II_A which are identified as positive GREs having sequence of 15 bps in the order GGTACAnnnTGTTCT. GCs-mediated gene repression is associated with a negative GRE (nGRE) of a variable sequence ATYACnnnTnATCn. Such GREs are correlated with genes like IL-1, IL-2 pro-opiomelanocortin, α-fetoprotein and prolactin. Flanking nucleotides in the GR binding sequence also regulate gene expression differentially (Fig. 3.3) (Truss and Beato 1993; Kelly et al. 1997; Petersen et al. 1988; McKay and Cidlowski 1999; Scheller et al. 2003; De Bosscher and Haegeman 2009; Zannas et al. 2015; Schöne et al. 2016). The differential transcriptional regulation of gene expression by GR via binding to the DNA regulatory response is a classical feature of the GR signaling pathway which is also synonymously termed GR genomic action (Lamia et al. 2011). The composition of the transcriptional interaction of GR with other factors at the various DNA regulatory site elicit a response of GCs which is coordinated to the basal transcriptional machinery, like TATA binding protein (TBP) and RNA polymerase II (Pol II) resulting in either activation or repression of gene expression. Considerable discrete transcriptional models set forth to explain the diverse glucocorticoid receptor transcriptional regulation. Simple glucocorticoid response element (GRE), composite GRE and tethering GRE accounts for the transactivation mediated glucocorticoid receptor transcriptional modulations. Transrepression by glucocorticoid receptor is accounted by negative GRE (nGRE), simple nGRE, composite nGRE, tethering nGRE and competitive nGRE (Newton and Holden 2007).

The binding of the glucocorticoid-receptor complex to the nuclei was higher in young mice as compared to adult mice. The differential interaction with the nuclei suggests a relationship between GR-mediated gene expression and GR binding ability (Ranhotra and Sharma 2001). GR mediated gene expression is however further dependent on the glucocorticoid response element. A number of epigenetic clock CpG sites were located within GRE are associated with stress via GC signaling which are pronounced in advancing age. Dexamethasone induced dynamic changes in methylation of the CpGs and a good transcription in the genes neighbouring epigenetic clock CpGs. Analysis of these dexamethasone-regulated genes showed enriched association with aging-related diseases which include coronary artery disease, arteriosclerosis and leukemia (Zannas et al. 2015) (Table 3.1). Gassen et al. (2016) proposed a model whereby excessive life stress, in part via its effects on glucocorticoid signaling, may alter the landscape of the aging epigenome and contribute to the development of aging-related diseases. This model has been recently supported by studies showing that lifetime stress and stress-related phenotypes may accelerate epigenetic aging.

Glucocorticoid receptor interaction with the GRE, nucleoprotein complex and histones along with a cohort of DNA-binding factors also regulate the accessibility of the chromatin complex. Surprisingly, the accessibility of GR to the constitutive regulatory sequences is not dependent on subsequent hormone treatment but the sites are opened prior to binding. However, chromatin remodelling of GCs inducible sites is hormone dependent. DNA binding protein such as AP-1 tagged the chromatin landscape which maintains an accessible chromatin organization which abets the recruitment of GR (Biddie et al. 2011). In contrast, progesterone receptor (PR) inhibits the ability of GR to remodel the chromatin and formation of the pre-initiation complex (Fryer et al. 1998). GR and NF-κB enhances long range chromatin contact (Kuznetsova et al. 2015). Such remodelling of the chromatin by GR in association with other factors like AP-1, NF-κB and PR could have age-related mechanistic action and play a role in aging phenomenon.

Studies in our laboratory indicate that the chromatin organization and remodelling is regulated by GR in an age-dependent manner. The chromatin organization of aged mice was found to be a compactly organized as compared to young mice which imply a correlation of GCs differential gene expression during aging. Aged chromatin organization seems to show a reduced response to GCs as compared to young mice which could plausibly results in various physiological and pathological alterations (Ranhotra and Sharma 2001). Glucocorticoid-mediated gene expression is dependent on the duration of GR binding to the DNA regulatory sequences. Physiological GCs level seems to regulate the interaction of GR with the regulatory elements. Pulsatile hormone stimulation results in transient GR nuclear translocation and GR-regulatory element interaction whereas constant hormone stimulation results in perpetual, more binding and interaction at sites not found during pulsatile stimulation. GCs pulsatile events are also correlated with pulsatile recruitment of RNA pol II and other proteins to GR transcriptional apparatus (Fig. 3.3). GCs inducible genes like *Per1* show an ultradian expression both at the primary and mature RNA level which is highly correlated to the ultradian activity of GCs. In aged mice, studies on GCs ultradian phenomenon shows a disrupted GCs pulsatile and a decrease pulse in peak phases which may disrupt the transient and ultradian GR binding to GRE and transcriptional activity (de Kloet and Sarabdjitsingh 2008; Spiga et al. 2014; Stavreva et al. 2016; Merkulov et al. 2016).

3.3.4 Glucocorticoids Non-genomic Signaling in Aging

Glucocorticoids non-genomic action is understood to implicate the molecular interaction between GR and various other signaling players. The molecular interaction subsequently regulates the downstream effect of these signaling partners with GCs signaling pathway. Glucocorticoid receptors physical interaction with various signaling players like NF-κB, AP-1, MSK1, JNK and Raf plausibly suggest a relationship among them in aging apart from various pathological conditions (Kharwanlang and Sharma 2011; Steelman et al. 2011). JNK signaling seems to induce lifespan extension in flies like Drosophila by cellular and systemic changes which influence metabolism, cell survival and tissue homeostasis. NF-κB and AP-1 are involved in inflammation which is another hallmark phenomenon in aging. Mitogen-and stress-activated protein kinase 1 (MSK1) is associated with striatal atrophy in aging (Martin et al. 2011; Biteau et al. 2011).

3.4 Management of Glucocorticoids Induced Age-Related Complications via GR Signaling Pathway Modulation

The wide spectrum of glucocorticoids effects on physiological processes like intermediary metabolism, inflammation, psychological problems and other processes could have underlying mechanisms that are age-related. Unregulated GC signaling in aging results in various diseases like cardiovascular, Alzheimer's disease, cancer, bone related disorders, age-related obesity, cognitive decline, type 2 diabetes with retinopathy, neuropathy, nephropathy and other pathological conditions (Hibberd et al. 2000; Reynolds et al. 2010; Hasan et al. 2012; Kharwanlang and Sharma 2013; Mueller et al. 2016). Modulation on the GC signaling pathway, HPA axis, and HSD enzymes by diet, selective GR modulators (SGRM) and nutraceuticals could maintain a fine tuning on the correlation of GC signaling pathway with age that could possibly minimize age-related disorders. In fact, population having low risk for chronic diseases like coronary heart disease and cancer consumes plant products. Mediterranean diet, low glycemic index diet, moderately low carbohydrate intake and vegetarian diets show a positive correlation with weight control, diabetes prevention, and cardio metabolic management. Diet is assumed to have major positive and negative effects on health and disease, which influences both the development and prevention of age-related diseases (Dutta and Sharma 2004; Ogle et al. 2012; vel Szic et al. 2015).

Nutraceuticals could also play an important role in overcoming geriatrics GCs therapeutic side effects like diabetes, arterial hypertension, osteoporosis, depression and cataracts. Nutraceuticals are food products that are physiologically beneficial and combat disease processes. They have both nutritional and pharmaceutical values rendering health and medicinal benefits (Ogle et al. 2012; Kaur et al. 2015).

Nutraceutical plant products having anti-aging activity like curcumin in turmeric, capsaicin in capsicum, glycyrrhizic acid in licorice, ginsenoside Rg1 in gingseng and resveratrol in red grapes were found to regulate GCs signaling. Under stressful conditions resveratrol was found to reduce GCs level and have anti-depressant effect possibly through regulation on the HPA axis. Resveratrol was also found to inhibit the activity of HSD1 enzymes in adipocytes thereby downregulating the level of active GCs in the adipocytes. This could account to the anti-obesity activity of resveratrol (Ge et al. 2013; Tagawa et al. 2013; Nade et al. 2014). Curcumin was found to alleviate the glucocorticoid induced osteoporosis by reactivating the dexamethasone inhibited Wnt signaling pathway (Chen et al. 2016). Modulation of HSD enzymes to treat GCs excess syndrome could be achieved by curcumin (Liang and Li 2011). Curcumin prevents GCs induced neurotoxicity (Xu et al. 2011). Curcumin and capsacin were found to maintain GCs and glucocorticoid receptor interaction in stressful environment and as well increase the binding of hormone receptor complex to the nuclei which could have implications on the anti-inflammatory effect of curcumin and capsaicin through GCs signaling pathway (Kharwanlang 2015). Ginsenoside maintains GCs efficacy probably through the GR signaling pathway in association with NF-κB. Ginsenoside enhances the effect of GCs in treating systemic lupus erythematosus in mice with lesser side effects. It potentiates GCs anti-inflammatory action with reduced hyperglycemic effect (Du et al. 2011; Li et al. 2014; Feng et al. 2015). Glycyrrhizic acid regulates HSD enzymes in GCs induced osteoporosis, thereby improving bone structure and strength. Glycyrrhetinic acid, another component of licorice blocks inflammation via dissociation of GR-Hsp90 complex. Glycyrrhizic acid and 18β-glycyrrhetinic acid also restore sensitivity in glucocorticoid resistance (Whorwood et al. 1993; Kao et al. 2010, 2013; Ramli et al. 2013).

Dietary restriction (DR) sans deficiency in essential nutrients is widely known to influence various physiological processes and is one of the means to delay aging and extend the mean and maximum lifespan in various species. In aged mice, DR was found to increase the level of GR and as well heightened the activation of the receptor as assayed by binding of the hormone-receptor complexes to the nuclei. DR may regulate metabolic processes via GR signaling pathway which might be beneficial in aging (Dutta and Sharma 2003, 2004, 2006). Selective glucocorticoid receptor modulators (SGRMs) are compounds exhibiting the good effects of GCs but abrogate GCs side effects. Compound C108297 partially inhibit CRH gene expression in the hypothalamus but does not disinhibit the HPA axis. It also enhances memory consolidation. Curcumin also displays GCs induced gene expression via differential GR-mediated gene transcription by inhibiting some genes whereas does not interfere other GCs-induced genes, thereby displaying selective GR modulation in gene regulation context. The response on GCs induced gene expression by using SGRMs could potentially augment the use of GCs in therapeutic intervention in geriatric care (Fig. 3.4) (Aoyagi and Archer 2011; Zalachoras et al. 2013).

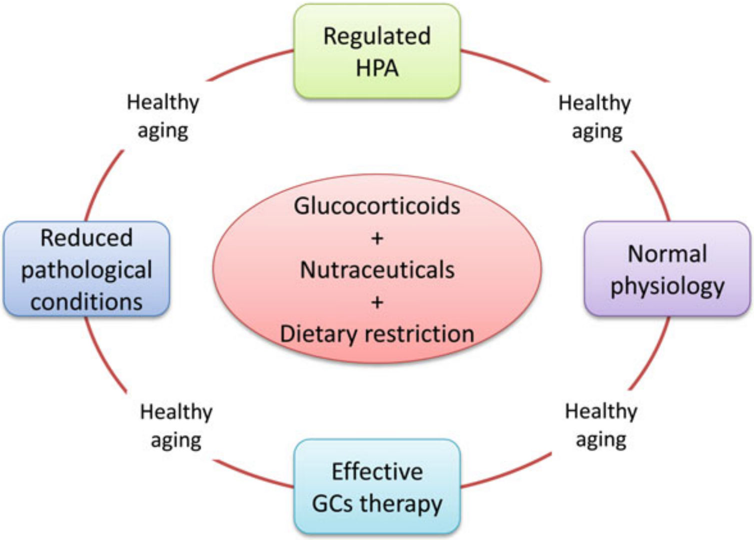

Fig. 3.4 Plausible interventions in glucocorticoid signaling for healthy aging. Nutraceuticals, dietary restriction and selective GR modulators (SGRMs) have positive outcomes on GCs signaling. Their usage during aging could potentially benefit aged for achieving normal physiology, reduced disease state and effective GCs therapy sans side-effects

3.5 Conclusion

In conclusion, glucocorticoids are indispensable for a healthy life and as well a disease-free aging. Understanding the various mechanisms of glucocorticoids molecular action ranging from GCs secretion, systemic circulation, bioavailability, genomic and non-genomic actions in aging could possibly open up new smart therapeutic and dietary interventions. Frequent intake of nutraceuticals like curcumin, resveratrol, capsaicin, ginsenoside and glycyrrhizic acid on the glucocorticoid signaling show a positive response in healthy aging. Dietary restriction which is known to maintain healthy life during aging in addition to extending lifespan up-regulates glucocorticoid receptor activity which plausibly keeps the organism receptive to stress during aging and thereby maintaining homeostasis. Selective GR modulators application in clinical science along with GCs could manage some of the side effects of GCs in old age. As GCs are indispensable in the therapy of many pathological conditions and with the increase in aging population, the induction of nutraceuticals, selective GR modulators and dietary restriction in GCs therapeutic in clinical science (Fig. 3.4) could ease the burden on the health management for upkeep of healthy aged population.

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Chapter 4
Zinc, Insulin and IGF-I Interplay in Aging

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Abstract Zn plays an important part in many biological processes including the insulin- and insulin growth factor (IGF) signaling (IIS) pathway which, in turn, is an evolutionary conserved pathway involved in many functions that are necessary for metabolism and growth. Notably, the IIS pathway play also a major role in aging. The overall cellular response and the outcome on the longevity of the organism depends also by the mechanisms involved in the regulation of Zn homeostasis that need to act in synergy just like a symphony orchestra. A likely conductor of this orchestra is the IIS pathway, which in turn is affected itself by the different ability to modulate Zn homeostasis of each cell within the tissues. The multiple ways by which Zn action affects insulin and IGF-1 activities and how these hormones modulate Zn homeostasis is reviewed in this chapter. While the mutual interaction between Zn and IIS on the modulation of longevity appears to be still unclear and characterized by contradictory findings, there is consistent support to draw a picture where a functional cell is able to disentangle IIS-mediated "nutritional Zn signals", which activate growth promoting pathways, functional integrity and cell division, from "stress response Zn signals" which, in turn, activate a cascade of signals to regulate transcriptionally and post-transcriptionally a multitude of cellular functions that include oxidative stress responses and the secretion of soluble factors.

Keywords Zinc · IGF-1 · GH · Insulin and insulin growth factor signaling pathway · Longevity · Aging

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4.1 Introduction

The role of Zn as essential nutritional factor for all form of life has been clearly established since long time. This is the natural consequence of the multiple functional relevance of Zn binding proteins (Zn proteome) which ranges from 5% in Bacteria and Archea to 8.8% in Eukaryota (Andreini et al. 2006a), with a notable 10% presence in humans (Andreini et al. 2006b). In most cases, the functional role of Zn regards its structural and catalytic role in proteins, including a vast number of transcriptional and nucleic acid metabolism regulators. More recently, an emerging role of Zn ions, as signaling factor in intra- and intercellular communication has emerged (Maret 2015). The process by which free Zn at nanomolar concentration is released and transduce various signals is very similar to the one observed for calcium in the millimolar range. This process is regulated by a huge machinery involving Zn sensors, buffering and Zn transporters proteins (in humans at least 24 members) that is conserved in all form of life (Cousins et al. 2006). Hence, it is not surprising that this element is essential for all cell functions including immune and antioxidant response as well as growth and duplication.

This last function appears to be primarily related to the presence of several Zn-dependent steps on the insulin- and insulin growth factor (IGF) signaling (IIS) pathway which, in turn, is an evolutionary conserved pathway involved in many functions that are necessary for metabolism and growth. Notably, the IIS pathway play also a major role in aging. Its downstream targets include master regulators of aging and longevity, such as the TOR complex, which is activated by IIS, and the FOXO family of transcription factors, which is repressed by IIS. Importantly, while flies and nematodes display a single receptor that regulate the IIS pathway, mammals evolved with two well characterized but highly overlapping hormonal pathways regulated by the insulin and IGF1 receptors (Russell and Kahn 2007). The first is controlled by insulin and it is mainly involved in control of metabolism (e.g. glucose uptake and synthesis), whereas the latter (IGF1 receptor) controls growth and development (Kim and Accili 2002). Experimental animal models and genetic human studies provide concrete evidence that reducing the function of the IIS pathway play a positive role in longevity that sometimes is accompanied by early deficits in growth and reproduction. However, excluding the case of the fat-specific insulin receptor knockout mice, which display a slight increase in median and maximal lifespan, loss of function or dramatic reduction of insulin signaling is related to metabolic dysfunction and shortened lifespan in mammals (Russell and Kahn 2007). Conversely, inhibition of IGF1 signaling by IGF1 receptor haploinsufficiency or by reduced levels of IGF1 as well as by reduction of function of mediators of both insulin and IGF-1 signaling (e.g. IRS1, IRS2, p66, Klotho) induce a longevity phenotype (Junnila et al. 2013).

A likely mechanism by which reduced IIS signaling increase lifespan is an increased protection against oxidative stress and cellular defense mechanisms (Holzenberger et al. 2003). These are in general mediated by inhibition of TOR and subsequent stimulation of autophagy as well as by activation of FOXO transcription factors which promote the expression of antioxidant and tumor suppressor genes. It thus may appear a paradox that Zn may act both in promoting the IIS pathway and the cellular antioxidant and defense mechanisms. We here review the biological role of Zn and its relationship with insulin and IGF-1 as well as the related pathways in order to provide a rationale to explain the apparent contradictions emerged in our understanding of the role of this trace element in aging.

4.2 Biological Functions of Zn

Zn plays an important part in many biological processes and there is extensive experimental evidence that both excess and deficiency of Zn causes alterations in almost all biological function. Experimental and clinical models of Zn deficiency are very important to define the processes that are "*in primis*" modulated by this essential trace element.

Zn deficiency "in vitro" results in impaired cell division that cannot be overcome by mitogens (MacDonald 2000). At organism level the impact of Zn on growth and reproduction is even more evident. The consequences of Zn deficiency on growth, reproduction, physical performance and diseases have been widely studied in humans and animal models. Zn was first known to be essential for growth and reproduction in rats (Todd et al. 1934) by providing a diet containing only 0.3 mg/kg (versus an established requirement of 12 mg/Kg), and a similar experiment was reproduced in mice (Day and Skidmore 1947). Zn deprivation in yeasts (*S. cerevisie*) is known to induce cell cycle arrest (Walker 1998) while an amount in the nutrient medium of 5–15 μM seems optimal for the growth rate. Growth of worms (*C. elegans*) is not appreciable at concentration of Zn around and below 1 μM in the medium (Davis et al. 2009) while it is maximized at 30 μM Zn. The impact of nutritional Zn in growth and reproduction is so well recognized that the first observation of Zn deficiency in humans regarded just these aspects (Prasad 2012). Although Zn may directly regulate DNA synthesis through the activity of transcription factors and polymerases, it is likely that these effects are mediated by its influence on the hormonal regulation of GH as well as on the IIS pathway.

Another area of intensive research around Zn regards the impact of this trace element on immune function. Zn deficient humans and rodents appears in general less able to fight off infection compared to Zn adequate controls (Blewett and Taylor 2012). While there are limited studies around these aspects in worms and flies, the impact of Zn deficiency on the human innate and adoptive immunity has been deeply investigated (Haase and Rink 2014). Innate immune system is particular sensitive to Zn deficiency. Most functions of monocytes and macrophages as well as phagocytosis of granulocytes and cytotoxicity of natural killer cells have been shown to be impaired by Zn deficiency in humans (Ibs and Rink 2003). An important molecular aspect of this regulation regards the modulation of the transcriptional regulation of cytokines by nuclear factor-κB (NF-κB) by Zn signals (Maares and Haase 2016), which are requires to mediate NF-κB activation in monocytes. Similarly, human adaptive immune system is affected by Zn deficiency mainly through an impaired thymus growth (the organ where maturation on T cells take place) and lymphopoiesis, thymulin activity (a Zn dependent thymic peptide necessary for T-cell differentiation and function) and lymphocyte ability to respond to antigens (Maares and Haase 2016). The impact of Zn in thymulin activity has been proven also in marginally Zn deficient mice (Dardenne et al. 1984) and rats (Chandra et al. 1980). Similar conclusions were drawn also regarding functional impairments of lymphocytes in rodents (Shi et al. 1998; Moore et al. 2001). However, conversely to data observed in adult mice (Fraker et al. 1978), Zn deficiency induced thymic atrophy and lymphopenia in rats is no more evident when data are corrected for body weight and organ weight, respectively (Hosea et al. 2003, 2004). These differences have been attributed to the different type of malnutrition associated with these Zn deficiency models: wasting (acute, with altered body composition) in adult mouse and stunting (chronic, with reduced linear growth) in growing rats (Blewett and Taylor 2012). The use of pair-fed and marginally Zn deficient controls to separate the role of malnutrition in rats has further provided evidence that Zn deficiency per se is not related to increased glucocorticoids and the associated lymphopenia. Compromised immunity in Zn deficiency appears to arise also from reduced Zn signaling. Indeed, IL-2 receptor stimulation in primary human T-cells and murine T-cell lines results in release of free Zn from lysosomes to the cytoplasm that induce ERK signaling and proliferation of T cells (Kaltenberg et al. 2010).

Interesting, the above described function of Zn in immunity appears to overlap with more than one function attributed to IGF and IGF receptors in immune cells, including priming and promotion of phagocytosis in monocytes (Balteskard et al. 1998), activation of NF-κB (Balaram et al. 1999) and stimulation of cytotoxic activity of NK cells (Ni et al. 2013).

Zn is also studied as an essential component of numerous proteins involved in the defense against oxidative stress. While being redox inactive under physiological conditions, Zn is strongly associated with the oxidative stress response. Indeed, oxidative stress and the related lipid, protein and DNA damage is a well characterized hallmark of Zn deficiency (Eide 2011). In addition to the structural and catalytic role of Zn in the antioxidant enzyme Cu, Zn superoxide dismutase (SOD1), the most important Zn-dependent mechanism that promote the intracellular antioxidant defense is the mobilization of free Zn signals. This is mostly mediated by Zn-binding “guardian proteins”, named Metallothioneins (MTs), that sense oxidative and nitrosative stress by their Zn-binding sulfhydryl groups with subsequent release of free Zn ions (Maret 2009). Human MTs superfamily includes 19 isoforms 11 of which are known to be functional (i.e., MT1A, MT1B, MT1E, MT1F, MT1G, MT1H, MT1 M, MT1X, MT2A, MT3, and MT4) (Laukens et al. 2009). The mouse genome includes only four MT genes (Mt1, Mt2, MTt3 and Mt4), and likewise four MT genes are present in the Drosophila genome (MtnA, MtnB, MtnC and MtnD), while two MTs encoding genes have been characterized in *C. elegans*, (mtl-1 and mtl-2).

The transcription of most MT isoforms is mainly under control of the metal transcription factor 1 (MTF-1) which is involved in their feedback regulation. Indeed, released Zn ions from MTs activate MTF1 that binds the metal responsive elements (MRE) in the DNA and induce transcription of MRE responsive genes including MTs themselves. The high evolutionary conservation of the MTF-1 gene (Lichtlen et al. 2001) suggests that this is a very important mechanism of transduction of oxidative stress signals common to nematodes, flies, mammals and many other species. In addition to MTs, target genes of MTF-1 include the γ-glutamylcysteine synthetasehc (γ-GCShc), which is involved in the synthesis of the antioxidant peptide glutathione (Lichtlen et al. 2001). Other target genes include α-fetoprotein, the liver-enriched transcription factor C/EBPα and tear lipocalin/von Ebner's gland protein, all of which have a role in the cell stress response. MRE elements can be also identified by TRANSFACT (predicted transcription factor targets) (Matys et al. 2006) in the promoter of human FOXO1 and FOXO4, suggesting the idea that Zn signals may be involved in the activation of their transcription. However, experiments in hepatoma cells and immune cells indicate that Zn signals may act immediately by inhibiting the phosphatase and tensin homolog deleted on chromosome 10 (PTEN) (Plum et al. 2014) and stimulating the PI3 K signaling (downstream the IIS pathway), which results in Akt-dependent FoxO phosphorylation and inactivation (Walter et al. 2006). In addition, autophagy, the process whereby cells degrade cytosolic proteins and organelles to recycle their components, was previously identified as required for growth of yeasts in low Zn conditions (North et al. 2012). Hence, the relationship of Zn with the IIS pathway appears to counteract the role of this trace element in antioxidant and cellular defense. The relationship is further complicated by a potential feedback regulation, as expression of MTs appears to be also supported by FOXO transcription factors. In particular, mRNA expression of MTs was reported to be upregulated in response to the *C. elegans* FOXO transcription factor, DAF-16, (Barsyte et al. 2001) as well as by the mammalian FOXO3 (Greer et al. 2007).

Finally, it is mandatory to mention that in addition to MTs and MTF-1 there is a multitude of Zn transporters that is involved in the regulation of Zn fluxes across cell membrane and intracellular compartments. Actually 14 members of the ZIP family and ten members of the ZnT family have been identified in humans (Lichten and Cousins 2009). ZIP proteins function in the uptake of Zn into the cytosol of the cell from the extracellular space or intracellular compartments, while ZnT proteins function in the efflux of Zn from the cytosol of the cell to the extracellular space or intracellular compartments. In the last years, emerging evidence has suggested a role for Zn transport proteins in facilitating glycemic control and glucose homeostasis.

In this context, it is useful to review our current knowledge around the influence of Zn, Zn treatments and Zn binding proteins on the longevity of different species, especially where a link with the IIS pathway has been studied or hypothesized.

4.3 Zinc in Biogerontology

Available data on the biology of Zn points out its strong interplay with the IIS pathway, including its downstream growth-signaling pathways regulated by the TOR family of kinases (Longo and Finch 2003). In general, experimental manipulations that upregulate these pathways promote aging, while those that downregulate these pathways extend life span and delay the onset of age-related pathologies. The mechanisms involved in the "accelerated aging" promoted by enhanced growth signaling include, among others, the inhibition of cellular detoxification pathways (Partridge et al. 2011), autophagy (Madeo et al. 2015) and oxidative stress response (Baumeister et al. 2006) as well as the promotion of DNA replication stress (Burhans and Weinberger 2007).

According to the results obtained in experimental models of Zn deficiency it is reasonable to conclude that Zn promotes the growth-signaling pathways, which is apparently in contrast with the antioxidant properties of Zn observed in experimental and clinical studies. How can we disentangle and find rationale explanations to these pleiotropic functions attributed to Zn in order to clarify the role of this trace element in aging?

A good starting point to answer this question is to review our current knowledge about Zn treatments and transgenic models on Zn binding proteins that affect longevity in various species.

4.3.1 Zn Supplements and Longevity

While Zn deficiency leads to functional defects in growth and development and decreased immune function, exposure to elevated Zn can have toxic effects. However, various impact of supplemental Zn on lifespan have been obtained in different models and with different doses of Zn.

One of the first observation was made on the impact of additional Zn in yeasts (Steenbergen et al. 1969). Yeasts were reported to growth well in a medium containing 1 μM Zn, while the presence of additional Zn (from 10 to 100 μM) increased death rate and reduced lifespan. Similarly, in trematodes (cercariae and schistosomules of Schistosoma mansoni) the longevity of the parasites was found to be inversely proportional to Zn concentration from 0.05 to 5 mM (Asch and Dresden 1977).

A very recent study performed in nematodes demonstrates that Increasing Zn levels in vivo from early development affect lifespan while Zn deprivation exert a beneficial effect. Excess dietary Zn supplementation (from 200 μM up to 500 μM) decreased dose dependently the mean and maximum lifespan in *C. elegans* (Kumar et al. 2016). More importantly, reducing Zn levels in vivo with the Zn chelator TPEN (from 50 μM up to 200 μM) increased dose dependently the mean and maximum lifespan. These effects where observed only when worms where exposed during early development, while treatments performed after day 5 of adulthood did not altered the lifespan. The study also demonstrates that the negative effects of Zn on lifespan are dependent on daf-16 (the sole ortholog of the FOXO family of transcription factors in the nematode *C. elegans*), hsf-1, and skn-1, which are genes that regulate the response to stress. In particular, DAF-16 is downstream of the IIS pathway which is regulated by Zn at different levels. These finding also indicate that the effects of Zn excess on longevity are not simply a matter of toxicity, but are affecting lifespan through their impact on longevity signaling pathways.

Conversely to the data on “wild type” models of longevity, food supplementation with Zn (4 mM ZnCl2) exerts a beneficial effect on lifespan of parkin mutant flies (Saini and Schaffner 2010) with an extension from 11 days on normal food (NF) to 23 days under Zn supplementation. However, control flies under the same regimen of Zn supplementation showed a reduction of lifespan. This was the case both of flies raised on Zn supplement and maintained on the same regimen, as well as of flies raised on Zn and maintained on normal food, but not of flies raised on Zn and maintained on normal food. Interestingly, on normal diet, the Zn content in heads of parkin mutant flies was much lower than in controls, consistent with a mutant model of Zn deficiency. Moreover, transcript levels of the metallothionein MtnB are higher in parkin mutants and is further increased (24 fold) by Zn supplementation. Moreover, the transcription levels of the Zn exporter ZnT35C of the parkin flies was reduced compared to controls and did not responded to Zn supplementation only in the mutant. Depressed expression in mutant flies was additionally observed also for other transporters involved in the uptake of Zn indicating a general Zn dyshomeostasis that is corrected by Zn supplementation.

This is even more evident in the mouse model of acrodermatitis enteropathica (a human genetic disease of acute Zn deficiency) generated by a conditional knockout of Zip4 (Slc39a4, the Zn transporter involved in the disease). Zn supplementation with high doses exerts a strong positive beneficial effect in the lifespan of these mice. These mice under Zn supplementation (250 mg/L in drinking water) starting on the day that the knockout is initiated were rescued from early death and survived for the whole length of the experiments (approximately 30 days) (Geiser et al. 2012).

There are also examples of a positive impact of Zn supplementation on the lifespan of normal mice. An antioxidant mixture including 5 mg/kg of elemental Zn in the form of gluconate (other components were 7.5 mg beta carotene, alpha 15 mg tocopherol acetate, 50 mg ascorbic acid, 25 mg quercetin 3β-rutinoside, 25 μg sodium selenite) enriched to a standard diet (from ‘Voronovo’, Moscow region, Russia) induced a 10–16% increase in the lifespan of C57BL/6 mice when starting the diet at 2 and 9 months of age, but not when starting at 16 and 23 months of age (Bezlepkin et al. 1996). This data leads to the idea that increased lifespan is the net result of the antioxidant mixture.

However, in the same period, it was shown that Zn treatment (22 mg/L in drinking water) in Balb/c mice housed in non-SPF conditions and starting at age of 18 months induced a median lifespan extension (from 27 to approximately

30 months) with an increase in 10% of maximal lifespan (from 30 to 33 months) (Mocchegiani et al. 1998).

Several years later, a detailed survival study in the long living MT1 overexpressing mice and in the respective controls (C57BL/6J) under supplementation with a high dose of Zn (380 mg/L) and starting in old age was performed (Malavolta et al. 2012). While Kaplan Meier analysis did not showed significant impact on median lifespan, Zn treatment induced an increase of 14% in the S0 parameter of the Piantanelli mathematical model of survivorship (Piantanelli et al. 2001) in the C57BL/6J mice. This effect was not observed in MT1 overexpressing mice. The advantage of using the model of Piantanelli is the possibility to draw a biological meaning from the analysis of the survival curves. Since S0 describe the extent of the variability of physiological functions as consequence of genetic and environmental factors, the observed difference in S0 suggests that Zn do not affected all C57BL/6J mice in the same way thus increasing the heterogeneity of the population. This is in line with the observed variations (likely due to stochastic and microenvironmental factors) of laboratory mice (Vedell et al. 2011). In this case, it might be hypothesized that these stochastic and microenvironmental variations regards Zn homeostatic mechanisms, drinking behaviors of mice, subclinical diseases and infections or other microenvironmental factors related to the non-SPF conditions which affect the individual outcome of Zn supplementation.

In conclusions, there is reasonable support to the idea that Zn supplementation, in absence of a well-defined Zn deficiency or Zn dyshomeostasis, may negatively affect longevity when administered in the early phases of growth. This is likely due to the impact of Zn on the IIS signaling pathway which has a well-defined antagonistic pleiotropic function in aging. Conversely, Zn treatment (even at high doses, but below the toxicity threshold) in adult and old organisms seems to have not mean negative effects on lifespan, but the treatment may induce an increased heterogeneity and likely a different response within the same experimental population. Part of this heterogeneity may be attributed to differences in the machinery that regulates Zn homeostasis. In this context, it is useful to look at longevity data of species with alteration in Zn regulating mechanism.

4.3.2 Zn Regulatory Mechanisms and Longevity

The first report of an impact on lifespan of a mutation of a Zn binding proteins was performed in Drosophila. A 30% increase in lifespan was reported in Drosophila by simultaneous overexpression of Cu-Zn-SOD and catalase (Orr and Sohal 1994). While this is not the case of a protein that regulates Zn homeostasis, it deserves to be cited in the context of the known regulatory activity of Zn on cellular stress response.

The main player in the free Zn regulated transcriptional activity is the Zn sensor MTF-1. Mutant drosophila with a defective MTF-1 display shortened lifespan, whereas MTF-1 overexpression results in resistant flies with prolonged longevity on iron or cadmium-supplemented media but shortened life-span on Zn-supplemented medium (Bahadorani et al. 2010). Hence, Zn responsive elements may play an important role in aging and life-span determination then what is commonly believed.

The most prominent target of MTF-1 are MTs, the oxidative stress sensors and Zn-buffers of the cell. In line with the reported role of MTF-1 in drosophila, a longevity phenotype has been shown in MTs transgenic mice that overexpress the MT-1 isoform (MT1-tg) in the C57BL/6J background (Malavolta et al. 2012), as well as in cardiac-specific MT transgenic mice that overexpress the human MT2 isoform in the FVB background (Yang et al. 2006), while MT knockout mice in the 129/Sv genetic background have shorter mean and median lifespan compared to the wild type (Kadota et al. 2015). However, Zn supplementation in old age seems to not affect the longevity phenotype of MT1-tg mice (Malavolta et al. 2012).

It is also worth of mentioning a human study on a polymorphism in the MT1a gene coding region. This SNP was found to be associated with the longevity of the centre-Italian female population (Cipriano et al. 2006). Interestingly, this SNP (which is in the coding region) was also indirectly shown to participate in the process of binding and release of Zn from MTs.

The feedback relationship between MT and MTF-1 make it extremely complicate to draw meaningful conclusion on the biological mechanisms affected by these factors and their role in longevity. In synthesis, MTs sense oxidative stress and release Zn. MTF-1 sense the Zn released by MTs and induce MTs synthesis. The newly synthesized MTs bind Zn to reduce its levels. The consequence is that in particular experimental settings we might observe an increase or a decrease of free Zn depending on the timing of the observations and the pre-existing status of the cell Zn homeostatic machinery (e.g. upregulated or downregulated MTs and Zn transporters). Hence, the intracellular Zn status is not a static, but rather a very dynamic phenomenon which may be represented by Zn waves. Therefore, it is likely that the overall cellular response and the outcome on the longevity of the organism depends on the intensity and length of these Zn waves that are played by different cells and tissues all over the organism, just like a symphony orchestra. A likely conductor of this orchestra is the IIS pathway. In turn, this conductor is affected by the different professional level of the members of the orchestra (e.g. the ability to modulate Zn homeostasis of each cell within the tissues) and by the overall features of the theatre where the orchestra play (e.g. the environmental conditions, including Zn intake). A schematic representation of this metaphoric interpretation is drawn in Fig. 4.1. Several interconnection links Zn, MTF-1 and MTs to insulin and IGF-1 activity as well as to their regulation and feedback responses. In the next chapters these interconnections have been extensively review.

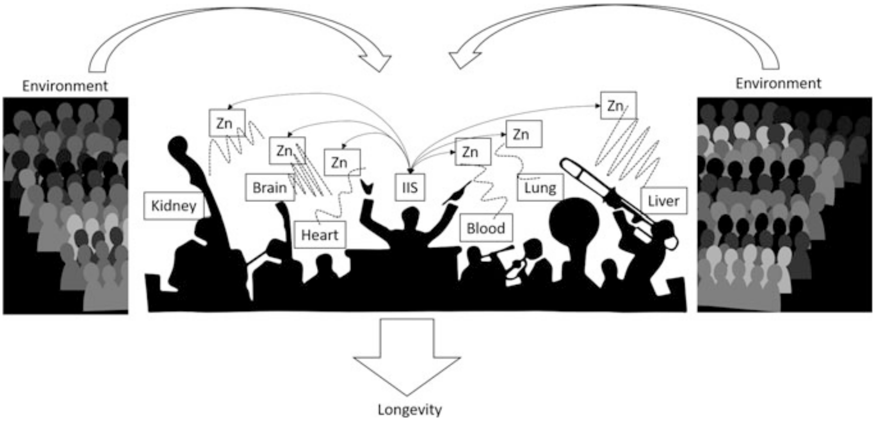

Fig. 4.1 Metaphoric interpretation of the relationship by IIS and Zn homeostasis. Zn status is not a static, but rather a very dynamic phenomenon which may be well represented by the intensity and length of "free Zn" waves inside cells from different tissues and organs. Cells play their own instruments (e.g. the Zn homeostatic machinery which is cell/tissue specific) just like a symphony orchestra to deliver a "music" representing health, functional status and finally the longevity of the organism. A likely conductor of this orchestra is the insulin/IGF-1 signaling (IIS) pathway. In turn, this conductor is affected by the different professional level of the members of the orchestra (e.g. the ability to modulate Zn homeostasis of each cell within the tissues) and by the overall features of the theatre where the orchestra play (e.g. the environmental conditions, including Zn intake)

4.4 Zinc Insulin Interplay

4.4.1 Zinc Action on Insulin Activity

Insulin is an ancient conserved molecule from nematodes to humans (Blundell and Humbel 1980). The role of Zn in the modulation of insulin action has been described in many experimental rodent models, clinical and epidemiological studies (Taylor 2005). Zn is required for insulin storage and secretion (Figlewicz et al. 1984). The hormone biosynthesis occurs in the pancreatic β-cells, where proinsulin is transported to the Golgi apparatus and it is packaged into secretory granules and then converted to native insulin by selectively cleaving the C-peptide (Coffman and Dunn 1988). In the presence of Zn ions that are transported into secretory granules, insulin undergoes a maturation process and aggregates further to form 2-Zn-hexameric complexes (Coffman and Dunn 1988; Hill et al. 1991). In vitro study on INS-1E cell line has demonstrated that Zn supplementation within the physiological concentration range induces insulin secretion, while Insulin content is reduced by Zn chelation (Nygaard et al. 2015).

During insulin exocytosis from the β-cell, Zn is co-secreted with insulin into the islet extracellular space. Zn is released from insulin when it reaches the higher pH of blood, and these Zn ions provide an "off-switch" for glucagon release from the α-cell during glucose deprivation by closure of the α-cell ATP dependent K+

channels (Slucca et al. 2010; Hardy et al. 2011a). Moreover, the activity of several gluconeogenic enzymes, including phosphoenolpyruvate carboxykinase and glucose 6-phosphatase, is Zn dependent (Cameron et al. 2010), further highlighting the key role of Zn in regulating glucose metabolism.

Several potential mechanisms have been suggested for the role of Zn affecting insulin action and glucose homeostasis. Zn exhibits insulinomimetic activities on target tissues promoting lipogenesis and glucose transport (Tang and Shay 2001; Keller 2004; Haase and Maret 2005). For example, Zn exerts an insulin-like effect on glucose transport mediated by phosphoinositol-3-kinase and Akt signaling pathways in target tissues (Tang and Shay 2001). Additionally, insulin-responsive aminopeptidase (IRAP) is a Zn dependent enzyme that seems to be required for the maintenance of normal glucose transporter (GLUT) 4 levels to ensure glucose uptake into tissues (Keller 2004). The cation has also a role as an inhibitor of protein tyrosine phosphatase 1B activity (PTP1B, a negative regulator of insulin signaling) (Haase and Maret 2005) ameliorating high-fat-diet induced insulin resistance and lipid disorders in mice (Ma et al. 2011). In addition, free Zn signals can inhibit PTEN (Plum et al. 2014), which in turn inhibits the downstream PI3 K/Akt-cascade, thus resulting in the overall activation of the IIS pathway. This raises the prospect that Zn plays stimulatory activity along the pathway that increase insulin activity.

On the other hand, suboptimal Zn status decreases insulin secretion from the pancreas (Fung et al. 2015) and this condition during pregnancy and lactation decreased insulin sensitivity and increased weight gain in rat offspring (Jou et al. 2010). In addition, severe Zn deficiency induces hyperglycemia and hyperinsulinemia (Hall et al. 2005), directly implicating Zn in systemic glucose regulation. Consistent with this role of Zn in glucose metabolism, individuals with type 2 diabetes often have low serum Zn concentrations (Quilliot et al. 2001; Al-Maroof and Al-Sharbatti 2006; Basaki et al. 2012).

4.4.2 Influence of Zinc Machinery on Insulin Activity

It is well recognized that Zn homeostasis depends on a complex network of MTs and Zn trasporters. The liver is the main organ for MTs synthesis in the body, but a certain amount of MTs can be also synthesized and secreted from pancreatic islets and adipose tissue (Davis and Cousins 2000; Meugnier et al. 2007). A link between MTs and the regulation of insulin metabolism has also been directly demonstrated in MT-1/-2-knockout mice, which became mildly obese and developed hyperinsulinaemia (Beattie et al. 1998). It has been additionally proposed that type 2 diabetes (T2D) subjects with reduced MTs levels might be more susceptible to oxidative damage and hyperglycemia (Maret and Krezel 2007) and increments of MTs dysfunctional proteins in ageing and T2D may contribute to a decreased antioxidant defense and T2D progression (Mocchegiani et al. 2008b). Mt1 overexpression in pancreatic islet significantly protects mice from acute streptozotocin -

induced ROS at young age, but conversely impairs glucose stimulated insulin secretion and promotes the development of diabetes in older age (Chen et al. 2015). However, studies using advanced transgenic technology showed that tissue-specific MT overexpression can effectively ameliorate diabetic hyperglycemia and cardiovascular disorders (Chen et al. 2001). Regarding MTs induction by hyperglycemia, contrasting results have been found. Chen and Song (Chen and Song 2009) report that serum MT levels did not differ between T2D patients and controls. In addition, MT content in adipose tissues didn't differ in streptozotocin-, high-fat-diet-induced hyperglycemic mice or ob/ob mice as compared to control mice. On the contrary, differences are found in liver MTs expression, being them increased in streptozotocin- and high-fat-diet-induced hyperglycemic mice and decreased in ob/ob mice as compared to control mice (Chen and Song 2009). Another study shows an upregulation of several MTs isoforms including MT1X and MT2A in adipose tissue and skeletal muscle of volunteers during a hyperglycemic-euinsulinemic clamp with infusion of somatostatin to inhibit endogenous insulin release (Meugnier et al. 2007), while other authors report a decrement of MT levels in the skeletal muscle of T2D patients compared with control subjects (Scheede-Bergdahl et al. 2005).

An oligonucleotide microarrays analysis has shown that MT-1A expression is modulated in rat islets after 96 h of culture exposed to several glucose concentrations (Bensellam et al. 2009). Bellomo and coauthors (Bellomo et al. 2011) show decrement in MT-1 and MT-2 after just 2 h of incubation, with high (16.7 mM) glucose concentration, in mouse pancreatic β-cells associated with an upregulation of some Zn importers (Zip-6, Zip-7, Zip-8).

Increased pancreatic MTs expression induced by Zn supplementation or by genetic manipulation can prevent diabetes induced by streptozotocin (Ohly et al. 2000) as well as the associated DNA damage in β cells and the subsequent alteration of the glycemic control (Chen et al. 2001). Similarly, in vivo, intraperitoneal injections of Tat-MT fusion protein in streptozotocin-treated mice increase radical-scavenging activity, decrease apoptosis, and reduce endoplasmic reticulum stress in the pancreas, thus delaying the development of diabetes (Park et al. 2011). Additionally, the presence of exogenous Zn7-MT-2A to beta-cell line, induces insulin secretion and increases the intracellular insulin content (Nygaard et al. 2014).

With regard to Zn transporters, one of the most highly investigated Zn transporters in the regulation of insulin activity and in the etiology of diabetes is ZnT8. This transporter is almost mainly expressed in the beta cells of the pancreas, where it plays a critical role in the transport of Zn into insulin secretory vesicles and is fundamental for the synthesis, storage, and action of insulin (Murgia et al. 2008; Nicolson et al. 2009; Kambe 2011; Davidson et al. 2014). However, ZnT8 is also expressed in other secretory cell types, such as thyroid cubical cells and adrenal gland cortex cells, suggesting for this Zn transporter a more widespread role in endocrine secretion (Murgia et al. 2008). It has been shown that the overexpression of ZnT8 in INS-1 cells induces glucose-stimulated insulin secretion (Chimienti et al. 2006). Conversely, a decreased ZnT8 expression in beta cells resulted in reduced insulin secretion after a hyperglycemic stimulus (Fu et al. 2009). Similarly, it has been shown that mice with a beta cell pancreatic-specific ZnT8 knockout have glucose intolerance (Wijesekara et al. 2010), while global ZnT8 null mice have abnormalities in diet-induced glucose tolerance and insulin secretion (Pound et al. 2009; Hardy et al. 2011b). However, results on Znt8 KO mice have been highly variable due to the mixed 129SvEv/C57BL/6J genetic background of the mice studied. When these mice are studied on the pure C57BL/6J genetic background, despite a marked reduction in islet Zn content, the absence of ZnT-8 does not have a substantial impact on mouse physiology (Pound et al. 2012). Interestingly, female C57BL/6J Slc30a8 KO mice had reduced ($\sim$ 20%) fasting insulin levels but normal fasting blood glucose, which may suggest a positive role in the long term prevention of a diabetes-like disease. ZnT8 is suggested to be associated with an increased risk of type 2 diabetes (T2D) due to a non-synonymous polymorphism (Arg325Trp, rs13266634) in the SLC30A8 gene, (Scott et al. 2007; Diabetes Genetics Initiative of Broad Institute of Harvard and MIT, Lund University, and Novartis Institutes of BioMedical Research et al. 2007; Zeggini et al. 2007). The Arg risk allele has been associated with higher proinsulin levels (Majithia et al. 2011), lower glucose-stimulated insulin secretion in INS-1E cells (Kim et al. 2011), and with increased hepatic insulin clearance in humans (Tamaki et al. 2013). Further SNPs in SLC30A8 gene have been implicated in T2D susceptibility or protection (Lin et al. 2010; Flannick et al. 2014). Interestingly, while previous functional study of SLC30A8 suggested that reduced Zn transport increases T2D risk, a recent study proposes that loss-of-function mutations in humans protects against T2D (Flannick et al. 2014). These studies combined with the recent results of the ZnT8 KO in the C57BL/6J mice advocate towards the development of experimental intervention studies with ZnT8 inhibitors in order to better clarify the potential therapeutic role of this Zn transporter in the disease.

Other Zn transporters may play a role on glucose homeostasis such as Zip6 and Zip7, which are two of the most highly expressed ZIP members in human and mouse pancreatic β cells and together cooperate in regulating cytosolic Zn under basal conditions and in response to glucose to replenish cellular and intragranular Zn during/after insulin secretion (Bellomo et al. 2011; Liu et al. 2015). Zip6 and Zip7 knockdown β cells have impaired glucose-stimulated insulin secretion and increased ROS production, suggesting the crucial role of these Zn transporters in maintaining normal insulin exocytosis in pancreatic β cells (Liu et al. 2015).

Zip4 seems to be not essential for proper glucose homeostasis and insulin secretion in mice, but its over-expression in beta cells influences increments in cytoplasmic and granular Zn pools and stimulates glucose dependent insulin secretion in vitro (Hardy et al. 2015).

Zip10 is normally localized to the plasma membrane but, under Zn deficient conditions, it moves to intracellular compartments (Lichten et al. 2011). The expression of Zip10 transcripts has been demonstrated in pancreatic α and β cells (Gyulkhandanyan et al. 2008) supporting a possible role for ZIP10 in glucose homeostasis.

Zip14, an ubiquitus Zn transporter involved in hypozincemia subsequent to the acute-phase response to inflammation (Liuzzi et al. 2005), is widely expressed in liver, pancreatic, and adipose tissues (Tominaga et al. 2005; Maxel et al. 2015). Zip14 seems to have an impact on glucose homeostasis and lipid metabolism, as ZIP14 knock-out mice show an altered glucose homeostasis with hypoglycemia, increased insulin and liver glucose levels as well as increased adipose mass (Aydemir et al. 2012). Moreover, hepatocyte endosomes from Zip14 knock-out mice are Zn deficient and this phenomenon is associated with increased hepatic glycogen synthesis and impaired gluconeogenesis and glycolysis. Zip14 is also downregulated in adipose tissue during chronic obesity and is inversely correlated with several metabolic markers of lipid and glucose metabolism (Maxel et al. 2015).

Among SLC30 family, ZnT-3 has been described in beta-cells, (Smidt et al. 2009), and it is localized in insulin containing granules close to the plasma membrane (Smidt et al. 2016). Znt3 is up-regulated by glucose and by Zn depletion in pancreatic β-cell lines, while Knock-out mice show an altered glucose metabolism after streptozotocin treatment (Smidt et al. 2009). Besides, siRNA-mediated knock-down of ZnT3 and ZnT8 affects β-cell survival (Petersen et al. 2011). By contrast Znt3 over-expression reduces insulin synthesis and secretion in pancreatic β-cells and protect from apoptosis after glucose exposure (Smidt et al. 2016).

Another Zn transporter implicated in the accumulation of Zn in the insulin-containing granules, is ZnT5 (Suzuki et al. 2005). It is expressed preferentially and abundantly in the pancreas and the localization of ZnT-5 in secretory granules and its upregulation in pancreatic β-cells after glucose treatment (Smidt et al. 2009) suggest that ZnT-5 may be involved in supplying Zn for the formation and stabilization of insulin Zn complexes.

ZnT7 is expressed in the Langerhans islets of the mouse pancreas overlapping with insulin and the main localization is in the perinuclear region of the β-cells (Huang and Yan 2010). It has been shown that ZnT7 over-expression in β-islets increases total cellular insulin content and the basal insulin secretion, but not the intracellular Zn levels, differently from β-cells with ZnT8 over-expression, suggesting that the two Zn transporters may play distinct roles in the regulation of insulin secretion (Huang and Yan 2010).

Conversely Znt7 knockdown, in male Znt7 KO mice fed with the high fat diet, leads to severe glucose intolerance and insulin resistance (Huang et al. 2012).

The involvement of so many Zn transporters in insulin activity suggests that Zn homeostasis plays a previously unidentified role in glucose metabolism. This concept would raise a whole new area of research into the pathophysiology of insulin resistance and introduce a new class of drug target with utility for diabetes pharmacotherapy (Myers 2015) and with a potential role in animal and humanlongevity.

4.4.3 Insulin Action on Zn Homeostasis

Apart from the consistent research demonstrating the modulation of insulin action by Zn signaling, there is wide evidence showing that insulin itself acts on Zn homeostasis both regulating extracellular Zn transporters (serum albumin), and intracellular Zn binding proteins (MTs). In fact, insulin can modulate Zn stimulating albumin production in cultured rat hepatocytes (Uchida et al. 1991) as well as restoring the impaired albumin synthesis in diabetic rats (Peavy et al. 1978). Moreover, Albumin expression is significantly decreased in livers after ablation of the insulin receptor or Akt (Chen et al. 2016).

A recent study shows an inverse relationship between serum insulin concentration and MTs mRNA levels in adipose tissues as well as a decrement in MT1 and MT2 mRNA gene expression after insulin exposure in adipocytes suggesting that the effect of nutritional status on MT1 and MT2 is mediated by insulin (Szrok et al. 2016). However, while the role of Zn in insulin activity has been extensively investigated, there is a paucity of data related to how insulin affect Zn homeostasis, in particular regarding the influence of this hormones on the activity of Zn transporters.

4.5 Zinc IGF-1 Interplay

4.5.1 Modulation of IGF-1 Action by Zn Signals

IGF-1 is an anabolichormone produced mainly by the liver and local expressed in peripheral tissues (Sherlock and Toogood 2007; Maggio et al. 2013) in response to the pituitary growth hormone (GH). Correct functioning of the GH–IGF axis is required for successful human development as well as for maintenance of healthy adults (Rosenfeld 2005). IGF-I production and GH secretion pattern gradually decrease with aging (somatopause) (Hoffman et al. 1993), with a consequent reduction of bone and muscle mass and strength, an increased fat mass, dyslipidemia, arterial hypertension, cardiovascular diseases and cognitive decline (De Marinis et al. 2002). Moreover, IGF-1 has been shown as a sensitive marker of nutritional status (Estívariz and Ziegler 1997), especially in the elderly and a statistically significant positive association is found between Zn intake and plasma levels of IGF-1, IGF binding protein (IGFBP-3) and IGF-1 to IGFBP-3 ratio (Giovannucci 2003). Dietary Zn supplementation increases growth and circulating levels of IGF-I (Ninh et al. 1996; Imamoğlu et al. 2005; Rodondi et al. 2009; Hamza et al. 2012). Conversely, dietary Zn deficiency causes growth retardation associated with decreased circulating IGF-I concentrations, inhibition of the anabolic actions of IGF-I and with a decreased expression of the IGF-I and GH receptor genes (Ninh et al. 1995, 1998). The exact mechanism of the effects of Zn deficiency and Zn supplementation on GH secretion, serum IGF-1 levels and growth is not well delineated.

In animal models, force-feeding a Zn-depleted diet to rats for 14 days results in a 28% decrease in serum IGF-1 compared with rats fed a Zn-adequate diet, although there was no difference in caloric food intake (Roth and Kirchgeßner 1994). However, growth retardation caused by dietary Zn deficiency cannot be reversed by IGF-I infusion or by inducing food intake, despite normalization of circulating IGF-I (Browning et al. 1998; Ninh et al. 1998), suggesting a state of GH resistance rather than GH deficiency in case of Zn deficiency (Hamza et al. 2012). Hence, the decrease of serum IGF-I is not the only mechanism responsible for the growth retardation caused by Zn deprivation, but also by inhibition of the anabolic actions of IGF-I. The anabolic effects of IGF-I are enhanced by Zn ions and some sites in the IGF-I action could be Zn-mediated. Thus, in an in vitro model of rat fibroblasts (Rat-1 cells), zin chelation inhibits IGF-1 mitogenic action. This inhibitory effect of Zn availability is associated with a marked decrease of the mitogen-activated protein kinase (MAPK) expression, a crucial factor for the IGF-1 induced mitogenic action in RATt-1 cells (Chesters et al. 1989). Zn affects intracellular signaling cascades associated with IGF-1 either directly or indirectly (MacDonald et al. 1998; MacDonald 2000). Binding of IGF-1 results in autophoshorylation of the receptor protein and activation of its intrinsic tyrosine kinase activity (De Meyts et al. 1994). The first cytosolic proteins to be activated by the IGF-1 receptor tyrosine kinase are insulin response substrates proteins IRS1 and IRS2. The tyrosine phosphorylation of these proteins initiates three distinct signaling pathways: phoshoinositol-3 kinase, mitogen- activated protein kinase and protein kinase C (MacDonald et al. 1998). Phosphoinositol-3 kinase is mainly thought to influence substrate uptake and fuel metabolism in cell, whereas mitogen-activated protein kinase activation results in nuclear association and induction of transcription factors that direct cell proliferation. Failure to activate the phosphorylation cascade within cells in response to IGF-1 might occur in Zn deficiency, which would explain the inhibition of DNA synthesis caused by zin depletion. Furthermore, protein kinase C is a metalloenzyme (Hubbard et al. 1991), and Zn facilitates the binding of protein kinase C to the cytoskeleton (Forbes et al. 1990).

A statistically significant correlation between Zn intake and plasma IGF-1 concentrations in men has been reported in humans (Larsson et al. 2005). Prepubertal male and female children with idiopathic short stature (IDSS) who receive chronic Zn supplementation in their diet have been shown to have increased plasma IGF-1 and IGFBP-3 levels despite an unaltered GH response to clonidine relative to controls (Imamoğlu et al. 2005). Chronic supplementation has also been shown to have no effect on GH levels in women undertaking exercise when analysed over three separate menstrual cycles (Singh et al. 1999). Thus, Zn supplementation may be beneficial in selected categories of subjects with low baseline Zn serum levels. A moderate Zn deficiency is also often observed in elderly subjects (Mocchegiani et al. 2008a). The age-related decline in plasma IGF-I may be exacerbated by Zn deficiency condition (Giovannucci 2003). Low Zn intake was also found to be associated with low IGF-I concentrations in healthy postmenopausal women and the effects of Zn appeared to be specific and independent of protein intake (Devine et al. 1998). Zn supplementation accelerates the serum IGF-I response to essential amino acids-whey protein administration, and is associated with a decrease in the serum levels of a biochemical marker of bone resorption and improved functional (Rodondi et al. 2009). The increase in serum IGF-I levels induced by protein supplements was accelerated, with a significant difference detectable after only one week of Zn addition. This could represent a clinically relevant advantage, since the potential benefits of correcting low IGF-I concentration could be achieved at a very early stage of intervention. A rapid improvement would be particularly desirable in the phase of recovery after surgery or in the rehabilitation phase of undernourished elderly. In patients with T2D who had low levels of IGF-1, Zn supplementation significantly increased IGF-1 concentrations (Jayawardena et al. 2012). The relationship between Zn and IGF-1 in aging population warrants further investigation in order to confirm whether Zn could be identified as a modulator of IGF-1 bioactivity in frail older persons at risk of mobility limitation.

4.5.2 Modulation of Zn Homeostasis by IGF-1

The relationship between Zn and IGF-1 has been widely studied, mostly around the regulation of IGF-1 and IIS pathway in particular nutrient conditions, such as Zn deficiency/supplementation (MacDonald 2000; Alves et al. 2012; Prasad 2013; de Medeiros Rocha et al. 2015). However, other studies explored how Zn homeostasis might be regulated by IGF-1 in condition of down/up- regulation of IIS pathway (Cheruvanky et al. 1982; Aihara et al. 1985).

IGF-1 is a nutrient regulated growth factor that mediates many of the anabolic effects of growth hormone (GH). Although the liver is believed to be the main source of circulating IGF-1, it is produced in most organs and exerts biological effects on various cell types. Among its functions, it stimulates amino-acid transport, protein synthesis, and body growth (Laron 2001). Experiments in vitro have also provided evidence that IGF-1 can protect endothelial and hematopoietic cells from different form of stress by upregulating the activity of glutathione peroxidase (Higashi et al. 2013) and manganese superoxide dismutase (MnSOD) (Floratou et al. 2012). Systemic delivery of recombinant human IGF-1 in mouse models of Type 1 diabetes and multiple sclerosis suppressed the progression of autoimmune responses by stimulating the proliferation of regulatory T (Treg) cells (Bilbao et al. 2014). Both in laboratory animals and humans, GH and IGF-1 levels decrease with age so that the initial studies (approximately three decades ago) around these hormones concluded that their decrease contributes to many aspects of aging including, but not limited to, accumulation of fat mass, cardiovascular dysfunction, as well as the decline in immune function, cellular protein synthesis, and muscle mass (Sonntag et al. 2012). Nevertheless, subsequent studies revealed that GH/IGF-1 deficiency positively influence the lifespan of several organisms, such as flies, worms, mice and rats. However, there is still a strong controversial regarding the role played by GH/IGF-1 in humans. A meta-analysis of 12 studies found a U-shaped association between IGF-1 levels and all-cause mortality in the general population (Burgers et al. 2011).

Nutritional status is one of the key regulators of the circulating and tissue IGF-1. In fact, serum levels of IGF-1 are positively associated with energy intake from lipids, consumption of red meats, fats, and oils while negatively associated with energy intake from carbohydrates (Kaklamani et al. 1999). The relevance of these associations is still not completely clear. In general, there is evidence that IGF-1 levels decrease both during treatment with a putative beneficial effect on health, e.g. short term fasting and energy restriction (Barzilai and Bartke 2009), as well as in the case of malnutrition and frailty (Maggio et al. 2013).

Zn, being one of the major micronutrients in our body (the most abundant after iron), shares with IGF-1 the involvement in several pathways, in particular those related to growth, development as well as stress and immune response. Among all proteins that mediates the biological role of Zn, MTs appears to play a major role in the control of the fluctuations of Zn ions as a consequence of their Zn-buffering and redox-sensor properties mediated by bisulphite bonds (Maret and Vallee 1998). Typically, MTs have a high affinity to both divalent essential metals such as Zn and Cu, and nonessential (or toxic) metals such as Cd and Hg which give rise to metal-thiolate clusters (Kagi and Schaffer 1998). In addition to the metal capture, MTs also release metals in the cytoplasm under stress conditions such as increased nitric oxide or reactive oxygen species, in order to promote stress response and maintain tissue integrity (Lynes et al. 2014). MTs expression occurs mainly through the activity of MTF-1, a conserved transcription factor that senses free Zn (Wang et al. 2010) and activates MRE to initiates the transcription of MTs (Andrews 2000). This autoregulatory loop maintains narrow optimal limits of intracellular free Zn and helps to reduce oxidative stress, as MTs can act by themselves as free radicals scavengers (Thornalley and Vasák 1985). In vitro evidence found the ZnMT complex is the main intracellular Zn protein fraction after extracellular Zn addiction, confirming the strict relationship between MTs production and Zn homeostasis (Malavolta et al. 2007). Several studies have been carried on Zn homeostasis and MTs in the context of aging research (Yang et al. 2006; Giacconi et al. 2007; Bahadorani et al. 2010; Sato et al. 2010). Survival studies performed on mice carrying genetic modification to overexpress MTs genes have shown a longevity phenotype (Yang et al. 2006; Malavolta et al. 2012, 2016) while a shortened lifespan is shown by MT KO mice (Kadota et al. 2015). Interestingly, a multitude of dietary compounds claimed to increased lifespan in mice, flies and worms also induced an increase of MTs (Mocchegiani et al. 1998; Kampkötter et al. 2008; Sun et al. 2010; Saul et al. 2010; Lee et al. 2010). An association study in humans also found a genetic variants in the coding region of MT1A involved in female longevity (Cipriano et al. 2006). MTs also plays a protective role against the effects of high fat/high calorie diets (Beattie et al. 1998; Dong et al. 2007; Sato et al. 2010) and much evidence has shown that MTs expression is induced by CR and IIS inhibition in invertebrate systems and mice (Murphy et al. 2003; Ebadi et al. 2005; Swindell 2007; Leiser and Miller 2010).

Although the system by which MTs are overexpressed in conditions of CR or IIS downregulation has not been fully explained, it is likely that the mechanism involve a mobilization of free Zn, which in turn is the main factor in MTs induction. Hence, during CR or IIS repression Zn ions could be released by intracellular stores (including MTs itself) and sensed by MTF1. The subsequent induction of MTs and other MRE responsive genes involved in the antioxidant response might thus play a role in the longevity phenotype expressed during these experimental conditions. The observed relocation of Zn pool, evinced by the increased erythrocyte Zn pool in children with GH deficiency and their normalization following Zn treatment, is in line with the suggested interplay between GH and Zn (Aihara et al. 1985).

This mechanism could be also mediated by the participation of the FOXO family genes. In fact, it has been proposed that loss of IIS induces a kind of hormetic response that confers both stress resistance and increased lifespan (Gems and Partridge 2008). This response is mediated in worms by genes acting downstream the DAF-16 transcription factor including heat shock proteins (HSPs), catalase and superoxide dismutase (SOD) (Murphy et al. 2003) as well as MTs (Barsyte et al. 2001; Murphy et al. 2003; Barsyte et al. 2001). A similar involvement of MTs in this pathway is well demonstrated in mice. Indeed, a meta-analysis of microarray data revealed that the expression of MT1 is increased in hepatic tissue obtained from multiple long-lived dwarf mouse models, including the Ames, Snell, Little, and GHR-KO dwarf mice (Swindell 2007). Overall, these observations suggest that MTs and the related longevity phenotype may be regulated by the same endocrine mechanisms shown in models of IIS repression.

A specific role of GH/IGF-1 in the regulation of the ZnMT complex has not been well studied, but this possibility is supported by the fact that expression of MT1 and MT2 was found increased from 4 to sevenfold in the hepatic tissue of long-lived GHRKO mice (Swindell 2007; Swindell et al. 2010). Moreover, a normalization of MT1 and MT2 expression was reported in Ames mice treated with GH for a six-week period (Swindell et al. 2010). Interestingly, this effect was observed only in ad lib-fed mice, but not in CR-fed mice confirming the conserved relationship between MTs, GH/IGF-1 axis and nutritional status.

However, several key issues remain unclear. First, it is uncertain whether altered MTs expression in dwarf mice is a direct or indirect consequence of GH/IGF-1 inhibition and if there are other factors that act in synergy with Zn ions to induce MTs.

In this context, it is noteworthy to mention that the absence of GH signals in long-lived dwarf mice lead to increased adiposity, and leptin derived from adipose tissue could induce MTs gene expression (Kondoh et al. 2002). Consistent with this possibility, it has been reported that serum leptin levels are elevated sevenfold in old male Snell dwarf mice (Flurkey et al. 2001). In this scenario, increased MTs in dwarf mice could be the result of an indirect mechanism such as the systemic effects of the GH/IGF-1 downregulation on circulating levels of leptin and other hormones

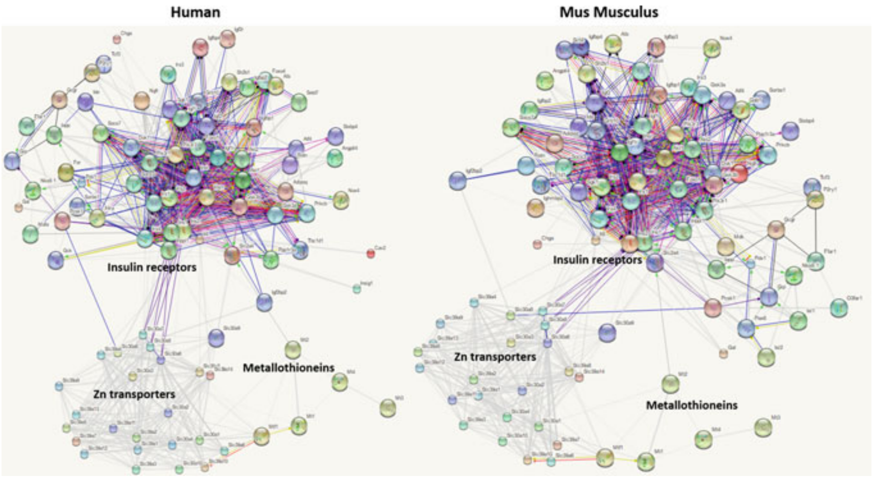

Fig. 4.2 Zinc IIS protein interacting Network. A picture describing the interaction network among insulin/IGF-1 signaling (IIS) pathway, Metallothioneins (MTs) and Zinc transporters in human and mouse proteome. The figure was drawn searching on the STRING database (http://string-db.org/) (Szklarczyk et al. 2015) for protein-protein interaction using the entry terms "Insulin", "Zinc transporters", "Metallothionein" and "Metal Transcription Factor" and including all the suggested genes for each genome (some interactions far from the Zn transporter and MTs network have been removed from the picture for clarity). Full link to the two network are reported in supplementary data

such as glucocorticoid or corticosterone. Unfortunately, these studies did not measure neither plasma Zn nor intracellular Zn.

A particular attention should be additionally deserved to the action of IGF-1 on albumin levels. In fact, a study on the mechanisms involved in the hypoalbuminemia induced by renal disease found that both plasma albumin concentration and its synthesis rate correlated positively with IGF-1, and both were independent of protein catabolic rate and all nutrition-related variables (Kaysen et al. 1995). This observation can be related to the age-related decrease of IGF-1 and the recent finding that plasma Zn can decrease independently of nutritional status during aging (Giacconi et al. 2016).

4.6 Conclusions

In this chapter is described the strict relationship of Zn and its regulatory mechanisms with the IIS pathway. A picture describing the interaction network among IIS pathway, MTs and Zn transporters in human and mouse is described in Fig. 4.2. the figure was drawn searching on the STRING database (Szklarczyk et al. 2015) for protein-protein interaction using the entry terms "Insulin", "Zinc transporters", "Metallothionein" and "Metal Transcription Factor" and including all the suggested genes for each genome (some interactions far from the Zn transporter and Metallothionein network have been removed from the picture for clarity).

The picture emphasizes both in mice and humans a direct interaction among the insulin receptors (INS1 and INS2) with the Zn transporters Slc30a7, Slc30a5 and Slc30a6. A direct interaction with Ins2 is also displayed for Slc30a8 along with its binding activity to Pcsk1 (the proprotein convertase subtilisin/kexin type 1, involved in the processing of hormone and other protein precursors). Slc30a9 appears also to connect several player in the IIS, including Akt1, Slc2a4 (an insulin-regulated facilitative glucose transporter), inositol polyphosphate phosphatase-like 1 (which plays a central role in regulation of PI3 K-dependent insulin signaling). MTs, in particular Mt2 is involved in the activation of Slc2a4. Moreover, several Zn importers (Scl39a4, Slc39a14, Slc39a8) appear to be linked to the IIS through their interaction with interleukin 6 (IL-6).

The relationship between Zn homeostasis and IIS appears thus to be extremely complex and involves direct interactions (e.g. binding) as well as indirect interactions (e.g. mediated by inflammation). Current evidence suggests that Zn is involved both in the promotion of growth and proliferation, that are downstream effects of IIS signaling, as well as on promotion of antioxidant response and cellular defense, which are normally inhibited by the IIS pathway. Although it is quite difficult to draw a conclusion about a definitive role of Zn and its regulatory mechanisms in aging (e.g. MTs), it is possible to attempt an explanation to this Zn/IIS dichotomy.

Our idea is that a functional cell is able to disentangle “nutritional Zn signals”, which activate growth promoting pathways, functional integrity and cell division, from “stress response Zn signals” which, in turn, activate a cascade of signals to regulate transcriptionally and post-transcriptionally a multitude of cellular functions that include oxidative stress responses and the secretion of soluble factors. This is dependent on the length of duration of the Zn signals, on the cell type, on its architecture and on the microenvironment in which the cell resides. Hence the question what is the role of Zn in aging might be reversed changing the subject in how different cells use Zn to modulate aging and lifespan. This concept is in agreement with the increased expression of MTs in longevity models of down-regulated IIS pathway as well as with the longevity phenotype of MTs overexpressing mice. These data can also be translated in human studies with Zn supplementation, as it is likely that Zn supplementation may have differential and even opposite effects in different diseases, age conditions and also in dependence of individual’s genetic variations. The first attempt to take into account these aspects was performed with the Zincage project where Zn supplementation was performed considering plasma Zn levels and IL-6 polymorphism which is thought to indirectly affect Zn homeostasis trough inflammatory pathways (Mocchegiani et al. 2008a).

Another interesting aspect of the interplay between Zn and IIS pathway for translational intervention in humans is the paradoxical decrease of GH and IGF-1 in human aging and models of premature aging. This is part of the defensive response of the organism that attempt to minimize growth and metabolism and to favor cellular defense in the context of the increasing damage associated with aging.

However, this defensive response may become excessive, aggravate aging and induce pathologies in older individuals. Here, Zn supplementation may be useful as co-stimulatory factor of the IIS pathway with the potential to additionally "keep high" the pathways related to defensive mechanisms. By the way, results of supplementation with Zn in elderly subjects suggest that the treatment is safe and may have beneficial effects in some aspects of immunity and functional recovery.

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Supplementary Data

Full link to the STRING NETWORKS described in Fig. 4.2. Copy and paste the URL to see interactive and high resolution picture.

Human:

http://version-10.string-db.org/cgi/network.pl?channel1=on&channel2=on&channel3=on&channel4=on&channel5=on&channel6=on&channel7=on&channel8=off&block_structure_pics_in_bubbles=0&direct_neighbor=1&hide_disconnected_nodes=on&hide_node_labels=0&limit=0&network_display_mode=svg&network_flavor=actions&targetmode=proteins&identifiers=10090.ENSMUSP00000059420%250D10090.ENSMUSP00000000220%250D10090.ENSMUSP00000030170%250D10090.ENSMUSP00000065764%250D10090.ENSMUSP00000114076%250D10090.ENSMUSP00000043401%250D10090.ENSMUSP00000075283%250D10090.ENSMUSP00000073134%250D10090.ENSMUSP00000108982%250D10090.ENSMUSP00000020704%250D10090.ENSMUSP00000025842%250D10090.ENSMUSP00000107388%250D10090.ENSMUSP00000034214%250D10090.ENSMUSP00000043837%250D10090.ENSMUSP00000059129%250D10090.ENSMUSP00000034211%250D10090.ENSMUSP00000027493%250D10090.ENSMUSP00000085763%250D10090.ENSMUSP00000096686%250D10090.

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Chapter 5
Growth Hormone and IGF-1 Axis in Aging and Longevity

Isao Shimokawa

Abstract The growth hormone (GH)-IGF-1 axis, which regulates postnatal growth and metabolism, progressively declines after puberty. This decline causes alterations in body composition and thus physical frailty in aging animals. By contrast, attenuation of the GH-IGF-1 axis consistently increases lifespan in a range of animals. Studies using mutant animals reveal key molecules for longevity in cytoplasmic IGF-1 signaling, including mechanistic target of rapamycin (mTOR) and forkhead box, subgroup O (FoxO) transcription factors. Dietary calorie restriction, a robust experimental intervention to extend lifespan in animals, also inhibits the GH-IGF-1 axis. Studies in human dwarf cohorts report lower incidences of cancers and cardiovascular diseases, though there is no scientific evidence of extended lifespan in these people. Genome-wide studies in long-lived people indicate an association between longevity and minor alleles of genes that lead to a reduction in IGF-1 signaling. Evolutionary views suggest a trade-off relation between growth and longevity. Therefore, it is rational to conclude that the GH-IGF-1 axis is the central pathway that regulates lifespan and thus aging in animals.

Keywords Aging · Lifespan · Growth hormone · IGF-1 · Dietary restriction · Dwarfism · Centenarian

5.1 Introduction

Growth hormone (GH) secreted from the pituitary gland is essential for postnatal growth in mammals (Butler and Le Roith 2001). The anabolic effect of GH is mostly mediated by IGF-1, although GH also exerts direct effects on peripheral tissues. GH receptor (GHR) is expressed not only in the liver but also in many

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peripheral organs. Three-quarters of the circulating IGF-1 is estimated to originate from the liver, with the remainder probably derived from other tissues including adipose tissues. IGF-1 receptor is also expressed in many tissues. GH and IGF-1 mostly work in concert to affect tissue growth and metabolism, but work independently in some physiological conditions.

Synthesis and pulsatile release of GH from the pituitary gland are governed mainly by the opposing actions of hypothalamic GH-releasing hormone (GHRH) and somatostatin (Butler and Le Roith 2001). Secretion is also modulated depending on nutritional conditions, e.g., long-term reduction in calorie intake or protein deficiency inhibits GH release and lowers plasma concentrations of IGF-1.

Twenty-four-hour secretion of GH, which reaches a peak around puberty, has begun to decrease in young adults (Savine and Sonksen 2000; Sattler 2013). In most adults, GH secretion has progressively declined by the age of 60 to levels indistinguishable from those of adult GH deficiency patients with organic lesions in the pituitary gland. GH deficiency with aging causes a decrease in lean muscle mass and an increase in fat mass. These alterations in body composition can cause physical frailty as well as an increased risk of cardiovascular diseases in elderly people. As a result, clinical trials of GH administration have been conducted in elderly people to improve such conditions (Rudman et al. 1990).

By contrast, loss-of- or reduction-of-function mutations of genes involved in GH signaling consistently extend lifespan in a range of vertebrates and invertebrates (Bartke 2008) Dietary calorie restriction (DR), a simple but robust experimental intervention to extend lifespan in animals, inhibits the GH-IGF-1 axis (Shimokawa 2015). Genome-wide human studies also report a potential relationship between longevity and reductions in IGF-1 (van Heemst et al. 2005). This chapter describes the antagonistic pleiotropic effects of GH in the aging process.

5.2 Decline of the GH-IGF-1 Axis and Replacement Therapy in Elderly People

Acquired GH deficiency (GHD) in adults due to structural defects in the pituitary gland causes alterations in body composition such as reduced skeletal muscle and increased total and trunk fat mass (Sattler 2013). Adults with GHD also show worsened cardiovascular disease risk factors including insulin resistance, increments of blood total and LDL cholesterol, and high blood pressure. GH replacement therapy improves some, but not all, of these factors (Sattler 2013). In elderly people, GH levels, and thus circulating IGF-1 concentrations, are as low as those in GHD adults. However, whether the alterations in body composition in elderly people are caused by the reduced GH-IGF-1 axis and thus whether GH replacement can improve the aging-related changes in body composition remained unclear. To address this question, Rudman et al. (1990) conducted clinical trials in which biosynthetic human GH (hGH) was administered for 6 or 12 months in healthy people aged 61–81 years. The doses of hGH were adjusted to elevate plasma IGF-1 levels according to the range shown in young people. The 6-month trial resulted in increases in lean muscle mass and decreases in adipose tissue mass with no significant changes in body weight or bone density. There were also no adverse effects such as edema or hypertension. This study supports the hypothesis that reduced bioavailability of GH results in an altered body composition causing physical frailty in elderly people. In contrast, a subsequent 12-month study involving administration of hGH to a larger number of subjects identified a high frequency of side effects such as carpal tunnel syndrome, gynecomastia, and hyperglycemia (Cohn et al. 1993). A systematic review based on 31 articles describing randomized, controlled trials in 18 unique study populations indicates that GH treatment in elderly overweight (mean BMI 28 kg/m^2) people increased lean body mass and decreased fat mass and total cholesterol without significant changes in body weight (Liu et al. 2007). However, the people treated with GH were significantly more likely to experience soft tissue edema, arthralgias, carpal tunnel syndrome, and gynecomastia. Some subjects also showed impaired fasting glucose and diabetes. The review concludes that GH administration cannot be recommended as an anti-aging therapy in elderly people because it increases the rates of adverse events, even though it induces small beneficial changes in body composition (Liu et al. 2007).

5.3 Loss-of- or Reduction-of Function Mutations of Genes in the GH Signal Transduction Pathway Consistently Extend Lifespan in a Range of Experimental Animals

Despite the aging–related decline of the GH-IGF-1 axis in animals, a large body of evidence indicates an extension of lifespan by inhibition of the GH-IGF-1 axis in mice (Fig. 5.1) and invertebrates.

GH secretion is stimulated by GHRH and inhibited by somatostatin. The *little* mouse strain, in which the GHRH receptor gene is spontaneously mutated, lives longer than wild-type mice (Flurkey et al. 2001). Disruption of the *Ghrh* gene in mice also increases lifespan in both males and females (Sun et al. 2013). *Little* mice and *Ghrh* knockout (KO) mice both show dwarfism and obesity (an increase in fat pad/body weight), and have similar phenotypes.

Mutations of paired-like homeodomain factor 1 (*Prop1*) and POU domain, class 1, transcription factor 1 (*Pou1f1 or Pit-1*) genes cause a deficiency of GH, prolactin (PRL), and thyroid stimulating hormone (TSH), because these factors are required for development of anterior pituitary cells (Li et al. 1990; Sornson et al. 1996). Ames mice with a mutation of the *Prop1* gene and Snell mice with a mutation of the *Pit-1* gene display similar phenotypes including dwarfism and extended lifespan, compared with wild-type littermates (Brown-Borg et al. 1996; Flurkey et al. 2002). Although the potential effects of PRL and/or TSH deficiency on lifespan are not eliminated in these models, most researchers believe GH to be a key factor in the regulation of lifespan and aging, because overexpression of the GH gene in mice

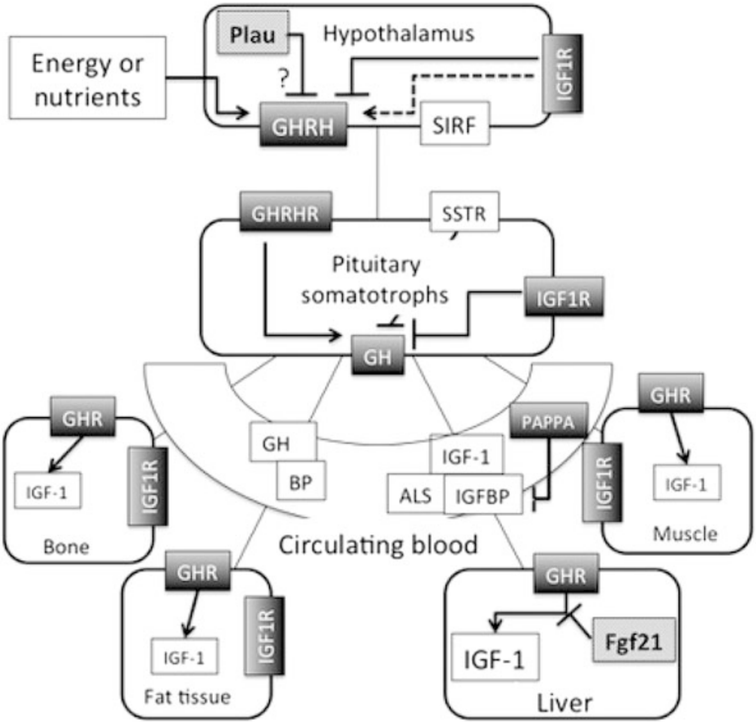

Fig. 5.1 Regulation of the GH-IGF-1 axis. Deletion of genes encoding ligands or receptors (*dark rectangles*) in the axis consistently extends lifespan in mice. Overexpression of genes and thus proteins (*shaded rectangles*) also inhibits components of the GH-IGF-1 axis, leading to prolonged lifespan. IGF-1 signaling in the hypothalamus during the adult phase inhibits GHRH neurons (*solid lines*); during the early postnatal phase, IGF-1 promotes development of the GHRH-GH axis. There are no reports indicating an involvement of somatostatin (SIRF) or its specific receptors (SSTR) in the regulation of lifespan or aging in mammals

produces premature aging phenotypes and shortens lifespan (Steger et al. 1993). A recent study also indicates that twice-daily treatment with bovine GH in Ames mice aged 2–8 weeks reverses their long lifespan phenotype, indicating not only the importance of but also the critical stage of reduction in GH for longevity in mice (Panici et al. 2010). Moreover, there are no reports to indicate extension of lifespan by PRL or TSH deficiency in mice. The one exception to this is a study involving the chemical removal of TSH in rats, which reported an extension of lifespan compared with wild-type control rats (Ooka and Shinkai 1986).

The significance of isolated GH deficiency on lifespan has been confirmed by gene disruption of GH receptor/binding protein (GHR/BP) in mice (Coschigano et al. 2000). Male and female *Ghr*$^{-/-}$ mice, in which plasma IGF-1 and IGFBP-3 are very low, outlived wild-type control mice.

Disruption of the IGF-1 receptor gene (*Igf1r*) is lethal at birth. Heterozygous mutants, *Igf1r*$^{+/-}$, show a modest reduction in body weight (8% in males; 6% in females) compared with wild-type mice after weaning (Holzenberger et al. 2003). Lifespan is extended in female *Igf1r*$^{+/-}$ mice but not male *Igf1r*$^{+/-}$ mice. Female, but not male, *Igf1r*$^{+/-}$ mice also display resistance to oxidative stress induced by intraperitoneal injection of paraquat, compared with wild-type mice. A reduction in cytoplasmic IGF-1 signaling is demonstrated by a comparison of MEF cells derived from *Igf1r*$^{+/-}$ mice and those from wild-type mice. The original study was conducted on mice with the genetic background of 129/SvPas. In a subsequent study using C57BL/6J mice, Xu et al. (2014) confirmed the lifespan extension in female *Igf1r*$^{+/-}$ mice only; however, the life-prolonging effect was smaller in C57BL/6J females than in 129/SvPas females (11% increase in C57BL/6J *Igf1r*$^{+/-}$ mice versus 33% increase in 129/SvPas mice). This difference between mouse strains in the extent of lifespan extension by *Igf1r*$^{+/-}$ mutation could be due to differences in plasma IGF-1 levelsand activation of IGF-1 signaling between the two strains. Plasma levels of IGF-1 were lower in C57BL/6J mice than in 129/SvPas mice (Xu et al. 2014). Similarly, indices of activation of IGF-1 signaling in tissues such as phosphorylated IGF-1R and IRS-1 co-immunoprecipitated with IGF-1R were lower in C57BL/6J mice than in 129 Sv/Pas mice. Even in control wild-type mice, the strain with lower plasma IGF-1 and thus less activated IGF-1 signaling lived longer than the strain with higher IGF-1 levels. Therefore, the life-prolonging effect of the *Igf1r*$^{+/-}$ mutation seems to be smaller in C57BL/6J mice. Xu et al. (2014) also reported that oxidative stress hyper-activates IGF-1 signaling in tissues. They speculated that hyper-activation of IGF-1 signaling injures tissues; however, *Igf1r*$^{+/-}$ mutation limits damage and promotes survival by blocking an acute overreaction of IGF-1. This is a possible mechanism underlying the life-extending effect of reduced IGF-1 signaling.

Heterozygous mice for brain-specific deletion of *Igf1r* (*bIgf1r)* also show modest dwarfism. The body weight of *bIgf1r*$^{+/-}$ mice at 90 days of age is approximately 90% of the control mice in both males and females (Kappeler et al. 2008). In these *bIgf1r*$^{+/-}$ mice, development of hypothalamic GHRH neurons and pituitary GH secreting cells is impaired. Subsequently, the total GH content in the pituitary gland remains low throughout development. Plasma IGF-1 does not show any pubertal increase whereas control mice display the normal surge. The survival rates of *bIgf1r*$^{+/-}$ mice are greater in the first half of life in both males and females compared with control mice; however, there is no such increase in survival (lifespan) in the last quarter of life (Kappeler et al. 2008). Thus, the life-extending effect of a brain-specific reduction in IGF-1R could be minor.

Three-quarters of circulating IGF-1 derives from the liver. Svensson et al. (2011) tested the effect of liver-specific IGF-1 inactivation (*LI-Igf1*$^{-/-}$) on lifespan. Using the LoxP and Mx-Cre system, the *Igf1* gene was inactivated at 1 month of age in C57BL/6 mice. Serum IGF-1 is reduced by 20% in *LI-Igf1*$^{-/-}$ mice compared with wild-type mice. *LI-Igf1*$^{-/-}$ male and female mice display reduced body weight mostly due to a decrease in body fat, but not lean mass. The mutant mice show a compensatory increase in GH levels, which are 3.1-fold greater in *LI-Igf1*$^{-/-}$ mice than in wild-type mice. This compensatory change in GH levels may cause the alterations in body composition in *LI-Igf1*$^{-/-}$ mice. Energy expenditure is modestly increased in *LI-Igf1*$^{-/-}$ mice. Finally, the mean lifespan is 10% greater in female mice but not male mice (Svensson et al. 2011).

The mechanism underlying sexually dimorphic responses in lifespan and oxidative stress to reduced IGF-1 signaling remains to be elucidated. In addition, if GH levels are increased in *Igf1r*$^{+/-}$ mice and *LI-Igf1*$^{-/-}$ mice from a negative feedback mechanism through the pituitary gland, this might have some adverse effects, particularly on male mice.

Circulating IGF-1 forms a ternary complex with one of the IGF binding proteins (IGFBPs) and acid-labile subunit (ALS) (Butler and Le Roith 2001). Of the six different IGFBPs, IGFBP3, produced by the liver under the regulation of GH, is the major carrier for IGF-1 in the blood. The remaining IGFBPs are expressed in a tissue-specific manner and regulate bioactivity in specific tissues and cells. Pregnancy-associated plasma protein A (PAPPA) is a metzincin superfamily metalloproteinase in the IGF system (Boldt and Conover 2007). PAPPA cleaves IGFBP4, leading to an increase in IGF-1 bioavailability and mitogenic effectiveness in vitro. *Pappa*-KO mice show dwarfism due to an increase in IGFBP4 expression that reduces the bioavailability of IGF-1 (Conover and Bale 2007). Plasma concentrations of GH and IGF-1 tend to show a 70% reduction in *Pappa*-KO mice compared with those of wild-type mice, though this is statistically insignificant. Male and female *Pappa*-KO mice also live longer than wild-type control mice (Conover et al. 2010).

In long-lived mouse models, in which circulating levels, bioavailability, or cytoplasmic signal transduction of IGF-1 is reduced, the extent of lifespan extension varies depending on the timing of gene disruption, gene dose, sex, and genetic background of mice.

In rats, the effect of inhibition of the GH-IGF-1 axis on lifespan and aging remains controversial. A modest suppression of the GH-IGF-1 axis by overexpression of an antisense of the *Gh* gene in Wistar rats results in dwarfism in adolescent rats heterozygous for the transgene (*tg*/−) versus wild-type rats (−/−) (Shimokawa et al. 2002). Compared with wild-type rats, dwarf (*tg*/−) rats show a 30% decrease in mean food intake and body weight with a 40% reduction in plasma IGF-1 (Shimokawa et al 2002). By contrast, dwarf rats originally derived from the Lewis strain do not live longer than control rats (Sonntag et al. 2005). However, GH administration after weaning for 10 weeks in dwarf rats from the Lewis strain extends lifespan compared with those without GH treatment and control normal-size rats (Sonntag et al. 2005). This rat model, in which GH is administered at a young age, is considered to represent adult onset GH deficiency, because the plasma IGF-1 concentration returns to the same level as that in control dwarf rats at 2 weeks after termination of GH replacement. This work indicates the importance of GH during adolescence, a contradictory finding to that from dwarf mice (Panici et al. 2010).

Spontaneous dwarf rats derived from the Sprague-Dawley (SD) strain are reported to live longer than control SD rats (Kuramoto et al. 2010). Unfortunately, this study does not contain a longevity group of control SD rats. Instead, the lifespan data from these dwarf rats are compared with multiple data sets from SD rats published from the other institutes.

In summary, the role of GH and IGF-1 in lifespan extension remains elusive in rats.

5.4 Lifespan Extension by Single Gene Mutations Related to the Reduction of the GH-IGF-1 Axis

Since the report on lifespan extension in Ames dwarf mice in 1996, a number of single genes have been reported to extend lifespan in mice if they are spontaneously mutated or genetically engineered (Shimokawa et al. 2008). Many of the genes are clustered in GH-IGF-1 signaling, as described above. Some of the gene mutations also seem to affect GH-IGF-1 signaling secondarily.

αMUPA transgenic mice, overexpressing the urokinase-type plasminogen activator gene (*Plau*, also known as *uPA*) in the brain, live longer than wild-type controls (Miskin and Masos 1997). Plau is a serine protease that activates plasminogen by proteolytic cleavage into plasmin. Transgenic *Plau* is also expressed in the hypothalamic paraventricular nuclei, which are involved in regulation of appetite and energy expenditure. αMUPA mice display reduced food intake and body weight, and plasma IGF-1 is reduced by 30% compared with wild-type mice. In αMUPA mice, body temperature is reduced and the incidence of spontaneously occurring tumors and carcinogen-induced neoplastic lesions is decreased. These phenotypes resemble those of DR mice and some phenotypes are also displayed in GH-IGF-1-reducing mice (Miskin et al. 2005). Therefore, brain-specific, particularly hypothalamic, overexpression of Plau may affect GHRH neurons and their downstream signaling.

Overexpression of the *Fgf21* gene in mice results in an extension of lifespan (Zhang et al. 2012). Fgf21, a hormone secreted by the liver, increases during periods of fasting, and sensitizes insulin actions (Potthoff et al. 2012). Fgf21 blocks somatic growth by induction of GH resistance. *Fgf21*-overexpressing mice show a decrease in circulating IGF-1 despite elevated GH levels, as compared with wild-type mice. Correspondingly, *Fgf21*-overexpressing mice are smaller than wild-type mice (Zhang et al. 2012). These hormonal alterations in *Fgf21* transgenic mice are similar to those of *GHR/BP*-KO mice (Coschigano et al. 2000). Gene expression analysis of *Fgf21* transgenic and DR mice has confirmed an overlap of genes significantly regulated in the liver, suggesting that Fgf21 signaling is a part of DR (Zhang et al. 2012).

Sirtuin 6 (Sirt6) acts in the DNA double-strand break repair system via deacetylation of CtBP-interacting protein (CtIp) and ADP ribosylation of poly ADP-ribose polymerase 1 (PARP1) (Gertler and Cohen 2013). Deletion of the *Sirt6* gene in cells induces genomic instability (Mostoslavsky et al. 2006). *Sirt6*-KO mice exhibit premature aging phenotypes similar to those of *XPA/CA* or *XPA/TTD* null mice (Mostoslavsky et al. 2006). Conversely, overexpression of *Sirt6* extends the lifespan of male mice but not female mice (Kanfi et al. 2012).

In *Sirt6*-overexpressing mice, plasma IGF-1levels are lower than those in wild-type mice. In addition, IGFBP1 is increased in *Sirt6*-KO mice. Decreased levels of phosphorylated IGF-1R and Akt indicate attenuation of IGF-1 signaling in tissues. Molecular mechanisms underlying the inhibition of IGF-1 by *Sirt6* overexpression remain elusive.

Three long-lived mouse models also support the significance of inhibition of the GH-IGF-1 axis for longevity.

5.5 Genes Downstream of IGF-1 Signaling that Lead to Lifespan Extension

IGF-1 binds to specific tyrosine kinase receptors and activates downstream signaling molecules such as insulin receptor substrate (IRS) proteins, PI3K, and then Akt (O'Neill 2013). Akt phosphorylates multiple substrates such as mechanistic target of rapamycin (mTOR, activated), glycogen synthesis kinase 3 beta (GSK3β, inactivated), and FoxO transcription factors (inactivated). A number of genes encoding these cytoplasmic molecules are also reported to regulate lifespan in mice.

Whole body or brain-specific deletion of the *Irs2* gene is reported to extend lifespan in mice (Taguchi et al. 2007). Brain-specific *Irs2*-KO mice, either homozygous or heterozygous, show hyperinsulinemia and mild glucose intolerance. However, the reduced IRS2 prevents aging-related decreases in FoxO1 and SOD2. Taguchi et al. (2007) speculated that attenuation of IRS2 signaling in the brain shielded against the harmful effects of hyperinsulinemia. However, controversial findings are reported by another group in *Irs2*$^{-/-}$ mice (Selman et al. 2008). In their experiment, *Irs2*$^{-/-}$ mice exhibited diabetic phenotypes and died earlier than wild-type mice. By contrast, it is reported that deletion of the *Irs1* gene extends lifespan in female mice.

Akt1$^{+/-}$ mice are also reported to outlive wild-type mice with 8% increase of lifespan in males and 14% increase in females (Nojima et al. 2013). In *Akt1*$^{+/-}$ mice, the mTOR pathway, which regulates ribosomal biogenesis, protein synthesis, and mitochondrial activity, is attenuated. Glucose tolerance and insulin sensitivity are comparable between *Akt1*$^{+/-}$ and wild-type mice, although *Akt1*$^{+/-}$ mice display lower body weight and decreased body fat content compared with wild-type mice. The total protein and phosphorylated form of FoxO3 do not differ between *Akt1*$^{+/-}$ and wild-type mice (Nojima et al. 2013).

The mTOR pathway exerts main effector proteins such as eukaryotic translation initiation factor 4E (eIF-4E)-binding protein 1 (4EBP1) and 70-kDa ribosomal S6 kinase (S6K) (O'Neill 2013). When activated, the mTOR pathway phosphorylates 4EBP1 and S6K and promotes protein translation and synthesis. Deletion of the S6K peptide 1 (*Rps6kb1*) in mice leads to extension of lifespan, though the effect is significant in females but not in males (Selman et al. 2009). *Rps6kb*$^{-/-}$ mice show a dwarf phenotype without reductions in plasma IGF-1 or pituitary GH. Motor functions, bone volume, and glucose tolerance are reported to be improved in *Rps6kb*$^{-/-}$ mice. The genetic model is a counterpart of a non-genetic, rapamycin (an mTOR kinase inhibitor) model for longevity (Harrison et al. 2009).

FoxO transcription factors are mammalian orthologs of DAF-16 in *C. elegans* (Greer and Brunet 2005). DAF-16 is needed for extension of lifespan following reductions in DAF-2 (mammalian IGF-1 receptor) and AGE-1 (a subunit of PI3K) signaling (Kenyon et al. 1993); that is, insulin-like signaling in *C. elegans* inhibits DAF-16 in nourished conditions. Target genes of FoxOs regulate cell cycle, DNA repair, stress resistance, apoptosis, autophagy, and metabolism in response to cellular and genotoxic stress (Greer and Brunet 2005). Negative energy balance is also a stimulator of FoxOs. In mice, under standard ad libitum feeding conditions, single deletion of the *Foxo1*, *Foxo3*, or *Foxo4* gene resulted in minor alterations in the incidence of neoplasms and in lifespan. Only triple knockout of the *Foxo1*, *Foxo3*, and *Foxo4* genes caused the cancer-prone phenotype and shortened lifespan, suggesting functional redundancy of FoxOs in the regulation of cancer and lifespan (Paik et al. 2007). However, under 30% DR conditions, in which wild-type mice live longer and are protected against neoplastic processes, FoxO1 and FoxO3 play differential roles in the life-extending and antineoplastic effects. In *Foxo1*$^{+/-}$ mice, the antineoplastic effect of DR is diminished, though lifespan is extended to the same extent as that of wild-type mice (Yamaza et al. 2010). In contrast, *Foxo3*$^{+/-}$ mice show a significant reduction in cancer incidence by DR in a lifespan study but little extension of lifespan (Shimokawa et al. 2015). These studies clearly demonstrate distinct roles for Foxo1 and Foxo3 in mammals. Isoform-specific functions of DAF-16 are also reported in *C. elegans*. A meta-analysis of direct FoxO targets across tissues and organisms using data of mammals, *C. elegans*, and Drosophila revealed evolutionarily conserved targets, enriched in growth factor signaling, metabolism, stress resistance and proteostasis (Webb et al. 2016). This study also identified candidate cofactors at conserved FoxO targets, e.g., CREB and ETS family factors.

A series of lifespan studies using mutant mice indicate a central role of the GH-IGF-1 axis and subsequent cytoplasmic signaling that inhibits mTOR and activates FoxO3 for longevity in mammals. DR inhibits GH secretion and lowers plasma concentrations of IGF-1. Therefore, it is probable that DR exerts its effects in part via the GH-IGF-1 axis and subsequent cytoplasmic signaling.

5.6 Evidence in Humans Indicating a Relation Between Longevity and Inhibition of the GH-IGF-1 Axis

Mouse and invertebrate models consistently indicate that reduction or deficiency of GH-IGF-1 signaling leads to longer lifespans compared with those of wild-type controls. This raises the question of whether similar mutations result in the extension of overall and healthy lifespans in humans.

A 22-year study of an Ecuadorian cohort with *GHR* gene mutations and IGF-1 deficiency, i.e., Laron syndrome, revealed that subjects showed a low incidence of cancer and diabetes compared with control relatives (Guevara-Aguirre et al. 2011). Subjects with Laron syndrome were obese, with lower serum insulin and increased insulin sensitivity. Subjects in the cohort displayed high mortality from common diseases of childhood. There was no evidence of extended lifespan in these subjects, although a small fraction (10%) of the cohort lived longer than 80 years (Guevara-Aguirre et al. 2011). A study of an Israeli cohort of Laron syndrome also indicates that these subjects have a lower incidence of cancers, despite the high prevalence of obesity (Shevah and Laron 2007).

Subjects in an Itabaianinha cohort with dwarfism from a mutation of the gene encoding the GHRH receptor also show a reduction in lean mass and an increase in percentage of fat mass (Oliveira et al. 2010). Some of the risk factors for cardiovascular diseases are also increased. However, these people do not display insulin resistance or evidence of premature atherosclerosis. A study from this cohort reports that serum adiponectin is increased without any changes in serum leptin (Oliveira et al. 2010). Insulin levels are also lower compared with control subjects. These hormonal changes may delay progression of atherosclerosis.

Congenital deficiency of the GH-IGF-1 axis in humans may induce protective effects against cancers, insulin resistance and atherosclerosis. Some studies describe a greater fraction of the extant population in older ages; however, there is no scientific evidence for extension of lifespan.

In a cohort of Ashkenazi Jewish centenarians, their female offspring show a smaller stature with high serum IGF-1 concentrations, when compared with offspring-matched controls (Suh et al. 2008). Male offspring of centenarians do not show these traits. Sequence analysis of the *IGF-1* and *IGF-1R* genes of female centenarians with a short stature revealed a number of variants of the *IGF-1R* gene but not the *IGF-1* gene. Immortalized lymphocytes established from the female centenarians carrying mutations of *IGF1R* show significant reductions in IGF1R levels compared with lymphocytes from female centenarians without gene mutations. IGF-1 signaling is also attenuated in lymphocytes from the *IGF1R* mutation carriers of female centenarians, compared with those from female centenarians with no mutations. The study found overrepresentation of those mutations in the *IGF1R* gene among female centenarians relative to controls. These findings suggest correlation between longevity and reduced IGF-1 signaling in females. These traits (reduction-of-function mutations of the *IGF-1R* gene, high concentrations of serum IGF-1—a compensatory increase in IGF-1, and extended lifespan) in female centenarians are similar to those in *Igf1r*$^{+/-}$ female mice (Holzenberger et al. 2003).

Human genetic studies have suggested an association between polymorphisms in IGF-1 signaling genes and longevity (Willcox et al. 2008; Ziv and Hu 2011; Di Bona et al. 2014). In an Italian cohort (Bonafe et al. 2003), long-lived people (aged 86–109 years) had lower BMI, fasting glucose, plasma insulin, insulin resistance (HOMA) and plasma free IGF-1 levels than young people (aged 17–85 years). The study indicates that *IGF1R* and *PIK3CB* (phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit beta) polymorphisms affect IGF-1 plasma levels. Carriers of the A allele at the *IGF1R* locus show lower plasma free IGF-1 compared with the GG genotype. Carriers of the T allele at the *PIK3CB* locus also display lower plasma IGF-1 than the CC genotype. Finally, carriers of the A allele of *IGF1R* were overrepresented among long-lived people compared with young people. These polymorphisms in the *IGF1R* gene contrast with those in the *IGF1R* gene mutations reported in the cohort of Ashkenazi Jewish centenarians regarding the levels of plasma IGF-1 (Suh et al. 2008). However, both studies conclude that the attenuation in IGF-1 signaling could result in longer life in humans.

In a genetic analysis of the population-based Leiden 85-plus study (subjects were 85 years old and over), variant allele carriers of the *GH1* single nucleotide polymorphism (SNP) were 2 cm shorter in body height and showed reduced mortality, compared with wild-type allele carriers (van Heemst et al. 2005). Other selected polymorphisms that lead to reduced insulin/IGF-1 activity such as the IGF-1 CA repeat also showed a trend of lower mortality but did not reach statistical significance. These findings were noted only in females.

A genome-wide meta-analysis of up to 30,844 adults of European ancestry from 21 studies confirms that the known longevity-associated *FOXO3* variant rs2153960 is a genome-wide significant SNP for lowering IGF-1 concentrations (Teumer et al. 2016). The study also reports the IGF-1 decreasing allele of SNP rs934073, an eQTL of *ASXL2*, to be associated with higher adiposity and higher likelihood of survival beyond 90 years.

A study on regulation of GH secretion in offspring of long-lived families indicates that 24-h total GH secretion is lower and that the secretion is more tightly feedback- or feedforward-controlled in these offspring compared with control subjects (van der Spoel et al. 2016). However, no significant differences were observed in circulating levels of IGF-1 and IGFBP3 between offspring and controls.

In conclusion, human genome studies have identified alleles for reduced GH and/or IGF-1 signaling, some of which are overrepresented in long-lived people. These findings suggest common mechanisms for regulation of aging and longevity among animals including humans.

5.7 Evolutionary Perspective on Regulation of Aging and Lifespan

Within a species, individuals with lower plasma concentrations of IGF-1 are smaller in body size but live longer than those with relatively higher levels of IGF-1 and thus bigger body size. The correlation between body size and plasma IGF-1 concentration is significant in female mice but not male mice (Miller et al. 2002).

A study on genetically-diverse inbred mouse strains at the Jackson Aging Center showed a negative correlation between median lifespan and plasma IGF-1 levels, particularly in long-lived strains (Yuan et al. 2009). A subsequent study revealed that in female mice, IGF-1 levels at 7 weeks of age significantly correlated with the age of vaginal patency, i.e., sexual maturation (Yuan et al. 2015) and IGF-1 levels at 52 and 76 weeks of age negatively correlated with longevity. Thus, female mice with lower IGF-1 levels delay sexual maturation but outlive mice with higher IGF-1 levels. Using the QTL method and combining human GWAS results, proprotein convertase subtilisin/kexin type 2 (Pcsk2) was found to regulate female sexual maturation and IGF-1, and thus *Pcsk2* may play a role in regulating aging and longevity in mammals (Yuan et al. 2015).

A comparative study of 36 mammalian species demonstrates a negative correlation between plasma IGF-1 and body mass, i.e., larger mammals have lower IGF-1 concentrations. Although there is no significant correlation between plasma IGF-1 levels and maximum lifespans among these species, it is tempting to speculate on the presence of a trade-off between growth rate and longevity. Larger animals with lower IGF-1 levels grow more slowly but live longer than animals with higher IGF-1 levels, who instead grow quickly to sexual maturation and have smaller body size. This trend is particularly notable in females.

5.8 Conclusion

The evolutionary view of aging predicts the presence of pleiotropic genes that regulate growth or sexual maturation and lifespan. Multiple genes or genetic loci are involved in the regulation of circulating GH and IGF-1 levels, and thus lifespan or aging is under complex genetic control. In considering extension of healthy lifespan in humans, we should avoid environments, including eating habits, which elevate circulating IGF-1. Experimental studies have identified key molecules, mTOR and FoxO3, downstream of IGF-1 signaling. The prospect of optimizing mTOR and FoxO3 activities in humans to not only increase lifespan but also reduce age-related disorders represents a fascinating avenue of clinical investigation.

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Chapter 6
Thyroid Function in Healthy Ageing and Longevity

Naveen Aggarwal and Salman Razvi

Abstract Thyroid hormones influence all major organs/systems and adequate levels are important for optimal function. Thyroid disease is common and its prevalence affects up to 1 in 7 older individuals. Reference ranges that determine ‘normal’ thyroid function has been predominantly obtained from younger populations. But, recent data from observational studies suggest that serum thyrotropin levels increase in older people and this change does not seem to be associated with adverse outcomes. In addition, thyroid hormone requirements change with age, particularly in older people. Moreover, older patients on replacement therapy are more susceptible to the effects of thyroid hormone excess. Therefore, careful consideration is required in the interpretation of thyroid function test results as well as in managing thyroid disease in the older population.

Keywords Endocrine system · Thyroid · Population · Gender · Race · TSH · Elderly · Cognition · Depression · Levothyroxine

6.1 Introduction

The population of the world is ageing, more so in high income countries at present. The pace of population ageing in many developing countries today is substantially faster than occurred in developed countries in the past. Between 2015 and 2030, the number of people in the world aged 60 years or over is projected to grow by 56%,

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from 901 million to 1.4 billion, and by 2050, the global population of older persons is projected to more than double its size in 2015, reaching nearly 2.1 billion. Globally, the number of people aged 80 years or over, the "oldest-old" persons, is growing even faster than the number of older persons overall. Projections indicate that in 2050 the oldest-old will number 434 million, having more than tripled in number since 2015, when there were 125 million people over age 80 (United Nations 2015).

With ageing, changes occur in all body systems including the endocrine system. These changes may be due to the change in amount of hormones secreted or change in sensitivity of target organs, or both. For example, gonadotropin secretion increases after menopause. In addition, there may also be some change in the rate of metabolism of other hormones (e.g. increased peripheral degradation of thyroid hormones) (Mariotti et al. 1995).

The interest in thyroid function in the elderly has been increasing with the recognition that thyroid status is associated with disability, cognitive function, cardiovascular disease risk and longevity. The effects of overt thyroid dysfunction are well documented in all age groups. However, the effects of subclinical thyroid disease in an older population are still unclear, mainly due to lack of randomised control trials (RCTs). Much of the research in the past has focused on younger individuals with subclinical hypothyroidism (SCH).

6.2 Defining 'Normal' Thyroid Function in Elderly

The definition of what constitutes 'normal' thyroid function in the elderly is unclear. It has been known for a long time that serum thyrotropin (TSH), T4 and T3 levels change with ageing (Woeber 1985; Hesch et al. 1976, 1977; Herrmann et al. 1974; Pawlikowski et al. 1975; Westgren et al. 1976; Rubinstein et al. 1973; Jeske and Thorner 1977; Hansen et al. 1975; Lipson et al. 1979; Canaris et al. 2000) (Fig. 6.1). The first Whickham survey showed that TSH levels increased markedly in females after the age of 45 years, while no similar change was observed in males. Interestingly, when individuals with thyroid antibodies were excluded from the sample, this rise in TSH with age in females was virtually abolished (Tunbridge et al. 1977). However, the number of individuals aged 75 or more were quite small in this seminal study, thus limiting the ability to detect a significant increase in TSH in this older age-group. The 20-year follow-up Whickham survey showed that with increasing age, the incidence of positive anti-thyroid antibodies and hypothyroidism also increased (Vanderpump et al. 1995). Due to advancement in assay techniques (the follow-up Whickham survey used more sensitive assays) longitudinal change in serum TSH and thyroid hormones couldn't be compared in a meaningful manner. The larger and more recent United States NHANES III survey showed that TSH concentrations and the prevalence of anti-thyroid antibodies are higher in women, increase with age, and are greater in whites and Mexican Americans than in blacks. The rise in serum TSH, serum thyroid peroxidase (TPOAb) and thyroglobulin

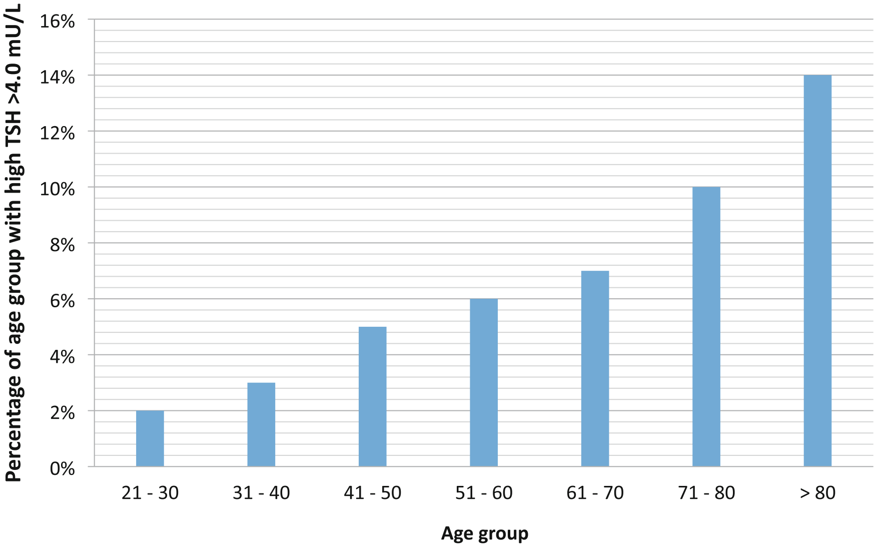

Fig. 6.1 Percentage of adults in population based studies with high serum TSH levels (>4.0 mU/L) by age. Adapted from Colorado Thyroid Prevalence study (Canaris et al. 2000) and NHANES III (Hollowell et al. 2002)

antibodies rise was seen with age in both men and women (Hollowell et al. 2002). In a subsequent further analysis of a carefully selected reference NHANES III sub-population after exclusion of individuals with other factors that could impact on thyroid function (such as pregnancy, positive anti-thyroid antibodies and certain drugs), it was demonstrated that a progressive increase in the median as well as the 97.5 centile for TSH concentration occurs with age. This analysis suggested that the 97.5 centile is 3.6 mU/L in 20–39 year old individuals, and 5.9 and 7.5 mU/L in those who are 70–79 and 80 years old and older, respectively (Surks and Hollowell 2007). They also demonstrated that about 70% of older patients who would be classified as having SCH (TSH greater than 4.5 mU/L with normal thyroid hormone levels) were within their age-specific reference range. Another analysis of the NHANES dataset to evaluate factors influencing the TSH reference range concluded that for each age group of each ethnicity studied, the inclusion of antibody-positive subjects increased TSH medians and upper limits (97.5 centiles) (Spencer et al. 2007). Subsequently, it has been suggested that age-based reference ranges for TSH should be considered (Surks and Boucai 2010; Aggarwal and Razvi 2013).

The Busselton survey was a longitudinal study conducted over a period of 13 years in Western Australia. It showed that serum TSH increased (mean increase of 0.32 mU/L over 13 years) with no significant change in thyroxine (T4) concentrations in this time period. The rise in TSH was most marked in the elderly population (Bremner et al. 2012). Similarly, another longitudinal thyroid function evaluation in a very elderly subgroup (mean age 85 years) of the Cardiovascular

Health All Stars Study found that serum TSH increased by 13% over 13 years of follow-up and was associated with a 1.7% increase in free thyroxine (FT4) and a 13% reduction in total T3 levels. Higher FT4 levels, but not TSH, were associated with mortality (Waring et al. 2012a).

The relationship between thyroid hormone and the pituitary hormone TSH is fundamental to our understanding of thyroid pathophysiology and laboratory diagnosis of thyroid diseases. For a long time it had been thought that the best fit to describe the TSH–FT4 relationship was log-linear (Fish et al. 1987). So, for a 2-fold change in serum FT4 levels there is a 100-fold inverse change in serum TSH levels (Fatourechi 2009). However, more recent cross-sectional studies have challenged this view and suggest that the log-linear relationship between TSH and T4 is incorrect, or at best an over-simplification. One analysis of two laboratory data sets including 3223 and 6605 individuals, reported that the log TSH–FT4 relationship was better described by a nonlinear model based on the error function than by a linear relationship (Hoermann et al. 2010). Another study of 5117 individuals older than 65 years reported a nonlinear fourth-order polynomial relationship between log TSH and FT4 (Clark et al. 2012). A third study analysing a large laboratory database of 152,261 individuals found the log TSH–FT4 relationship to be best explained by two conterminal negative sigmoid curves (Hadlow et al. 2013). Interestingly, recent data suggest that the complex relationship between log TSH and FT4 is not only non-linear but also changes with age and differs by gender (Brown et al. 2016). According to this analysis, TSH is higher in men and in older people, whereas the TSH response to low FT4 is more robust in younger people.

The other factor to consider is iodine status. All the above cross-sectional and longitudinal studies (Whickham study, NHANES III, Busselton and CVHS All Stars Surveys) were conducted in iodine sufficient areas. In contrast to these findings, a cross-sectional study performed in an area of borderline sufficient iodine intake showed that serum TSH concentrations decreased gradually with age throughout life whereas FT4 levels increased only in participants older than 60 years (Hoogendoorn et al. 2006). However, iodine status itself was not measured in this study, although, the authors hypothesized that this decline in TSH could be the result of development of thyroid autonomy after longstanding iodine insufficiency. An earlier study performed in a previously iodine deficient area in Germany had shown a lower reference range (0.25–2.12 mU/L) for serum TSH in people without thyroid disease (Volzke et al. 2005).

The notion of normal thyroid function is further challenged by some studies which show that SCH and subclinical hyperthyroidism may correct spontaneously over time. A study from Birmingham, UK, showed that over 1 year follow-up, TSH returned to normal spontaneously in 5% of people aged 60 years or more with SCH and in 76% of patients with low but detectable TSH (Parle et al. 1991). In another similar and larger study in adults across all age groups, over a 5 year period, TSH normalized without any intervention in more than 50% of patients with elevated or decreased serum TSH level (Meyerovitch et al. 2007).

Selecting a normal range of TSH for a population has implications for the diagnosis of subclinical thyroid diseases (both hypothyroidism and

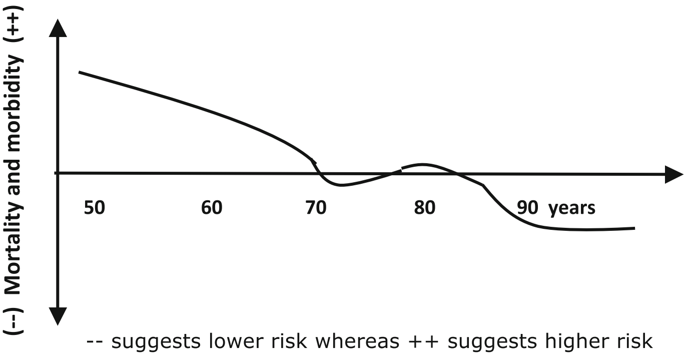

Fig. 6.2 Hypothetical graph showing risk of morbidity and mortality association with mildly high TSH levels at various ages

hyperthyroidism) as well as related issues such as screening, association with co-morbidities especially cardiovascular risks, and treatment. Based on the currently available evidence associating morbidity and mortality of a mildly raised serum TSH across various age groups, it is reasonable to consider treatment in younger individuals with SCH (<70 years) and to monitor those that are older for progression of disease (Fig. 6.2). Recent guidelines from the European Thyroid Association on management of SCH (Pearce et al. 2013) and subclinical hyperthyroidism (Biondi et al. 2015) suggest a different management strategy depending on the age of the patient. Thus, current data support the view that serum TSH increases slightly with age but the data on FT4 is conflicting. Prospective large studies are required to confirm whether age-specific reference ranges should be utilized when reporting thyroid parameters.

6.3 Thyroid Function Association with Outcomes in Older Individuals

Interest in long-term outcomes of thyroid diseases is of clinical importance due to the high prevalence of the condition: 0.2–2.0% for overt disease and between 4 and 20% for subclinical diseases (Biondi and Cooper 2008). The clinical relevance and implications of mild thyroid abnormalities have been debated for decades.

6.3.1 Thyroid Status and Cardiovascular Risks

The association between hypothyroidism and atherosclerosis is well recognized. A case-control *post mortem* study conducted by Belgian researchers in 1967 that included 25 autopsies from inadequately-treated hypothyroid patients and age- and sex-matched control autopsies. This seminal study found that the presence and severity of coronary artery disease as well as left ventricular hypertrophy and dilatation was much more common in hypothyroid patients than in euthyroid individuals (Vanhaelst et al. 1967). A few years later in 1977, British researchers found a weak association between women with SCH and minor electrocardiogram changes (Tunbridge et al. 1977).

Since then, SCH has been demonstrated to be associated with atherosclerosis and its risk factors in some studies. SCH is also associated with impaired left ventricular diastolic function at rest, systolic dysfunction on effort, and enhanced risk for atherosclerosis and myocardial infarction (Biondi et al. 2002). Furthermore, SCH is associated with adverse lipid profile (Caraccio et al. 2002; Monzani et al. 2004; Razvi et al. 2007), increased carotid intima-media thickness (Monzani et al. 2004) and endothelial dysfunction (Razvi et al. 2007; Taddei et al. 2003), with all these parameters reversing with levothyroxine replacement.

There are several studies that show that the association between SCH and ischemic heart disease (IHD) is dependent on the age of the subjects. The Cardiovascular Health Study cohort showed that in people above age of 65, SCH is not associated with increased risk of cardiovascular disease, mortality or heart failure, although the latter was significantly higher in those with serum TSH > 10 mU/L (Cappola et al. 2006; Rodondi et al. 2008). The Health, aging and Body Composition Study of individuals aged 70–79 years also showed similar results (Rodondi et al. 2005). A meta-analysis divided studies into those that recruited participants below and above the age of 65 years. Interestingly, the incidence and prevalence of IHD and cardiovascular mortality was higher in the younger age group but not in the group above 65 years of age (Razvi et al. 2008). Another recent study in patients >65 years age has shown no association between persistent or transient subclinical hypothyroidism and incident IHD, heart failure or cardiovascular death (Hyland et al. 2013). Furthermore, an observational study of *real life* practice performed from data obtained from the United Kingdom General Practitioners Research Database, showed that treatment of SCH with levothyroxine was associated with fewer IHD events in younger individuals (40–70 years) but this was not evident in older people (>70 years) (Razvi et al. 2012). The Leiden 85+ study in which 599 people were followed from age of 85 years through age 89 years (mean follow up 3.7 years) showed that increasing levels of TSH and decreasing levels of FT4, both representing lower thyroid function, were associated with a survival benefit mainly due to reduced IHD events (Gussekloo et al. 2004). Several other longitudinal studies of older individuals with a mildly raised or high normal serum TSH have either shown no impact or have shown a modest benefit on cardiac outcomes/mortality (Bremner et al. 2012; Pearce et al. 2016; Yeap et al. 2013; Ogliari et al. 2016; Ceresini et al. 2013). However, a participant-level meta-analysis of multiple prospective cohorts of tens of thousands of individuals (n = 55,287) showed that SCH had a higher risk of adverse cardiac outcomes with more severe disease (serum TSH $\geq$ 10 mU/L) and that age did not seem to influence this observed cardiovascular risk (Rodondi et al. 2010). It is of interest to note that individuals with the more prevalent milder form of SCH (serum TSH levels below 10 mU/L) had no increased cardiovascular or mortality risk.

There have been a number of observational studies reporting an association of subclinical hyperthyroidism with IHD (Parle et al. 2001; Iervasi et al. 2007), atrial fibrillation (AF) (Cappola et al. 2006; Iervasi et al. 2007; Sawin et al. 1994; Auer et al. 2001; Gammage et al. 2007) and cardiac dysfunction (Rodondi et al. 2008; Biondi et al. 2000). A recent meta-analysis on this topic (Collet et al. 2012) pooled individual data from 10 prospective cohort studies and concluded that endogenous subclinical hyperthyroidism is associated with increased risks of total- and IHD-related mortality, and incident AF, with highest risks of IHD-related mortality and AF being associated with TSH level is low (<0.10 mU/L).

These results indicate that a higher TSH in the elderly may not have any adverse effects on the cardiovascular system and may even be protective. On the other hand, a low TSH, particularly if suppressed, is more likely to be associated with adverse vascular outcomes. Large adequately designed trials are required to confirm that the association between serum TSH levels and adverse vascular outcomes in the elderly is causal. At this juncture, one European study has commenced recruitment investigating whether levothyroxine treatment improves outcomes in SCH patients aged 65 years or older (TRUST Trial).

6.3.2 Thyroid Status and Cognitive Function

Overt hypothyroidism is well known to be associated with cognitive impairment and depression (Davis and Tremont 2007; Dugbartey 1998; Kamil and Joffe 1991; Nemeroff and Loosen 1987). Some studies have explored the relationship between thyroid function in older euthyroid individuals and cognition (Gussekloo et al. 2004; van Boxtel et al. 2004; Prinz et al. 1999; Volpato et al. 2002; Wahlin et al. 1998, 2005). A relationship between thyroid function within the reference range and cognition has been noted but it is unclear as to what the most sensitive marker for the thyroid status is (TSH, T4 or T3). Furthermore, there is also variation regarding the particular domain of cognition affected by changes in thyroid hormone concentrations.

A number of studies have shown the adverse effect of SCH on cognition in younger age groups (Monzani et al. 1993; Baldini et al. 1997; del Ser Quijano et al. 2000) but results in older people have been conflicting. One study in individuals with mean age of 74 years showed that people with SCH had worse performance on verbal recall and cognitive scores but working memory and processing speed were unaffected (Cook et al. 2002). The PAQUID survey of individuals aged 65 years or more showed that increased TSH levels were significantly linked with the presence of symptoms of depression but not with impairment of cognitive function (Manciet et al. 1995). There have been other studies which do not support any association between SCH and cognitive impairment (Osterweil et al. 1992; Luboshitzky et al. 1996, Park et al. 2010; de Jongh et al. 2011).

Similarly, the results in the few RCTs investigating improvement in cognition with levothyroxine replacement in SCH have also been conflicting. Three small RCTs in middle aged individuals showed improvement in cognitive function with levothyroxine replacement therapy in people with SCH (Nystrom et al. 1988; Jaeschke et al. 1996; Samuels et al. 2007). Two larger RCTs with longer follow up have not shown any benefit in cognition with levothyroxine replacement (Jorde et al. 2006; Parle et al. 2010); with the latter study specifically being in the elderly population aged 65 years or over.

A recent systematic review has considered the association of subclinical hyperthyroidism with dementia (Gan and Pearce 2012). It concluded that there is a substantial body of evidence to support the association between subclinical hyperthyroidism and cognitive impairment. It also concluded that at present, there is lack of evidence to suggest that antithyroid treatment might ameliorate dementia.

6.3.3 Thyroid Status, Depression and Disability

Some studies have shown an association of subclinical thyroid disease with depression (Cook et al. 2002; Samuels et al. 2007) but other findings have been inconsistent. A recent study did not support deleterious effects of subclinical thyroid disorders on physical or cognitive function, depression, or mortality in an older population (de Jongh et al. 2011). Another observational study showed that SCH is not associated with metabolic derangement, cognitive impairment, depression or poor quality of life in elderly subjects (Park et al. 2010). The Leiden 85+ study of individuals >85 years also did not show a consistent association between thyroid status and disability or depressive symptoms (Gussekloo et al. 2004). A RCT of levothyroxine treatment concluded that in SCH, where the serum TSH level is in the 3.5–10.0 mU/L range, there is no neuropsychological dysfunction or increased symptoms of hypothyroidism compared with healthy controls, and, more importantly, treatment with levothyroxine had no effect (Jorde et al. 2006). Interestingly, one observational study of individuals aged 70–79 years showed a slight functional mobility advantage with higher TSH (Simonsick et al. 2009).

6.3.4 Thyroid Status and Bone Health

In 1891, von Recklinghausen reported a “worm eaten” appearance of the long bones in a young woman who died from hyperthyroidism (von Recklinghausen 1891). Since then, it has become increasingly clear that thyroid hormone has important effects on bone and calcium metabolism via osteoclastic and osteoblastic function (Britto et al. 1994). TSH may also have a direct effect on bone formation and bone resorption, mediated via the TSH receptor on osteoblast and osteoclast precursors (Abe et al. 2003). Various studies have examined the association of thyroid function with bone mineral density (BMD) and/or fracture risk. Individuals with serum TSH below the 2.5 centile had significantly lower BMD as compared to controls with serum TSH in the normal range. Conversely, the postmenopausal women with serum TSH above the 97.5 centile had significantly higher BMD at the femoral neck than women with serum TSH in the normal range (Grimnes et al. 2008).

A study using quantitative ultrasound demonstrated that SCH does not affect bone turnover but has an impact on bone structure (Nagata et al. 2007). In women aged >65 years, low TSH was not associated with BMD or accelerated bone loss (Bauer et al. 1997). However, the study group, analysing data from the same cohort, also showed that low serum TSH levels were associated with increased risk for new hip and vertebral fractures (Bauer et al. 2001). Another study in men older than 65 years showed that patients with subclinical hyperthyroidism or hypothyroidism were at increased risk of hip fracture (Lee et al. 2010). The MrOS study concluded that although neither TSH nor FT4 are associated with bone loss, lower serum TSH may be associated with an increased risk of hip fractures in older men (Waring et al. 2013). There have been two meta-analyses on the effects of long term thyroxine replacement (Faber and Galløe 1994; Uzzan et al. 1996). Both of these showed that levothyroxine therapy leading to suppressed TSH is associated with significant bone loss in postmenopausal women but not those that were premenopausal.

6.4 Thyroid Status and Longevity

As populations age, one of the important questions that remains to be answered is the association between thyroid status and longevity. There have been few studies exploring the effect of thyroid disease on mortality and longevity in the elderly population. It is important to point out that these results differ from studies in younger population and they should not be extrapolated to them.

As mentioned earlier, the Leiden 85+ study showed that higher TSH concentrations and lower free thyroxine levels were associated with a survival benefit (Gussekloo et al. 2004). In this study, participants with low levels of TSH at baseline had highest mortality rate, and participants with high TSH levels and low FT4 levels had the lowest mortality rate. The authors speculated that lower thyroid function may lead to lower metabolic rate which in turn could cause caloric restriction. Lower metabolic rate and caloric restriction have both been shown to be associated with improved survival in several animal studies (Mobbs et al. 2001; Longo and Finch 2003; Blanc et al. 2003).

A study in very elderly Ashkenazi Jews (median age of 98 years) demonstrated that centenarians have significantly higher median serum TSH concentrations compared with younger Ashkenazi controls (median age 72 years) as well as in a population of thyroid disease-free individuals (median age, 68 years) from the U.S. NHANES 1998–2002 (Atzmon et al. 2009a). Similar results were noted in another study in Zoetermeer in which low serum FT4 was associated with a better 4-year survival in men aged 73–94 years (van den Beld et al. 2005). The Milan 75+ study evaluated the longitudinal association between thyroid parameters and 10-year all-cause mortality risk in older outpatients with normal TSH. It concluded that among older euthyroid outpatients, higher TSH and lower FT4 levels were associated with decreased mortality risk in men but not in women. They suggested that sex and age should be taken into account when assessing thyroid status (Ogliari et al. 2016). A study of 643 individuals aged 85 years who were followed for up to 9 years did not show any significant relationship between mortality and thyroid function, except for reverse T3 levels, after adjustment for other comorbidities (Pearce et al. 2016). However, other studies have failed to show similar results (de Jongh et al. 2011; Waring et al. 2012b).

On the other hand, various studies have shown subclinical hyperthyroidism to be associated with either higher mortality or to not have any adverse effect. The Amsterdam Longitudinal Ageing (de Jongh et al. 2011) and Zoetermeer (van den Beld et al. 2005) studies have not shown any change in mortality in their subjects with subclinical hyperthyroidism. Previously, a landmark study by Parle et al. showed that a single measurement of low serum TSH in individuals aged 60 years or older is associated with increased mortality from all causes, and in particular mortality due to circulatory and cardiovascular diseases (Parle et al. 2001). A meta-analysis showed that endogenous subclinical hyperthyroidism is associated with increased risks of total as well as IHD mortality, with highest risks of CHD mortality and AF when TSH level is lower than 0.10 mU/L (Collet et al. 2012). The Kangbuk Samsung study was a large study conducted in over 200,000 middle aged South Korean men and women. It concluded that FT4 and FT3 levels within the normal range were inversely associated with the risk of all-cause mortality and cancer mortality, particularly liver cancer mortality (Zhang et al. 2014). The Chianti study was conducted in 951 adults aged 65 years or more living in a mildly iodine deficient area. It concluded that subclinical hyperthyroidism is an independent risk for all-cause mortality in older adults, suggesting careful monitoring of low to normal thyrotropin level in elderly euthyroid individuals (Ceresini et al. 2013).

These results are not entirely unexpected as similar results have been shown in animal studies. It has been reported that rats in which hyperthyroidism was induced had a shorter life span (Robertson 1928; Ooka and Shinkai 1986). Wistar rats with induced hypothyroidism had a longer life-span then euthyroid rats (Ooka et al. 1983). Ames and Snell dwarf mice have low levels of prolactin, growth hormone and thyroid hormones and have lower body core temperature and slower metabolic rates consistent with hypothyroidism and live 40–70% longer then euthyroid mice (Brown-Borg 2007).

Some recent studies have explored the genetic basis of longevity with raised TSH. In the Leiden Longevity Study, when compared with their partners, the group of offspring of nonagenarian siblings showed a trend toward higher serum TSH levels in conjunction with lower FT4 levels and lower FT3 levels (Rozing et al. 2010). In their extension to this study, the researchers found that lower mortality in the parents of nonagenarian siblings was associated with higher serum TSH levels, lower FT4 levels, and lower FT3 levels in the nonagenarian siblings (Rozing et al.

2010b). In the study of Ashkenazi Jews, a heritable phenotype characterized by raised serum TSH which is associated with human longevity was identified. Carriers of rs12050077 and rs10149689 single nucleotide polymorphism in the TSH receptor gene had higher serum TSH, possibly contributing to decreased thyroid function and longevity (Atzmon et al. 2009b).

6.5 Mechanisms Responsible for Changes in Thyroid Function with Ageing

Thyroid hormones play a key role in energy homeostasis and metabolism (McAninch and Bianco 2014). Several theories of ageing link energy metabolism to the pathophysiology of ageing. The “rate of living theory” proposes that the positive association between lifespan and size of the animal is due to differences in resting metabolic rate (Jansen et al. 2015). Similarly, the linked “free radical theory of ageing” suggests that free radicals generated as by-products of oxidative metabolism play an important role in the negative correlation between lifespan and resting metabolic rate (Harman 1956). Another model proposes that ageing may involve depletion of functional stem cell reserves which could be slowed by reducing tissue turnover (Campisi 2003). Thyroid hormones influence metabolism, growth, development and tissue turnover.

A possible mechanism that could explain the increased TSH secretion in older individuals is increased thyroid hormone turnover due to augmented uptake in tissues or hormone clearance. Deiodination is the enzymatic process by which an atom of iodine is cleaved from the thyroid hormone moiety that leads to its activation or deactivation. Deiodination could contribute to changes in peripheral thyroid hormone turnover and may be altered with ageing, comorbidities and drugs (Gereben et al. 2008). Currently it is not possible to measure deiodinase enzymes but surrogate measures such as ratio of FT3/FT4 or rT3/FT3 in serum can be used. However, no significant differences in either of these ratios have been noted in the offspring of nonagenarians with high serum TSH compared to their partners. The same study also showed that, similarly, TSH bioactivity determined by an in vitro process and resting metabolic rate measurement were similar in people with a higher serum TSH compared to their partners with a lower TSH value (Jansen et al. 2015). One possible explanation could be increased production of biologically inactive TSH isoforms by the pituitary with age (Estrada et al. 2014). Although not directly studied but the evidence for the relative increase in biologically inactive TSH is borne by the lack of increase in thyroid hormone levels with age (Hollowell et al. 2002). It is therefore unclear as to the exact mechanism(s) by which changes in thyroid function occur in older individuals.

6.6 Levothyroxine Requirements in Older Hypothyroid Individuals

Hypothyroidism and its treatment with thyroid hormones are more commonly encountered in older individuals (Taylor et al. 2014). In elderly hypothyroid patients (>65–70 years) without overt cardiovascular disease, levothyroxine therapy can be initiated at the full replacement dose of 1.6 mcg/kg/day (Roos et al. 2005). The dose of levothyroxine that normalises serum TSH level is lower in older patients (Rosenbaum and Barzel 1982) due to changes in thyroxine turnover with age related reduction in lean body mass. Other factors such as decreased absorption, concomitant medication use, and other comorbidities could also affect T_4 metabolism.

The elderly are more susceptible to the ill-effects of thyroid hormone excess such as AF (Sawin et al. 1994) and osteoporotic fractures (Bauer et al. 2001; Flynn et al. 2010). Therefore, careful adjustments of levothyroxine dose at regular intervals are required in this population to avoid iatrogenic hyperthyroidism.

No definitive RCT of levothyroxine treatment in elderly hypothyroid patients comparing different TSH target values is currently available. A small feasibility RCT of 6 months has shown that hypothyroid patients aged ≥ 80 years that are allocated a lower levothyroxine dose aiming for a higher target TSH value are likely to continue treatment as those that are on their usual dose (Razvi et al. 2016). Therefore, in addition to the lower dose requirements related to thyroxine metabolism, based on the current evidence, it is reasonable to raise the target serum TSH up to 6 or 7 mU/L in persons greater than age 70–80 years particularly if they are at risk of cardiac arrhythmias or osteoporotic fractures.

6.7 Conclusions

Thyroid hormones have an essential role in the functioning of nearly all tissues in the body at all stages. Thyroid function changes with age and these alterations are more pronounced at both ends of the life span. Current evidence suggests that a slight lowering of thyroid function in older individuals, as evidenced by a marginally raised serum TSH and low normal FT4, may not be associated with an adverse outcome and may, in fact, be beneficial. On the other hand, high thyroid function, as evidenced by a low TSH level needs careful monitoring and treatment considered if there is evidence of end-organ damage (such as osteoporosis or AF), or if serum TSH is suppressed. Despite major advances in our understanding of thyroid function and ecology, mainly due to improvements in assay techniques and high quality epidemiological studies, several unresolved issues remain. It is currently unclear what the precise underlying mechanisms are behind the changes in thyroid function that are observed in older individuals. Moreover, it is uncertain whether these changes are part of healthy aging or are a bio-marker of underlying disease.

More research is required to fully understand why thyroid function changes in older individuals and whether modulation of thyroid hormones is advantageous for healthy aging and longevity. It is widely believed that mild thyroid hormone deficiency (or subclinical hypothyroidism) is more common in the elderly. But, if it is 'normal' and indeed desirable to have a slightly low thyroid function in older people then the current use of uniform reference ranges across all adult ages may need to be revised. Age-specific reference ranges may be required to diagnose thyroid disease with special reference to subclinical thyroid disease as well as to target serum TSH in patients on thyroid hormone replacement. And, in the future, it is possible that manipulation of thyroid function for health and longevity may be routinely practiced.

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Chapter 7
TGF-β in Development and Ageing

Harris Pratsinis, Eleni Mavrogonatou and Dimitris Kletsas

Abstract Transforming Growth Factor-β (TGF-β) is the prototype of a growth factor family playing central roles in a wide variety of functions both at the tissue and the organismal levels, ranging from the regulation of cell proliferation, and the enhancement of extracellular matrix accumulation to immunosuppression and a dual role in cancer development. Here, we present a brief description of TGF-β in terms of structural and functional aspects, including the major signal transduction pathways activated by it. The role of TGF-β as an inducer of cellular senescence in normal, malignant and stem cells is documented, as well as, its ability to induce or reinforce senescence when acting in an autocrine or paracrine fashion, as part of the senescence-associated secretory phenotype. The interplay of TGF-β with the two major intracellular protein degradation systems, i.e. autophagy and the ubiquitin-proteasome system, is also discussed, given their associations with senescence and age-related pathologies. The importance of the TGF-β signaling axis in organismal development is documented through the presentation of a variety of knock out approaches. Furthermore, we present its contribution to the programmed cellular senescence during development, as a newly recognized means for tissue patterning and remodeling. Finally, the role of TGF-β in age-related diseases and in longevity is discussed, based on data regarding gene polymorphisms, as well as, the plasma levels of this growth factor.

Keywords Transforming growth factor-β · Stress · Signaling · Senescence · Ageing · Proteolysis · Polymorphism

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7.1 Transforming Growth Factor-β: Structure, Receptors and Signaling Pathways

The term Transforming Growth Factor (TGF) was introduced in 1980 to describe two polypeptides (α and β) capable of "transforming" normal cells to malignant ones based on the criterion that in the presence of these factors the otherwise normal cells could grow in an anchorage-independent fashion (Roberts et al. 1980). TGF-α was found later to be a member of the Epidermal Growth Factor (EGF) family (Groenen et al. 1994), while TGF-β was shown to induce anchorage-independent growth actually through the potent induction of extracellular matrix (ECM) molecules and not due to malignant "transformation" (Ignotz and Massague 1986); however the names were already established.

TGF-β exists in five different isoforms numbered successively from TGF-β1 to TGF-β5, and only the three first of them are expressed in mammals (Massague 1990). Actually, the so-called TGF-β superfamily encompasses 33 members (Moustakas and Heldin 2009) encoded by 42 open reading frames in human, 9 in fly, and 6 in worm, which can be classified in two subfamilies, the TGF-β/Activin/Nodal subfamily and the BMP (bone morphogenetic protein)/GDF (growth and differentiation factor)/MIS (Müllerian inhibiting substance) subfamily, based on sequence similarities and the specific signaling pathways activated (Shi and Massague 2003). For reasons of brevity, the present chapter will focus on the three mammalian TGF-β isoforms, and especially on the prototype TGF-β1. This is a homodimer with molecular mass of 25 kDa; each chain of 112 amino-acids is linked to the other through a disulfide bridge (Sporn and Roberts 1992). TGF-β chains are synthesized in the form of a 390 amino-acid precursor containing an amino-terminal hydrophobic signal sequence, which is cleaved upon secretion, but it remains non-covalently associated with the TGF-β homodimer forming a biologically latent complex, hence it is called latency-associated peptide (LAP) (Fig. 7.1) (Massague 1990). The activation of TGF-β, i.e. its release from LAP, is a very important regulatory step induced by physical, biochemical, and mechanical factors, e.g. extreme pH or heating (Lawrence et al. 1985), protease treatment (Jenkins 2008), reactive oxygen species (ROS) (Barcellos-Hoff and Dix 1996), or application of traction and strain forces (Hinz 2015; Keski-Oja et al. 2004), to name some of them. The intricacy of this system is increased by the existence in various cell types of four latent-TGF-β-binding proteins (LTBPs) co-secreted with latent TGF-β in the form of a large complex (Horiguchi et al. 2012).

All members of the TGF-β superfamily exert their effects on cells through binding to type I and type II transmembrane receptors (TβRs) that form heterotetrameric complexes stabilized in the presence of the dimeric ligand (Fig. 7.1) (Kang et al. 2009). TβRs possess a cytoplasmic kinase domain that has strong serine/threonine kinase activity, hence the formation of the above complex leads to phosphorylation of the dormant TβRI by the constitutively active TβRII, starting an intracellular signaling cascade effected by members of the Smad family (Moustakas

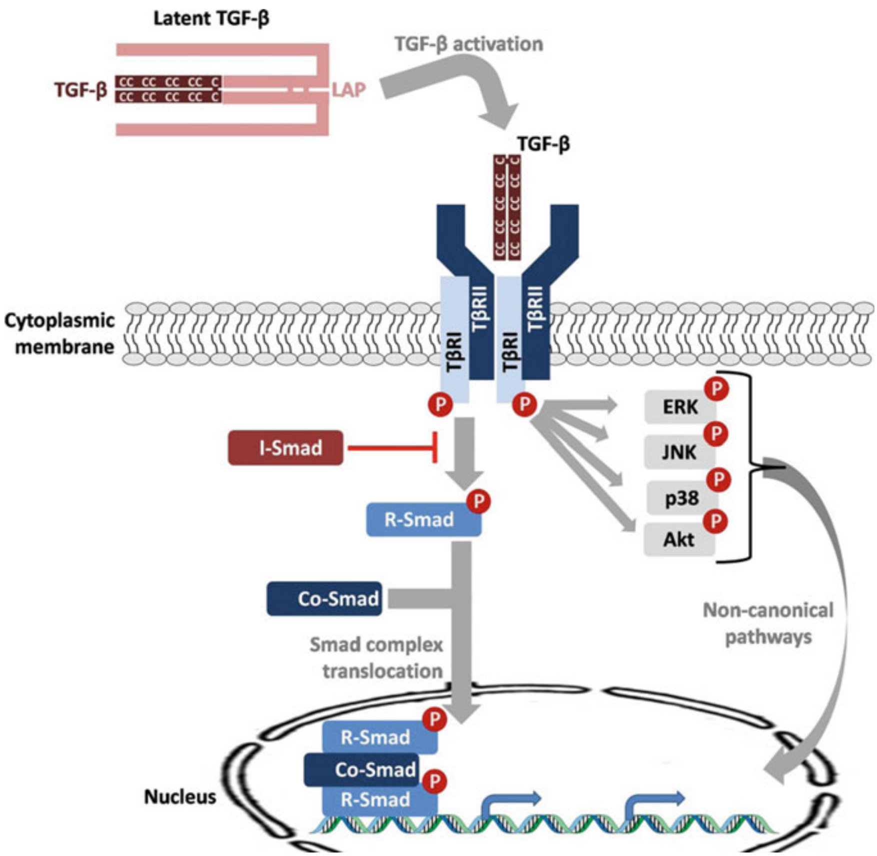

Fig. 7.1 TGF-β activation and signaling. TGF-β is released from its latent form, binds to the heterotetrameric receptor complex and triggers several signaling pathways and predominately the "canonical" Smad pathway

and Heldin 2009). Interestingly, TβRs possess also a weaker tyrosine kinase activity, which classifies them as being dual specificity kinases and can explain signaling through other substrates beyond Smads, e.g. Mitogen-Activated Protein Kinases (MAPKs) (Lee et al. 2007). Depending on the cell type, other transmembrane proteins are also involved in the regulation of TβR signaling, such as betaglycan (also called TβRIII), endoglin or neuropilin-1, collectively termed as co-receptors (Heldin and Moustakas 2016).

The Smad family encompasses the receptor-activated Smads or R-Smads (Smad2 and Smad3 for the TGF-β subfamily and Smad1, Smad5, and Smad8 for the BMP subfamily, see above), the common-mediator Smad or Co-Smad, i.e. Smad and the inhibitory Smads or I-Smads (Smad6 and Smad7) (Heldin et al. 2009). Phosphorylation of R-Smads by TβRI leads to their association with the Co-Smad and transfer of this complex to the nucleus to regulate gene transcription, while I-Smads negatively regulate signaling strength and duration (Fig. 7.1) (Horbelt et al. 2012). Smad signaling is regulated both transcriptionally and post-transcriptionally, among others through ubiquitylation and sumoylation (Lee et al. 2003; Mavrakis et al. 2007).

As already mentioned, beyond the so-called "canonical" pathway of Smad proteins (Kardassis et al. 2009), TGF-β-superfamily-members' signaling can also involve one or more of the MAPKs ERK1/2, JNK, and p38, the Src tyrosine kinase, the PI 3-K kinase, PP2A phosphatases and Rho family members (Fig. 7.1) (Heldin and Moustakas 2016).

7.2 TGF-β Functions

TGF-β is a ubiquitous factor, since nearly every cell type has the ability to secrete it, as well as the ability to respond to it via the presence of TβRs on the cell surface (Smith et al. 2012). Moreover, it is considered to be the prototype of the pleiotropic and multifunctional growth factor, affecting a variety of functions both at the cellular and the organismal level, sometimes in contradictory ways. For example, regarding cell proliferation TGF-β is inhibitory for most cell types (e.g. epithelial or endothelial) but for fibroblasts it can act both as an inducer or an inhibitor (Massague 1990; Sporn and Roberts 1992). Furthermore, while it is inhibitory for fetal skin fibroblasts, TGF-β stimulates the proliferation of fibroblasts from adults (Armatas et al. 2014; Giannouli and Kletsas 2006; Pratsinis et al. 2004). Even for the very same cell strain, TGF-β can either stimulate or inhibit proliferation depending on its concentration (Battegay et al. 1990) or on the cell culture density (Stathakos et al. 1993).

A characteristic feature of TGF-β is its ability to increase gene expression, as well as, secretion for most of the ECM proteins, including collagen types I, II, III, IV, V and X, fibronectin, thrombospondin, osteopontin, tenascin, elastin, hyaluronic acid, biglycan and decorin (Roberts et al. 1992). At the same time, TGF-β interferes with enzymes degrading these ECM proteins, thus enhancing further their accumulation (Massague 1990). The potency of TGF-β in inducing ECM deposition explains both its beneficial function in the wound healing process and its detrimental ability to induce fibrosis in a variety of tissues (Schiller et al. 2004).

Another very important characteristic of TGF-β is its interference with the immune system: it inhibits the proliferation and/or the functions of most types of immune cells, such as B- and T-cells, dendritic cells, and natural killer (NK) cells, it suppresses the differentiation of effector Th cells and the development of type I macrophages and neutrophils and it blocks hematopoiesis (Yoshimura et al. 2010; Yang et al. 2010). Hence, TGF-β may be considered as an anti-inflammatory agent, a fact supported by the observation that the main phenotype observed in the TGF-β1-null mice is massive multifocal inflammation leading to death (Kulkarni and Karlsson 1997). However, depending on the context, TGF-β can also exert pro-inflammatory effects, mainly due to its ECM-inducing and chemotactic properties leading to topical inflammatory cell recruitment and adhesion, as in the case of the early cutaneous wound healing stages (Wahl 1992; Wang et al. 2006).

The duality of TGF-β effects is probably most evident in its role in cancer: the so-called "TGF-β paradox" refers to its tumor suppressive effects in early-stage tumors as opposed to its promotional actions regarding later-stage malignancies (Tian and Schiemann 2009). Initially, many genes implicated in TGF-β signaling were considered as tumor suppressors, the most characteristic example being *Smad4*, originally discovered to be homozygously deleted in almost one third of pancreatic cancers, hence named also "*DPC-4*", i.e. "*Deleted in Pancreatic Cancer-4*" (Hahn et al. 1996). The tumor suppressive role of TGF-β is mainly based on its already mentioned anti-proliferative effects on epithelial cells (Massague 2008). On the other hand, TGF-β's ability to promote tumor progression is associated with the loss of balance between some of its conflicting activities e.g. the pro-tumor attenuation of host immunosurveillance versus the suppression of tumorigenic inflammation (Yang et al. 2010), as well as, with TGF-β mediated stromal-epithelial interactions in the tumor microenvironment (Bierie and Moses 2006). Moreover, TGF-β is a major inducer of epithelial-to-mesenchymal transition (EMT), a key-process during embryogenesis and development (Moustakas and Heldin 2007), which however is also offering to cancer cells increased invasive, migratory and stem cell properties allowing them to disseminate and propagate at distant sites (Fuxe and Karlsson 2012).

7.3 TGF-β and Cellular Senescence

During ageing of cells in vitro significant variations in the expression of TGF-β and its receptors have been observed. Human skin fibroblasts rendered senescent due to serial replications were found to secrete more total (i.e. latent) TGF-β than early passage cells, however the activation mechanism in senescent cells seemed to be less efficient resulting in a much lower (approx. an order of magnitude) amount of active growth factor, a fact associated with the catabolic phenotype of late passage cells (Zeng et al. 1996). On the other hand, in the human fetal lung fibroblast strain IMR-90 all three isoforms of TGF-β, as well as, both TβR types were found to be overexpressed at the protein level during replicative senescence in vitro; although a direct assessment of the active factors was not performed in this study, the higher levels of connective tissue growth factor (CTGF—a known target of TGF-β signaling) observed in senescent cells suggested higher levels of active TGF-β, as well (Kim et al. 2004). Overexpression of TGF-β1 (but not TGF-β2) at both the mRNA and protein levels was also observed in IMR-90, as well as, in human skin fibroblasts, due to stress-induced premature senescence (SIPS) following non-cytotoxic hydrogen peroxide treatment (Frippiat et al. 2001). Interestingly, inhibition of this overexpression through neutralizing antibodies against TGF-β1 or TβRII led to the reversal of the SIPS phenotype (Frippiat et al. 2001), while later it was found to be regulated by the retinoblastoma protein (Rb) and p38 MAP kinase phosphorylation, which in turn was kept in phosphorylation state due to the high TGF-β1 levels establishing a closed regulatory loop supporting the SIPS phenotype (Frippiat et al. 2002). A similar mechanism involving TGF-β1 (but also TGF-β2, and -β3) was also observed in SIPS induced by non-cytotoxic ultraviolet B (UVB) treatment, and in this case both latent and active TGF-β levels were found to be increased (Debacq-Chainiaux et al. 2005). In a reverse approach, stimuli leading to the extension of human fibroblast replicative lifespan were correlated with the secretion of lower active TGF-β levels (Pratsinis et al. 2002). Also in a different cellular model, i.e. mouse keratinocytes, the use of another stress provoking SIPS, i.e. oncogene overexpression following infection with the v-ras^{Ha} retrovirus led to increase of the secretion of both the total and the active forms of TGF-β1, while blocking TGF-β1 secretion or TβRII reversed SIPS (Tremain et al. 2000).

Most of the above mentioned studies support the idea of an enhanced TGF-β and/or TβR expression as a result of senescence, but some of them also indicate that the signaling axis of TGF-β is a mediator of senescence. Indeed, there are many reports supporting the role of TGF-β as an effector of other primary stimuli that eventually lead to cell senescence, usually oxidative stress or DNA damage. In this direction, it has been shown that chronic exposure of human peritoneal mesothelial cells to elevated glucose levels may result in TGF-β1-mediated accelerated senescence, an effect partially reduced by anti-TGF-β1 neutralizing antibodies (Ksiazek et al. 2007). Furthermore, subcytotoxic concentrations of desferroxamine mesylate (DFO), an iron chelator, were found to induce a senescence-like arrest of hepatocyte cell lines, associated with induction of p27^{kip1} through TGF-β, again in a reversible manner (Yoon et al. 2002). Ionizing radiation, a known DNA-damaging agent, provokes premature senescence of human breast stromal fibroblasts in vitro, as well as, in the breast tissue in vivo. These senescent cells were shown to overexpress the cell surface proteoglycan syndecan 1 as a result of autocrine TGF-β-secretion, and the concomitant involvement of the Smad pathway and the transcription factor Sp1 (Liakou et al. 2016). These senescent fibroblasts express a pro-inflammatory phenotype creating a favorable milieu for tumor cell growth. Similarly, fibroblasts from the area of oral squamous cell carcinomas are becoming senescent due to the exposure to ROS produced by the cancer cells and the subsequent increase in the production of autocrine acting TGF-β1 and -β2 (Hassona et al. 2013). Hence, members of the TGF-β family seem to belong to the senescence-associated secretory phenotype (SASP), a term introduced to explain the role of the senescent cells in tissue homeostasis (Coppe et al. 2008). Indeed, TGF-β has been identified as a major component of SASP with a regulatory role for other components, through the use of a variety of experimental approaches (Acosta et al. 2013; Hassona et al. 2014; Olivieri et al. 2013). Interestingly, TGF-β has been shown to induce the production of ROS in a variety of cell types (Thannickal et al. 1993; Thannickal and Fanburg 1995) through the up-regulation of NADPH oxidase 4 (NOX4) (Hecker et al. 2009). Therefore, TGF-β secreted by senescent cells–along with other cytokines that are SASP elements—can further induce the so-called cytokine-induced senescence to adjacent bystander cells through NOX4 and ROS (Hubackova et al. 2012, 2016).

Although the examples above support a detrimental role for TGF-β in cancer through paracrine tumor-stroma interactions, there are also numerous observations supporting its favorable role, through the direct induction of cancer cell senescence. TGF-β can induce two independent pathways of cellular senescence (involving telomerase down-regulation or not) in the cancer cell line A549, although it lacks the *p15*^{INK4b} and *p16*^{INK4a} genes (Katakura et al. 1999). TGF-β enforces senescence in Myc-transformed hematopoietic tumor cells through induction of Mad1 and repression of Myc activity (Wu et al. 2009). In mouse keratinocytes oncogene-induced replicative senescence has been shown to depend on TGF-β1 secretion, and inactivation of this factor's secretion or defects in its signaling accelerate malignant progression of squamous cell carcinomas following skin grafting of these cells (Tremain et al. 2000). Similarly, the TGF-β-pathway seems to be instrumental in head and neck squamous cell carcinomas (HNSCCs), since the *type I TGF-β receptor/Pten* double conditional knockout mice develop full-penetrance HNSCCs involving among others senescence evasion (Bian et al. 2012). In human breast cancer cells the cytokine bone morphogenetic protein-7 (BMP-7), which belongs to the TGF-β superfamily, can induce telomere shortening and senescence through a mechanism involving BMP receptor type-II and Smad3 leading to repression of the *human telomerase reverse transcriptase* (*hTERT*) gene promoter activity (Cassar et al. 2016). In this study, TGF-β or activin receptors were not found to contribute to *hTERT* repression, however, in telomerase-immortalized human mammary epithelial cells a dominant-negative type II receptor of TGF-β abrogated autocrine TGF-β signaling and suppressed H-Ras-V12-induced senescence-like growth arrest (Lin et al. 2012). In normal, finite lifespan human mammary epithelial cells Ras transformation leads to oncogene-induced senescence, which requires TGF-β signaling and can be abrogated either by the expression of a dominant-negative TGF-β type II receptor or by the presence of a TGF-β type I receptor inhibitor (Cipriano et al. 2011).

Recently, a special interest has been raised regarding senescence of mesenchymal stem cells, since this can be a limitation in their use for clinically feasible therapeutic approaches (Li and Pei 2012; Kregar Velikonja et al. 2014). In this context, it was reported that TGF-β1 and -β2 are responsible for the induction of senescence during long-term culture of human mesenchymal stem cells (Ito et al. 2007). In particular, TGF-β1 dose-dependently induces characteristic senescence markers, such as SA-β gal and p16^{INK4a}, as well as, the lipid peroxidation product 4-hydroxynonenal and simultaneously down-regulates the antioxidant enzyme superoxide dismutase 2 and Id1, a negative regulator of p16^{INK4a}, in bone marrow derived mesenchymal stem cells, an effect mediated—at least partially—through the production of mitochondrial ROS (Wu et al. 2014). On the other hand, it has been suggested that multipotent mesenchymal stromal cells isolated from mononuclear cells through plastic adherence do not enter premature senescence after TGF-β1 treatment, but their increased proliferation rates in response to this growth factor lead them to reach replicative senescence earlier than untreated control cultures (Walenda et al. 2013).

In general, the above data point to a very important correlation of the TGF-β axis with cell senescence. On the other hand, the role of TGF-β in senescence induction is not always straightforward due to the complexity of the senescence process and the compensatory mechanisms activated at the cellular level. For example, Hic-5, a TGF-β-inducible focal adhesion adaptor protein with similarity to paxillin, was initially considered to induce senescence in immortalized cells (Shibanuma et al. 1994, 1997). Recent studies, however, indicate that at least in myofibroblasts TGF-β1-induced Hic-5 functions as a negative feedback mechanism to limit senescence by promoting the ubiquitin-proteasome system-mediated degradation of Nox4 (Desai et al. 2014).

7.4 Intracellular Proteolysis and Ageing: The Role of TGF-β

Ageing is characterized by a progressive decline in the maintenance, repair and turnover pathways that lead to the accumulation of molecular damages and thus to a failure of homeodymanics (Rajawat et al. 2009; Rattan 2006). Among these pathways are included those involved in the removal, recycling and renewal of malformed or damaged proteins, i.e. two major proteolytic systems, the ubiquitin-proteasome degradation system and the lysosomes, both known to decline with ageing (Carrard et al. 2002; Ciechanover 2013; Ward 2002). The lysosomal degradative system is responsible for the removal of the majority of these damaged or altered proteins, as well as of damaged organelles, and it is combined with the autophagy process. Autophagy ("eat oneself" in Greek), an evolutionary conserved process, appears in three forms: macroautophagy, chaperone-mediated autophagy and microautophagy (Rajawat et al. 2009). The classical form, i.e. macroautophagy, briefly consists of several steps involving autophagosome formation, elongation, closure, fusion with lysosomes and finally degradation (Wu et al. 2016). Several lines of evidence from various models indicate that autophagic activity decreases during organismal ageing while lifespan extension depends on an effective autophagy (Young and Narita 2010). Even more, experimental manipulations known to extend lifespan, such as calorie restriction or the negative regulation of insulin signaling and the mTOR pathway, are linked with the activation of autophagy, suggesting that this process represents an anti-ageing effector (Bergamini et al. 2007; Cuervo et al. 2005; Hansen et al. 2008; Salminen and Kaarniranta 2009; Young and Narita 2010).

Recent evidence suggest a complex relation between TGF-β and autophagy (Wu et al. 2016). First, the reduction of specific autophagy effectors (e.g. LC3) or the use of specific autophagy inhibitors such as bafilomycin A1 increases TGF-β1 expression in obstructed kidneys (Ding et al. 2014). On the other hand, the effect of TGF-β on autophagy seems to be cell type-specific. In human hepatocellular carcinoma cells TGF-β was found to activate autophagy via both the Smad and the

JNK pathways (Kiyono et al. 2009). In addition, TGF-β rescues hepatic stelate cells from serum starvation via autophagy (Fu et al. 2014).

As mentioned above, TGF-β is a well known stimulator of extracellular matrix accumulation. In this line, in primary human atrial myofibroblasts it induces a fibrogenic response via autophagy (Ghavami et al. 2015). However, in lung fibroblasts it reduces autophagy via mTOR stimulation, while rapamycin (an mTOR inhibitor) increases autophagy and reduces fibrosis (Patel et al. 2012). In mouse mesangial cells, TGF-β activates the TAK-MKK3-p38 pathway leading to collagen synthesis; interestingly, the same pathway activates autophagy leading to intracellular collagen degradation, thus indicating a dual role in kidney fibrosis (Kim et al. 2012). Based on the above, more studies are needed for the elucidation of the role of TGF-β in autophagy in ageing and in various age-related diseases.

On the other hand, the ubiquitin-proteasome system represents the other major protein degradation system, comprised by the E1 ubiquitin activating enzyme, the E2 ubiquitin conjugating enzyme and the E3 ubiquitin ligase, leading to the multiubiquitination of proteins and their targeting to the proteasome for degradation (Ciechanover 2013). The role of this system in cellular senescence, in vivo ageing and age-related diseases is well described (Tsakiri and Trougakos 2015; Chondrogianni et al. 2015). Several lines of evidence indicate also a complicated involvement of the ubiquitin-proteasome system in TGF-β signaling. The TGF-β signaling inhibitors Smad7, SnoN and—to a lesser extent—Ski are degraded by the proteasome (Bonni et al. 2001; Kavsak et al. 2000; Sun et al. 1999). Conversely, TGF-β, in parallel with Smad activation, initiates the ubiquitination and degradation of Smad2 and Smad3 (Lo and Massague 1999; Fukuchi et al. 2001). Finally, momo- and oligoubiquitination of Smad4 enhances its ability to form a complex with the R-Smads (Moren et al. 2003), while its polyubiqitination leads to the degradation via the proteasome (Wan et al. 2002). Collectively, the ubiquitin-proteasome system can affect TGF-β signaling positively or negatively, and it is the timing and the cellular context that determines the final outcome (Zhang and Laiho 2003; Zhang et al. 2002).

7.5 The Role of TGF-β in Development

7.5.1 TGF-β in Development

An active TGF-β signaling cascade consisting of functional ligands, receptors and intracellular effectors has been shown to be necessary for proper development and disease prevention. The important role of each specific component of the TGF-β pathway (TGF-β isoforms, TGF-β receptors and Smads) during development has been established by knocking out the respective genes.

Deficiency in all TGF-β isoforms and receptors is generally embryonally lethal or leads to death early in life after birth. Despite intrauterine mortality observed for a high proportion of homozygous TGF-β1 null mice at midgestation as a result of defects in yolk sac vasculogenesis and hematopoiesis (Dickson et al. 1995), more than one third of the fetuses are actually born and are similar to their wild-type or heterozygous littermates (Kulkarni et al. 1993). Animals grow normally for the first two weeks, before developing a wasting syndrome that leads them to death after approx. 20 days due to the incidence of an inflammatory disorder accompanied by the production of autoantibodies (Diebold et al. 1995; Shull et al. 1992; Kulkarni et al. 1993). This multifocal inflammation has been suggested to be the result of an impaired IFN-γ signaling (McCartney-Francis and Wahl 2002) and is characterized by the presence of massive lesions in multiple organs, such as the heart, the lungs, the pancreas, the colon and the salivary glands (Dang et al. 1995; Kulkarni et al. 1993; Letterio et al. 1994). TGF-β2$^{-/-}$ mice are characterized by congenital heart, pulmonary, craniofacial, limb, spinal column, eye, inner ear, urogenital system defects and die just before or during birth (Sanford et al. 1997). Disruption in both alleles of the gene encoding TGF-β3 leads to delayed lung maturation and defective palatal shelf fusion, which is intrinsic and does not stem from primordial craniofacial malformations (Kaartinen et al. 1995; Proetzel et al. 1995). Homozygous TGF-β3-deficient mice die within the first 24 h of birth.

TβRII$^{-/-}$ embryos are identical to their siblings until day 8.5 *postcoitum* and present a normal morphology accompanied by growth delay and severe anaemia at day 9.5. At that stage, histological analysis has revealed a problematic hematopoiesis and vasculogenesis of the yolk sac, which is most probably the causative factor of the growth retardation observed after day 10.5 and of the consequent embryonic death met at day 13.5 of gestation (Oshima et al. 1996). Lethal TβRII$^{-/-}$ phenotype resembles that of TGF-β1$^{-/-}$ embryos that do not make it to birth also due to defects in yolk sac vasculogenesis and hematopoiesis (Dickson et al. 1995) and could be attributed to a non-functional TGF-β1 signaling (Oshima et al. 1996). TβRI$^{-/-}$ mice die during embryogenesis at around embryonic day 10.5 due to abnormal vessel formation of the yolk sac and placenta and due to elimination of the circulating red blood cells, without though any disturbance being observed in their hematopoietic potential (Larsson et al. 2001), in contrast to what has been reported for TβRII$^{-/-}$ embryos (Oshima et al. 1996). The vast majority of homozygous TβRIII-null mice die during late gestation, while the really small percentage of surviving homozygous pups are healthy but generally infertile (Stenvers et al. 2003). TβRIII$^{-/-}$ embryos present an extensive damage to the liver and to the heart and an impaired coronary vessel development (Stenvers et al. 2003; Compton et al. 2007) that negatively affect erythropoiesis and cardiac function, ultimately leading to embryonic death. Homozygous deficiency for endoglin (*eng*$^{-/-}$) is lethal in mice by 10.5 days *postcoitum*, while the main characteristics of *eng*$^{-/-}$ embryos are smaller size, fragility and defective hematopoiesis and angiogenesis of the yolk sac (Arthur et al. 2000; Li et al. 1999b), as has also been observed in TβRII$^{-/-}$ mice (Oshima et al. 1996) and in the TGF-β1$^{-/-}$ embryos that fail to survive to term (Dickson et al. 1995), supporting the necessity of TGF-β1 signal transduction for the development of vessels. In addition, it has been demonstrated that disruption of endoglin hinders hemangioblast development, affecting both hematopoietic and endothelial lineages (Perlingeiro 2007). *eng*$^{-/-}$ mutants also show cardiac defects (Bourdeau et al. 1999), which have later been reported to be the primary event that leads to vessel malformations in the absence of endoglin (Nomura-Kitabayashi et al. 2009).

Knocking out Smad2, Smad4, Smad6 and Smad7 results in early embryonic or perinatal mortality, while only Smad3-null mice are viable. Homozygous Smad2 mutant mice possess a smaller size than their siblings, present defects in egg cylinder elongation and disability to establish a posterior-anterior axis, fail to develop ectoderm and endoderm and solely generate extraembryonic mesoderm (Nomura and Li 1998; Waldrip et al. 1998; Weinstein et al. 1998). Smad2-null embryos die at approx. 8.5 days *postcoitum* (Weinstein et al. 1998). In contrast to Smad2 deficiency which is detrimental and lethal, Smad3 ablation by targeted disruption of exon 8 has not an apparent outcome on embryonic development and Smad3$^{-/-}$ mice are viable (Datto et al. 1999; Yang et al. 1999). This discrepancy may arise either from the ability of the almost identical Smad2 to compensate the lack of Smad3 (which is not inverted since both of Smad2 alternatively spliced forms cannot be substituted at once) or from the different expression patterns of the two proteins, with Smad2 playing a key role during development and Smad3 being important in the adult organism (Datto et al. 1999). Homozygous Smad3-null mice show accelerated wound healing due to a higher rate of re-epithelialization possibly stemming from an enhanced proliferation ability in response to TGF-β and a Smad3-independent migration pathway adopted by keratinocytes (Ashcroft et al. 1999), whereas embryonic fibroblasts deriving from mice lacking Smad3 do not respond to the anti-proliferative effect of TGF-β (Datto et al. 1999). On the other hand, homozygous Smad3-deficient mice are growth-retarded and die between one and eight months from a multifocal inflammatory disease and chronic infection provoked by immune dysregulation (Yang et al. 1999). Alternatively, Smad3-deficient mice arising from the targeted mutation of exon 3 develop at the age of approx. five months spontaneous colorectal malignancies that are highly invasive and metastatic (Zhu et al. 1998). Given that Smad4 is implicated as a common partner in many signaling pathways launching from all TGF-β ligands and receptor-activated Smads, disruption of Smad4 not surprisingly results in a severely defective phenotype and early embryonic lethality at around day 7.5 of embryogenesis (Sirard et al. 1998; Yang et al. 1998). Homozygosity for the loss of Smad4 leads to an abnormal development of the visceral endoderm and a secondary deficiency in epiblast proliferation that hampers mesoderm formation (Yang et al. 1998), while Smad4$^{-/-}$ embryos are characterized by a reduced size ascribed to an impaired proliferation capacity rather than to apoptosis (Sirard et al. 1998). Targeted disruption of the locus encoding inhibitory Smad6 leads to cardiovascular abnormalities, such as hyperplasia of the heart valves and abnormal septation of the outflow tract (Galvin et al. 2000) and defects in skeletal development (Estrada et al. 2011). First attempts for knocking out Smad7 by disrupting exon 1 led to viable and smaller mice showing alterations in B cell responses (Li et al. 2006), severe renal fibrosis and inflammation in a unilateral ureteral obstruction model (Chung et al. 2009) and in a rat model of diabetes (Chen et al. 2011). Since loss of function of

Smad7 is not complete using this approach—because the resultant truncated Smad7 contains the necessary MH2 domain (Li et al. 2006)—, next attempts for knocking out Smad7 were performed by disrupting exon 4 that comprises the entire MH2 domain (Chen et al. 2009; Tojo et al. 2012). This strategy resulted in perinatal lethality due to defects in cardiovascular development, whereas surviving animals suffered from impaired cardiac function and growth retardation (Chen et al. 2009; Tojo et al. 2012).

7.5.2 TGF-β in Programmed Cellular Senescence in the Course of Embryonic Development

As mentioned above, the TGF-β signaling system (ligands, receptors and intracellular signaling molecules) seems to play crucial roles in embryonic development, while it can provoke premature cellular senescence. Recently, two groups reported a specific type of cellular senescence in the later stages of embryonic development focusing especially in the apical ectodermal ridge of the limb, the closing neural tube of the hind-brain, the developing mesonephros and the endolymphatic sac of the inner ear (Munoz-Espin et al. 2013; Storer et al. 2013). The characterization of these cells indicated that they bear classical characteristics of senescent cells, such as increased SA-β gal activity, decreased expression of the proliferation marker Ki-67, increased expression of the senescence-associated heterochromatin markers HP1γ and H3K9me3 and they were found arrested in the G1 phase of the cell cycle. However, they lack specific markers observed in typical senescent cells related to a DNA damage response, such as γH2Ax expression, while the DNA-damage-signaling kinases ATM and ATR seem not to be involved in this process. Even more, conventional markers of cellular senescence, such as the expression of p53 and the INK4 family of cell-cycle inhibitors (e.g. $p16^{INK4a}$) do not play a significant role. This process of programmed senescence is mediated by $p21^{WAF1}$ in a p53-independent manner. By studying wild-type and p21-null mouse embryos using DNA microarrays it was found that important development pathways, including TGF-β are linked with this phenomenon. Furthermore, phosphorylated Smad2 was observed in the tissues bearing senescent cells, while a TGF-β receptor inhibitor reduced SA-β gal activity and $p21^{WAF1}$ expression. At the functional level, these senescent cells are involved in macrophage-mediated apoptosis leading to elimination of embryonic structures, as well as in the balance of different cell types during development. Further studies in p21-null mice indicated that in late stages of development and in adult animals in most of the cases no major defects appear, most probably due to compensatory mechanisms underlying the robustness of embryonic development. Still, developmental deficiencies are observed suggesting that these compensatory processes cannot fully replace the absence of this programmed senescence. Overall, these findings allow the suggestion that this type of cellular senescence appeared in evolution as a remodeling mechanism and later adopted some features of stress responses as to adapt in tumor suppression and ageing (Munoz-Espin et al. 2013; Storer and Keyes 2014; Storer et al. 2013).

7.6 TGF-β Gene Polymorphisms and Plasma Levels in Diseases and in Longevity

Due to the implication of TGF-β in multiple pathophysiological processes gene organization and plasma levels have been studied in several disease states, as well as in long-lived individuals. In the TGF-β1 gene nine polymorphisms have been identified: (i) C-988A, G-800A, C-509T reside in the promoter region; (ii) an insertion (C) is found in the 5′ UTR atposition +72; (iii) two SNPs are in the signal sequence [codon + 10 (T/C) or T869C and codon + 25 (G/C) or G915C], one in exon 5 (T263I or C788T), and one in each of introns 4 and 5 (713-8delC and C861-20T), respectively [reviewed in Bosco et al. (2013)]. TGF-β1 polymorphisms have been associated with several diseases, such as hypertension (Li et al. 1999a; Suthanthiran et al. 2000), heart failure (Holweg et al. 2001), liver cirrhosis (Lee et al. 2011), osteoporosis (Langdahl et al. 1997; Yamada et al. 1998), cerebral amyloid angiopathy (Hamaguchi et al. 2005) or Alzheimer's disease (Bosco et al. 2013). On the other hand, genetic variability was studied in a cohort of Italian centenarians. It has been found that a particular haplotype combination (G-800/C-509/C869/C915)—composed by two alleles located in the 5′ region and two missense polymorphisms, associated with the transcriptional activity of TGF-β1—was significantly lower in these extremely long-lived individuals in comparison to younger controls (Carrieri et al. 2004).

The circulating levels of TGF-β1 have also been estimated in donors of several age levels. Initial studies have shown that serum TGF-β1 levels were not associated with the age in a cohort of healthy UK donors ranging from 17–68 years old (Young et al. 1999). However, data from a group of Swedish octogenarians and nonagenarians indicated significant higher plasma levels of active TGF-β1 as compared with younger donors (Forsey et al. 2003). In agreement, the levels of active TGF-β1 in the plasma of Italian centenarians (both men and women) were also higher than in young individuals (Carrieri et al. 2004). Having in mind the increase of the levels of inflammatory cytokines in the elderly, it can be suggested that the high levels of the anti-inflammatory TGF-β1 can counterbalance this phenomenon and can possibly contribute to increased longevity.

Collectively, the data presented here indicate that TGF-β exerts multiple functions, thus having a central position in tissue homeostasis. Interestingly, in a number of cases such as in cancer development, TGF-β can play opposing roles suggesting that a very delicate balance of its presence and action, depending on the overall context of cell and tissue interactions, directs the final outcome. Although numerous reports indicate also a direct involvement of TGF-β in various diseases and pathologic conditions—for a review see (Heldin and Moustakas 2016)—this was not the focus of the present chapter. On the other hand, the data discussed here clearly show the importance of TGF-β in developmental and ageing procedures.

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Part III
Neuroendocrine and Rhythms

Chapter 8
Hormones of Hypothalamus in Aging

Gurcharan Kaur and Jyoti Parkash

Abstract Hypothalamus being the master regulator of the vertebrate endocrine system undergoes many adjustments/alterations which body makes during the course of aging. Moreover, the endocrinological basis of aging in male and female organisms is very complex, with multiple hormones along the hypothalamic-pituitary (HP) axis interacting with each other via different feedback loops to maintain homeodynamic state. Also the sensitivity of the hypothalamus to the external stimuli decreases with age mainly due to its lack of sensitivity towards the feedback system The endocrine system is although severely affected by aging but all the organ systems are not affected at the same time or in the same way. During aging cellular protein synthesis machinery as well as immune functions are diminished and gradually physiological functions decline. There is also an increase in fat mass, a loss of muscle mass and strength, and a decrease in bone mineral density profile that contribute to declining health status with increasing age. The hallmarks of aging such as Genomic instability, Telomere attrition, Epigenetic alterations, Loss of proteostasis, Dysregulated Nutrient Sensing, Mitochondrial dysfunction, Altered intracellular communication, Cellular senescence etc. are well reported in literature. In this chapter we have compiled information and discussed various hormonal changes that occur with age in hypothalamus and pituitary gland and how these two master regulators gradually lose their sensitivity with the increasing age.

Keywords Hypothalamus · Regulation · Receptors · Thyrotropin · Oxytocin · Vasopressin · Somatostatin · Reproduction · Menopause · Neurotransmitters

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8.1 Introduction

Hormones are known as the body's distant messengers/regulators which determine the way the body functions, and are produced by many different parts of the body. The hypothalamus, a part of the basal brain, is the source of many releasing hormones. Understanding these "brainy hormones" will help to take control of our body and health profile. The hypothalamus produces releasing hormones which in turn control the production of trophic hormones from the pituitary gland. These two endocrine regulators work in harmony to control the other endocrine glands to release their respective hormones. Because of these reasons, function of hypothalamus is directly responsible for overall hormone health. So in case the hypothalamus is damaged due to traumatic brain injury or genetic factors, overall hormonal health becomes the target.

The hypothalamus produces different hormones to regulate various endocrine glands in the body (see Fig. 8.1)

- **Thyrotropin-Releasing Hormone**—Stimulates production of the thyroid hormone, which in turn controls the cardiovascular system, brain development, muscle control, digestive health and metabolism

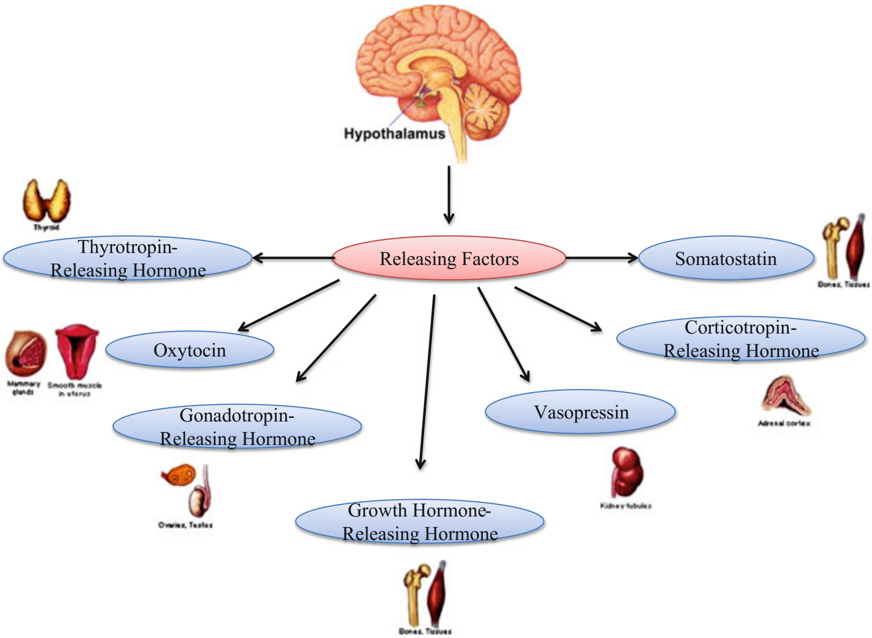

Fig. 8.1 Hypothalamus secretes various releasing hormones, which control the production of trophic hormones from pituitary gland

- **Oxytocin**—A hormone that controls parturition, human behaviors and the reproductive system
- **Gonadotropin-Releasing Hormone**—Stimulates the release of hormones which control reproductive function, puberty and sexual maturation
- **Growth Hormone-Releasing Hormone**—Controls growth and physical development in children as well as metabolism in adults
- **Anti-Diuretic Hormones (Vasopressin)**—The hormones that regulate water levels in the body, including blood volume and blood pressure
- **Corticotropin-Releasing Hormone**—Controls the body's response to physical and emotional stress, and is responsible for suppressing the appetite and stimulating anxiety
- **Somatostatin**—Inhibits growth and thyroid-stimulating hormones

Aging is a multi-faceted and multi-factorial decline of the body functions, and is associated with degenerative changes in multiple organ systems. The rate and extent to which these degenerative changes occur depends on genetics, the presence of other disease processes, and the accumulated effects of socioeconomic factors, lifestyle, and environmental factors (Araujo and Wittert 2011). Aging is very well reported to cause reproductive decline in mammals, including hormonal abnormalities and infertility (Kunimura et al. 2017). Reproductive aging refers specifically to changes in germ cells and the hormone-producing cells that support them, both within the gonad as well as at the hypothalamic and pituitary level. With advanced aging, there are reproductive changes, which are more pronounced in females. In comparison, aging effects on the male reproductive axis are much less pronounced, and reproductive potential is maintained until very late in life. During the reproductive aging process, every level of the hypothalamic-pituitary-gonadal (HPG) axis undergoes changes in structure, function, and synthesis/release of regulatory hormones. In addition, ovarian and testicular hormones exert feedback actions on the hypothalamus and pituitary, which may also change with aging. Although reproductive aging occurs within the broader context of somatic aging, but how the reproductive systems of females and males change with age is also very important in terms of physiological functions, hormones, and underlying mechanisms.

8.2 Aging and Thyroid Hormones

Thyroid gland functioning and Hypothalamo-hypophyseal-thyroid (HPT) axis are known to undergo numerous changes with the ageing process (Maudsley et al. 2012; Stan and Morris 2005). Thyroid functions have been extensively studied in relation to the cognition and its pathological conditions and TSH is the main biomarker in cognition studies. The level of TSH declines with age and impacts the processes such as maturation of neurons, mitochondrial enzyme activity (Tan and Vasan 2009). Congenital hypothyroidism is a common pathological condition related to aging process which is the outcome of insufficient secretion of the thyroid gland hormones and often mental deficiencies are observed (De Escobar et al. 2004). Most of the animal studies have reported a decrease in circulating T4 levels with old age, whereas, changes in TSH and T3 are less consistent with age and are more gender and strain specific (Stan and Morris 2005).

8.3 Aging and Oxytocin

Literature reports very few studies on the role of ageing on the mammalian oxytocin system and majority of these reports relied on the autopsy studies of the neural tissue of the PVN and the supraoptic nuclei. Autopsy studies of the aged person suffering from Alzheimer's disease showed mixed resuts with some groups showing decrease in oxytonergic activity while others failed to show any such effect (Wierda et al. 1991; North et al. 1992). One more study of the aged rodents showed the decrease in central oxytonergic activity mainly including the reduced oxytocin responses to stress (Keck et al. 2000) as well as the reduction in the oxytocin receptors numbers frome various regions of the brain (Arsenijevic et al. 1995). Moreover, although animal studies have provided sufficient evidence for age-related decreases in oxytonergic activity but human reports have so far provided mixed evidence. There are also reports on age-related changes in hormonal and neurotransmitter systems interacting with the oxytonergic system, particularly reduced levels of gonadal steroids and dopaminergic functions (Rehman and Masson 2001), which may be responsible to alter the functional consequences for the oxytocin system. Huffmeijer et al. (2012) proposed that even if ageing adversely affects the human oxytonergic system, but this does not necessarily reflect that the oxytonergic system is not involved in social behavior and social cognition. Recently Sannino et al. (2016) and Colonnello et al. (2016) have reviewed the role of oxytocin in three temporal distinct periods of life-early postnatal period, puberty/adolescence, and old age. The available literature reports suggest that oxytocin potentially contributes to maintain social capacities of aged people and may help to ameliorate socially emotional deficits as well as symptoms of neurodegenerative diseases.

8.4 Growth Hormone Level as a Marker of the Aging Process

GH is the most widely associated candidate hormone as a biomarker of aging as the level of this hormone decreases with age. Both the secretion of GH by the pituitary gland, and its receptors become unresponsive to the hormone with age. This reduction in the GH level is also suggested to be responsible for age-dependent accumulation of adipose tissue and reduced muscle mass with decrease in the mineral content in bones (Corpas et al. 1993). The existence of a potential hypothalamic releasing hormone regulating GH secretion from pituitary was demonstrated by Reichlin (1961), who for the first time identified that lesions of the VMN in the rat hypothalamus resulted in poor growth and a reduction in GH content of the pituitary. Further this evidence was supported by observations that the addition of rat hypothalamus extract increased GH secretion from cultured rat pituitaries, but rat cerebral cortex extract had no effect at all (Deuben and Meites 1964). A close association between acromegaly and extra-pituitary tumours was also recognized in the 1960s. It was in 1982 when three different peptides [GH-releasing factors, GRF(1–44)NH_2, GRF(1–40)OH and GRF(1–37)], were purified and sequenced from pancreatic tumours removed from acromegaly patients (Guillemin et al. 1982). Two of these peptides were subsequently identified within the human hypothalamus (Bőhlen et al. 1983) with the major form being GRF (1–44) NH_2 and given the name GHRH. GH plays very important role in controlling IGF-1 biosynthesis and exerts important actions on insulin secretion and also on responsiveness of target tissues to action of insulin. A major role of IGF-1, insulin and homologous molecules in the regulation of lifespan has been conclusively reported in wide range of organisms from worms and insects to mammals.

Suh et al. (2008) reported that mutations of the human IGF-1 receptor which reduced cellular responses to IGF-1 and resistance was associated with shorter stature and extended longevity. These key findings strongly suggests that the reduced somatotropic signaling can lead to increased life expectancy in the human. Further reduced levels of IGF-1, partial IGF-1 resistance and mutations suppressing IGF-1 signaling downstream from the IGF-1 receptor also suggests that GH signaling can accelerate aging and shorten lifespan although reported only in females (Selman et al. 2008). Age-related reductions in GH secretion in rats and humans have been suggested to be the result of decrease in GHRH secretion (Russell-Aulet et al. 1999). Old rats show lower pituitary GH mRNA, GH content as well as down regulation GHRH receptor expression. Many recent studies in animal models and human subjects provide a great body of evidence on the role of an attenuation of the GHRH/GH/Insulin-like growth factor-1 (IGF-1) axis in the control of mammalian aging (Reviewed by Steyn et al. 2016).

8.5 Reproduction and Gonadotropin-Releasing Hormone (GnRH)

Hypothalamus is the key regulation center of reproduction and produces the decapeptide Gonadotropin Releasing Hormone (GnRH). Mammalian reproduction is regulated by interactions between the hypothalamus, pituitary gland and gonads. Each component of the reproductive system is regulated by feedback mechanisms coordinating the processes resulting to gonadotropin secretion, gamete production and maintenance of the species (Conn and Crowley 1994; Ojeda et al. 2006). In most mammalian species, GnRH neurons are distributed in the preoptic area and adjacent sites in the rostral region of the hypothalamus, rather than concentrated in a discrete nucleus. These scattered neurons are believed to form a diffuse neural network that functions coordinately as a GnRH pulse generator (Knobil 1990). The generation of pulsatile GnRH release at the median eminence is the central and essential element governing reproductive function, and depends on the coordinated activities of the 1500 or so GnRH neurons that are located in the hypothalamus (Wray 2001, 2010; Herbison et al. 2008).

8.6 The GnRH System

GnRH was discovered in the early 70s when two groups, Dr Schally's and Dr Guillemin's, respectively, published the primary structure of a decapeptide, named Luteinizing Hormone Releasing Hormone (LHRH) and capable to release LH. This discovery was rewarded with the Nobel Prize few years later (1977). As this molecule was able also to induce Follicle- Stimulating Hormone (FSH) release, it was called GnRH for gonadotropin-releasing hormone. Pioneer experiments had already started to put in evidence the importance of the GnRH system in the control of reproduction, even before its discovery by Schally and Guillemin. In fact, in 1950s, Donovan and Harris demonstrated that cutting the pituitary stalk in female ferret caused the loss of female cyclicity, but reconnections of the portal vessels between median eminence and pituitary reversed this condition (Donovan and Harris 1954). The expression pattern of GnRH peptide was evidenced by the production of the first GnRH antibody that permitted the presence of GnRH fibers at the level of median eminence (Barry and Dubois 1974).

GnRH cells arise from the nasal placode and migrate into the brain to become integral members of the hypothalamic-pituitary-gonadal axis. Disruption of either the development or regulationof the GnRH system results in reproductive dysfunctions. In 1989, two independent groups (Schwanzel-Fukuda and Pfaff 1989; Wray et al. 1989) proposed that GnRH neurons arise from an extra-CNS region (the olfactory placode) and during prenatal development migrate from nasal regions into the forebrain along olfactory/vomeronasal axons. Once GnRH neurons have reached the hypothalamus, they project their axons to the median eminence. Here they release GnRH hormone into the pituitary portal vessels to induce the secretion of pituitary gonadotropins into the general circulation.

Pulsatile LH release in rodents is altered by aging, and the age-related decrease in pulsatile LH secretion is controlled by LH pulse generator. Pulsatile LH release decreases during aging in ovariectomized female rats, due to absence of feedback pulse and as a result increased LH pulse (Scarbrough and Wise 1990). As gonadectomized aged animals exhibit markedly reduced pulsatile LH secretion, it was proposed that the age-related reduction of LH pulses is not the result of change in sensitivity of the gonadal steroid negative feedback, but rather may be due to deterioration of the LH pulse generating system.

Hypothalamic arcuate nucleus (ARC) express 3 different peptides, kisspeptin, neurokinin B (NKB), and dynorphin, termed KNDy neurons and their coexpression has been reported in several species, including rats, mice, goats, sheep, and humans (Wakabayashi et al. 2010). Several parallel evidence suggests that ARC neurons coexpressing these three different peptides are the LH pulse generator. Kisspeptin, a neuropeptide encoded by the Kiss1 gene, is a strong activator of GnRH neurons and is required for episodic GnRH release (Goodman and Lehman 2012). Recent study revealed that the attenuated LH secretion in aging female and male rats was associated with reduced numbers of kisspeptin, NKB, and dynorphin neurons in the ARC. These results suggest that the decreased NKB and dynorphin expression in aged animals causes the reduction of kisspeptin release from ARC kisspeptin neurons. Therefore, reduced expression of kisspeptin, NKB, and dynorphin in the ARC and/or change in pituitary responsiveness may cause attenuated LH levels in aged rats (Kunimura et al. 2017).

8.7 Aging, Menopause and Reproduction: Role of Steroid Feedback Mechanism

Age-related changes in the hypothalamus and pituitary are known to play a critical role in reproductive aging in rodents, and it has been hypothesized that parallel changes may contribute to reproductive aging in women (Zapantis and Santoro 2002). In this section, we will focus on experimental evidence of what is known about hypothalamic aging and regulation by steroid hormones during reproductive aging in female models. The decline in ovarian function is associated with dramatic fluctuations in ovarian feedback on the central components of the reproductive axis during the menopause transition in women thus making it very difficult to determine the potential contribution of aging of the hypothalamus and/or pituitary per se to reproductive senescence. The cessation of ovarian function may then create an open-loop setting so that the hypothalamic and pituitary components of gonadotropin secretion can be examined as a function of aging. A 30–40% decrease in both LH and FSH levels has been reported after menopause thus providing evidence that aging itself influences the hypothalamic and/or pituitary components of the reproductive axis.

The GnRH system has been investigated for age associated changes and the data suggests little to no loss of GnRH perikarya numbers and no change in their distribution. On the other hand, some properties of GnRH neurons may change with aging such as their morphology, cytoarchitecture, and ultrastructure, thus suggesting that alterations in properties of cells such as their ability to synthesize or release the GnRH peptides may occur. Finally, GnRH transcriptional activity, studied by co-expression of GnRH perikarya with the immediate early gene Fos or its gene product, is decreased in middle-aged compared to young rats during the preovulatory surge (King and Rubin 1994). As a whole, these studies suggest a loss of activation of GnRH neurons in middle-aged rodents, prior to a loss of ovarian follicular complement. The fact that GnRH changes precede ovarian changes suggests at least some causality of the hypothalamus in reproductive senescence in rodents.

Females of most other spontaneously ovulating species have estrous cycles that may differ substantially in length and in hormonal and physiological changes from these processes in women. Rodents are an established laboratory model of reproductive aging, but rats and mice differ fundamentally from humans both in having a short estrous cycle (4–5 days) compared to the much longer menstrual cycle (−28 days) and in that their ovaries maintain viable follicles until relatively late in life (Kermath and Gore 2012). Despite the presence of viable follicles, rodents undergo a transition from regular reproductive (estrous) cycles to irregular cycles to acyclicity at middle age, (LeFevre and McClintock 1988) indicating that the neuroendocrine system is driving this process independently of follicular loss (Kermath and Gore 2012). Further-more, transplantation studies of ovaries from aged to young ovariectomized rodents show that ovaries from old donors can begin to function in the young hosts, and undergo folliculogenesis and ovulation. These findings suggest that reproductive failure with aging in rodents is not limited by the ovary. Therefore, rat and mouse models are useful in that they have enabled insight into how the hypothalamus changes with aging, both independent of and dependent upon gonadal steroid hormonal changes.

Basic research on reproductive aging is hampered by limitations in the availability of aging animals; the high cost of maintaining and/or purchasing such animals; and morbidity and mortality associated with aging. Thus, alternative models have been developed such as ovariectomy or orchidectomy to model loss of gonadal hormones. While these experimental models are extremely important, they are typically conducted in young animals. Because young and aged animals (and humans) differ in both hormone-dependent and -independent manners, there is a great need to conduct experiments in age-appropriate models. Another experimental limitation in females is that few species experience menstrual cycles or undergo a true menopause, and those that do typically experience menopause relatively later in life than in humans. For example, in the rhesus macaque it has been estimated that females undergo menopause at the equivalent age of a 65–90 year-old woman, compared to the typical age of 45–55 years in women. Thus, new models of menopause are needed that better approximate the hypothalamic, pituitary, and ovarian changes in women.

The onset of irregular cycles marks the transition from the late reproductive years to the menopause transition. Large-scale cohorts have provided important insights into the overall pattern of hormonal changes that occur through this final transition to the end of reproductive life. The fact that the pituitary response to GnRH is attenuated with aging may also contribute to the approximately two-thirds of cycles in the year before the final menstrual period that are either anovulatory or have prolonged follicular phases (Van Voorhis et al. 2008), resulting in irregular bleeding patterns along with varying breast tenderness, hot flashes, sleep disturbance, and possible mood changes.

In women, ovarian aging per se is central to reproductive senescence. Studies to date suggest that both genetic and environmental factors play a role, alone or in combination, but such studies are in their infancy. Future research that seeks to understand the genetic and environmental factors that prolong the health of and their supporting structures or hasten their demise will be required to extend reproductive capacity to meet the social needs of women in industrialized countries. There is now evidence that neuroendocrine aging occurs in women as well as animals. It will be important to determine the degree to which modification of these changes can extend reproductive life. Markers of ovarian aging have been identified in infertility populations, but further studies will be required to determine if these markers can predict menopause in the general population. Finally, further studies on the effects of the loss of reproductive hormones on non-reproductive systems including the brain (cognition, sleep, vasomotor symptoms), metabolism, bone, and cardiovascular disease will be critical to healthy aging in women as will be development of individualized approaches to treatment that optimize risk and benefit based on genetic and environmental information.

Thus, new models of menopause are needed that better approximate the hypothalamic, pituitary, and ovarian changes in women. Aging has more modest effects on the HPG axis of men than women. The most significant changes occur peripherally at the level of the testis, with steroidogenesis being impacted much more than spermatogenesis. Further studies are needed to dissect the impact of age versus age-related diseases on the HPG axis, as well as to determine the role of changes in semen parameters to the modest decline in fertility seen in older men. Further studies will be required to determine whether optimal aging in men is associated with age-related norms or is better achieved with replacement to levels in young men. Perimenopause is a midlife transition state that leads to reproductive senescence in women. Worldwide, >850 million women are currently aged 40–60 years, 88% of whom will transition through perimenopause at an average age of 51.4 years with a Gaussian distribution of 40–58 years. Regardless of ethnicity, geographic loca-tion or culture, all women who reach the age of 60 years with their reproductive organs intact will transition through perimenopause state to menopause with an average duration of 1–5 years from start to completion (Reviewed by Brinton et al. 2015).

8.8 Role Played by Different Neurotransmitters

Glutamate is the predominant excitatory neurotransmitter in the brain, and a well-known stimulatory regulator of GnRH Glutamate's actions on GnRH neurons occur through N-methyl-D-aspartate receptors and non-NMDARs (Eyigor and Jennes 2000). While initially controversial, it is now accepted that GnRH neurons co-express different classes of glutamate receptors (Miller and Gore 2002), and sub-populations of GnRH neurons respond to NMDA and non-NMDA pharmacological agents in electrophysiological studies of relevance (Iremonger et al. 2010).

The co-expression of GnRH neurons with glutamate receptor protein has reported stoichiometric changes in the ratio of receptor subunits, especially NR2a and NR2b, in aging compared to young female rats (Miller and Gore 2002). Thus, the stimulatory drive to GnRH neurons from glutamate decreases in the aging rodent hypothalamus. Interestingly, a pharmacological study on the interplay among glutamate, kisspeptin, and GnRH signaling has reported that glutamate and kisspeptin may modulate each other's activity on GnRH cells and contribute to the loss of GnRH output with aging (Neal-Perry et al. 2009). There are a few reports about another key amino acid neurotransmitter in the brain, GABA, and its regulation of the GnRH system during reproductive aging. In general, this data suggest increased GABAergic signaling in the aging hypothalamus. Measuring GABA release in the POA using microdialysis showed higher levels in middle-aged as compared to young female rats. Elevated gene expression of an enzyme involved in GABA bio-synthesis, glutamic acid decarboxylase 67 (GAD67), was shown in middle-aged compared to young rats (Grove-Strawser et al. 2007).

8.9 Male Reproductive System and Aging

Reproductive aging in men exhibits a number of striking differences from the female. While the association between advanced maternal age and declining fertility has been unequivocally established, the role of paternal age is more controversial (Langheinrich et al. 2012). The impact of aging on fertility and spermatogenesis in men has been comprehensively reviewed in (Handelsman 2006). The most fundamental difference between reproductive aging between males and females is that unlike the fixed complement of follicles seen in women, the germinal epithelium of the male can continue to generate fresh gametes throughout life. Accordingly, the reproductive lifespan of men may be as much as several decades longer than their female counterparts. Second, while aging in the female is characterized by an inexorable decline in ovarian function with follicle exhaustion following a relatively predictable time course, the process in the male is more modest and gradual and shows a high degree of variability. Third, while gametogenesis and steroidogenesis are very tightly coupled in the ovary and are both susceptible to the impact of aging, the same is not true in the male in whom aging tends to have a greater impact on testosterone production than spermatogenesis (Kidd et al. 2001).

Although aging in males cause a modest decline in testosterone levels, data from several studies highlight that age-related increases in obesity and chronic illness as well as smoking and marital status have a significant impact on circulating testosterone levels (Travison et al. 2007). Obesity is considered to have a greater impact on testosterone levels than age alone and that a 4–5 kg/m^2 increase in body mass index (BMI) is associated with declines in total serum testosterone comparable to that seen with approximately 10 years of aging (Travison et al. 2007). These data imply that the apparent age-related decline in testosterone may not be an inevitable consequence of the aging process but rather reflect health and lifestyle factors and thus be potentially preventable and/or reversible. Aging in men is associated not only with a reduction in absolute levels of testosterone but also a blunting of the characteristic diurnal rhythm in the secretion of this steroid in young, healthy males. The physiological significance of the diurnal rhythm in testosterone secretion is unknown.

Reproduction is the most important function of an organism's life not only for perpetuation but also for species survival. One key feature of the Reproductive-Cell Cycle Theory of aging is that the hormones that regulate reproduction act in an antagonistic pleiotrophic manner through cell cycling signaling to control aging (Atwood and Bowen 2011). Reproductive hormones promote growth and development of species to achieve reproduction early in life, but later in life as age progresses, hormonal control in order to maintain reproduction, become dysregulated and drive senescence and aging. Atwood and his team suggest that since hypothalamic-pituitary-gonadal axis hormones regulate cell division, differentiation and death, therefore may be involved in the regulation of growth and development early in life with an aim to achieve reproduction, but as reproductive function starts declining progressively, the associated endocrine dyscrasia (dyotic signaling) drives senescent phenotype, age asssociated diseases and ultimately removes non-reproductive individuals from the gene pool.

8.10 Aging and Disease: Role of Hormones

Endocrine dyscrasia which is associated with gonadal cell loss is known to initiate senescence and age-related diseases. It is very important to understand the pathophysiology of age-related diseases as they account for ~80% of all deaths, the remainder 20% is largely the result of accidents and infections. Infections again are positively correlated with age (CDC National Vital Statistics Report 2009). Functional HPG hormone signaling is necessary for normal growth and development during embryogenesis, fetal life, childhood, adolescence, and for the maintenance of general health during adult reproductive lifespan. The loss of sex steroids following menopause in females and during andropause in males results in unopposed elevations in GnRH, gonadotropin [LH and follicle-stimulating hormone (FSH)]. This may lead to dysregulation of cell cycle events keeping in view the proliferation and differentiation properties of these hormones (Bowen et al. 2004; Cole 2009; Gallego et al. 2009).

The signaling resulting from reproductive endocrine dyscrasia in women commences around the time of menopause (~51 years of age), while in males andropause commences around 30 years of age when testosterone levels start declining at the rate of 1% every year between the ages of 30–80 (Belanger et al. 1994) with corresponding increases in circulating gonadotropins. Thus, males are under the influence of these lower testosterone and higher LH levels for many decades before they enter into hypogonadism phase (the clinical definition of andropause). Several studies in the recent past present evidence at molecular, epidemiological and clinical aspects towards reproductive endocrine dyscrasia-induced cell cycle changes as important risk factors of age-related diseases such as AD/dementia (Atwood et al. 2005), stroke (Wilson et al. 2007), osteoporosis (Sun et al. 2006), heart disease (Lee et al. 2009) and cancer. The basis for this association are also attributed to the identification of receptors for HPG axis hormones in many different body tissues (reviewed in: Bowen et al. 2004; Vadakkadath Meethal and Atwood 2005). HPG axis hormones and receptors are evolutionarily conserved throughout reproductive organisms as the orthologs having been identified in flies (*D. melanogaster*), worms (*C. elegans*), yeast (*Saccharomyces cerevisiae*) and plants (Bowen et al. 2004; Vadakkadath Meethal et al. 2006). Therefore reproduction is the most important function of an organism from the perspective of species survival.

8.11 Conclusion

In this chapter we have made an attempt to compile information on the various hormonal changes that occur with age and how hypothalamus and pituitary gland slowly lose their sensitivity with the increasing age. Much is still to be done to understand the basic mechanisms of hormones and aging and to understand the mysterious link of age associated diseases. Neurodegenerative diseases such as AD, PD, and HD may not only due to the changes in some biochemical and molecular factors but also attributed to lack of hormonal activity due to the gradual alterations/loss of the endocrine mechanisms with aging. Several studies in literature affirm that the age related changes in the hypothalamus are manifested in many disorders. We also need to carry out more extensive research to understand how the structure-activity relationship of many commercially available synthetic hormonal drugs correlates to their potential beneficial effects in humans suffering with various age related diseases. Women, as they age, become prone to the cumulative effects of menopause, adiposity, and inflammation that is associated with the depletion of estrogens and bioenergetic dysregulation. The association between hypometabolism and the neurological symptoms of the perimenopause suggests that this transition state is a critical period in the neuroadaptive landscape of ageing in the female brain and may provide a window of opportunity to intervene and prevent age-related neurological diseases in post-menopausal women. This will be of importance when determining the efficacy of sex-hormone replacement strategies and diets rich in sex-hormone active compounds when it comes to preventing AD in women.

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Chapter 9
Environmental and Physiological Cues on the Hypothalamus During Aging

Jan O. Nehlin

Abstract The hypothalamus is a specialized tissue in the brain responsible for the central regulation of hormone production in the body linking the nervous system with the endocrine system. The hypothalamus regulates development, growth, and metabolism, and is considered to have a key role in the progression of whole-body aging. Multiple environmental and physiological signals can adversely affect hypothalamic function leading to cellular senescence. The timing and duration of these signals, the heterogeneity of the hypothalamic neurons involved and the individual genetic background, together, determine the optimal functional health span of the hypothalamus. Epigenetic effects on hypothalamus regions in early life may already influence health outcomes later in life. The consequences of detrimental changes anytime during lifetime could have a tremendous impact on health and metabolic function and ultimately lifespan. A summary of the possible molecular causes of aging of the hypothalamus as well as the impact that age-related disorders have on the functional regulation of hypothalamic neurons is discussed. A great number of physiological and environmental cues, with relevance to aging, influence hypothalamus function.

Keywords Aging · Hormone · Hypothalamus · Lifespan · Senescence · Stress

9.1 Introduction

The hypothalamus is a brain organ located below the thalamus and sits just above the brainstem. It consists of four regions and three zones (Fig. 9.1). The hypothalamic-pituitary system governs the neuroendocrine control of many complex functions including growth, reproduction, osmoregulation, stress and metabolism

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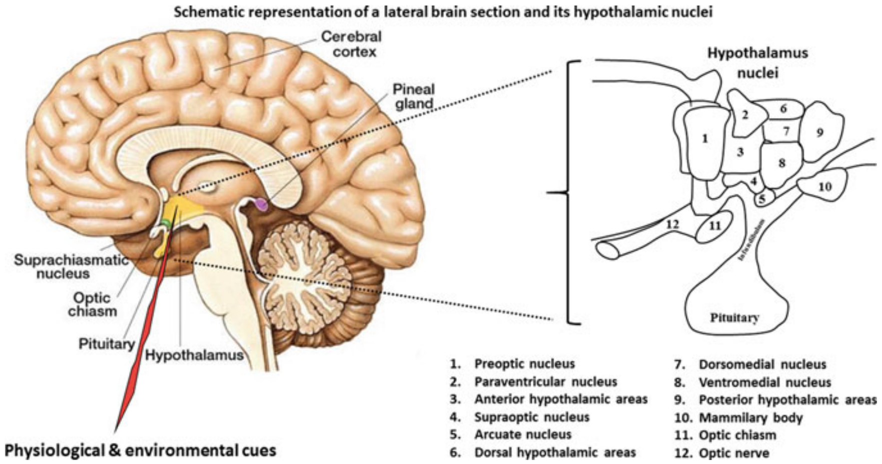

Fig. 9.1 Schematic representation of a lateral brain section and its hypothalamic nuclei. The hypothalamus is composed of three longitudinally oriented cell columns, or zones, that run the entire rostrocaudal length of the hypothalamus. The three areas or zones are the periventricular and intermediate zones (medial) and lateral zones. These zones can be further subdivided into four nuclear groups, or regions, based on the rostral to caudal position, starting with the pre-optic region, the supraoptic or anterior region, the tuberal or middle hypothalamus and the mammillary or posterior hypothalamus. There are eleven major nuclei in the hypothalamus, grouped based on their locations in the hypothalamic zones and regions. Each nucleus has a specialized function [Based on Braunstein, G.D. Chap. 19 In: DeGroot and Jameson (2006)]

(DeGroot and Jameson 2006; Norman and Litwack 1997), and is considered to be a vertebrate innovation and seminal event that emerged prior to or during the differentiation of the ancestral agnathans or jawless fish (Sower et al. 2009). The development of the hypothalamo-pituitary axis is a highly complex and organized process that involves multiple signaling molecules and transcription factors arranged in a spatio-temporal fashion (Biran et al. 2015; MacKay and Abizaid 2014; McCabe and Dattani 2014). Developmental abnormalities in specific hypothalamic circuits can lead to obesity, sleep disorders, anxiety, depression and autism (Biran et al. 2015). Hypothalamic dysfunction is intimately associated with aging (Batrinos 2012). Hypothalamic dysfunction can also be a driver of Alzheimer's disease pathology with a dual role of the hypothalamus as both a culprit and target of the disease pathology (Ishii and Iadecola 2015). Many pathologic processes have been described that give rise to hypothalamic syndromes (DeGroot and Jameson 2006).

The hypothalamus main function is to maintain the body's metabolic balance by means of its numerous hormones and contributes to compensate the physiological changes that inevitably take place during the aging process. Several input signals are relayed from the body to the hypothalamus to allow it to monitor environmental cues and internal body functions, in a timely and trouble-free manner (Saper and Lowell 2014).

Blood pressure and gut distension visceral sensory information, skin temperature, light and darkness visual information, smells and emotions, sensory information, body weight, appetite, hunger and thirst, libido, etc. are relayed to the hypothalamus either directly or through nerve projections from other brain areas (DeGroot and Jameson 2006). As a result, these input signals generate a hypothalamic response as output signals that control energy metabolism, from feeding through digestion, metabolic control, and energy expenditure; fluid and electrolyte balance, from drinking through fluid absorption and excretion; thermoregulation, from choice of environment through heat production and conservation, and fever responses; wake-sleep cycles and emergency responses to stressors in the environment; and reproduction, from reproductive hormone control through mating, pregnancy, birth, milk production and suckling, etc. (Saper and Lowell 2014). The output signals can be either neural or endocrine, acting as compensatory signals to bring the state of the body back to the individual set-points. The neural signals towards the autonomous sympathetic system in the spinal cord are relayed by lateral hypothalamus neurons to the medulla, influencing typically autonomic systems such as heart rate, digestion, vasoconstriction, sweating, etc. The endocrine signals are relayed by neurons that connect with the anterior or posterior pituitary gland, to secrete a variety of hormones that affect a great number of body organs and systems (DeGroot and Jameson 2006; Norman and Litwack 1997; Saper and Lowell 2014).

The hypothalamus operates in concert with the pituitary gland to control the secretion of various hormones by the release of its hormone-releasing hormones and inhibiting hormones that stimulate or inhibit, respectively, the production of hormones in the anterior pituitary. This nervous-endocrine system interface facilitates the amplification from the femto- and pico-molar concentrations of hypophysiotropic factors/hormones to the nano-molar concentrations of pituitary hormones, and temporal smoothing, from the ultradian pulsed secretion of hypophysiotropic hormones to the circadian rhythms of pituitary hormone secretion. Importantly, the function of this interface is modified by feedback, usually negative, via the nervous system and via the endocrine system (Lim et al. 2000).

Hypothalamic disease may cause insufficient or inhibited signaling to the pituitary leading to hormonal deficiencies. A list of causes of hypothalamic dysfunction is listed in Table 9.1 [Adapted from Braunstein, Chap. 19, In: DeGroot and Jameson (2006)].

A recent updated review of hypothalamus function has challenged the early stimulus-secretion coupling paradigm. The hypothalamus contains no more than a few thousand parvocellular neurons and controls more known body functions than any other brain region. The activity of hypothalamic neurons is modified by inputs leading to heterogeneous activity indicating that only small numbers of neurons can induce pituitary hormone pulsatility. Moreover, secretion of hypothalamic factors can vary following neuron excitation according to the physiological status, which might also lead to declining neuroendocrine output with age. The factor's release

Table 9.1 Causes of hypothalamic dysfunction

Congenital acquired	Congenital genetic (familial or sporadic)	Tumors: Primary intracranial	Infectious: Bacteria	Trauma	Vascular	Immunologic	Functional
Developmental malformations	Hypothalamic hypopituitarism	Angioma of the third ventricle	Meningitis	Bleeding	Aneurysm	Idiopathic diabetes insipidus	Diencephalic epilepsy
Anencephaly	Familial diabetes insipidus	Craniopharyngioma		Birth injury	Arteriovenous malformation	Paraneoplastic syndrome	Drugs
Porencephaly	Prader-Willi syndrome	Ependymoma	**Infectious: Mycobacteria**	Head/brain injury	Pituitary apoplexy	Infections and swelling (inflammation)	Hayek-Peake syndrome
Agenesis of the corpus callosum	Bardet-Biedl and associated syndromes	Ganglioneuroma	Tuberculosis	Post-neurosurgical	Subarachnoid hemorrhage		Idiopathic syndrome of inappropriate
Septo-optic dysplasia	Wolfram's syndrome	Germ cell tumors				**Hormonal**	Secretion of antidiuretic hormone
Suprasellar arachnoid cyst	Pallister-Hall syndrome	Glioblastoma multiforme	**Infectious: Spirocheta**	**Degenerative**	**Nutritional/Metabolic**	Hypothyroidism	Kleine-Levin syndrome
Colloid cyst of the third ventricle	Hemochromatosis (iron buildup)	Glioma	Syphilis	Glial scarring	Anorexia nervosa or bulimia	Adrenal insufficiency	Periodic syndrome of Wolff
Hamartoma	Kallmann syndrome	Hamartoma		Parkinson's disease	Kernicterus	Gonadal deficiency	Psychosocial deprivation syndrome
Aqueductal stenosis	Cushing's syndrome	Hemangioma	**Infectious: Vira**		Wernicke-Korsakoff syndrome	Growth hormone deficiency	
Trauma		Lipoma	Encephalitis		Weight loss		**Other**
Intraventricular hemorrhage		Lymphoma	Jakob-Creutzfeldt disease		Malnutrition	**Infiltrative**	Radiation eg. X-rays, UV, etc.
		Medulloblastoma	Kuru		Hypothalamic obesity	Histiocytosis	Porphyria
		Meningioma	Poliomyelitis			Leukemia	Chemical exposure e.g. toluene
		Neuroblastoma	Varicella			Sarcoidosis	
		Pinealomas	Cytomegalovirus infection				
		Pituitary turners					
		Plasmacytoma					
		Sarcoma					
		Metastatic tumors					

into the blood can be modified by alterations in the juxtaposition of nerve terminals with the vasculature and tanycytes (see Sect. 9.3.3) in the median eminence. A range of endocrine axes defects in hypothalamic-vasculature-pituitary function arise with age which could be target for interventions (Le Tissier et al. 2017).

Age progression has profound impacts on physical and mental health. Age-related gene expression is brain region-specific, genotype-dependent, and associated with both mean and dispersion changes. The genes affected are involved in apoptosis, mRNA splicing, aminoacid biosynthesis, and neurotransmitter transport (Brinkmeyer-Langford et al. 2016). Single-cell RNA sequencing of adult mouse hypothalamus helped define 11 non-neuronal and 34 neuronal cell clusters with distinct transcriptional signatures that will contribute to dissect cell-type specific functions (Chen et al. 2017a).

Analyses of hypothalamic regions including total unilateral hypothalamus, suprachiasmatic nucleus (SCN), supraoptic nucleus (SON), paraventricular nucleus (PVN), dorsomedial nucleus (DM), ventromedial nucleus (VM), medial mammillary nucleus (MMN), and lateral hypothalamic area (LHA) during aging in rhesus monkeys concluded that there was no age-related difference in neuron number, glia number, or volume in any area in either sex except the PVN of male monkeys, which showed a significant increase in both neuron and glia numbers with age. Neuron loss cannot account for age-related deficits in hypothalamic function and provides further evidence of the absence of neurodegeneration and cell death in the normal aging rhesus monkey (Roberts et al. 2012). Also, most pituitary hormones are altered by aging in humans, often in a manner dependent on sex, body composition, stress, comorbidity, intercurrent illness, medication use, physical frailty, caloric intake, immune status, level of exercise, and neurocognitive decline (Veldhuis 2013).

Research into hypothalamus function is constrained by its relative inaccessibility, its central role in whole-body functions and incomplete knowledge of all the various highly-specialized cell subsets within the organ. Most knowledge about its function stems from genetic and pharmacological studies in mice and rat animal models. Progress has also been achieved in establishing immortalized cell lines from both mouse and human hypothalamic areas. The generation and use of hypothalamic cell models would allow the study of the mechanisms underlying the function of individual hypothalamic neurons and to gain a more complete understanding of the overall physiology of the hypothalamus (Dalvi et al. 2011; Mayer et al. 2009). More recently, functional assays have been used to select for e.g. mouse hormone leptin-responsive hypothalamic neuronal clones based on the phosphorylation levels of STAT3 induced by leptin, after having become immortalized. This helps to analyze the molecular nature of signal disturbances in defined areas of the hypothalamus that occur in disease states and during aging (Iwakura et al. 2016).

9.2 Possible Molecular Causes of Aging of the Hypothalamus

9.2.1 Telomere Shortening and Regeneration

Telomere shortening occurs following cellular division and is a hallmark of replicative senescence (Harley et al. 1990). The question arises whether cells in the hypothalamus undergo telomere shortening and if there is any cellular regeneration in the organ. The amount of radiocarbon present in the brain due to nuclear bomb testing during 1955–63 is a remarkably accurate indicator of when a person was born, with an error margin of 1.6 years. It was determined that the tissue ^{14}C concentrations in brain areas such as the cortex or the olfactory bulb correspond to the atmospheric levels at the time of birth of the individuals, establishing that there is very limited, if any, postnatal neurogenesis. These results showed that such neurons are as old as the person (Bergmann et al. 2012; Spalding et al. 2005). Limited postnatal cell turnover in a specific tissue results in stable cell populations with telomere lengths well above the telomere length required to elicit cell senescence. The capacity to regenerate e.g. adult mouse adult pituitary in response to injury by means of stem cell/progenitor cell activation exists (Fu et al. 2012), among tanycytes in the rat hypothalamus (Chauvet et al. 1998) and Fgf10-expressing tanycytes in the mouse hypothalamus that add new neurons to the arcuate nucleus regulating appetite and energy balance (Haan et al. 2013) (see Sect. 9.3.3). Hypotalamic neurogenesis persists in the aging mouse brain controlled by insulin-like growth factors (IGF) (Chaker et al. 2016) but very little is known about the extent of regeneration in human hypothalamus (Goodman and Hajihosseini 2015).

The effects of epidemiological and environmental factors, physiological factors, iatrogenic factors, genetic and epigenetic factors can result in telomerase dysfunctions that can lead to chromosomal instability and/or cellular senescence. The association between telomere dysfunction and the hypothalamic–pituitary–adrenal (HPA) axis and GH-IGF-1 system has been reviewed (see also Sect. 9.3.1): most of the evidence linking the telomere system and HPA function has been evaluated in psychiatric diseases (chronic stress, post-traumatic stress disorders and major depression) where hypercortisolism is often present (Aulinas et al. 2013). Stress reactive HPA axis with greater cortisol responses to an acute stressor and/or dysregulated patterns of daily cortisol secretion were associated with shorter telomere length in peripheral blood mononuclear cells from elderly but there seems to be very limited knowledge in the literature regarding impact of environmental insults on the telomere length of hypothalamic cells (Tomiyama et al. 2012). Brain tissue appears to retain long telomere length at least in the case of a 115-year old supercentenarian (Holstege et al. 2014). Studies of telomere length in the human pituitary gland, neighboring the hypothalamus, showed that it was highly preserved throughout adult life to centenarian age (Ishikawa et al. 2012). Further studies of hypothalamic cell subsets will allow further characterization of their telomere lengths during aging (Chen et al. 2017a).

9.2.2 DNA Damage and Oxidative Damage

It may seem surprising that the brain as a whole does not seem to accumulate mutations with age, but certain regions of the mouse brain such as the hippocampus and the hypothalamus are much more susceptible to DNA damage and showed increased mutational loads with advancing age (Busuttil et al. 2007). Base-excision repair (BER) contributes to repair DNA oxidative lesions, and its function decreases with age in the rat brain including the hypothalamus. Interestingly, caloric restriction helped to maintain the activity of BER enzymes in old brains at the level of younger brains (Kisby et al. 2010).

One of the major theories of aging is the oxidative stress (or free radical) theory (Harman 1956). Reactive oxygen species (ROS) are produced mainly in the mitochondria, where aerobic metabolism takes place. The incomplete reduction of oxygen leads to the generation of different radical species such as the superoxide radical (O_2^-). An excessive production of free radicals and ROS in the central nervous system and its related glands, including the hypothalamic-pituitary axis, might be a crucial factor in the aging of these structures and the aging process in general. There is evidence of an imbalance between the production of oxidants and protective antioxidant systems leading to ROS accumulation that might cause cellular oxidative damage in the hypothalamus. Accumulating evidence from in vitro and in vivo models indicates that activation of the Growth hormone/Insulin-like growth factor-1 (GH/IGF-1) system increased the production of ROS and decreased the levels of antioxidants. In addition, an increase in lipid peroxidation with age in the hypothalamus was associated with protein dysfunction and cellular damage. Also, neuronal vulnerability caused by an age-dependent increase in levels of glucocorticoids has been associated with chronic stress and cognitive impairment (Vitale et al. 2013).

A significant age-dependent increase in the generation of free radicals was observed in the hypothalamus and adrenal glands, as well as on lipid peroxidation in the rat hypothalamus, as well as a significant reduction in glutathione peroxidase (GPx) activity and total antioxidant capacity (Rodrigues Siqueira et al. 2005). Also, in diabetic mice, an increase in the levels of enzymes involved in oxidative stress and apoptosis in the hypothalamus has been reported (Baquedano et al. 2016). However, an appropriate quantity of ROS is necessary to maintain insulin signal transduction in the hypothalamus, as evidenced by the findings that insulin-induced phosphorylation of insulin receptor beta (IRβ) and Akt is mediated via ROS which are predominantly produced by NADPH oxidase in the mouse hypothalamus (Onoue et al. 2016).

9.2.3 Mitochondria Defects

Age-related changes in brain energy metabolism and in mitochondrial functionality are considered as factors associated with physiological aging. An enhanced oxidative metabolism during aging has been documented, concomitant to an increase in HPA activity in response to an increase in external stress stimuli during aging. Among the tricarboxylic acid (TCA) cycle enzymes in non-synaptic mitochondria, the activity of succinate dehydrogenase was greatly increased with age. Within the electron transport chain, the activities of cytochrome c reductase and cytochrome oxidase were increased in the old hypothalamus. The activity of glutamate dehydrogenase was also enhanced in old age. Different functional areas of the brain seemed to have a variable biochemical profile (Villa et al. 2012). Attenuated mitochondrial activity and biogenesis capacity during aging is a key factor in progression of age-related diseases (Yuan et al. 2016). Peroxisome proliferator-activated receptor-gamma coactivator-1β (PGC-1β) coordinates mitochondrial biogenesis and function as well as fatty acid metabolism. Ablation of PGC-1β was associated with constitutive activation of the mTORC1 pathway (see Sect. 9.3.3) and with increased basal protein levels of the endoplasmic reticulum chaperone GRP78/BIP in the hypothalamus and cortex of mice fed chow diet (Camacho et al. 2012). Thus, nutritional stress can impose changes to mitochondria dynamics.

Inside the mitochondria, succinate is one of the most prominent intermediates of the TCA cycle. Extra-mitochondrial succinate triggers succinate receptor (SUCN1 or GPR91)-mediated signaling pathways in many peripheral tissues including the hypothalamus. Thus, succinate availability may play an important role in hypothalamus aging (Chen et al. 2015).

Malonyl-CoA, an intermediate in fatty acid synthesis, serves as an indicator of energy status in the hypothalamic neurons that express orexigenic and anorexigenic neuropeptides. It regulates food intake and energy expenditure. Malonyl-CoA is an inhibitor of carnitine palmitoyl-CoA transferase-1 (CPT1), an outer mitochondrial membrane enzyme that regulates entry into, and oxidation of fatty acids, by mitochondria (Lane et al. 2008).

A reduced efficiency of hypothalamic OXPHOS complex I assembly and activity in the anorectic anx/anx mouse model and signs of increased oxidative stress were recorded in the hypothalamus, possibly as an effect of the decreased hypothalamic levels of fully assembled complex I, due to defects in the Ndufaf1 gene, encoding a complex I assembly factor (Lindfors et al. 2011).

9.2.4 Autophagy and Proteostasis Impairment

Autophagy is a conserved cellular turnover process that degrades unwanted cytoplasmic material within lysosomes and is impaired with age. A role for autophagy in hypothalamic agouti-related peptide (AgRP) neurons in the regulation of food intake and energy balance was reported. AgRP is a potent appetite stimulator. Starvation-induced hypothalamic autophagy mobilized neuron-intrinsic lipids to generate endogenous free fatty acids, which in turn up-regulated fasting-induced AgRP levels. Inhibiting autophagy resulted in failure to upregulate AgRP in response to starvation and in constitutive increases in hypothalamic levels of anorexigenic pro-opiomelanocortin and its cleavage product α-melanocyte-stimulating hormone that typically contribute to a lean phenotype (Kaushik et al. 2011; Singh 2011).

Loss of autophagy in hypothalamic pro-opiomelanocortin (POMC: precursor polypeptide to adenocorticotropic hormone ACTH, α-MSH and opioid) neurons decreased α-melanocyte-stimulating hormone (α-MSH) levels, promoted adiposity, impaired lipolysis and altered glucose homeostasis. A reduction with age in POMC hypothalamic autophagy, lipolysis and α-MSH levels was revealed (Kaushik et al. 2012).

Neuropeptide Y (NPY), a neurotransmitter produced in the AgRP neurons, stimulated autophagy in mice hypothalamic neurons and was associated with the concerted activation of the PI3 K, MEK/ERK, and PKA signaling pathways. NPY levels decreased with age (Aveleira et al. 2015a). Since both hypothalamic autophagy and NPY levels decreased with age, modulation of NPY levels could help to ameliorate age-related dysfunctions (Aveleira et al. 2015b).

Cold-induced activation of autophagy in pro-opiomelanocortin (POMC) neurons activated lipophagy and cytosolic lipases in a complementary manner to mediate lipolysis in brown adipose tissue (BAT) and liver in mice (Martinez-Lopez et al. 2016).

Growth hormone (GH) secretion decreased spontaneously during lifespan, and the resulting GH deficiency participates in aging-related morbidity. This deficiency does not involve a defect in the numbers, distribution or activity of hypothalamic GH-releasing hormone (GHRH) neurons but due to an abnormal enlargement of GHRH-positive axons, suggesting neuropeptide accumulation leading to failure of hormone secretion from GHRH neurons. Upon closer examination, ultrastructural changes were detected such as selective impairment of secretory vesicle trafficking in aging GHRH nerve terminals, electron dense precipitates preferentially associated with typical secretory vesicles (100–120 nm in diameter) present in large number within axonal profiles and autophagic vacuoles of various sizes. This appears to be the mechanism most likely to account for the hypothalamic dysfunction of the GH/IGF-1 axis during aging (Alonso et al. 2007). In fact, reduced secretion of GHRH in humans may be the cause of GH secretion decline with age (degli Uberti et al. 1997). The effects of gender, aging, sleep, exercise, glucose levels, nutritional status and negative feedback on GH secretion by the hypothalamus were explored early on (Lim et al. 2000).

The somatotropic axis consists of hormonal signaling pathway from the hypothalamus to the somatotrophs of the anterior pituitary gland resulting in the release of growth hormone (GH), which in turn stimulates the production of insulin-like growth factor-1 (IGF-1) in the liver. Previously, it was believed to only regulate directly proliferation, growth, and the counterregulatory effects on glucose metabolism, but in recent years it has been shown to influence aging-related processes, age-related disease, and ultimately longevity. GH deficiency was implicated in delaying aging and extending life. The natural physiological decline of GH in humans and mammals in general may be a protective mechanism that reduces the incidence of age-related diseases such as diabetes and cancer. Dietary restriction extended lifespan via reductions in somatotropic signaling. A summary of somatotropic axis interventions aimed at extending lifespan has been presented (Brown-Borg 2015). In the long-lived Ames dwarf mice, that are GH-deficient and GH-resistant, disruption of GH signaling modulates hypothalamic inflammation that resulted in hypothalamic changes that contributed to longevity. Disruption of the GH receptor, specifically in liver, with a mutation that reduced circulating IGF-1 but did not lead to lifespan extension, had no effect on hypothalamic projections or inflammation, suggesting an effect of GH, rather than peripheral IGF-1, on hypothalamic development (Sadagurski et al. 2015)

The overall ultrastructure of neurons in the anterior, middle and posterior hypothalamus in young and aged monkeys showed no major differences. Upon closer examination, old neurons from the supraoptic, paraventricular and infundibular regions exhibited a spiral arrangement of the smooth surfaced endoplasmic reticulum, presence of neurosecretory granules, progressive accumulation of lipofuscin with age and degenerative changes in the mitochondria. The young hypothalamus showed more lipofuscin accumulation in the posterior part than in the anterior. In contrast, the aged hypothalamus revealed increased accumulation of pigment in the anterior part as compared to the posterior. Lipofuscin was found close to mitochondria (Glees et al. 1975). Accumulation of lipofuscin with age is a well-known hallmark of aging (Brunk and Terman 2002a) and is associated with inefficient autophagocytosis, which leads to accumulation of damaged mitochondria and senescence (Brunk and Terman 2002b).

Recently, evidence to date was put forward to propose that defective autophagy and proteostasis occurs in the hypothalamus with age. Defective hypothalamic autophagy via the IκB kinase β (IKKβ)/NF-κB signaling pathway directs the central pathogenesis of obesity (Meng and Cai 2011). Hypothalamic neurons were damaged in obesity and during aging due to defective regulation of proteostasis and inflammation. Proteostasis defects included alterations in protein folding, the unfolded protein response, RNA stress granules, the ubiquitin-proteasome system and endoplasmic reticulum stress (Cavadas et al. 2016).

9.3 Physiological and Environmental Cues, with Relevance to Aging, Influencing Hypothalamus Function

9.3.1 Stress

The major neuroendocrine response to stress is mediated by activation of the hypothalamic pituitary adrenal (HPA) axis. Parvocellular neurons of the hypothalamic paraventricular nucleus (PVN) are triggered to release the neuropeptides corticotropin releasing hormone/factor (CRH/CRF) and vasopressin (VP), that stimulate the release of adrenocorticotropic hormone (ACTH) from the so-called corticotrophs of the anterior pituitary gland. ACTH acts upon the cortex within the adrenal glands stimulating the secretion of glucocorticoids (cortisol in humans), that exert a negative feedback on CRH producing neurons to restrain HPA axis activity (Aguilera 2011; Lim et al. 2000). A cohort of parvocellular cells interspersed with magnocellular PVN neurons expressing secretagogin represent a distinct CRH-releasing neuron population. Secretagogin's loss of function occludes ACTH release from the pituitary and lowers peripheral corticosterone levels in response to acute stress (Romanov et al. 2015).

A normal acute stress response shall be rapid and temporary. An increase in glucocorticoid activity and central levels of CRH during aging can have damaging effects and contribute to pathologies associated with advancing age such as depression, anxiety, neurodegeneration, immune and metabolic disorders. The "glucocorticoid cascade hypothesis" and the "glucocorticoid vulnerability hypothesis" propose that impairment of the negative feedback control of the HPA axis lead to long-term exposure of the brain to glucocorticoids that would lead to brain damage (Vitale et al. 2013). A summary of the age- and gender-dependent regulation of the Hypothalamic-Adrenocorticotropic-Adrenal Axis was reviewed (Veldhuis et al. 2013). The pathophysiology of HPA dysfunction and its association with aging-related diseases like depression, cognitive deficits, and Alzheimer's disease in some older individuals has been assessed (Gupta and Morley 2014).

A variety of interactions between physiological biomarkers of HPA axis activity and psychosocial factors contribute to stress resilience in some elderly (Gaffey et al. 2016).

The HPA axis activity throughout life can influence biological aging, not only through inappropriate glucocorticoid secretion but also through alterations in the production of the regulatory peptides, CRH and VP (Aguilera 2011). When the HPA function in the elderly was evaluated by analysis of basal serum levels of adrenal steroids and the response to stimulation with a low-dose ACTH, it was found that there was a decrease of major T lymphocyte subsets together with an increase of NK cells and activated T lymphocytes, and a decline in the steroid hormones dehydroepiandrosterone (DHEA) and dehydroepiandrosterone-sulphate (DHEAS) levels. A close correlation between immune changes with aging and DHEA response to ACTH stimulation was found (Martinez-Taboada et al. 2002).

CRH/CRF, together with its three family members, urocortins (UCNs) 1, 2, and 3, integrates the neuroendocrine, autonomic, metabolic and behavioral responses to stress by activating its cognate receptors CRFR1 and CRFR2, present not only in the pituitary but also in other brain areas and peripheral tissues including reproductive organs and the immune system (Dedic et al. 2017). The adverse effects of glucocorticoids on the immune system has been recently reviewed, and interestingly, many age-associated diseases are included such as cataracts, osteoporosis, obesity, impaired wound healing, hypertension, glucose elevation and immune suppression (Cain and Cidlowski 2017). The discovery that immunosurveillance of senescent cells by immune cells can help to delay the aging process (Sagiv et al. 2016) suggests that the detrimental effects of glucocorticoids on senescence immunosurveillance is one possible mechanism that can explain the accelerated aging in chronically stressed individuals. Also, exaggerated neurobiological sensitivity to threat is perceived as a mechanism linking anxiety with increased risk for diseases of aging (O'Donovan et al. 2013). Hyper-excitation of the stress axis might activate the tumor suppressor p53. This reinforces the loss of stem cell proliferative capacity and interferes with the feedback mechanism by which the glucocorticoid receptor turns off neuroendocrine pathways and resets the axis (Scrable et al. 2009).

Genetic defects in the cAMP–protein kinase A (PKA) signaling pathway can lead to major activation of cortisol secretion in the adrenal cortex. Chronic exposure to hypercortisolism (excessive cortisol production), causes endogenous Cushing's disease, a rare disorder that is associated with many co-morbidities such as systemic hypertension, diabetes, osteoporosis, impaired immune function, and psychiatric disease, all of which severely reduce quality of life and life expectancy (Lodish and Stratakis 2016; Sbiera et al. 2015). Treatment of Cushing syndrome with centrally acting drugs, adrenal steroidogenesis inhibitors, and glucocorticoid receptor antagonists, and possibly several novel molecules being investigated (Cuevas-Ramos and Fleseriu 2014) could also be of interest in the management of the HPA axis with age.

Pseudo-Cushing's states (PCS) have also been described. PCS are a heterogeneous group of disorders, either physiological or non-physiological, that lead to increased cortisol production and stigmata of hypercortisolism. PCS physiological conditions include surgery associated stress, severe illness, emotional stress, intense aerobic exercise and caloric restriction. Stress-related cortisol secretion is associated with lack of diurnal cortisol rhythm, failure to adequately suppress cortisol levels after dexamethasone administration, abdominal obesity, hypertension, hyperlipidemia and insulin-resistance. Non-physiological conditions associated with PCS are amongst others chronic alcoholism and alcohol withdrawal syndrome, major depression, poorly controlled diabetes mellitus, polycystic ovary syndrome (PCOS) and obesity (Androulakis et al. 2000).

A systematic review and meta-analysis, based on studies investigating levels of cortisol, adrenocorticotropic hormone (ACTH) and corticotropin-releasing hormone (CRH) in depressed participants older than 60 and compared with healthy controls, was presented. Older participants suffering from depression showed a high degree of dysregulation of HPA axis activity that could be explained by the presence of physical illnesses, alterations in the CNS and immune-endocrinological alterations (Belvederi Murri et al. 2014).

Epigenetics effects in early life could influence HPA axis activity and stress sensitivity during adulthood and probably in old age. Epigenetic reprogramming of important stress-response genes in the brain persisted throughout adult life. One such gene, *NR3C1*, encodes the receptor for hormones called glucocorticoids. This receptor mediates stress responses through a feedback loop whereby its activation in the hippocampus inhibits neuronal activity in the hypothalamus. Early-life deprivation in rats or a history of early-life abuse in humans were both associated with epigenetic silencing of nuclear receptor subfamily 3 group C, member 1 (NR3C1) encoding glucocorticoid receptor, resulting in heightened sensitivity of the hypothalamus and elevated release of stress hormones (McGowan et al. 2009). Another mechanism might involve direct effects of glucocorticoids on the NF-κB pathway. Although glucocorticoids are typically anti-inflammatory, it has been suggested that they can have the opposite effect in some brain regions and stimulate NF-κB signaling (see Sects. 9.2.4 and 9.3.2).

9.3.2 Inflammation

The systemic inflammation that is associated with aging, also known as "inflammaging", leads to activation of Nuclear Factor-κB (NF-κB) in microglia of many brain areas, especially the hypothalamus, in mice. NF-κB stimulates the production of TNF-α, which, in turn, stimulates NF-κB activity in hypothalamic neurons. These responses result in epigenetic repression of the gene encoding gonadotropin-releasing hormone (GnRH), resulting in reduced GnRH release, which is associated with multiple age-associated phenotypes such as bone loss, skin atrophy, muscle weakness and memory loss. GnRH treatment significantly, although partially, reversed aging-impaired neurogenesis in the hypothalamus. Thus, immune and hormonal responses being integrated in the hypothalamus contribute to the control of systemic aging. This inflammatory-exerted pathway might also mediate the effects of a variety of environmental and physiological stressors (Zhang et al. 2013).

Chronic caloric excess can lead to hypothalamic microinflammation, which in turn participates in the development and progression of metabolic syndrome disorders such as obesity, glucose intolerance, and hypertension. Therefore, NF-κB-dependent hypothalamic microinflammation is considered a common mediator of metabolic syndrome and systemic aging and aging-related diseases. Many studies in rodents have linked neural, endocrine, or metabolic signals to influences on aging and/or longevity (Tang et al. 2015). Overnutrition is associated with chronic inflammation in metabolic tissues. A central regulator of innate immunity and related functions and mediator of metabolic inflammation, IKKβ/NF-κB, remains suppressed although enriched in the hypothalamus under conditions where nutrition is normal. Overnutrition activates IKKβ/NF-κB at least in part through elevated endoplasmic reticulum stress, which translates into central insulin and leptin resistance (see Sect. 9.3.3), leading to energy imbalance, obesity and type 2 diabetes. Thus, dietary obesity could be largely prevented by inhibiting IKKβ/NF-κB (Zhang et al. 2008).

Hypothalamic inflammation disrupts key signaling pathways that affect the central control of blood pressure leading to hypertension, the primary cause of significant morbidity and mortality associated with cardiovascular disease (Khor and Cai 2017). Inflammation of the hypothalamus can also lead to pancreatic islet dysfunction (Calegari et al. 2011).

Chronic inflammation arises from many kinds of insults, from acute infection to genomic instability. The concept that the hypothalamus can sense inflammation through immune pathways is a new one; just as the hypothalamus responds to nutrient status, its response to inflammation may enable the organism to rapidly adapt to physiological perturbations (Tang and Cai 2013; Zhang et al. 2013). Pro-inflammatory cytokines can also originate from senescent cells, as part of the senescence-associated secretory phenotype (SASP) or senescent secretome that can cause ravage locally and systemically (Malaquin et al. 2016) but no evidence of SASP within the hypothalamus has yet been reported.

The neuroendocrine and immune systems communicate through multiple hormonal and neuroanatomical routes. Disturbances at many levels can either directly result in disease, or indirectly lead to increased pathogenesis of- and susceptibility to- autoimmune and inflammatory diseases. A summary of the mechanisms behind the immunomodulatory role of the hypothalamus on the bone marrow, thymus and spleen, including the critically important feedback loops required to maintain balance for these bidirectional interactions and alterations that occur with age were reviewed (Barnard et al. 2008).

9.3.3 Food and Beverage Intake

Brain control of energy intake and expenditure involves a broad diversity and distribution of neuronal networks (Sousa-Ferreira et al. 2014; Waterson and Horvath 2015) with the hypothalamus occupying a central role.

The type of diet and intake of specific beverages can affect hypothalamic function. Several hypothalamic nuclei are involved in the regulation of food intake. The hypothalamus is also a crucial sensing area involved in the early detection of imbalanced diets. Numerous types of cells and neurons populate the hypothalamus. Some hypothalamic neurons secrete peptides that stimulate food intake (orexigenic neurons) whereas others produce appetite-suppressant molecules (anorexic/anorectic neurons). As stated previously (see Sect. 9.2.4), pro-opiomelanocortin (POMC)-expressing neurons and agouti-related peptide (AgRP)-expressing neurons in the arcuate nucleus (ARC) are examples of neurotransmitters controlling feeding. The peptide α-melanocyte stimulating hormone (α-MSH) secreted from POMC neurons activates, whereas AgRP inhibits, melanocortin 3 and 4 receptors (MC3R and MC4R). As a result, α-MSH suppresses feeding whereas AgRP promotes food intake. POMC and AgRP neurons are oppositely regulated by circulating metabolic cues: the saciety hormone leptin released by adipose tissue activates POMC neurons and inhibits AgRP neurons. Neurons expressing steroidogenic factor 1 (SF1) in the ventromedial hypothalamic nucleus (VMH) are also known to respond to changes in circulating leptin and other metabolic cues (Coppari 2012). AgRP neurons also control peripheral substrate utilization and nutrient partitioning (Joly-Amado et al. 2012).

POMC neurons promote satiety and the cannabinoid receptor 1 (CB1R) is critical for the central regulation of food intake. CB1R activation selectively increases β-endorphin but not α-melanocyte-stimulating hormone release in the hypothalamus. Thus, POMC neurons are also involved in the promotion of feeding by cannabinoids (Koch et al. 2015). The formation of POMC and AgRP projections to hypothalamic target sites is severely impaired by neonatal insulin during maternal high-fat diet (Vogt et al. 2014).

Leptin action on the above-named neurons contributes only modestly to the overall energy balance. Leptin receptor (LepRb) neurons in the lateral hypothalamic area, including those that contain neurotensin, mediate the action of leptin on orexin neurons and the mesolimbic dopamine system. A neuronal nitric oxide synthase (NOS1)-expressing LebRb neuron subset was shown to be a key site of action of leptin control of systemic energy balance/expenditure and body weight (Leshan et al. 2012) but not food intake (Rezai-Zadeh et al. 2014). The expression of islet amyloid polypeptide (Iapp, precursor to amylin), which is co-secreted with insulin, is induced by leptin in hypothalamic neurons and synergizes with leptin to regulate feeding and prevent obesity (Li et al. 2015). Histone deacetylase 5 (HDAC5) was found to mediate hypothalamic leptin action to control food intake (Kabra et al. 2016).

Hypothalamic SIRT1 is a key molecule in POMC and SF1 neurons that selectively and properly controls energy expenditure defenses against diet-induced obesity (Ramadori et al. 2011). Metabolic aging processes such as age-dependent decline in brown adipose tissue (BAT) and activity in fat depots, as well as reduced insulin sensitivity, is controlled by hypothalamic SIRT1 in the obesogenic and diabetogenic hypercaloric feeding environment (Coppari 2012). SIRT1 is a NAD$^+$-dependent protein deacetylase, member of the Sirtuin protein family (see below), with roles not only in energy metabolism, stress resistance, apoptosis, autophagy and inflammation, but is also involved in maintenance of genome integrity and aging (Herskovits and Guarente 2014; Yuan et al. 2016). Sirtuins are a family of NAD$^+$-dependent protein deacetylases and deacylases that regulate mammalian aging and longevity (Satoh and Imai 2014).

SIRT1 regulates senescence through deacetylation of target proteins when the NAD$^+$/NADH balance is disturbed by ROS and oxidative stress. Through AMPK, HIF-1α and PGC-1α, SIRT1 activates mitochondrial biogenesis promoting an increase in expression of mitochondrial genes critical for proliferation, fission and fusion, and ATP generation via OXPHOS (Yuan et al. 2016). Diet restriction significantly increased SIRT1 protein levels. Moreover, Nk2 homeobox 1 (Nkx2-1) as a partner of SIRT1, was shown to upregulate expression of the orexin type 2 receptor in POMC and SF1 neurons of the dorsomedial and lateral hypothalamic nuclei (DMH and LH) in control of energy expenditure, in response to diet restriction, augmenting the response to ghrelin, a gut hormone whose levels increase in these conditions. Thus, SIRT1 action in the hypothalamus, as a key mediator of the central response to low nutritional availability, contributed to extend lifespan and delay aging in mice (Satoh et al. 2013). Also, hypothalamic SIRT1 prevented age-associated weight gain by improving leptin sensitivity in mice (Sasaki 2015).

The level of hypothalamic NAD$^+$ is the critical target of control, and it is regulated by the feedback loop between the hypothalamus and adipose tissue. Hypothalamic NAD$^+$ could be affected by internal and external perturbations and thereby shifted over time. To ensure NAD$^+$ control, it is necessary a NAD$^+$-driven regulation of SIRT1 activity in a specific subset of hypothalamic neurons, such as SIRT1/NKX2-1-double-positive neurons in the DMH, a feedback loop between the hypothalamus and adipose tissue, and a feedback loop between the hypothalamus and skeletal muscle through the secretion of specific myokines (Imai 2016). Proanthocyanidins from grape seed extract restored the hypothalamic leptin-STAT3 signaling and POMC gene expression in rats subject to diet-induced obesity, in part by increasing SIRT1 expression and preventing hypothalamic inflammation (Ibars et al. 2017)

Forkhead box transcription factor 2 (Foxa2) also known as Hepatocyte nuclear factor 3-beta, a downstream target of insulin signaling, regulates the expression of orexin/hypocretin and melanin-concentrating hormone (MCH), orexigenic peptides, in the lateral hypothalamus (LH) neurons, to integrate metabolic signals, adaptive behavior and physiological responses, during fasting (Silva et al. 2009).

The obesity susceptibility gene *Cpe* which encodes Carboxypeptidase E (CPE), also known as CPH and convertase, is regulated by FoxO1 in POMC neurons the hypothalamus to link food intake with energy expenditure. CPE is involved in the biosynthesis of most neuropeptides and peptide hormones. Central insulin resistance leads to increased FoxO1 activity and inhibition of CPE, resulting in impaired POMC processing, which, in turn, promotes food intake and leads to further insulin resistance, setting off a self-perpetuating cycle (Plum et al. 2009). FOXO factors like FoxO1 maintain cellular quality control promoting the expression of genes involved in autophagy and the ubiquitin–proteasome system, and are therefore critical in processes and pathologies where damaged proteins and organelles accumulate, including aging and neurodegenerative diseases (Webb and Brunet 2014).

A large sex difference in physical activity, energy expenditure and the development of obesity was found to be driven by a subpopulation of POMC neurons, which constitutes approximately 40% of all POMC neurons in the hypothalamic arcuate nucleus. This indicated that a gender-specific strategy must be taken into consideration when designing anti-obesity therapies (Burke et al. 2016)

Ghrelin is a multifaceted gut hormone which activates its receptor, growth hormone secretagogue receptor (GHS-R) expressed in the ARC predominantly in NPY/AgRP-expressing neurons of the hypothalamus, inducing appetite and in the long term, increased body weight and adiposity. Ghrelin's orexigenic pathways rely on the most prominent energy/nutrient sensors known to date, such as AMP-activated protein kinase (AMPK), SIRT1, or mammalian target of rapamycin (mTOR), to increase food intake and modulate transcription factors controlling the expression and activity of different neuropeptides. Ghrelin also can interact with other signals such as dopamine, cannabinoids, opioids, or serotonin to induce appetite. Ghrelin can regulate glucose hemostasis by inhibiting insulin secretion and regulating gluconeogenesis/glycogenolysis. Ghrelin signaling decreased thermogenesis to regulate energy expenditure and also enhanced GH secretion and neuroprotection (Al Massadi et al. 2017).

The sole phosphorylation of the mediobasal hypothalamic eIF2α by GCN2 kinase signaling is sufficient to regulate food intake. Perturbation of eIF2α signaling results in age-related diseases (see also Sect. 9.3.8) (Maurin et al. 2014).

The peroxisome proliferator-activated receptor gamma coactivator-1-a (PGC-1α) is a transcriptional coactivator of many factors induced under conditions that cause energy expenditure, such as cold, fasting, and exercise. PGC-1α directly regulates the expression of the hypothalamic neuropeptide oxytocin, a known central regulator of appetite controlling energy intake. Oxytocin also regulates the neurovascular contact between hypothalamic axons and neurohypophyseal capillaries of the zebrafish, by stimulating endothelial morphogenesis (Gutnick et al. 2011).

Aging in rodents is associated with the general down-regulation of hypothalamic orexigenic peptides that stimulate food intake with no change in hypothalamic anorexic peptides. This may explain the decrease in appetite in senescent animals and in elderly humans, that can lead to the loss of weight observed at the end of lifespan (Kmiec 2010). The role of the adipocyte-derived hormone leptin signaling in various hypothalamic nuclei (dorsomedial hypothalamus, the ventromedial hypothalamus, and the arcuate nucleus) and its effects on the sympathetic nervous system to influence blood pressure, heart rate and brown adipose tissue thermogenesis was recently described. The mechanisms whereby leptin controls energy expenditure involving the interplay of different hypothalamic sites is being addressed (Pandit et al. 2017). Glucose-sensing neurons in VMH promote peripheral glucose metabolism, and dietary restriction, by reducing glucose metabolism in these neurons, they control glucose metabolism of the rest of the body, thereby increasing lifespan (Mobbs et al. 2013).

mTOR represents a molecular switch in the onset and progression of systemic aging. Over-stimulation of hypothalamic mTOR, resulting from chronic exposure to nutrients and activation of the pro-inflammatory NF-κB that inhibits expression of the GnRH gene, are responsible for the loss of sensitivity of the hypothalamus. Activation of HIF-1α which activates POMC gene expression appears to reverse the decline of hypothalamic function. The role of hypothalamic TOR complex in cellular energy sensing has been evidenced in the last years, focusing on the metabolic pathways where it is involved and the importance of this metabolic sensor in cellular and whole body energy management (Martinez de Morentin et al. 2014b)

Metabolic intermediates in fatty acid biosynthesis such as malonyl-CoA and long-chain acyl-CoA's, have been implicated as signaling mediators in the central control of body weight. Hypothalamic malonyl-CoA is suppressed during fasting and increases upon refeeding, is affected by blood glucose concentration and expression of orexigenic and anorexic neurotransmitters. Malonyl-CoA acts as the basic chain-elongating substrate for the formation of long-chain saturated fatty acids catalyzed by fatty acid synthase (FAS). Malonyl-CoA regulates fatty acid oxidation by inhibiting the enzyme carnitine palmitoyl transferase (CPT1c) which is highly-expressed in the hypothalamus. CPT1c can integrate carbohydrate and lipid nutrient sensing in the brain and can sense and respond to the nutritional environment. The challenge is to understand the role of CPT1c, its regulation and how its activity can lead to complex behavioral phenotypes (Wolfgang and Lane 2011).

Leptin did not only inhibit AMPK but also activated acetyl-CoA carboxylase (ACC), the key regulatory enzyme in fatty acid biosynthesis, that mediates the leptin up-regulation of malonyl-CoA levels specifically in the arcuate nucleus (Arc). Leptin also increased the level of palmitoyl-CoA (a major product of fatty acid biosynthesis) specifically in the paraventricular nucleus (PVN) in the hypothalamus (Gao et al. 2007).

Adiposity, as present in obesity, is associated with chronic low-grade systemic inflammation and increased inflammation in the hypothalamus, crucial structure involved in reward and feeding behaviors, reducing its integrity (Cazettes et al. 2011). Rats fed a high-fat diet, induced an inflammatory response in the hypothalamic areas that control feeding behavior and energy homeostasis by regulating downstream neurons and inducing apoptosis. Neurons expressing TLR4 receptor were protected suggesting that TLR4 exerts a dual function, activating pro-inflammatory pathways that play a central role in the development of resistance to leptin and insulin, and restraining further damage by controlling apoptotic activity (Moraes et al. 2009). Hypothalamic inflammation is associated with the breakdown of the circuitry that maintains balance between energy intake and energy expenditure (Cazettes et al. 2011).

Interestingly, NPY receptors such as hypothalamic Y1 and Y5 receptors are both required for the regulation of food intake and energy homeostasis in mice. Y1 or Y5 receptor knock-outs induced late-onset obesity, and the Y5 receptor knockout also induced hyperphagia (Nguyen et al. 2012). NPY in the dorsomedial hypothalamus regulates body weight by regulating food intake, body adiposity, thermogenesis, energy expenditure, and physical activity. NPY knock-down inhibited both brown fat thermogenesis and conversion of white-to-brown adipocytes in a white fat depot, resulting in reduced energy expenditure (Chao et al. 2011)

Diabetes type 2 is a metabolic disorder caused by overnutrition, obesity and lack of exercise that is commonly associated with the elderly (Barres and Zierath 2016). Hypothalamic neuropathy is considered the leading candidate that could contribute to the pathogenesis of obesity and diabetes mellitus. Pro-opiomelanocortin neurons, which regulate food intake and energy expenditure, are impaired in the arcuate nucleus, where there is an increase in local inflammatory signals and activation of innate immunity (Kalin et al. 2015).

Humanin, a 24-amino acid mitochondria-associated polypeptide, prevents apoptosis and has a role in neuroprotection. In addition, it has a central role in peripheral insulin action through inhibition of hypothalamic STAT3 leading to lower glucose levels in rats. Humanin expression levels decreased with age not only in the hypothalamus but in cortex and skeletal muscle, which may influence the pathogenesis of type 2 diabetes (Muzumdar et al. 2009).

Hypothalamic glucagon signaling inhibited hepatic glucose production which suggests that hypothalamic glucagon resistance contributes to hyperglycemia in diabetes and obesity (Mighiu et al. 2013).

In diabetic rats, structural and ultrastructural alterations in hypothalamus were accompanied by depression. The alterations included an abnormal accumulation of swollen and vacuolated mitochondria, myelin abnormalities and demyelination.

Cellular swelling was represented by local cytoplasm swelling, dilated rough endothelial reticulum (RER) fragments and swollen organelles mainly mitochondria, or by total cellular swelling Diabetic encephalopathy, characterized by impaired cognitive functions and neurochemical and structural abnormalities, may involve direct neuronal damage (Hernandez-Fonseca et al. 2009).

Sedentary lifestyles and calorie imbalance has promoted obesity worldwide, that is associated with hypersecretion of insulin by β-cells and chronic low-grade tissue inflammation, which consequently causes insulin resistance in peripheral tissues such as the liver, skeletal muscles, and white adipose tissue. However, hyperinsulinemia can also cause insulin resistance in the brain, and especially insulin-receptor (IR) expressing astrocytes in the hypothalamus are susceptible cells. IR-expressing astrocytes respond directly to a variety of nutrient and endocrine signals regulating entry of glucose and insulin across the blood-brain-barrier into the brain and control CNS and systemic glucose metabolism according to nutrient availability (Chen et al. 2017b). IR-expressing astrocytes control glucose-induced activation of hypothalamic pro-opiomelanocortin (POMC) neurons and the physiological responses to changes in glucose availability (Garcia-Caceres et al. 2016). Hypothalamic astrocytes are also known to respond to leptin signaling. Leptin-regulated feeding was diminished, whereas feeding after fasting or ghrelin administration was elevated in mice with astrocyte-specific leptin receptor deficiency (Kim et al. 2014)

Peroxisome proliferator-activated receptor gamma (PPARγ) is a ligand-activated transcription factor involved in adipocyte differentiation, fatty acid storage and glucose metabolism. PPARγ signaling also occurs within the central nervous system (CNS). PPARγ is expressed at moderate levels in the suprachiasmatic nucleus (SCh) and the ependymal of the third ventricle of the mouse hypothalamus (Liu et al. 2015). PPARγ signaling contributes to aging processes. PPARγ expression and activity are reduced during aging in rodents, and this may contribute to age-associated loss of function. Reductions in PPARγ signaling act to further promote aging processes. Treatments that increase PPARγ activity reverse age-related disturbances. Stress and aging have similar effects on brain such as altered neuronal activity, increased neuroinflammation and oxidative stress, and metabolic disturbances; PPARγ regulates all of these aspects of brain function, and is altered during stress and aging processes (Ulrich-Lai and Ryan 2013). Increased HPA axis activity has been reported in diabetic patients with poor glycemic control and is associated with some diabetic complications, including wound healing deficiency, neuropathy and retinopathy. Activation of PPAR-γ reduces HPA axis activity in diabetic rats by up-regulating PI3K expression (Torres et al. 2016).

The AMP-activated protein kinase (AMPK) is an important hypothalamic integrator of signals that control energy balance and metabolism (Lopez et al. 2016). AMPK participates in the regulation of cellular senescence and premature aging, and its responsiveness declines with age (Burkewitz et al. 2014; Salminen and Kaarniranta 2012). In the hypothalamus, AMPK is activated by fasting and its activity is inhibited in the arcuate and paraventricular hypothalamus (PVH) by the anorexigenic hormone leptin, and in multiple hypothalamic regions by insulin, high glucose and refeeding (Minokoshi et al. 2004).

The thyroid axis is a key modulator of both energy balance and lipid metabolism. Hyperthyroidism is a clinical disorder characterized by excessive production of thyroid hormones [triiodothyronine (T3) and tetraiodothyronine (T4)], which causes a hypermetabolic state characterized by increased energy expenditure and weight loss despite marked hyperphagia. Whole-body hyperthyroidism or administration of T3 decreased the activity of hypothalamic AMPK, increased sympathetic nervous system (SNS) activity and upregulated thermogenic markers in brown adipose tissue (BAT) (Lopez et al. 2010). Pharmacological activation of AMPK in the hypothalamus is sufficient to increase food intake (Andersson et al. 2004).

Ovarian estrogens play a major role in the regulation of energy balance. Estrogens regulate body weight and reproduction primarily through actions on estrogen receptor alpha (ERα). Female mice lacking ERα in SF1 neurons develop anovulation and infertility, while POMC-specific deletion of ERα inhibits negative feedback regulation of estrogens and impairs fertility in females. These results indicate that estrogens act on distinct hypothalamic ERα neurons to regulate different aspects of energy homeostasis and reproduction (Xu et al. 2011).

Decreased levels of estradiol (E2) after menopause or ovariectomy are associated with hyperphagia, reduced energy expenditure, and weight gain. E2 inhibits AMPK through estrogen receptor alpha (ERα) selectively in the ventromedial nucleus of the hypothalamus (VMH), leading to activation of thermogenesis in brown adipose tissue (BAT) through the sympathetic nervous system (SNS) in a feeding-independent manner (Martinez de Morentin et al. 2014a).

In mice subject to high-fat diet-induced obesity, leptin loses its inhibitory effect, leaving AMPK constitutively activated. A high-fat diet and pharmacological activation of AMPK, induce pRb phosphorylation and E2F target gene derepression in the Arcuate nucleus (ARC) hypothalamic POMC neurons. The high-fat diet-AMPK-pRB-E2F1 pathway promotes positive energy imbalance (Lu et al. 2013).

Tanycytes are hypothalamic radial glial cells occupying the floor and ventro-lateral walls of the third ventricle. The elongated tanycytes reside next to hypothalamic neuronal nuclei that regulate appetite and energy expenditure. Tanycytes proliferate postnatally but this capacity gradually decreases with increasing age. Age-related studies in rats spanning 3–24 months of age showed that the number of tanycytes declines by almost 30% with increasing age. Aged tanycytes may become phagocytic and engulf debris produced by nearby degenerating neurons, axons and their myelin sheaths (Goodman and Hajihosseini 2015). Tanycytes have many functions including neural stem cell/progenitor function and transport of chemical signals from the cerebrospinal fluid. One of these is leptin. Leptin is an adipose-derived saciety hormone that normally enters the brain to promote decreased food intake and increase energy expenditure. When leptin cannot fulfil its function to regulate body weight, obesity takes place. Interestingly, under physiological conditions, peripheral leptin is internalized by tanycytes through a leptin receptor-dependent mechanism transporting it across the blood-brain barrier. Then, tanycytes release leptin into the cerebrospinal fluid allowing leptin to reach energy-sensing target neurons in the mediobasal hypothalamus (MBH) to exert its catabolic/anabolic action. During diet-induced obesity tanycytes cannot release bound leptin reducing its access to MBH neurons. Activating the ERK pathway through EGF treatment releases leptin from tanycytes and restores its access to hypothalamic target neurons (Balland et al. 2014; Gao et al. 2014). AgRP neurons and the melanocortin system play a crucial role in the antidiabetic glucose-lowering actions by leptin, but not NPY and GABA, to correct hyperglycemia (Goncalves et al. 2014).

Dietary fat and aging lead to atypical transforming growth factor-β1 (TGF-β1) signaling in the hypothalamus, which disturbs whole-body glucose regulation. An excess of TGF-β1 is released predominantly from astrocytes and acts on TGF-βR2 receptors on hypothalamic neurons such as POMC neurons to induce pro-diabetic effects such as hyperglycemia and glucose intolerance. This event occurs not only in obesity but also in aging. TGF-β1 induces formation of RNA stress granules that sequester IκBα RNA, an inhibitor of pro-inflammatory Nuclear Factor-κB (NF-κB), and impair IκBα translation. The rapid decay of IκBα mRNA and subsequent reduction of IκBα protein levels lead to NF-κB activation, resulting in the dysfunction of POMC neurons. The resulting increased hepatic glucose production could promote the development of type 2 diabetes mellitus. Thus, TGF-β1 may be considered a direct mediator of obesity- and aging-related diabetes (Araujo et al. 2014; Yan et al. 2014). Acute activation of NF-κB and its upstream activator IkB kinase-b in the mediobasal hypothalamus can also rapidly elevate blood pressure in mice independently of obesity. POMC neurons were crucial for the hypertensive effects of the activation of hypothalamic IKK-β and NF-κB, which underlie obesity-related hypertension (Purkayastha et al. 2011)

A way to increase energy expenditure and weight loss in diet-induced obesity relies on the hormone Fibroblast growth factor-21 (FGF21). FGF21 stimulates sympathetic nerve activity to brown adipose tissue through a mechanism that depends on the neuropeptide corticotropin-releasing hormone/factor (CRF). Hypothalamic Crf mRNA was elevated 3 h after FGF21 injection, with a corresponding increase in plasma adrenocorticotropic hormone (ACTH). FGF21 acts on the hypothalamus to induce CRF and to stimulate sympathetic nerve activity (SNA), which in turn induces uncoupling protein-1 (UCP1) and lipolysis in brown adipose tissue (BAT). FGF21 also acts directly on BAT to stimulate glucose uptake and to mobilize oxidative substrates. These dual effects induce efficient energy expenditure (Owen et al. 2014). Thus, FGF-21 is considered a promising dietary restriction mimetic (Mendelsohn and Larrick 2012).

A potential route to treat hypothalamus glucose disorders is through therapy using implantation of neural stem cells. Neurogenesis of these cells towards POMCergic and GABAergic lineages was at least accountable. iPS-derived NSC when engineered with NF-κB inhibition were also effective in reducing obesity and glucose intolerance (Li et al. 2014).

The hypothalamus is not only a key regulator of food intake but also water intake and part of the reward system. An age-related dysfunction of the hypothalamic-neurohypophyseal-renal axis takes place that leads to multiple abnormalities in water homeostasis leading to deficits in renal function, thirst and responses to osmotic and volume stimulation (Cowen et al. 2013). High salt intake induces imbalance between pro- and anti-inflammatory cytokines and neurotransmitters in the hypothalamic paraventricular nucleus (PVN) with the increase in blood pressure or sympathetic activity, and is involved in the pathogenesis of hypertension. Oral CoQ10 supplementation can attenuate these effects (Gao et al. 2016).

The hypothalamus is a very alcohol-sensitive brain region and can modulate an individual's alcohol consumption. Alcohol is known to activate the HPA axis. Experiments in mice showed that chronic alcohol consumption during adolescence, even at moderate levels, may trigger gene expression changes in the CNS that parallel those found in dilated cardiomyopathy (DCM). Age-related DCM is the most frequent cause of heart failure. Changes in the expression of DCM pathway genes (β1AR-DHPR-Ca^{2+} branch) may serve as early warning signs for alcohol's harmful effect. If analogous effects would take place in adolescent humans, premature aging effects in the hypothalamus could occur (Zou et al. 2014). Orexigenic neuropeptides such as galanin, the endogenous opioid enkephalin, and orexin/hypocretin, appear to stimulate alcohol intake not only through mechanisms that promote food intake but also by enhancing reward and reinforcement from alcohol. In contrast, anorexigenic neuropeptides such as the endogenous opioid dynorphin, corticotropin-releasing factor, and melanocortins, which act in the hypothalamus to inhibit alcohol drinking as well as reward, counter the alcohol craving promoted by orexigenic neuropeptides. Excessive signaling from orexigenic neuropeptides or inadequate signaling from anorexigenic neuropeptides can therefore allow alcohol drinking to become dysregulated (Barson and Leibowitz 2016). For example, in individuals with a history of binge drinking, there is an increased risk of developing the metabolic syndrome and type 2 diabetes. In rat studies, binge drinking induced systemic insulin resistance by impairing hypothalamic insulin action and this effect was prevented by inhibition of brain protein tyrosine phosphatase 1B (PTP1B) (Lindtner et al. 2013).

The protective effects of dietary restriction or its mimetics during aging are mediated by the hypothalamus in *C. elegans* and *Drosophila*. The nutrient-sensing neurons of the ventromedial hypothalamus (VMH) in mammals mediate some responses to food deprivation. Dietary restriction decreases glucose metabolism in the hypothalamus and that this effect mediates the reduction of glucose metabolism in the periphery, thus slowing aging in these tissues. Possibly, VMH nutrient-sensing neurons could be targeted with calorie restriction mimetics to protect against age-related disease (Dacks et al. 2013).

Deep brain calcium live imaging in freely behaving mice reveals that appetitive and consummatory behaviors are encoded in distinct neurons in the lateral hypothalamus (LH), suggesting the existence of separate networks regulating motivation to eat and consume food (Jennings et al. 2015). Evidence has also been provided showing that inhibitory synaptic inputs from the extended amygdala preferentially innervate and suppress the activity of LH glutamatergic neurons to control food intake (Jennings et al. 2013).

In summary, nutrient sensing neurons in the hypothalamus play a critical role in modulating the aging process and age-related diseases, and that their epigenome is essential for functional homeostasis (Moreno and Mobbs 2016).

9.3.4 Circadian Rhythms

Light acts upon several non-image-forming functions including body temperature, hormonal secretions, sleep-wake cycle, alertness, and cognitive performance. Melanopsin (OPN4) is a blue light (short light wavelength ~460–480 nm) sensitive pigment retinylidene protein form of the G-protein-coupled receptor family expressed by intrinsically photosensitive retinal ganglion cells (ipRGC) that is expressed in 1–2% subset of all retinal ganglion cells (RGC). A monosynaptic pathway, the retinohypothalamic tract (RHT), conveys light information from ipRGC axons. RHT directly connects the ipRGC from the retina to the suprachiasmatic nuclei (SCN) of the anterior hypothalamus situated directly above the optic chiasm. SCN is the master circadian oscillator (biological clock) controlling melatonin secretion, pupillary constriction, and the regulation of the sleep-wake cycle or circadian rhythm. SCN sends efferent projections to other hypothalamic and non-hypothalamic structures. The ipRGC also sends direct connections to regions engaged in the regulation of the sleep-wake cycle such as the ventrolateral preoptic nucleus (VLPO; sleep-wake regulation core-region), the subparaventricular nucleus/zone (SPVZ) of the hypothalamus, which is involved in sleep regulation but also in motor activity, as well as the lateral hypothalamus (LH), which contains orexin/hypocretin neurons regulating feeding, sleep and wakefulness (Daneault et al. 2016). The age-associated changes in the hypothalamic SCN have been featured (Farajnia et al. 2014).

The circadian rhythm in the hypothalamic SCN is governed by SIRT1 (see also Sect. 9.3.3) which activates the transcription of two major circadian regulators, BMAL1 and CLOCK. During aging, SIRT1 levels in the SCN decrease which explains the loss of circadian rhythm with age (Chang and Guarente 2013). Thus, SIRT1 is a metabolic sensor that links food intake to modulation of the circadian clock. SIRT1 relays nutritional inputs to the circadian clock through the Sf1 neurons of the Ventromedial Hypothalamus (VMH). Under food restriction and absence of light, SIRT1 in the VMH contributes to activity behavior and circadian gene expression in the suprachiasmatic nucleus (Orozco-Solis et al. 2015). The circadian clock BMAL1 of the ventromedial hypothalamus computes light and feeding inputs to modulate circadian energy expenditure through the rhythmic activation of brown adipose tissue metabolism and dissipating energy as heat (Orozco-Solis et al. 2016). Interestingly, neurons of the dorsomedial hypothalamus (DMH) have a critical role in the expression of food-entrainable circadian rhythms in rats (Gooley et al. 2006).

A comprehensive summary of aging-associated changes in circadian function has been presented, linking such alterations to adverse health consequences in late life and promotion of the aging process (Gibson et al. 2009; Turner and Mainster 2008). Circadian aspects of energy metabolism and their relationship with aging (Froy 2013) and its control over glucose homeostasis (Kalsbeek et al. 2010) were also reported.

Age-related alterations have been detected in human eye lens (Yan and Wang 2016) and retina (Lei et al. 2011). Photoaging of RGC leading to their degeneration or functional alteration may be a primary cause of an altered circadian rhythm and further hypothalamic imbalances leading to shorter life span.

9.3.5 Sleep

The hypothalamus also regulates sleep, and the areas responsible for it deteriorate earliest during aging. The sleep and hormonal changes during aging have been studied in detail during many years (Mander et al. 2017; Rolls 2012). A decline in deep non-rapid eye movement (NREM) or slow wave sleep, and the characteristic brain waves associated with it, including both slow waves and faster bursts of brain waves known as “sleep spindles” are characteristic of old age, and this is related to memory decline in later life. Another deficiency in later life is the inability to regulate neurochemicals that stabilize sleep and help transition from sleep to waking states. These neurochemicals include galanin, which promotes sleep, and orexin/hypocretin, which promotes wakefulness. Galanin is thought to be involved in non-REM sleep onset, inhibiting the posterior hypothalamic arousal system. A disruption to the sleep-wake rhythm commonly leaves older adults fatigued during the day but frustratingly restless at night. Sleep need is either reduced or remains high in older adults, older adults innately get less sleep, less deep sleep, show less intense rebound sleep following deprivation, report less subjective sleepiness under sleep restriction conditions, and suffer a smaller increase in lapses of attention after sleep deprivation and restriction. In other words, sleep need remains, but sleep regulating and/or generating capacity may be impaired (Mander et al. 2017).

Endocrine-related alterations include a dramatic decrease in growth hormone secretion with age which correlates with a decrease in the amount of slow wave sleep. Lack of normal sleep patterns can result in an elevation of evening cortisol levels and an activated HPA axis which can promote restless nights and nocturnal awakenings (Copinschi and Caufriez 2013). The lateral hypothalamic area (LH), with its hypocretin/orexin expressing neurons, is crucial in the control of the sleep-wakefulness cycle (Herrera et al. 2017; Yamashita and Yamanaka 2017). The posterolateral hypothalamic neuropeptides orexin A and B (hypocretin 1 and 2) are important homeostatic mediators of central control of energy metabolism (feeding, thermoregulation), neuroendocrine and cardiovascular control and maintenance of sleep/wake states. Dysregulation or loss of orexin signaling has been linked to narcolepsy, obesity, and age-related disorders (Nixon et al. 2015). Selective and acute chemogenetic activation of vesicular GABA transporter-expressing ($VGAT^+$) neurons in the ventrolateral preoptic area that arises from the lateral hypothalamus was profoundly wake promoting, whereas acute inhibition increased sleep (Venner et al. 2016).

The brainstem and hypothalamic pathways that drive wake or sleep have been carefully studied, but the full understanding of the anatomy, physiology and dynamics of these circuits are still being explored (Bonnavion et al. 2016; Scammell et al. 2017).

A variety of neuropeptides present in the hypothalamus have been extensively investigated in terms of their physiological functions and many have been reported to be related to sleep regulation. A reciprocal interaction of the sleep-promoting growth hormone-releasing hormone (GHRH) and corticotrophin-releasing hormone (CRH), which promotes wakefulness and REM sleep, plays a key role in sleep regulation (Steiger et al. 2013). The neuropeptides hypocretin-1 and -2/orexin-A and -B (HCRT-1 and -2/OX-A and -B, respectively, regulate sleep–wake control through complex interactions between monoaminergic/cholinergic (wake-promoting) and gamma-aminobutyric acid-ergic (sleep-promoting) neuronal systems (Chow and Cao 2016).

Epigenetic effects on the sleep and circadian rhythm circuitries has been presented, resulting in increased DNA methylation, histone post-translational modifications and non-coding RNA defects (Qureshi and Mehler 2014). Recurrent sleep restriction, experienced by a substantial and rapidly growing proportion of children and young adults might contribute to accelerated senescence of the hypothalamus and its downstream functions (Copinschi and Caufriez 2013). Insomnia, a state of hyperarousal with difficulty falling asleep and maintaining sleep, is rather prevalent in modern societies. Among a number of clinical therapies to treat insomnia, hypocretin/orexin receptor antagonists are being developed to improve sleep (Chow and Cao 2016) but dietary compounds such as grape seed proanthocyanidin extract alone can modulate melatonin levels in plasma and the expression pattern of clock genes in the hypothalamus of rats (Ribas-Latre et al. 2015). Also, interventions such as exercise can elicit acute elevations in forebrain serotonin (5-HT, 5-hydroxytryptamine) concentrations. Reductions in central serotonin activity with aging might be involved in sleep-related disorders in later life. 5-HT might facilitate synthesis of hypnogenic compounds through stimulation of specific target cells, which could help promote sleep by acting as permissive substances in the preoptic region of the hypothalamus (Melancon et al. 2014).

9.3.6 Sex and Reproduction

Both genders are subject to significant changes in their hormonal profiles with age, especially those associated with reproduction. An age-related decline in testosterone concentration in some middle-aged and older men (>65 years), known to some as andropause, is associated with a cluster of symptoms and signs that resembles those observed in men with classical hypogonadism. Adiposity, chronic illness, weight gain, medications and genetic factors affect testosterone levels. The decline in testosterone production in older men is the result of abnormalities at all levels of the hypothalamic-pituitary-testicular axis, whereas gonadotropins, serum luteinizing hormone (LH) and follicle-stimulating hormone (FSH) levels show an age-related increase in longitudinal studies (Bhasin et al. 2000). The timing of permanent stop of menstrual periods in women, menopause, leading to ovarian follicular aging, is dependent on hypothalamic signaling, breast cancer susceptibility and BRCA1-mediated DNA repair (Day et al. 2015).

Gonadotropin-releasing hormone (GnRH) regulates the release of sex steroids involved in development and repro-duction (estrogens and progesterone in females and androgens in males) but it has other functions as well. GnRH given to mice abrogated aging effects and increased the production of new neurons in the hypothalamus and hippocampus (a part of the brain that regulates memory (Zhang et al. 2013). Estrogen-responsive neurons in the ventrolateral region of the ventromedial hypothalamus regulate female-specific activity but not reproduction or brown adipose tissue thermogenesis (Correa et al. 2015).

A decrease in gonadal sex steroids is a well-established marker of aging, but many other hormonal changes occur as well; and some of these age-regulated hormones (such as dehydroepian-drosterone) also regulate inflammation and other immune responses. Thus, interplay between the hormonal and immune systems occurs at multiple levels (Gabuzda and Yankner 2013).

Circulating and hypothalamic IGF-1 levels decrease with aging, suggesting a role for IGF-1 in the onset of reproductive senescence. IGF-1 receptor signaling is necessary for GnRH neuron activation under estrogen-positive feedback conditions and that decreased brain IGF-1 signaling in middle-aged females contributes, in part, to LH surge dysfunction by disrupting estradiol-sensitive processes that affect GnRH neuron activation and/or GnRH release. Pharmacological blockade of IGF-1 receptors attenuated and delayed the LH surge in young adult rats, prior to the onset of reproductive senescence (Todd et al. 2010).

Genetic and non-genetic factors influence sexual orientation. Various hypothalamic structures are structurally different in relation to sexual orientation (Swaab et al. 2003).

9.3.7 Radiation

In mice, low dose X-rays may potentiate the activity of the neurons in mouse hypothalamus, expedite their signal transduction, and down-regulate the functions of the HPA axis (Wan et al. 2001). Also, humans undergoing cranial radiation therapy are at increased risk for hypothalamic-pituitary dysfunction within 3 years (Madaschi et al. 2011).

UVB (290–320 nm) radiation can specifically affect various local neuroendocrine activities by stimulating the expression of corticotropin-releasing hormone (CRH), urocortin, pro-opiomelanocortin (POMC), and POMC-derived peptide (Jozic et al. 2015).

Eyes of mice exposed to ultraviolet B radiation (UVB), elicit a signal through inducible nitric oxide synthase (iNOS)-dependent ciliary ganglia involving the first branch of the trigeminal nerve to the hypothalamopituitary proopiomelanocortin system, resulting in upregulation of alpha-melanocyte-stimulating hormone (α-MSH) secretion and consequent stimulation of α-MSH-receptor containing cells such as melanocytes in the skin (Hiramoto et al. 2003). UVB exerts effects on body homeodynamics through activation of a local HPA axis in the skin, which in turn activates the central HPA axis and requires the pituitary gland for systemic effects. UVB stimulated CRH gene and protein expression in the arcuate and paraventricular nucleus of the hypothalamus in mice (Jozic et al. 2015; Skobowiat and Slominski 2016). Thus, skin can influence overall systemic levels of cortisol and help regulate local and central HPA axes in the context of homeostasis, skin injury, and inflammatory skin disorders (Jozic et al. 2015).

9.3.8 Neuropathies

Dementias such as Alzheimer's disease (AD) or vascular dementia are age-associated neuropathies that lead to a gradual decrease in brain function. During normal aging and in AD, changes take place in hypothalamic neurons such as sex-dependent activation of the AVP neurons in the supraoptic nucleus (SON) and in the paraventricular nucleus (PVN), which may be the basis of analogous changes in the prevalence of hypertension and hyponatraemia in the elderly. The activity of the corticotropin-releasing hormone (CRH) neurons in the hypothalamic PVN is increased. A dysfunctional circadian clock may underlie the disordered rhythms in AD (Swaab and Bao 2011).

Many structural and functional gender-specific differences have been reported in the human hypothalamus and adjacent structures that may be related to not only reproduction, sexual orientation and gender identity, but also in relation to sex differences in prevalence of psychiatric and neurological diseases. The neuronal hyperactivity in the infundibular nucleus resulting from a lack of estrogens in the menopause seems to protect females against Alzheimer changes, in contrast to males (Swaab et al. 2003).

Streptozotocin-induced diabetic rats exhibit an aberrant metabolism including hyperglycemia and hyperlipidemia following insulin deficiency, causing hippocampal atrophy, neurodegeneration, Aβ deposition, and declined dendritic spine density, and cognitive impairment at early onset. All symptoms are characteristics of accelerated brain aging with characteristics of Alzheimer's disease-like pathology. Insulin signaling in the brain promotes amyloidogenesis and insulin improves Aβ clearance through insulin-degrading enzyme, one of the main proteases involved in Aβ degradation (Wang et al. 2014). A role for circadian cortisol hypersecretion in the initiation of sporadic Alzheimer's disease has been proposed, involving neuroinflammation-mediated suppression of hypothalamic glucocorticoid receptor (GR) signaling (Notarianni 2014). Amyloid toxicity affects the HPA axis adaptive response to stress suppression of glucocorticoid receptor (GR) signaling by amyloid-β protein. The long-term increase in HPA axis activity (denoted by increased plasma corticosterone and ACTH, and hypothalamic CRH) are consistent with β-amyloid/$A\beta_{25-35}$ peptide-induced redox inactivation of central GR, causing inhibition of central glucocorticoids negative feedback through GR signaling insufficiency (Brureau et al. 2013; Notarianni 2014)

The potential toxic effects of β-amyloid in the hypothalamus controlling insulin action and glucose metabolism and the role of insulin resistance and glucose metabolism dysregulation in the development of Alzheimer´s disease was recently presented. Beta-amyloid's neurotoxic actions perturbs hypothalamic glucose regulation, leading to increased hepatic glucose production and altered glucose metabolism (Arrieta-Cruz and Gutierrez-Juarez 2016).

Another type of cells in the hypothalamus associated with diseased states is the dark microglia. These cells become abundant during chronic stress, aging, fractalkine signaling deficiency, and Alzheimer's disease pathology. They exhibit several signs of oxidative stress, including a condensed, electron-dense cytoplasm and nucleoplasm making them appear refractive opaque, accompanied by a pronounced remodeling of their nuclear chromatin. They could play a significant role in the pathological remodeling of neuronal circuits, especially at synapses (Bisht et al. 2016).

9.3.9 Other Epigenetic Modifiers

Epigenetics, comprising heritable changes in gene expression other than alterations of the nucleotide sequence, could affect hypothalamus function. As stated above, light, social cues, stress and energy balance stimulate relatively short- as well as long-term genomic modifications in the hypothalamus, which are mediated by epigenetic mechanisms (Stevenson 2017). Age-related learning and memory impairments due to epigenetic changes in the suprachiasmatic nucleus have been documented (Deibel et al. 2015). Polyphenolic compounds may be particularly promising therapeutic candidates to prevent age-related cognitive decline and depression by modulating the HPA axis activity, serotonergic transmission and hippocampal neurogenesis (Ogle et al. 2013).

The environment contains many potential disruptors of proper HPA axis functioning. For example, alcohol and tobacco consumption alter the hypothalamic pituitary adrenal axis DNA methylation patterns (Dogan et al. 2016). Endocrine disruptors such as Bisphenol A (BPA), used in plastics, is a potential obesogen that exerts its effects through developmental programming of the hypothalamic melanocortin circuitry, permanently altering the neurobiology of metabolic homeostasis (MacKay et al. 2017). BPA-related transcriptional changes were mainly confined to the hypothalamus (Arambula et al. 2016).

Early life exposure to estrogenic endocrine disruptors such as methoxychlor and estradiol benzoate has lifelong effects on neuroendocrine gene expression and DNA methylation, together with causing the advancement of reproductive senescence (Gore et al. 2011).

Pesticides in food and water can negatively affect male and female fertility, cause reproductive diseases, early menopause and senescence, and affect endocrine pathways in the hypothalamus (Rattan et al. 2017). Doses below the current no-observed-adverse-effect levels of various chemicals in our environment, can alter gene expression in the developing brain, and have serious consequences in the long-term.

9.3.10 Physical Damage

Trauma caused by surgery or accidents can have a detrimental effect on the hypothalamus and has many consequences. Destruction of the anterior portion of the hypothalamus in rats led to hypertrophy of the thymus and the serum level of the growth hormone (GH), secreted by the pituitary gland, markedly increased. Cells secreting GHRIH (growth hormone release inhibitory hormone), but not GHRH (growth hormone releasing hormone), were most likely removed. In other words, the development and aging of thymus appears to be under the balance of the positive (GHRH) and negative (GHRIH) signals of the hypothalamus: probably GHRH is high just after the birth and decreases with age thereafter with a concomitant increase of GHRIH, leading to the onset of thymic involution at around puberty. Thus, control of the hypothalamus-pituitary axis on the thymus can impact on aging of the immune system (Hirokawa et al. 1998).

9.4 Conclusions

The hypothalamus is a remarkable tiny organ (Saper and Lowell 2014) regulating an impressive amount of body functions (Le Tissier et al. 2017). Many efforts have been dedicated to understanding the mechanisms governing control of metabolism and energy turnover, which is illustrated by the number of hormones and peptides involved. The role of stress, inflammation, food and beverage intake, circadian rhythms, sleep, sex and reproduction, radiation, neuropathies, epigenetic modifiers, and physical damage on hypothalamus function and dysfunction have been addressed here, but many other physiological cues relayed from the body to the hypothalamus exist that were not possible to include. All physiological and environmental cues modulate the extent of responses required to ensure the proper whole-body function following those events. A lifetime of constant, and at times severe fluctuations in the pulsatile nature of secreted peptides and hormones leads eventually to functional exhaustion. The hypothalamus has a central role in aging. Many studies show that brain tissues preserve their telomere lengths with aging, possibly also the hypothalamus, suggesting that other mechanisms associated with the induction of a senescent state may be in place including defects in oxidative damage repair, proteostasis and autophagy, mitochondria dysfunction and epigenetic changes. Analysis of senescence in the hypothalamus has not yet been fully addressed. The state of endocrine hypothalamic function in old age is based on adaptation/compensation to changes that gradually trigger loss of function. Intervention studies have been addressed to improve hypothalamic control in disease states such as chronic stress, metabolic syndrome, diabetes, insomnia and Alzheimer's.

Very limited information is available regarding the short- and long-term effects of specific foods and beverages or individual dietary compounds on the metabolism and normal functioning of all subsets of hypothalamic neurons. The physiological cues influencing tanycytes and dark microglia remain largely unexplored. A great deal of knowledge on hypothalamus structure and function has been attained in recent years, with further progress awaited based on novel technological initiatives.

9.5 Future Perspectives

The impressive plethora of functions within a tiny organ such as the hypothalamus, together with its relative inaccessibility, makes it rather intricate to explore many properties that would help to understand the precise mechanism(s) leading to hypothalamic neuron senescence.

The advent of novel methodologies to visualize the location and function of neurons and their synapses including opto-genetics (Berlin and Isacoff 2017; Jennings et al. 2015) and quantitative two-photon fluorescence lifetime biosensor imaging (Kislin et al. 2017) together with circuit-mapping and precise phenotypic and behavioral screening would allow to better decipher the identity and interactions of specific subsets of hypothalamic neurons, and how they contribute to the pathophysiological processes leading to specific age-associated diseases. In this regard, refinement of the use of biomarkers of senescence in post-mitotic tissues such as the hypothalamus and subset-specific hypothalamic neuronal biomarkers would indeed contribute to analyze in depth the physiological state of the cells involved. Single-cell transcriptome analysis combined with protein interactome profiling and high-content imaging studies would be very useful.

The fate of existing neurons and the dynamics of tissue regeneration exhibited by hypothalamic tanycytes/progenitor neural stem cells before, during and after acute and chronic physiological and environmental cues/insults, would provide important information regarding the identity of neurons responding to the insult and the consequences of the response (Goodman and Hajihosseini 2015). The genetic and physiological determinants of the resilience of specific subsets of hypothalamic neurons to stress derived from HPA dysfunction, and their possible association with accelerated aging and longevity deserve further attention (Chen et al. 2017a; Gaffey et al. 2016).

Future hypothalamic hormone replacement therapies to heal or improve hypothalamic-dependent dysfunctions, need to address the amount and activity of individual-specific neuropeptides and neurohormones, time and site of delivery, the possible pleiotropic effects of agonists and antagonists, etc. Personalized stem cell-derived therapies for regenerative purposes using human inducible pluripotent stem cells and their fully-functional derivatives would need to explore safe and effective delivery options (Li et al. 2014).

The precise epigenetic changes taking place at various hypothalamic neurons such as DNA methylation and histone posttranslational modifications, resulting from of environmental insults awaits further analysis.

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Chapter 10
Melatonin in Healthy Aging and Longevity

Rüdiger Hardeland

Abstract Melatonin is a regulator of the circadian multioscillator system. It transmits the information ‘darkness’, contributes to internal and external alignment of rhythms and, presumably via sirtuin-1, to high amplitudes. During normal aging, both the circadian system and melatonin secretion deteriorate. These processes are aggravated by several diseases and disorders of different etiology, most strongly under conditions of neurodegeneration, especially Alzheimer’s disease. Preclinically, melatonin counteracts alterations that may contribute to the acceleration of aging. It supports mitochondrial electron flux, enhances intramitochondrial protection against free oxygen and nitrogen radicals, reduces the duration of permeability transition and attenuates free radical formation. In nontumor cells, it acts as an antiapoptotic agent and may prevent excessive peripheral mitophagy. Numerous preclinical studies have documented a remarkable neuroprotective potential. Despite encouraging results, the translation of findings to the human is sometimes problematic, e.g., because of the different association of melatonin with rest or activity in nocturnally active rodents and in man. These differences extend to the tractability of metabolic syndrome and diabetes type 2, because of the preferred times of food intake. An overexpressed risk allele of the melatonin receptor gene *MTNR1B* decreases glucose tolerance in humans. Insulin resistance is also of high interest to neuroinflammation. With regard to both pro- and antiinflammatory actions, the role of melatonin in human inflammaging remains to be clarified. Life extension by melatonin was only preclinically demonstrated and remained, in rodents, rather modest. In man, melatonin’s value should be sought in supporting healthy aging, which may have indirect effects on lifespan.

Keywords Light · Darkness · Sleep · Rhythms · Aging · Circadian · Inflammaging · Insulin resistance · Melatonin · Neurodegeneration

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10.1 Introduction

Healthy aging is not just a matter of caring about a favorable lifestyle after having reached an advanced age. At first glance, this statement may appear to be not more than a banality, but its intention is not at all to refer to unhealthy habits in youth. Instead, some biological facts are meant that become apparent in midlife and indicate changes towards problems to develop during senescence. These alterations may not necessarily be caused by extremely unhealthy habits, but can also result from genetic dispositions or some diseases. Moreover, disorders and diseases that develop during midlife can be indicative of previous aberrations in the physical state. In numerous middle-aged humans, the onset of various undesirable changes becomes obvious, such as first signs of metabolic syndrome, a prediabetic state, moderate sleep disturbances, mood disorders, or decreased sexual function. These deviations that may aggravate by age are frequently associated with decreases in circadian amplitudes, internal coordination of rhythms and levels of melatonin (Hardeland 2012, 2016, 2017). For the relationship between circadian oscillators and melatonin see Fig. 10.1. Interestingly, the interindividual variability of the losses in melatonin and circadian functionality is remarkably high. This is particularly well documented in the case of melatonin (Reiter 1995; Mishima et al. 1999; Bubenik and Konturek 2011). Diseases and disorders represent one cause of these differences between individuals.

It is a remarkable fact that reductions of melatonin levels have been described in numerous entirely different pathologies. These include neurological/behavioral cases, neurodegeneration, various types of painful or stressful conditions, metabolic and cardiovascular diseases, and also some forms of cancer, as summarized elsewhere (Hardeland 2012). While a return to normal is possible in some pain- or stress-related disorders, this cannot be expected in neurodegenerative diseases and may not be easily achieved in metabolic diseases. In particular, this concerns the complex of obesity, metabolic syndrome, prediabetic and diabetic states, including insulin resistance in the central nervous system and various associated aspects of inflammation. These metabolic deviations seem to be related to a midlife onset of melatonin reduction that may not allow full restoration by conventional treatments. The decrease of melatonin relatively early in life has even been discussed as a risk factor for developing type 2 diabetes (Anonmyous 2013; Hardeland 2016). Surprisingly, a risk factor allele of the melatonin receptor gene *MTNR1B* (G allele carrying the SNP rs10830963) has been shown to become increasingly and supranormally expressed in beta cells around midlife (Lyssenko et al. 2009). At this time, melatonin is already substantially decreased, in particular, in subjects developing a diabetic state. The upregulation of this gene, which encodes a variant of the receptor MT_2, causes an extreme suppression of cAMP formation, which would be required for insulin secretion (Tuomi et al. 2016). This example is of particular interest with regard to the importance of type 2 diabetes as a widespread disease and

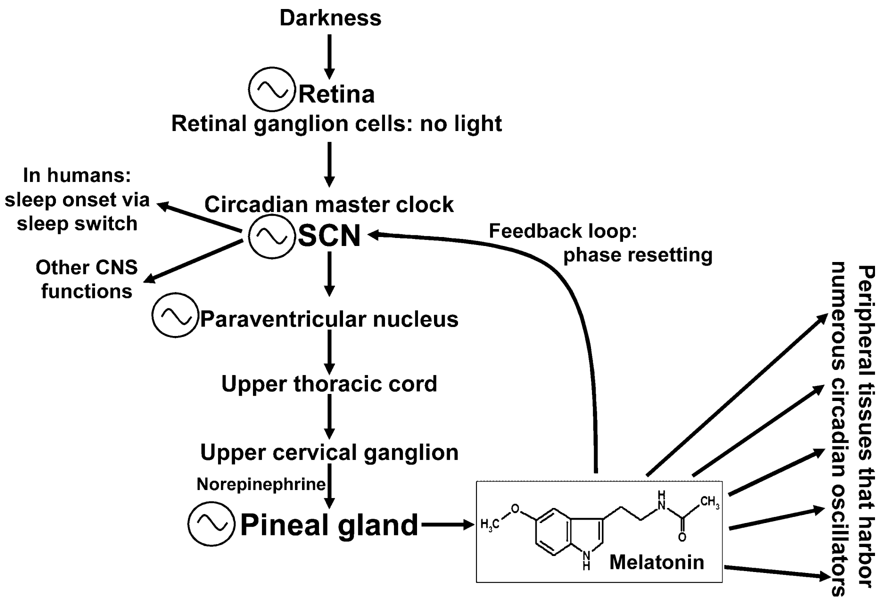

Fig. 10.1 The relationship between circadian oscillators and melatonin synthesis in the mammalian pineal gland. *SCN* suprachiasmatic nucleus. Photic information is mainly transmitted to the SCN via photoreception by melanopsin-containing retinal ganglion cells. Encircled waves indicate presence of circadian oscillators. Oscillators in paraventricular nucleus and pineal gland can be classified as slave oscillators of the SCN. Melatonin is released from the pineal gland to both the circulation and, via the pineal recess, directly to the third ventricle of the brain. Please note that this scheme only refers to the pineal gland and not to the numerous extrapineal sites of melatonin synthesis, which, in total, generate amounts of melatonin that exceed by orders of magnitude those found in the pineal gland, but normally contribute poorly to the circulating levels

its associated secondary health problems, especially with regard to successful aging. The reason for why the strong upregulation occurs in midlife of risk allele carriers may be sought in two age-related changes, which are mutually associated and cannot be easily dissected. The first one is the decrease of melatonin, to which target cells such as the pancreatic beta cells may respond in a compensatory way by increasing *MTNR1B* expression. The second one concerns the deterioration of the circadian system, which is profoundly intertwined with melatonin. On the one hand, the mammalian circadian pacemaker, the suprachiasmatic nucleus (SCN), controls via a neuronal pathway melatonin secretion by the pineal gland, but melatonin additionally feeds back to the SCN (Stehle et al. 2003). Therefore, the reduction of melatonin secretion may be interpreted as an age-associated decrease of circadian amplitudes. This conclusion would be well in accordance with numerous observations and has been discussed as a key feature of chronobiological deteriorations in the course of aging (Hardeland 2013a, 2017). On the other hand, the reduced melatonin feedback to the SCN may additionally weaken the circadian system and further contribute to reduced amplitudes. Moreover, it seems to be of importance to not restrict such considerations to the relationship between SCN and pineal gland, since melatonin has numerous effects in the periphery, including influences on peripheral circadian oscillators that depend to a variable degree on the SCN (Hardeland et al. 2012; Hardeland 2013b).

The complexity of the circadian system deserves particular attention in the aging processes, with consequences to the either good or poor functioning of the body as an entity. This system is, in fact, a multioscillator machinery based on countless cellular oscillators that have to be coordinated, coupled, and internally brought into physiologically favorable phase relationships. In this regard, important observations have been made in aging rats (Yamazaki et al. 2002). The most remarkable findings concern the strongly deviating behavior of the different peripheral oscillators. In one category, aging did not cause substantial changes, whereas a second category comprised oscillators that showed phase advances. Surprisingly, a third category had lost their rhythmicity, which could, however, be experimentally re-initiated. As a consequence, one can conclude that an aging mammal loses the optimal phase relationships between oscillators, and this internal misalignment is also associated with complete losses of rhythmicity in other functions. In this context, it may be of importance that melatonin is capable of enhancing the expression of the aging suppressor sirtuin 1 (SIRT1) in various senescent tissues, as recently summarized (Hardeland 2017). This effect should have a substantial influence on peripheral oscillators, especially if they display reduced amplitudes or may have lost their rhythmicity. The nexus between SIRT1 upregulation by melatonin and oscillator properties is apparent in the role of this sirtuin as an accessory oscillator component in both peripheral clocks and the SCN, along with the observation that SIRT1 enhances circadian amplitudes (Nakahata et al. 2008; Bellet et al. 2011; Chang and Guarente 2013). All these observations that indicate connections between melatonin, the antiaging factor SIRT1 and circadian oscillators are in good agreement with epidemiological data that demonstrate associations of numerous aging-related diseases and disorders with polymorphisms or altered expression levels of oscillator genes and also of genes involved in melatonin signaling (summarized in: Hardeland et al. 2012; Hardeland 2012). The chronobiological role of melatonin seems to represent an important aspect of its contribution to healthy aging, but this has not to be misunderstood as an exclusive mode of action. Various additional effects are of relevance, too. These comprise actions on metabolic sensing, mitochondrial function including their localization and integrity, antioxidative and antiapoptotic actions, modulation of the immune system, prevention of neuronal overexcitation and counteraction of brain inflammaging (Hardeland 2013a; Hardeland et al. 2015). The relevance of these findings will be discussed in the light of contributions to healthy aging, which should, however, not be misinterpreted in terms of direct lifespan extension.

10.2 The Multiplicity of Melatonin's Actions: Systemic Consequences

The pleiotropy of melatonin, which acts systemically as an orchestrating regulator molecule, has several consequences. It influences numerous physiological functions that are, at first glance, unrelated and not necessarily directly interacting. However, when perceiving the both hierarchical and dynamic aspects of a system that is to a large extent based on oscillations, the obvious pleiotropy gains plausibility. Very often researchers have expressed their doubts on the manifold beneficial effects ascribed to melatonin. Even though not every report will pass a thorough reexamination, the multitude of health-improving actions is by no means illogical, but simply the consequence of orchestration. Melatonin is known to control and modulate many regulatory molecules including other hormones, paracoids, cytokines, ion channels, metabolic sensors and transcription factors (Pandi-Perumal et al. 2006; Hardeland et al. 2011). Therefore, it acts from a higher hierarchical level than most other factors. Moreover, it contributes substantially to the circadian dynamics of the body, as it mediates circadian information and also influences phases and amplitudes of oscillators. With regard to the countless cellular functions under circadian control by output signals of the local intracellular oscillator machineries, the chronobiological role of melatonin has to have numerous cell biological and, ultimately, physiological consequences.

However, the involvement in a multitude of functions within the organism has also a dark side. This becomes apparent from the moment on when such an orchestrating regulator loses its efficacy, because of aging-associated reduced synthesis and secretion, dysphased rhythmicity, suppression by external factors such as light at night, stress, or pain. Flattening of rhythms including that of circulating melatonin are a common feature of aging, wherein losses of melatonin may contribute to a reduced coupling and, thus, misalignment of oscillators (Hardeland 2017). As mentioned above, the age-associated reductions in melatonin are interindividually highly variable. Nocturnal melatonin levels have been reported to range between >100 and 35 pg/mL in subjects of 21–25 years, between >60 and <20 pg/mL around 51–55 years, and to have declined down to 30–10 pg/mL in senescent persons of 81–86 years (Reiter 1995; Karasek and Reiter 2002; Bubenik and Konturek 2011). Although individuals can be found whose melatonin levels exceed these ranges, the tendency is well documented. A specific aspect of melatonin's contribution to a good physical condition may be deduced from the fact that insomniacs typically exhibit lower melatonin levels than age-matched subjects with normal or only moderately disturbed sleep (Pandi-Perumal et al. 2005). Such findings have led to the assumption that age-associated insomnia may be related to the decrease of nocturnal melatonin. Sleep difficulties are experienced by the majority of humans at a certain age and certainly cause reductions in fitness and daytime functioning. However, one should be aware that the disturbances of sleep represent only a single physiological dysfunction that is easily perceived. With regard to the pleiotropic properties of melatonin there is good reason to assume that many more functions are also affected without being perceived by the individual, but presumably reduce physical fitness as well.

Under these aspects, the idea of a melatonin replacement strategy has been discussed many times. In the case of sleep promotion, the efficacy has, however, remained relatively moderate, for reasons mentioned elsewhere (Hardeland 2009a, 2012, 2017). As discussed there, it is important to consider differences between effects requiring short or long actions. Concerning short actions, melatonin is highly effective in reducing sleep onset latency, but much less in supporting sleep maintenance, which needs prolonged actions. It is also important to be aware that the chronobiotic, i.e., synchronizing properties of melatonin only require short actions. Therefore, desired improvements in the functioning of the circadian multioscillator system may be achieved by melatonin treatment, although many details remain to be worked out. Moreover, the multitude of protective effects that have been reported and may contribute to healthy aging should be seen as a good reason for continuing research on the medical suitability of melatonin in the field of gerontology. This may not only be a matter of treatment of senescent subjects, but should rather comprise the attenuation of unfavorable changes in midlife concerning reductions in melatonin secretion and circadian amplitudes.

10.3 Protection Mechanisms and the Limits by Experimental Approaches

10.3.1 Antioxidative, Antinitrosative and Antinitrative Protection

After the discovery of hydroxyl radical scavenging by melatonin (Tan et al. 1993), the, at that time, newly discovered function of antioxidative protection has been in the focus of many researchers and this molecule was often perceived more as a protective compound rather than a regulator. However, the property of radical scavenging was frequently overrated with regard to the concentrations required for attaining a substantial efficacy. Although melatonin is believed to represent an especially potent scavenger, and although the first interaction can initiate a cascade of nonenzymatically formed metabolites which can scavenge a total of up to 10 free radicals (Rosen et al. 2006), the levels of melatonin in the circulation and in most cells should be much too low to explain an efficient antioxidative protection as experimentally observed. Direct scavenging may only substantially contribute to antioxidative protection in cells or organelles that synthesize or accumulate melatonin. Outside the animals, much higher levels of melatonin that are relevant to direct radical detoxification can be found, but this remains beyond the topic of this article. Therefore, other mechanisms of protection have become into focus and have led to the concept of radical avoidance (Hardeland 2005).

The indirect, largely regulatory effects of melatonin that contribute to antioxidative protection in mammals comprise numerous mechanisms. These may be summarized as (1) upregulation of antioxidant enzymes; (2) downregulation of prooxidant enzymes; (3) enhanced synthesis of reduced glutathione (GSH) and reduction of oxidized glutathione (GSSG), especially in mitochondria; (4) support of electron flux through the mitochondrial electron transport chain, an effect that decreases electron dissipation and, thus, formation of superoxide anions ($O_2 \cdot^-$), the primary free oxygen radical, as well as other reactive oxygen species deriving thereof; (5) downregulation of inducible NO synthase (iNOS) and inhibition of neuronal NOS (nNOS), effects that reduce inflammatory responses, especially in the microglia, contribute to the prevention of neuronal overexcitation, and counteract mitochondrial dysfunction; (6) additional antiexcitatory actions via $GABA_c$ and $mGlu_3$ receptors, GABAergic facilitation and effects on Ca^{2+} and K^+ channels; (7) support of the circadian system, which may be of importance because mutants of oscillator genes were shown to exhibit enhanced oxidative damage. Details of these numerous indirect antioxidant effects have been summarized by Hardeland (2005, 2009b, 2013a), Hardeland and Poeggeler (2008), and Hardeland et al. (2011, 2015).

Because of its crucial importance with regard to numerous diseases, some aspects of mitochondrial protection shall be discussed in this place. Blockades within the electron transport chain (ETC) can be caused by actions of reactive oxygen and nitrogen species. $O_2 \cdot^-$ radicals are steadily formed in mitochondria at a certain rate by electron leakage. Their detoxification product, H_2O_2, can become a source of the highly reactive hydroxyl radical (·OH), but the formation of this strongly damaging intermediate is also possible and presumably much more important via another pathway: $O_2 \cdot^-$ easily combines with ·NO to peroxynitrite ($ONOO^-$), another reactive compound that generates free radicals, such as ·OH and $\cdot NO_2$, as a consequence of protonation, or carbonate radicals ($CO_3 \cdot^-$) and $\cdot NO_2$, by decomposition of a CO_2 adduct ($ONOOCO_2^-$). As the affinity of $O_2 \cdot^-$ to ·NO is in the same range as that to the mitochondrial superoxide dismutase (MnSOD), peroxynitrite formation is rather likely at elevated ·NO levels, and as high quantities of CO_2 are produced in the citric acid cycle, the generation of $ONOOCO_2^-$ and, thus, carbonate radicals is probable. Carbonate radicals represent another oxidizing intermediate, which has, by virtue of resonance stabilization, a considerably longer lifetime and, thus, reaches more distant sites than the extremely short-lived hydroxyl radical. Blockades of mitochondrial electron flux can be caused by several of the compounds mentioned. ·NO, its congeners and derivatives thereof can cause nitrosation of ETC components, either directly or indirectly by transnitrosation from nitroso glutathione or nitroso cysteine. Combinations of electron/hydrogen-abstracting radicals and $\cdot NO_2$ can lead to tyrosine nitration in ETC proteins. Further details of these reactions of reactive nitrogen species, their products and interaction partners have been summarized elsewhere (Hardeland 2009c, 2011). Moreover, respirasomal proteins and membrane lipids can be oxidized by

$\cdot OH$ or $CO_3\cdot^-$. Therefore, the downregulation of $\cdot NO$ formation by melatonin represents an important contribution to the avoidance of oxidative and nitrosative/nitrative damage. Moreover, melatonin has been shown to efficiently scavenge carbonate radicals (Hardeland et al. 2003). Again, the efficacy of $CO_3\cdot^-$ detoxification should depend on melatonin concentration. However, melatonin has been shown to accumulate in mitochondria (Messner et al. 1998; López et al. 2009; Venegas et al. 2012), which might imply a possible protection at local physiological levels. At least, mitochondrial actions of melatonin should be relevant at pharmacological doses. These considerations are also relevant to the inhibition of the mitochondrial permeability transition pore (mtPTP), which is observed at an IC_{50} of 0.8 μM melatonin (Andrabi et al. 2004). An additional effect of melatonin on mtPTP opening concerns its duration. Intense openings lead to so-called superoxide flashes. In astrocytes, melatonin was shown to suppress long-term openings that would lead to a persistent breakdown of the mitochondrial membrane potential ($\Delta\Psi_{mt}$) and may induce apoptosis, but it still allowed intense short-term openings that did not lead to apoptosis (Jou 2011). These transient openings may be less important in terms of superoxide rather than calcium release and are thought to prevent mitochondrial calcium overload. Another aspect of mitochondrial protection concerns the counteraction of cardiolipin peroxidation, which represents a crucial step in the release of cytochrome c, which initiates apoptosis. The antagonizing effect of melatonin was observed in the context of both aging and diseases (Petrosillo et al. 2008, 2010; Paradies et al. 2010; Hardeland 2013a). It should be briefly mentioned that mitochondrial protection does not only prevent apoptosis via the intrinsic pathway but also mitophagy. The role of melatonin in the control of mitophagy does not seem to be uniform and obviously varies according to the type of insult. Low-grade oxidative insults may be suppressed by melatonin, with the result of attenuated mitophagy (Coto-Montes et al. 2012; Hardeland 2013a). However, under conditions of traumatic brain injury, melatonin was reported to stimulate mitophagy via the mTOR pathway (Lin et al. 2016). It seems that mitophagy is a favorable procedure to eliminate damaged mitochondria under some conditions of neuroinflammation, which may be mitigated by melatonin. However, mitophagy can become problematic in chronic low-grade neuroinflammation, especially as the repeated elimination of these organelles predominantly causes losses in peripheral mitochondria, which lead to reduced neuronal connectivity by synaptic ATP depletion.

It should be briefly mentioned that some aspects of protection against oxidation and nitration are correspondingly relevant to blood vessels, in which oxidized and tyrosine-nitrated proteins contribute to atherosclerotic plaques. Especially the possibly important role of peroxynitrite-derived free radicals is poorly perceived, especially those formed by decomposition of the peroxynitrite-CO_2 adduct, $ONOOCO_2^-$. Under conditions of reduced vascular perfusion in atherosclerosis, $\cdot NO$ is released in attempts of dilating the vessel and can combine with the relatively abundant $O_2\cdot^-$, especially in the presence of foam cells, which additionally produce $\cdot NO$ by upregulated iNOS. The resulting peroxynitrite can easily combine with CO_2, which is insufficiently eliminated in underperfused vessels.

The nonclassic nitration of aromates by combinations of $CO_3\cdot^-$ and $\cdot NO_2$ has been demonstrated and was more effective than combinations of $\cdot OH$ and $\cdot NO_2$, presumably because of the short halflife of $\cdot OH$ (Guenther et al. 2005). Beneficial effects of melatonin against vascular tyrosine nitration might be reconsidered under this point of view.

10.3.2 Antiapoptotic Actions

In addition to the antiapoptotic effects mentioned with regard to protection of mitochondria, inhibition of the intrinsic pathway of apoptosis has been demonstrated in countless publications and has been multiply reviewed (Sainz et al. 2003; Hoijman et al. 2004; Pandi-Perumal et al. 2006; Acuña-Castroviejo et al. 2007; Koh 2008a; Wang 2009; Maity et al. 2009; Luchetti et al. 2010; Tuñón et al. 2011; Hardeland et al. 2011; Kim et al. 2011; Mohseni et al. 2012; Hardeland 2013a; Jumnongprakhon et al. 2014). Typical findings were upregulation of antiapoptotic factors such as Bcl-2 and Bcl-xL and downregulation of their proapoptotic counterparts Bax and Bad, as well as inhibition of Bad dephosphorylation and of poly ADP ribose polymerase (PARP) cleavage. The frequently also observed decreases in cytochrome c release, apoptosome formation and activities of caspases 3 or 9 have to be seen as downstream effects that depend on prevention of apoptosis initiation by melatonin rather than direct actions on these factors. Upstream pathways of apoptosis initiation have been more rarely investigated, mostly in the context of severe forms of brain injury (Koh 2008b, c, d) and high-grade inflammation such as colitis (Mazzon et al. 2006) or nephrosis (Pedreañez et al. 2004). In brain injury models, the role of Akt (protein kinase B), which is modulated by melatonin, was addressed and dephosphorylation of Akt downstream factors, such as mTOR, p70S6 kinase, pAFX and pFKHR, shown to be inhibited. In colitis and nephrosis, Fas ligand expression was reported to be reduced by melatonin.

The relevance of the antiapoptotic actions has to be seen on the background of the experimental approaches, which are all based on strong challenges or injuries. Although the knowledge of these effects can be of value for the treatment of acute diseases or brain injury, the significance of melatonin in counteracting apoptosis in the basal, subclinical processes of aging is not yet evident. Although the inhibition of apoptosis has been studied in neurodegenerative diseases (Wang 2009), this would require extensive direct investigation in a gerontological context beyond acute clinical conditions. Nevertheless, clarification might be of particular interest, especially with regard to low-grade inflammation, as it is typical for inflammaging (cf. Hardeland et al. 2015).

Importantly, melatonin is not generally an antiapoptotic agent, but can also behave in a proapoptotic way. Interestingly, melatonin discriminates between nontumor and tumor cells and has been called, for this reason, a ‘smart killer’ (Lanoix et al. 2012). Induction of apoptosis in tumor cells by melatonin was found to be frequently associated with oxidative stress, sometimes ER stress, as well as upregulation of proapoptotic and downregulation of antiapoptotic factors of the Bcl family (Sainz et al. 2003; Sánchez-Hidalgo et al. 2012; Uguz et al. 2012; Bonmati-Carrion et al. 2013; Chuffa et al. 2016; Li et al. 2016). Along with other oncostatic effects such as inhibition of growth and tumor cell migration, these findings may be of future therapeutic value. However, it is to date far from being clear whether physiological levels of melatonin may contribute to the prevention of tumorigenesis.

10.3.3 Neuroprotection

Neuroprotection by melatonin has been extensively studied. However, many of these reports concern protection against rather dramatic insults, such as experimental ischemia/reperfusion, various types of traumatic brain injury, high-grade neuroinflammation and administration of neurotoxins with properties of either inducing mitochondrial dysfunction or excitotoxicity. Evidence is mainly based on preclinical experiments that have been amply documented and repeatedly reviewed (Reiter 1998; Skaper et al. 1999; Cheung 2003; Reiter et al. 2003, 2005, 2007, 2010; Beni et al. 2004; Chandrasekaran et al. 2004, Kilic et al. 2005; Macleod et al. 2005; Mayo et al. 2005; Das et al. 2010; Singhal et al. 2012; Shinozuka et al. 2013; Pita et al. 2013; Gonzales-Portillo et al. 2015; Andrabi et al. 2015; Watson et al. 2016). Although respective pathological conditions in humans are life-threatening and although, after a successful treatment, the remaining consequences may still reduce lifespan, a strong relationship to normal aging and to possibilities of improving health and longevity is not obvious. Moreover, some of the experimental settings are far apart from clinical reality or the etiology of respective diseases. This is most obvious in Parkinson’s disease (PD), in which studies were usually based on toxicological damage to the nigrostriatum. However, such approaches aim to mimic the motor deficits that appear at a relatively late stage of the disease, which, in fact, starts much earlier but not in the nigrostriatum and can be recognized by prodromal nonmotor symptoms, such as olfactory loss, REM sleep behavior disorder and constipation, not rarely in association with depression (Postuma and Berg 2016). Therefore, the use of neurotoxins fails to meet the etiological basis of this disease. Nonetheless, melatonin may have a particular value in preventing or reducing damage to the central nervous system in the course of normal aging.

Therefore, the main focus of this section shall be laid upon low-grade inflammation, as it occurs subclinically in the course of aging and is actually known by the term of inflammaging. However, these slow, lingering changes may gain clinical relevance at advanced age and can be severely aggravated in neurodegenerative diseases, in which additional proinflammatory mechanisms and agents are involved.

Before discussing the pertinent actions of melatonin, a few age-associated alterations shall be mentioned that drive inflammaging. First, inflammaging is, at least in part, the consequence of immunosenescence, which comprises numerous changes in the abundance and proliferative potential of leukocyte subtypes, the balance between pro- and antiinflammatory cytokines and the production of antibodies. Details that may be of relevance to melatonin's actions have been summarized elsewhere (Hardeland 2013a; Hardeland et al. 2015). In the course of immune remodeling, which is necessary as a consequence of thymic involution but also includes some compensation mechanisms, new phenotypes develop that especially deviate with regard to the pro- vs. antiinflammatory balance. In many individuals, this balance is shifted towards the proinflammatory side and may result in a so-called immune risk profile (IRP) that is characterized, besides other deviations, by the prevalence of proinflammatory cytokines. In the worst case, immunosenescence leads to misdirected reactions of enhanced inflammatory responses and autoimmune diseases. Notably, enhanced inflammation generally causes an increased oxidative burden, up to oxidative stress. With regard to melatonin, its properties as both an immune modulatory agent and antioxidant indicate an influence on the severity of changes towards inflammaging. Interestingly, centenarians have been found to not exhibit a typical IRP, but instead to sometimes display an opposite phenotype referred to as an 'inverted IRP' (Strindhall et al. 2007). However, this should not be misunderstood in terms of a complete inversion, because both anti- and proinflammatory cytokines were found to be enhanced in this age group (Candore et al. 2010).

The second change relevant to inflammaging is related to the senescence-associated secretory phenotype (SASP), which is a consequence of the DNA-damage response (DDR) (Coppé et al. 2008, 2010; Young and Narita 2009). Damage of DNA causes, at least in differentiated cells, a mitotic arrest which allows the cells to persist in the tissue and to participate in the tissue-specific metabolism. However, these cells, which are typically nonimmune cells, start to secrete, besides other factors, proinflammatory cytokines which attract and activate immune cells. These contribute to low-grade inflammation, but may even induce, by means of released oxidants, malignant transformation in previously nondamaged stem cells, which are not arrested by DNA damage (Davalos et al. 2010; Acosta et al. 2013). SASP is of general importance in many tissues and has been shown to also occur in the central nervous system, since it was demonstrated in astrocytes (Salminen et al. 2011; Mombach et al. 2015).

The third mechanism that contributes to low-grade inflammation in the brain is based on interactions between neurons and astrocytes with the microglia. Glutamatergic neuronal overexcitation has been shown to cause microglia activation (Campuzano et al. 2008; Arlicot et al. 2014; Lin et al. 2014; Nomaru et al. 2014). Conversely, microglia can lead to excitotoxicity (Takeuchi et al. 2005; Brown 2007; Brown and Neher 2010). Astrocytes, which are frequently coactivated with microglial cells, contribute to both overexcitation and microglia activation, by impaired glutamate uptake or enhanced NO formation, which acts as both a neuronal stimulator and an inflammatory signal. (Tilleux and Hermans 2007; Brown 2007; Salminen et al. 2011; Morales et al. 2014). Both neuronal calcium overload, which leads to enhanced mitochondrial electron dissipation, and inflammation-related oxidant release cause oxidative stress and, under conditions of simultaneously enhanced NO formation, also nitrosative/nitrative stress.

With regard to the complex interplay between neurons, astrocytes and microglia, the pleiotropy of melatonin, which acts on all these three cell types, is of protective value under conditions of low-grade neuroinflammation (Fig. 10.2). The antiexcitatory actions mentioned in 10.3.1 suppress overexcitation and mitochondrial dysfunction. As these effects are accompanied by inhibition of nNOS at already low melatonin concentrations (León et al. 2000; Acuña-Castroviejo et al. 2005; Entrena et al. 2005; Jiménez-Ortega et al. 2009), proinflammatory signaling by neuron-derived NO towards the microglia is substantially reduced. The additional downregulation of iNOS, which has been documented for various tissues (summarized in: Hardeland 2013a), but has been also specifically demonstrated in both astrocytes and microglia (Tocharus et al 2008; Tapias et al. 2009; Niranjan et al. 2012), can be concluded to interrupt proinflammatory activation at the sides of these other two cell types, too.

Two additional aspects of low-grade neuroinflammation shall be briefly addressed. The recently discovered role of insulin resistance in the brain as an early event of neuroinflammation, especially with regard to the etiology of Alzheimer's disease (AD) (Clark and Vissel 2013; Jiang et al. 2013; de Felice et al. 2014; de la Monte and Tong 2014; Ferreira et al. 2014; Arrieta-Cruz and Gutiérrez-Juárez 2016; Salameh et al. 2016), raises new questions concerning the relationship between melatonin and diabetes. Insulin resistance has been shown to be more generally associated with memory deficits and cognitive decline in elderly patients (de la Monte 2014a). Actually, AD has been suggested to represent kind of a 'type 3 diabetes' (de la Monte 2014b; Kandimalla et al. 2017), in which insulin resistance is not only related to AD-typical changes such as amyloidogenesis and tangle formation, but may be also causative to oxidative stress and mitochondrial dysfunction in the CNS (Verdile et al. 2015; Abolhassani et al. 2017).

With regard to melatonin in AD, various effects have been assumed to be beneficial (Rosales-Corral et al. 2012), but the evidence is largely based on preclinical findings. In addition to the general properties as antioxidant, protector of mitochondria, and partially antiinflammatory agent, some more AD-specific actions have been described, such as (1) reduction of tau hyperphosphorylation in various models (Deng et al. 2005; Srinivasan et al. 2006; Wang et al. 2007; Bender Hoppe et al. 2010; García-Mesa et al. 2012), (2) inhibition of fibrillogenesis under different settings based on A$\beta_{1\text{-}40}$ and A$\beta_{1\text{-}42}$ peptides (Pappolla et al. 1998, 2000; Poeggeler et al. 2001; Cheng and van Breemen 2005; Srinivasan et al. 2006), (3) inhibition of Aβ-induced damage to mitochondrial DNA (Pappolla et al. 1999) and reduced death in neuroblastoma or astroglioma cells (summarized by Srinivasan et al. 2006), (4) inhibition of amyloid plaque disposition in transgenic mouse models (Matsubara et al. 2003; Feng et al. 2004) as well as (5) reduction of Aβ peptide levels (Lahiri et al. 2004; García-Mesa et al. 2012). In the pheochromocytoma cell line P12 used as another model, melatonin decreased the expression of β-amyloid precursor protein (APP) mRNA (Song and Lahiri 1997) and the secretion of soluble Aβ (Lahiri 1999). More recently, melatonin was shown to directly enhance α-secretase

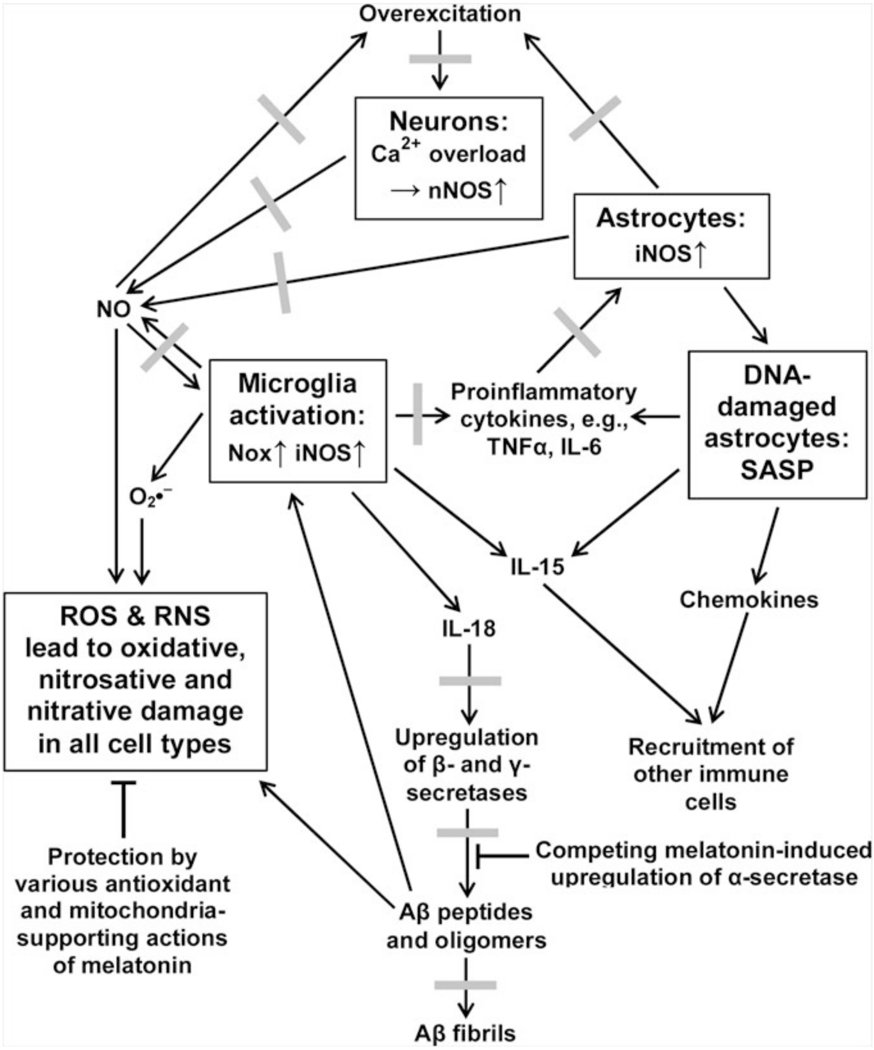

Fig. 10.2 A simplified overview of the major, multiply interacting neuroinflammatory processes and the described protective effects of melatonin that can prevent or interrupt the pathological changes, according to mainly preclinical studies. Blockades of proinflammatory effects are indicated by grey bars, other counteractions by T-shaped inhibition symbols. Abbreviations: *Aβ* amyloid-β; *IL* interleukin; *iNOS* inducible nitric oxide synthase; *nNOS* neuronal nitric oxide synthase; *Nox* NADPH oxidase; *RNS* reactive nitrogen species; *ROS* reactive oxygen species; *SASP* senescence-associated secretory phenotype; *TNF* tumor necrosis factor. ↑ indicates upregulation. The neuroinflammation-inducing role of insulin resistance has been omitted from the scheme, because of lacking information on the connections to the other pathways of interaction and, also, because of uncertainty concerning the influence of melatonin

activity, which leads to the formation of the nonamyloidogenic and neuroprotective fragment sAPPα (Shukla et al. 2015). This effect that competes with the formation of toxic peptides by β- and γ-secretases, was studied in human βAPP overexpressing HEK293 or N2a cells and involved melatonin receptor-dependent ERK1/2 activation. These findings were confirmed in human neuroblastoma SH-SY5Y cells, in which additional inhibitions of β- and γ-secretases were reported (Panmanee et al. 2015). All these findings are certainly encouraging, but one has to remain aware that they are still in a preclinical stage. If they will turn out to be translatable, they may be also of relevance to normal aging, since changes typical for AD have been observed, at inapparent severity, in subclinical aging. However, the distinction between nonpathological changes and the early stages of AD is not always easy. An additional problem concerns the uncertainties on the usefulness of melatonin treatment with regard to insulin resistance in humans, as already discussed in the Introduction 10.1. Although a beneficial role of melatonin cannot be ruled out for youthful subjects (Hardeland 2017), the pineal hormone seems to be highly problematic in type 2 diabetics of advanced age, and the same may apply for AD patients, whether or not one will be inclined to call this disease type 3 diabetes.

10.4 Modulation of the Immune System in the Light of Immunosenescence

The complexity of the immune system and the corresponding multitude of melatonin's actions within this system result in some ambiguities concerning the balance between pro- and antiinflammatory responses and reveal a conditionality that still requires further clarification. Some effects of melatonin have to be understood in terms of an immunoenhancement that may comprise the potential of more strongly reacting in an inflammatory way, but are not generally per se proinflammatory. This concerns, e.g., changes in the abundance or distribution of leukocyte subtypes, differentiation effects, release of growth factors and upregulation of MHC class II molecules (summarized in: Hardeland et al. 2011). In relation to aging, the relative importance of these actions does not seem to be uniform, especially as certain subsets of leukocytes as well as their proliferative potential are decreasing in the course of senescence (Hardeland 2013a). Even the proportions between antibody classes are known to change by age, since B cell immunosenescence leads to losses of naïve IgD^+ B cells, which are replaced by exhausted memory IgD^- B cells, to decreases in IgM and IgD, but to increases in IgG1 levels (Listi et al. 2006; Buffa et al. 2013). The well-known effects of melatonin on T cell differentiation, growth activation, mainly in favor of the T_h1 response (García-Mauriño et al. 1999; Raghavendra et al. 2001; Hardeland et al. 2011; Hardeland 2013a), can be assumed to become reduced in the course of thymic involution.

When melatonin effects were studied in leukocyte preparations or cultures of transformed myeloid or lymphocytic cell lines, proinflammatory responses prevailed (Carrillo-Vico et al. 2013). These consisted in upregulation of the proinflammatory cytokines IL-1β, IL-2, IL-6, IL-8, IL-12, IFNγ, and TNFα, and in downregulation of the antiinflammatory cytokine IL-10. Moreover, a direct activation of monocytes is known, which causes strongly enhanced production of reactive oxygen species and, thus, enhanced cytotoxicity (Morrey et al. 1994). However, these effects strongly contrast with observations made (i) under conditions of high-grade inflammation (Carrillo-Vico et al. 2013; Hardeland 2013a; Hardeland et al. 2015), which shall not be considered here in detail, and (ii) in the gerontological context. In the liver of old female rats, melatonin downregulated the proinflammatory cytokines TNFα, IL-1β and, especially, IL-6, whereas it strongly increased the antiinflammatory IL-10 (Kireev et al. 2008). In the liver of the senescence-accelerated mouse strain SAMP8, TNFα and IL-1β were downregulated and IL-10 upregulated (Cuesta et al. 2010). Corresponding results were obtained in the pancreas, in addition to antioxidative and antiapoptotic effects (Cuesta et al. 2011). Antiinflammatory actions were also observed in the heart of SAMP8 mice (Forman et al. 2010, 2011), observations that are well in accordance with findings on antioxidative actions of melatonin in the aging heart of the rat (Eşrefoğlu et al. 2011). Another nexus between melatonin and antiinflammatory effects may concern SIRT1, a relationship that may involve but possibly exceed the circadian interactions mentioned in the Introduction 10.1. SIRT1 has been reported to display antiinflammatory properties and its posttranslational inactivation has been concluded to promote inflammaging (Hwang et al. 2013). In the hippocampus (Chang et al. 2009) and pancreas (Cuesta et al. 2013) of SAMP8 mice, in aged murine neurons (Tajes et al. 2009), and in the dentate gyrus of old rats (Kireev et al. 2014), melatonin upregulated SIRT1. In the latter study, these changes were accompanied by reduced expression of iNOS, IL-1β and TNFα as well as decreases in proinflammatory and prooxidant signaling pathways. Generally, accumulating evidence indicates that melatonin upregulates SIRT1, at least in aging rodents, effects that strongly contrast with substantial downregulations observed tumor cells, a difference that has been explained on the basis of dysregulated circadian oscillators in cancer cells vs. weakened, low-amplitude oscillators in senescent animals (Hardeland 2014a, 2017). The downregulation of iNOS by melatonin, as reported in the study by Kireev et al. (2014), has been frequently disregarded in the gerontological context, but the reduction of the inflammatory signal NO may substantially contribute to antiinflammatory actions in aging. This effect on iNOS is presumably related to another antiinflammatory action of melatonin, which consists in the suppression of cyclooxygenase 2 (COX-2), since both changes seem to be mediated by the same signaling pathways (Mayo et al. 2005; Deng et al. 2006; Korkmaz et al. 2012). However, the relevance of melatonin-induced COX-2 suppression has not been sufficiently studied in aging.

Although these effects seem to indicate a predominantly antiiflammatory action of melatonin in aging, several reservations have still to be made. First, it remains to be shown whether or to what extent immunological effects by melatonin that have been obtained in aging rodents are applicable to humans. Moreover, it seems important to remain aware of the changes in immunosenescence, which exhibit a substantial interindividual variability. In a subpopulation of humans, in which a proinflammatory phenotype is developing or progressing, the immune stimulatory properties of melatonin may become problematic, whereas this may not be the case in subjects with an ‘inverted IRP’. Proinflammatory traits may, in particular, be critical in autoimmune diseases, in which a more or less generalized immune stimulation should be avoided, which may happen with melatonin. Therefore, simplifying statements on melatonin as an antiiflammatory agent are inappropriate with regard to the senescent human. In rheumatoid arthritis, melatonin has been shown to aggravate the symptoms (Maestroni et al. 2005; Cutolo and Maestroni 2005; Hardeland 2012), findings that should be taken as a caveat for melatonergic treatment of idiopathic inflammatory and, in particular, autoimmune diseases.

10.5 Problems of Translation from Rodents to Humans

10.5.1 Circadian Differences

In the pineal gland, melatonin is preferentially synthesized at night and rapidly released to both the circulation and the third ventricle of the brain. The cyclicity of melatonin formation and secretion is based on a high-amplitude circadian rhythm. Although circadian oscillations are by definition of endogenous nature, exogenous influences such as light at night can directly suppress melatonin synthesis, in addition to phase shifting that may also occur under the influence of light signals (for mechanisms see: Hardeland 2008). The coupling of the melatonin maximum to the dark phase exists in nocturnally as well as diurnally active vertebrates. In either category of animals, the information of ‘darkness’ is transmitted to brain areas including the SCN and to peripheral organs (cf. Fig. 10.1). The association with darkness implies a profound difference between nocturnally active rodents, which represent preferred laboratory animals, and day active species such as the human. In rats and mice, high melatonin is related to enhanced alertness, locomotor activity and food intake, whereas the opposite is the case in humans, in whom melatonin acts as a sleep-promoting compound. This difference between night-active laboratory animals and humans sets limits to the translation of results obtained in rodents.

Although it is quite obvious that effects of melatonin on sleep promotion, which is highly desired in humans with insomnia, cannot be based on experiments in rats or mice, in which the pineal hormone is associated with enhanced alertness, several experimental approaches have been tested in which sleep implicitly plays a role. This concerns especially the treatment of depression. Sleep disturbances are typically associated with mood disorders and are often prodromal to depressive episodes (Kupfer et al. 1981; Perlis et al. 1997a, b; Pandi-Perumal et al. 2009; Srinivasan et al. 2009). Countless studies have reported antidepressant-like effects of melatonin and synthetic melatonergic drugs in rats, which shall not be cited here to not exceed the scope of this article. Instead, it seems worth-while to dissect the principles of such approaches. If a study is simply aiming to reduce depressive symptoms by improving sleep, this cannot be successful in rodents because of their inverse relationship between melatonin and sleep. However, meaningful approaches should not be ruled out, as far as the intention is focused on improvements of the circadian system. Sleep difficulties are frequently a sign of deteriorating circadian clocks. In humans, several types of depression are also related to circadian dysfunction, in particular, bipolar disorder and seasonal affective disorder. This is also documented by associations with polymorphisms of various circadian oscillator genes (summarized in: Hardeland 2012; Hardeland et al. 2012). Additional associations have been reported for major depressive disorder, but an etiology involving circadian dysfunction may only exist in some its subforms. As soon as the relevant action of melatonin mainly consists in corrections of the circadian system, in terms of improving synchronization with the environment or, internally, within the multioscillator system, or in enhancing circadian amplitudes, such an approach may work in rodents as well as in humans, regardless of the differences in phase relationships between sleep and high melatonin. However, positive effects in rodents may not necessarily be obtained by applying behavioral treatments of inducing depression-like symptoms that are not related to the circadian system. Instead, circadian deviations that are potentially accessible to melatonergic treatment exist in aging rodents, which display deteriorations of the circadian system such as phase shifts, flattening, and even disappearance of rhythms (Yamazaki et al. 2002). Therefore, preclinical gerontological studies on improvements of the circadian system may be of value to the understanding of circadian deviations in human senescence and aging-associated disorders. Thereafter, pertinent findings may be tested with regard to their suitability in the treatment of subforms of depression. It seems important to distinguish between subforms with and without an etiology of circadian dysfunction. In the former but not in the latter case, attempts of readjusting circadian clocks, by melatonin in the evening or combinations with morning light, are worth to be tested in the individual, since treatment with conventional antidepressants, which are not free of side effects, may not be necessary. However, the caveat concerning rheumatoid arthritis and autoimmune diseases in general remains in the case of melatonin. It should be briefly mentioned that synthetic melatonergic agonists exist, such as agomelatine and TIK-30, which additionally act as 5-HT$_{2C}$ receptor antagonists, a property that is thought to exert direct antidepressant effects. However, the use of these synthetic drugs introduces toxicity problems (Hardeland 2014b), while the gain of this additional antidepressant property is rather moderate and this effect is not required for readjusting circadian rhythms (Hardeland 2012).

Generally, the circadian differences between nocturnal laboratory animals and humans should be always taken into account when designing experiments using melatonin, especially when the concept is to find strategies against human diseases or disorders. In the past, this has not always been done, sometimes because of a lacking awareness of circadian regulation of parameters to be studied. This concerns, e.g., also the immune system, which is in various aspects under circadian control, including inflammatory responses (Geiger et al. 2015; Lebailly et al. 2015; Kizaki et al. 2015; Trufakin and Shurlygina 2016; Sobolewska-Włodarczyk et al. 2016; Dumbell et al. 2016). With regard to the immunological actions of glucocorticoids, the high-amplitude circadian rhythms of these hormones differ between nocturnally and diurnally active species (Oster et al. 2017). Contrary to the general association of melatonin with darkness, glucocorticoid rhythms peak around the time of awakening, in both groups of organisms. This is also indicative of substantial temporal deviations in their immune systems as well as in the respective role of melatonin. In preclinical immunological experiments using melatonin this difference has been rarely considered. In the context of senescence and the extraordinary significance of the immune system for successful aging (DelaRosa et al. 2006; Strindhall et al. 2007; Ponnappan and Ponnappan 2011), this should be more consequently done in the future.

10.5.2 Metabolic Syndrome, Type 2 Diabetes and Insulin Resistance

Concerning the suitability of treatment with melatonin, this complex of diseases and their prodromal stages is, again, affected by circadian differences between rodents and humans that are related to the times of food consumption. The problem of adverse effects of melatonin on glucose tolerance and of enhanced melatonergic signaling by an overexpressed MT_2 receptor have already been briefly addressed in the Introduction 10.1. For many investigators, these observations have been surprising, since numerous preclinical studies have reported and underlined the beneficial actions of the pineal hormone in reducing cardiovascular problems in metabolic syndrome, in counteracting obesity, experimentally induced diabetes and insulin resistance (Leibowitz et al. 2008; Nduhirabandi et al. 2011; Agil et al. 2011, 2012; Kitagawa et al. 2012; Cardinali et al. 2013; Hatzis et al. 2013; Vinogradova and Anisimov 2013; Huang et al. 2013; Cano Barquilla et al. 2014; Kantar et al. 2015; Favero et al. 2015; Cardinali and Hardeland 2017). Similar results were obtained with synthetic melatonergic agonists such as piromelatine (=NEU-P11) and ramelteon, as summarized elsewhere (Hardeland 2010, 2017). Although the pathologies studied in the different models are mutually interconnected, one has to admit that they do not represent a uniform disease. Therefore, it may be possible that melatonin will turn out to be beneficial in humans, too, with regard to some of the aspects investigated in rodents. For instance, melatonin was reported to decrease the nocturnal blood pressure in hypertensive patients (Scheer et al. 2004; Cagnacci et al. 2005; Grossman et al. 2006) including those with diabetes of type 1 (Cavallo et al. 2004a, b) or 2 (Możdżan et al. 2014). However, this effect is fully in accordance with the chronobiological role of melatonin in humans, namely, to be associated with decreased blood pressure during sleep. Such a relationship is not that obvious in other findings concerning symptomatic improvements in metabolic syndrome of obese patients (Koziróg et al. 2011; Goyal et al. 2014). In the latter study, no ameliorations of fasting glucose were induced by melatonin, whereas other parameters improved.

With regard to these divergencies between the manifold aspects of metabolic syndrome and related pathologies, it seems, at the actual state of knowledge, preferable to focus on one aspect in which the chronobiological role of melatonin is more evidently different in nocturnal rodents and humans, namely, glucose tolerance. Apart from the well-documented prodiabetic role of the G allele of the melatonin receptor gene *MTNR1B* and its overexpression in beta cells, which inhibits insulin secretion (cf. 10.1), several reports have stated that melatonin impairs glucose tolerance also in nondiabetic volunteers (McMullan et al. 2013; Rubio-Sastre et al. 2014; Eckel et al. 2015). The contrasting results of antidiabetic actions in rodents and prodiabetic effects in humans are, in principle, well in accordance with the inverse phase relationships of melatonin to food consumption in these different organisms. A particular interpretation had been that melatonin may cause circadian misalignment, either when given in the morning or when high endogenous melatonin levels coincide with morning wakefulness because of a shortened sleep phase (Eckel et al. 2015). Although such disturbances of the internal circadian organization may aggravate the impairment of glucose tolerance, it has also been shown that melatonin reduces this parameter in both the morning and the evening (Rubio-Sastre et al. 2014). Recently, the *MTNR1B* risk allele has been reported to decrease glucose tolerance in nondiabetic young women more strongly than the wildtype allele (Garaulet et al. 2015). This effect was only demonstrable in the morning, but not significant in the evening, however, in very small cohorts.

These findings strongly indicate that the numerous data on antidiabetic effects of melatonin in rodents cannot be simply translated to the human. However, it might be that this conclusion is only valid for patients that have already developed the diabetic or a prediabetic state. The deleterious effects of melatonin in these groups of subjects are not easily compatible with some other findings, such as low melatonin as a diabetic risk factor, reduced melatonin levels in diabetics, and the occurrence of other, nonfunctional *MTNR1B* alleles that are also prodiabetic. Moreover, one might ask whether reductions of glucose tolerance by physiological nighttime levels of melatonin should be harmful instead of reflecting a minor, tolerable effect during the rest phase. As has been discussed recently (Hardeland 2017), the approximate coincidence of increased *MTNR1B* expression around midlife with age-related impairments of the circadian system and melatonin secretion might be understood as a circadian failure. Thus, melatonin might be even beneficial in the first half of life by improving the circadian system with regard to amplitudes and phase relationships, at least, as far as health conditions are not compromised by an unfavorable lifestyle that causes repeated circadian disruption or, e.g., obesity. It seems also important to aware of the difference between reductions of glucose tolerance by decreased insulin secretion, i.e., an effect in the beta cell, or by insulin resistance, i.e., a change in the target organs. With regard to insulin resistance in the central nervous system and its importance to neuroinflammation, the additional question arises as to whether melatonin may change insulin levels in the brain, including the not yet definitely clarified possibility of local insulin formation in the brain (cf. Lee et al. 2016). While diabetic states (and autoimmune diseases, too) are obviously a contraindication to melatonergic treatment, it remains to be clarified at which age and under which pathological conditions melatonin may be used in the elderly.

10.5.3 Limited Experimental Evidence for Lifespan Extension

The idea of extending the lifespan by melatonin has been followed over the years in several publications. As summarized and discussed by Poeggeler (2005), many of the earlier reports arrived at inconsistent results or may have been affected by methodological problems. In particular, the use of mouse strains that primarily die from specific diseases such as cancer might imply that a prolonged lifespan results from chemoprevention by melatonin rather than decelerated aging. Most of the earlier results that have demonstrated substantial prolongations of lifetime have been obtained in invertebrates (Poeggeler 2005). For instance, lifetime was extended by 50–100 percent in the rotifer *Philodina*. In mammals, all pertinent data remain in a much smaller range. A moderate prolongation of lifespan by melatonin was documented in the in the normally aging mouse strain SAMR1, in which mean and maximal lifetimes were extended from 20 to 23 and 25 to 26 months, respectively. More profound effects were obtained the largely isogenic, senescence-accelerated mouse strain SAMP8, with an extension of mean and maximal lifetimes from 16 to 22 and 23 to 27 months, respectively (Rodríguez et al. 2008). The higher efficacy of melatonin in SAMP8 raises, however, the question of whether melatonin predominantly counteracted the genetically caused acceleration of aging rather than decelerating the basic processes of normal aging. Another case of life extension under the pathological conditions of AD will be discussed in the subsequent section.

A further difference to the treatment of humans has to be seen in the mode of melatonin administration. In nocturnally active rodents, melatonin can be easily and continually given throughout the night via the drinking water. In humans, this is not possible and the developed prolonged-release formulations of melatonin do not yet afford sleep maintenance and may, thus, not be sufficient for mimicking the nocturnal melatonin peak in humans, a shortcoming that is not overcome by synthetic melatonergic drugs (Hardeland 2009a). Perhaps, new formulations may solve this problem. What can be done to date, is mainly the use of melatonin preparations that readjust the circadian system, which only requires short-term actions (Hardeland 2012). This may already have some influences on health-related parameters, but, if aging-decelerating processes would require the continuous presence of high nocturnal melatonin, the actually available preparations should be assumed to be insufficient.

10.5.4 Neurodegenerative Diseases

Apart from the aforementioned problem (10.3.3) that toxin-based models may not appropriately reflect the conditions and etiology of neurodegenerative diseases, another limit for translation to humans concerns the start of treatment. This is particularly obvious in AD. In transgenic Tg2576 AD mice, which may represent a model superior over toxicological approaches, reduction and delay of Aβ accumulation, preservation of cognitive performance as well as extension of lifespan were only convincingly observed when the treatment was started early in life, at 4 months of life (Matsubara et al. 2003) or 2–2.5 months (Olcese et al. 2009), but not at a later stage at 14 months (Quinn et al. 2005). In this progressed stage of the disease, the vicious cycle of self-reinforcing neuroinflammation can apparently no longer be halted and there seems to exist a point of no return, from which on the treatment with melatonin is no longer promising. The consequence to humans is rather a pessimistic one, because it seems unlikely that treatments will be started before the appearance of AD symptoms, when neuroinflammation has already exceeded a tractable stage. The only condition under which an AD-preventing or -delaying treatment with melatonin might be meaningful could be based on a screening of AD risk factors. According to the outcome, it would be necessary to convince carriers of risk factors and physicians that an early onset of therapy would be recommendable, something that would require a broader scientific basis than currently available. Although a late onset of treatment cannot be expected to substantially reduce disease progression, the use of melatonin in advanced stages may not be entirely useless, but its value is more or less restricted to palliative improvements. These include reduction of sundowning, mild but mostly transient improvements of memory and mood (Srinivasan et al. 2006), but usually not sleep (Singer et al. 2003). This latter observation is not that much surprising because AD is associated with a progressive deterioration of the circadian system including the SCN and its neuronal connections, changes that affect both sleep and the melatonin rhythm. The decay of the circadian system is evident in the loss of accuracy in the phase position of the remnants of the melatonin peak (Mishima et al. 1999).

Compared to other neurodegenerative diseases such as AD, Pick's disease, Friedreich's ataxia or Huntington's disease (Reiter et al. 1999; Srinivasan et al. 2006), the situation seems to be widely different in Parkinson's disease (PD). The unsuitability of some toxicological approaches with regard to the PD etiology (cf. 10.3.3) may be seen as a particular problem for translation. As summarized elsewhere (Hardeland 2012), several results and claims have been highly controversial. Melatonin secretion is, in most patients, only moderately reduced. Advance phase shifts of the melatonin rhythm that had been also reported were caused by dopaminergic treatment but not by the disease. Some moderate improvements of sleep and of depression have been reported for melatonin and its synthetic analog, agomelatine (Srinivasan et al. 2011). The entirely opposite suggestion by Willis (2005, 2008) to consider PD as a disease of melatonin overproduction has not been supported by levels of melatonin, of melatonin receptor expression and the missing relation to premotor symptoms (Hardeland et al. 2012). However, a report on observed improvements in PD patients by melatonin receptor antagonists (Willis 2005) should, at least, been taken as a caveat for the use of melatonin and other melatonergic agonists in this disease.

10.6 Conclusion: Healthy Aging Versus Extension of Lifespan

Numerous gene variants of circadian oscillator components, of melatonin synthesis and signaling have been shown to be associated with many, at first glance unrelated, diseases and disorders (Hardeland 2012; Hardeland et al. 2012). These findings indicate an important role of the circadian system and of melatonin, which is part thereof, in the maintenance of health. This is well in accordance with results on protective functions of melatonin summarized in this chapter. However, its seems to be important to also perceive the limits of melatonin treatment, e.g., in relation to sometimes proinflammatory effects, to adverse effects such as in diabetes type 2, and to the necessity of discriminating between actions in nocturnal rodents and the human. Nevertheless, melatonin appears as an agent that can support health and, more in particular, healthy aging. As melatonin feeds back to the SCN and as it additionally seems to act on, at least, some peripheral circadian oscillators, it is not unlikely that beneficial effects of melatonin are mediated to a certain extent via improvement of the circadian system, by contributing to the maintenance of high rhythm amplitudes, e.g., by upregulating SIRT1. It may optimize phase relationships between the various oscillators and favor entrainment to external time cues. In terms of resynchronization by chronobiotic actions (Armstrong and Redman 1991; Arendt and Skene 2005; Skene and Arendt 2006; Pandi-Perumal et al. 2006; Hardeland 2010, 2012; Hardeland et al. 2011, 2012; Golombek et al. 2015), the suitability of melatonin and other melatonergic agonists has been multiply demonstrated. To date, the most convincing effects concern the correction of circadian dysfunction in sleep and mood disorders, as far as they are caused by deviating spontaneous period lengths. Other health-promoting actions via the circadian system have not been sufficiently documented. However, it seems to be an important task for the future to follow this line. Additional effects such as mitochondrial protection, antioxidant, antiexcitatory and, when prevailing, antiinflammatory actions may contribute to the maintenance of health as well. However, it is widely unknown to what extent these experimentally well documented properties demonstrably contribute to human health in reality. In some human subpopulations of aged individuals affected by certain health problems, associations of melatonin or 6-sulfatoxymelatonin levels with measures of healthy aging or mortality were either not demonstrable with certainty or remained relatively modest (Goyal et al. 2014; Devore et al. 2016). At least, high nocturnal blood pressure, i.e., an adverse symptom that may reduce lifespan, was shown to be corrected by melatonin treatment in aged subjects (Obayashi et al. 2014). In total, the basis for judging the value of melatonin in improving health and eventually extending lifespan in humans is virtually absent. Although several studies have reported protection under acute disease conditions, there is no evidence based on the long-term use of melatonin in relation to normal human aging or any eventual delay of disease onset in whatever pathology.

What is remaining that is a remarkably large body of health-protecting effects in experimental animals under highly diverse conditions. Moreover, possibly important observations have been made on healthy aging in rodents, referred to as the melatonin Methuselah syndrome (Poeggeler 2005). In fact, animals treated with melatonin maintained a better health state, compared to untreated age-matched controls, displayed at advanced age a higher mobility, a glossy fur, absence of skin inflammation and low osteoporosis. The fact that several mouse strains die at an overproportional rate from cancer (Poeggeler 2005) may be seen in relation to melatonin deficiency that is present in many of these laboratory strains, and this might be regarded as another hint for the promotion of healthy aging by melatonin. However, there is no direct demonstration of a Methuselah syndrome in humans. Whether or not melatonin may extend lifespan is equally uncertain. As outlined in Sect. 10.5.3, the life-prolonging effects in mice had remained relatively moderate, except for conditions of aging-promoting pathologies. Thus, no realistic conclusions can be drawn on life extension in humans. However, the manifold reports on health-supporting and rescuing effects in preclinical studies may justify a certain degree of hope that melatonin may be beneficial to humans, too, despite the conclusion that several findings cannot be translated from rodents. Future work should especially consider the perspective of supporting the circadian system, to which melatonin may contribute. Finally, any contribution to healthy aging, which should be an aim of utmost importance in a progressively aging society, will indirectly extend the average lifespan, even if the basal processes of aging are not or only very moderately attenuated.

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Chapter 11
Hormones in Clock Regulation During Ageing

Anita Jagota and Neelesh Babu Thummadi

Abstract Organisms have evolved with an internal time keeping system to synchronize physiology and behaviour with nearly 24 h periodicity. In mammals, cognitive performance, energy metabolism, stress regulation, growth and development are governed by the circadian clock. SCN, the central pacemaker relays signals to the peripheral clocks via neuronal signals and humoral signals such as melatonin, glucocorticoids etc. The tight coordination between the circadian system and endocrine system is essential for healthy living and is regulated by several transcriptional and translational feedback pathways. Upon ageing, the circadian clock function and cognition diminishes with decline in endocrine function due to decrease in hormones as well as their receptors thus leading to disturbed stoichiometry of the homeostatic balance. Modulation of such pathways may play important role in extension of life span. The present chapter unravels the communication between the circadian clock and the endocrine system.

Keywords Suprachiasmatic nucleus · Melatonin · HPA axis · Ageing · Peripheral clocks · Longevity

11.1 Introduction

Ageing, an inevitable and unidirectional process that declines several physiologies, leads to various pathologies due to pleiotropic damage effect in several organisms. It is characterized by genomic instability, telomere shortening, epigenetic aberrations, loss of proteostasis, dysregulated nutrient sensing, mitochondrial dysfunction, cellular senescence, stem cell exhaustion, and altered intercellular communications (López-Otín et al. 2013).

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There are several theories to explain the causes of ageing but the widely accepted is free radical theory of ageing which states that in the course of life time the increased burden of free radicals would damage the biomolecules due to their high chemical reactivity causing several disorders. Concomitantly, there is neuroendocrine theory of ageing which proposes that as we age the neuroendocrine system slowly becomes less functional that causes blood pressure, impaired sugar metabolism and sleep abnormalities etc. (da Costa et al. 2016).

Ageing affects circadian rhythms, cognitive performance, hormone secretion, glucose metabolism, energy homeostasis, cell cycle, immunity etc. As ageing differentially affects various aspects of physiologies, it is more than difficult to decline the rate of age related deteriorations targeting a single event (Reiter et al. 2012).

For past few decades there is sharp rise in the elderly population for several reasons. But at what cost? This increase in the age is highly associated with several ailments like cancers, neurodegenerative disorders, cognitive disorders, metabolic disorders etc. Ageing is not curbed to few physiologies, there is no physiology in the body that has not been dampened with the advancing age. With the advancement in the molecular techniques, researchers are able to impinge the age-related disorders at the molecular level. The past decade has witnessed an awe-inspiring research on anti-ageing strategies ranging from hormonal therapy to chronotherapy and from caloric restriction to stem cell therapies. In this chapter we make an attempt to depict an interplay between ageing, cognition, longevity, circadian timing system and hormones (Fig. 11.1).

11.2 Circadian Timing System (CTS)

Adaptation is the foremost basic phenomenon for the survival of the organisms on the earth. To sustain in an environment that changes on the daily basis, organisms have evolved with an internal clock mechanism that maintains the internal

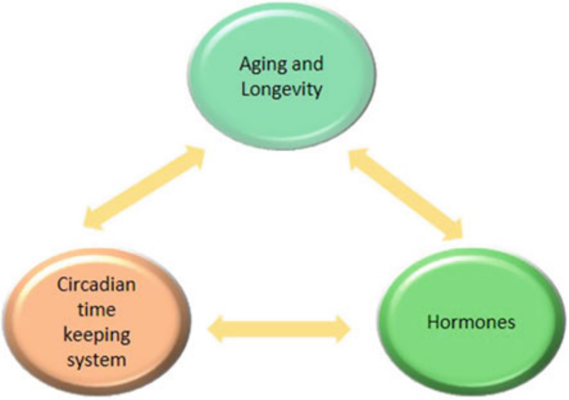

Fig. 11.1 Picture depicting the interplay between ageing and longevity, circadian time keeping system and hormones

homeostasis in phase with the external light-dark cycles. In mammals, the internal time keeper is present in Suprachiasmatic nucleus (SCN) (Jagota 2012; Takahashi 2016) (Fig. 11.2). SCN, the central pacemaker in mammals is entrained by light as major phasing factor or zeitgeber (time giver). Shift work, irregular feeding regimes, sleep disturbances, diminished neuro-behavioural performance and jet lag can influence the shift in the circadian rhythms.

SCN receives photic information from retina via glutamate and pituitary adenylate cyclase-activating polypeptide (PACAP) from retinohypothalamic tract (RHT). In SCN neurons, the glutamate-induced calcium influx immediately triggers several protein kinase pathways ultimately leading to phosphorylation of Ca^{2+}/cAMP-response element binding protein (CREB). Ca^{2+}/cAMP-response elements (CREs) are present in the promoters of several core clock genes, where phospho-CREB homodimers bind and activates transcription. *Period1* (*Per1*) and *Period2* (*Per2*) genes are important CREB clock targets with respect to photic signals and are readily induced in SCN neurons by nocturnal light exposure. The photic information received by the SCN is further processed and is transduced into neuronal and humoral signals to synchronize the physiological, cellular, molecular rhythms like sleep/wake cycles, hormone release, immunity, body temperature etc. (Jagota 2012; Takahashi 2016; Gnocchi and Bruscalupi 2017).

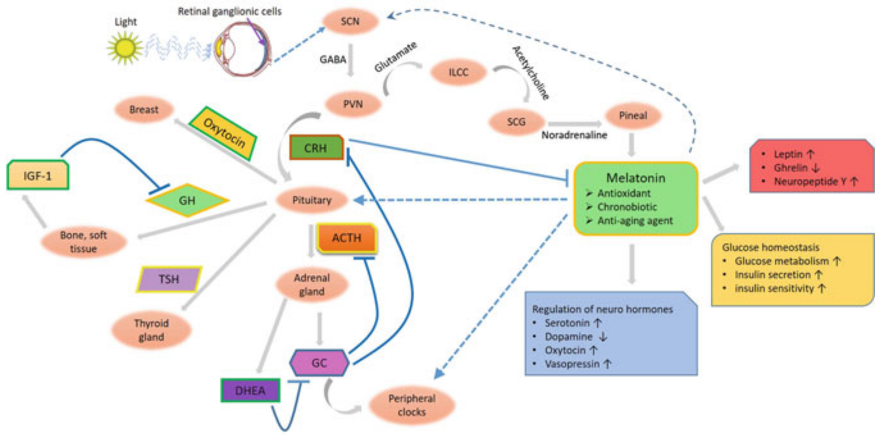

Fig. 11.2 Schematic representation of interlocked communication between the central clock and hormones: SCN via multi-synaptic pathways signals pineal and adrenal gland for the secretion of melatonin and glucocorticoids respectively through which it communicates with the peripheral clocks. The negative regulator of glucocorticoids, DHEA is also under the circadian control. Melatonin, the Pineal Hormone (messenger of darkness), regulates several other hormones. SCN also regulate hypothalamic–pituitary–thyroid (HPT) axis and GH–IGF1 axis; *SCN* suprachiasmatic nucleus, *PVN* paraventricular nucleus, *ILCC* intermediolateral cell column, *SCG* superior cervical ganglion, *CRH* corticotropin releasing hormone, *ACTH* adrenocorticotropic hormone, *GC* glucocorticoids, *DHEA* dehydroepiandrosterone, *TSH* thyroid stimulating hormone, *GH* growth hormone, IGF-1-Insulin growth factor-1

Within the neurons of SCN there exists a dedicated clock machinery to process the received photic information and to transduce it to other cells. Circadian machinery consists of tightly regulated transcriptional and translational feedback loops. The core clock components CLOCK and BMAL1 by heterodimerizing bind to the promoter region of clock controlled genes (*ccg*) that includes *Per* and *Cry* genes among others and induce the transcription (Liu and Chang 2017). During the day time PER proteins undergo ubiquitination and degrades. At the onset of the dark period, PER proteins cross the threshold level when they dimerize with CRY, translocate to the nucleus and inhibit the CLOCK and BMAL1 dimer thus repressing their own transcription along with the other *ccg* (Takahashi 2016). This cycle is crucial in generating and sustaining circadian rhythms in the mammals in accordance with the daily light/dark cycles (Albers et al. 2017).

In addition to this feedback loop, there exist an auxiliary feedback loop that comprises REV-ERBα and RORα. These two proteins competitively bind to the ROR elements in *Bmal1* promoter. Interestingly, RORα induce the transcription of *Bmal1* and REV-ERBα inhibits it (Jagota 2012; Takahashi 2016; Albrecht 2017).

Once the information is processed, the signals translocate through the autonomic neural connections and hormonal release (Jagota 2012).

11.3 Central and Peripheral Clocks

Central clock regulates peripheral organs, acting as autonomous clocks, through generation of temporal oscillations. Circadian clock machinery is not only present in SCN but also in almost every other cell (Yoo et al. 2004). Though peripheral clocks have clock machinery, the circadian rhythms become desynchronized and damp out in few days in the absence of SCN. The pacemaker property of SCN is demonstrated when kept as isolated culture with the circadian activity in metabolism, gene expression and electrical firing activity indefinitely at the single cell level. The circadian activity of peripheral clocks were demonstrated to be 4 h delay than the SCN proving the master and the slave relation between central and peripheral clocks (Balsalobre et al. 2000). Earlier reports from our laboratory also demonstrated the master and slave clock relation as the serotonin rhythms were established early in SCN compared to brain. Additionally, the rhythms disintegrated earlier in brain compared to SCN upon ageing (Jagota and Kalyani 2008). The circadian regulation of *ccg* differs with the type of tissue in order to synchronize different physiologies across the tissues (Tsang et al. 2016). It is the synchronization between the clocks that produce a coherent clock system that prepares the organism for the daily environmental challenges (Albrecht 2017). Feeding regimes, social interactions, drugs etc. can also act as time givers for both central and peripheral clocks (Pevet and Challet 2011; Reddy and Jagota 2014).

11.4 Ageing and Circadian Disruption

Ageing is associated with the disrupted circadian rhythms at level of gene expression, protein profile and biological processes. Age associated circadian disorders are associated with sleep disorder (Scullin 2016). Other reported age related attritions are disrupted locomotor activities as well as reduced re-entrainment capacity. Such age-related disorders are through the curtailed physiology of SCN in corroboration with its neurochemistry, electrophysiological output, altered phase-relationships with peripheral clocks. Additionally, ageing has been attributed for the disruptions in the hormonal rhythms, body temperature, immunity and other physiologies (Gnocchi and Bruscalupi 2017). The robust alterations in the clock genes expression in the SCN with ageing have been reported in rodent studies (Wyse and Coogan 2010) as well as from our laboratory (Mattam and Jagota 2014). Various types of cancers have been linked to chronodisruption (Reiter et al. 2012). Several studies point towards the circadian dysfunction associated accelerated ageing through aberrations in clock gene expression (Table 11.1). Disrupted circadian clock also leads to several metabolic disorders leading to age induced cognitive impairments.

Overexpressing SIRT1, a circadian regulator gene, in the brain, however showed improved mitochondrial function in skeletal muscle, as well as quality of sleep and increased life span (Satoh et al. 2013).

11.5 Interlocked Communication Between the CTS and Hormones with Ageing

Several hormones were discovered and understood for their crucial role in synchronizing the physiologies such as growth and development, digestion, excretion, sleep, sensory perception, respiration, metabolism, mood, stress, movement and reproduction across the tissues to help in sustaining the homeostasis. A healthy life is the result of strict regulation of hormones by the circadian clock. Several studies demonstrate that endocrine function declines with ageing and show phase

Table 11.1 Role of clock genes in aging and longevity: clock components deletion studies

Mice	Clock	Increased age related dermatitis, cataracts Reduced life span	Dubrovsky et al. (2010)
		Accelerated aging	Antoch et al. (2008)
	Bmal1	Accelerated aging Increased ROS	Kondratov et al. (2006)
		No change in lifespan, fertility, body weight, blood glucose, arthropathy Improved atherosclerosis, hair growth	Yang et al. (2016)

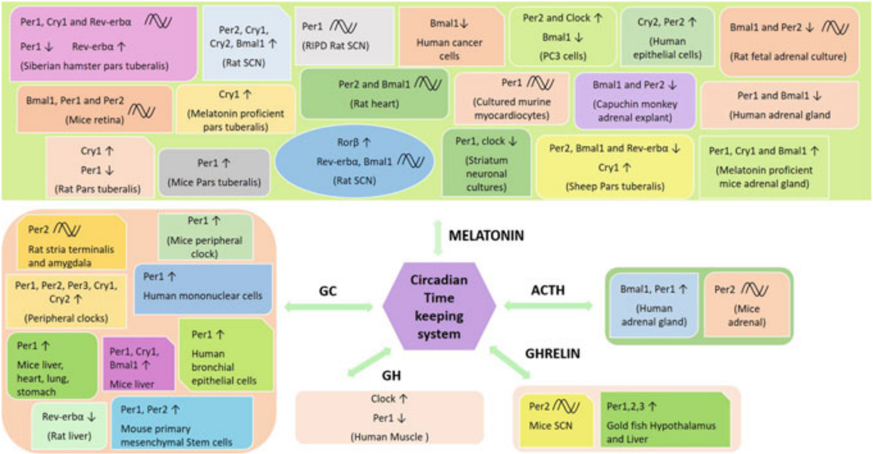

Fig. 11.3 Various Hormones involved in regulation of expression of various clock genes in circadian time keeping system; *GC* glucocorticoids, *ACTH* adrenocorticotropic hormone, *GH* growth hormone, *RIPD* rotenone induced parkinson's disease; *up arrow* increase in expression, *down arrow* decrease in expression, ∿ phase alteration

alterations with irregular hormonal cycles causing hormonal inefficiency and health threatening supporting neuroendocrine theory of ageing (Zjacić-Rotkvić et al. 2010).

11.5.1 Melatonin

Melatonin, the pineal hormone has been generally recognized for maintaining the sleep-wake cycles among other cognitive processes (Cardinali et al. 2012) is synthesized in pineal under the influence of SCN and then released into circulation to travel throughout the body and thus a universal time messenger (messenger of darkness) for all the tissues. In pineal, tryptophan gets converted into 5-hydroxytryptophan by tryptophan-hydroxylase and then into serotonin. Serotonin acted upon by arylalkylamine N-acetyltransferase (AANAT) yields N-acetylserotonin. Then hydroxyindole-O-methyl transferase (HIOMT) transfer a methyl group from 5-adenosylmethionine to the 5-hydroxy group of N-acetylserotonin to yield Melatonin (Jagota 2012; Tan et al. 2015). The synthesis of Melatonin is driven directly by the SCN through a multi-synaptic neural pathway (Fig. 11.2) that comprises of paraventricular nucleus (PVN) of the hypothalamus, sympathetic pre-ganglionic neurons of the intermediolateral cell column (ILCC) of the spinal cord, and noradrenergic sympathetic neurons of the superior cervical ganglion (SCG). All mammals, either diurnal or nocturnal, show a nocturnal upsurge of Melatonin, the messenger of darkness (Jagota 2012) and duration of

Table 11.2 Effect of various Hormones on clock genes expression

Effect of melatonin: clock genes expression			
Human	Adrenal gland	*Per1* and *Bmal1* ↓	Campino et al. (2011)
	Epithelial cells	*Cry2*, *Per2* ↑	Xiang et al. (2012)
Capuchin monkey	Adrenal explant	*Bmal1* and *Per2* ↓	Valenzuela et al. (2008)
Rat	SCN	*Rev-erb*, *Bmal1* Phase alteration	Agez et al. (2007)
	Fetal adrenal culture	*Bmal1* and *Per2* ↓	Torres-Farfan et al. (2011)
		Bmal1 and *Per2* phase alteration	Torres-Farfan et al. (2011)
	Heart	*Per2* and *Bmal1* phase alteration	Zeman et al. (2009)
	Pars tuberalis	*Cry1* ↑	Dardente et al. (2003)
		Per1 ↓	
Mice	Cultured myocardiocytes	*Per1* phase alteration	Zhou et al. (2002)
	Striatum neuronal cultures	*Per1* and *clock* ↓	Imbesi et al. (2009)
	Retina	*Bmal1*, *Per1* and *Per2* phase alteration	Hiragaki et al. (2014)
Melatonin proficient mice	Adrenal gland	*Per1*, *Cry1* and *Bmal1* ↑	Torres-Farfan et al. (2006)
	Pars tuberalis	*Cry1* ↑	Unfried et al. (2009)
Siberian hamster	Pars tuberalis	*Rev-erbα* ↑	Wagner et al. (2007)
		Per1 ↓	
		Per1, *Cry1* and *Rev-erbα* phase alteration	
C3H/HeN Mice	Pars tuberalis	*Per1* ↑	von Gall et al. (2005)
Sheep	Pars tuberalis	*Cry1* ↑	Johnston et al. (2006)
		Per2, *Bmal1* and *Rev-erbα* ↓	
Effect of melatonin: aging			
Rat	SCN	*Per2*, *Cry1*, *Cry2*, *Bmal1* ↑	Mattam and Jagota (2014)
Effect of melatonin: pathological conditions			
Human	Cancer cells	*Bmal1*↓	Hill et al. (2009)
	PC3 cells (prostate carcinoma cell lines)	*Per2* and *Clock* ↑	Jung-Hynes et al. (2010)
		Bmal1 ↓	

(continued)

Table 11.2 (continued)

RIPD Rat	SCN	*Per1* phase restoration	Mattam and Jagota (2015)
Effect of glucocorticoids: clock genes expression			
Human	Mononuclear cells	*Per1* ↑	Fukuoka et al. (2005)
	Bronchial epithelial cells		Burioka et al. (2007)
Rat	Peripheral clocks	*Per1*, *Per2*, *Per3*, *Cry1*, *Cry2* ↑	Balsalobre et al. (2000)
	Liver	*Rev-erbα* ↓	Torra et al. (2000)
	Stria terminalis and amygdala	*Per2* phase restoration	Segall et al. (2006)
Mice	Peripheral clocks	*Per1* ↑	Yamamoto et al. (2005)
	Liver	*Per1*, *Cry1*, *Bmal1* ↑	Reddy et al. (2007)
	Primary mesenchymal Stem cells	*Per1*, *Per2* ↑	So et al. (2009)
Effect of ACTH: clock genes expression			
Human	Adrenal gland	*Bmal1*, *Per1* ↑	Campino et al. (2011)
Mice	Adrenal gland	*Per2* phase alteration	Yoder et al. (2014)
Effect of growth hormone: clock genes expression			
Human	Muscle	*Clock* ↑	Sjögren et al. (2007)
		Per1 ↓	
Ghrelin: clock genes expression			
Rat	SCN	*Rorβ* ↑	Agez et al. (2007)
Mice	SCN	*Per2* phase alteration	Yannielli et al. (2007)
Gold fish	Hypothalamus and Liver	*Per1,2,3* ↑	Nisembaum et al. (2014)

melatonin release had been linked to the length of the night (Pevet and Challet 2011). Melatonin conveys the circadian as well as seasonal temporal information and exerts its functions via G-protein-coupled membrane receptors, MT1, MT2 and MT3 (quinone reductase 2) (Boutin 2016). These melatonin receptors exhibit variability in the number and availability across the tissues and the species. Melatonin has been also identified as most efficient free-radical scavenger regulating oxidative damage, anti-inflammatory, chronobiotic and having immune properties (Jagota 2012; Hardeland 2016). Extending its antioxidant property, melatonin is well understood as a stimulator of the antioxidant enzymes like glutathione peroxidase, glutathione reductase and catalase as well as regulator for NO

(Nitric oxide) rhythms (Manikonda and Jagota 2012; Vinod and Jagota 2016). Recently we reported its influence on *Socs1* expression rhythms (Vinod and Jagota 2017). Several researchers reported the melatonin influence on clock gene expression (Table 11.2; Fig. 11.3) and claimed that these genes could be the important modulators for melatonin as a chronobiotic.

The ageing has been related with decrease in sensitivity to melatonin in the SCN which can be related with decrease in melatonin receptors (Sanchez-Hidalgo et al. 2009; Jagota and Kalyani 2010). Interestingly, melatonin has been speculated to also act via non genomic action i.e. receptor independent action or formation of a complex with another molecule. The receptor independent action of molecule or radical scavenging has been related to the presence of melatonin in the vicinity of a reactive oxygen species when it is generated (Manikonda and Jagota 2012).

Light-at-night also has several potential negative consequences on the human health (Reiter et al. 2012). Based on the clinical implications, it can be predicted that shift workers face higher risk than the blind women (Spiegel and Sephton 2002). Circadian dysfunctions due to Melatonin irregularities have been linked with cancer initiation and growth as reported in human epidemiological studies on cancers such as breast, prostate, endometrial and colorectal cancers, etc. (Reiter et al. 2012). Interestingly, nude rats implanted with the human breast cancer xenograft (from MCF7) were reported with increased growth of xenografts upon constant exposure to light (Blask et al. 2003).

The immune modulatory effects of melatonin on immune cells have been well demonstrated and that immune cells express melatonin receptors (Dubocovich and Markowska 2005). The circadian melatonin rhythm and the immunity changes have been linked to levels of thyroid hormone in ageing mice (Pierpaoli and Yi 1990). Interestingly, melatonin has a varied immune regulatory effect based on the illumination regimes i.e. immunosuppressive in normal LD and immunostimulatory in constant darkness (Calvo et al. 2013). There is a close association between impaired nocturnal melatonin secretion and impaired cell-mediated immunity as reported in rheumatoid arthritis patients (Maestroni et al. 2002).

Melatonin was found to inhibit the B-cell apoptosis and this phenomenon was restricted to the early stages of B cell differentiation. Interestingly, melatonin is shown to favour T-helper (Th) type 1 response and nocturnal melatonin concentration associates with the rhythmicity of Th1/Th2 ratio (Cardinali et al. 2012). The immunostimulatory and anti-apoptotic properties of melatonin are via its action on Th cells and T lymphocyte precursors. At molecular level, melatonin has shown to attenuate *Cry1* expression, which may exacerbate mouse anti-type II collagen antibody-induced arthritis (Bang et al. 2012). Proinflammatory cytokines IL-1b, IL-2, IL-6, and TNF-α do possess a circadian rhythm with peak expression at night and early morning, the time period that corresponds shortly after the highest melatonin serum levels (Couto-Moraes et al. 2009). Additionally, melatonin has been reported as IL-2/IL-2 receptor modulator (Reiter et al. 2012).

Many researchers have demonstrated decrease in melatonin levels with ageing (Reddy and Jagota 2015; Scholtens et al. 2016). Additionally, in a human related study, excretion of 6-hydroxymelatonin sulfate (aMT6s), a melatonin metabolite has shown to be higher at night than in day in both young and healthy centenary subjects, probably suggesting the circadian rhythm of melatonin as marker for proper ageing. Melatonin, a wonderful multitasking molecule has an advantageous role as an anti-ageing drug due to its amphiphilic feature as it crosses the blood brain barrier (Reiter et al. 2012). Solid evidences to prove melatonin as potent candidate against undesirable facets of ageing, neurodegeneration come from several studies showing its administration leading to regeneration and longevity. Its administration resulted in regeneration of degenerated thymus in aged mice, enhanced longevity in drosophila and Balb/c or NZB. Further the timing of administration influenced the longevity (Reiter et al. 2012; Hardeland 2016). But the prolonged lifespan mice were severely affected with cancer incidences questioning the quality of extended lifespan. Melatonin has shown several anti inflammaging properties. Most of the anti inflammaging activities of melatonin are by reducing the microglial activity in brain (Hardeland 2016). SIRTUIN 1, a longevity protein has shown to be protected by the melatonin administration in sleep deprived rats (Yu et al. 2014). It has been reported recently that melatonin can inhibit the L1 retrotransposons, which otherwise might cause DNA damage (deHaro et al. 2014). Administration of melatonin in the older rats and rotenone induced Parkinson’s disease (RIPD) rat model differentially restored the mean levels, phase and rhythms of the circadian clock regulatory genes such as *Bmal1*, *Per1*, *Per2*, *Cry1*, *Cry2* (Mattam and Jagota 2014, 2015). Similarly, its beneficial effects have been reported in Parkinson’s disease (PD) (Carocci et al. 2014). Age induced cognitive performance with memory impairments and neuronal degeneration have been demonstrated to alleviate with melatonin administration (Shin et al. 2015). Some researchers have reported benefits of melatonin on sleep deprived induced memory deficits and sleep efficacy (Sun et al. 2016).

Melatonin plays protective role against cognitive deficits and neurodegeneration marker (Olcese et al. 2009). In a clinical study, the supplementation of melatonin has ameliorated the sundowning and slowed the evolution of cognitive deficits in Alzheimer’s disease (AD) patients (Cardinali et al. 2012). Melatonin has also shown to inhibit β-fibrillogenesis (Lin et al. 2013), reduce β-amyloid, abnormal nitration of proteins, and increase the survival of AD transgenic mice (Matsubara et al. 2003). Melatonin has been proven to inhibit Tau phosphorylation (Lin et al. 2013). Meta-analysis of randomized controlled trials (RCTs) in AD demonstrated melatonin is effective and safe in improving sleep quality (Wang et al. 2017).

11.5.2 Hypothalamic-Pituitary-Adrenal Axis (HPA)

Apart from adapting to the daily light/dark cycles, organisms have to face several external and internal stressors such as food scarcity, pathogen invasion, extreme temperatures, hurtful memories and injuries etc. during their life time. In mammals, the stress system is comprised of HPA axis and its end-effector glucocorticoids, and the locus coeruleus/norepinephrine–autonomic nervous systems and their end-effectors, norepinephrine and epinephrine. Circadian clock and HPA axis dynamically communicates with each other (Nader et al. 2010). The dysregulation of the stress system could be deleterious over the course of time (Haussmann and Heidinger 2015). HPA axis attritions and clock disorders share common metabolic syndromes such as obesity, diabetes (Nader et al. 2010).

11.5.2.1 Glucocorticoids

Glucocorticoids (GC), hormones produced by the adrenal gland cortex influence all organs and tissues and are extremely necessary for the preservation of several vital biologic activities such as the homeostasis of the CNS, the cardiovascular system, intermediary metabolism and the immune/inflammatory reaction which are known to influence mRNA expression of up to 20% of the expressed genome (Nader et al. 2010).

SCN can modulate the secretion of glucocorticoids through influencing the HPA axis (Bao et al. 2008) (Fig. 11.2) or through HPA axis independent pathway through activation of autonomic nervous system (Oster et al. 2006). Interestingly, glucocorticoids exhibit both pulsatile and ultradian rhythms (1–2 per hour) and circadian rhythms. The circadian peak of the glucocorticoids is strongly based on the activity phase of the animal wherein the peak is observed in the early morning in diurnal and in the early night in the nocturnal animals (Lightman et al. 2008).

Bmal1 plays significant role in rhythmic expression of glucocorticoids in adrenal gland (Son et al. 2008). Knockdown studies have shown that the release of glucocorticoids is tightly regulated by *Per1* and *Per2* (Nader et al. 2010). Glucocorticoids exerts circadian regulatory function through modulating several clock related genes in the peripheral clocks (Table 11.2; Fig. 11.3).

Glucocorticoids exert their functions through the glucocorticoid receptors (GR). Human glucocorticoid receptors undergo acetylation with peak at morning and nadir at evening (Charmandari et al. 2009). Circadian machinery maintains counter regulatory feedback to the HPA axis via CLOCK and BMAL1 by blocking the active sites of GR, which is very essential for the proper response to the stressors (Nader et al. 2010).

The basal level of circulating glucocorticoids in the aged humans and animals are controversial (Zambrano et al. 2015). But the rhythms of glucocorticoids are known to fluctuate in several age-related disorders like AD, Cushing’s syndrome, mood disorders and metabolic syndromes (Bao et al. 2008). The external administration of glucocorticoids has been reported to result in cardiovascular mortality, disrupted glucose homeostasis and loss of bone, enhanced ROS levels and cell death in the hippocampal layer (You et al. 2009), learning and memory impairments, neuronal apoptosis and increased amyloid precursor protein, β-secretase and caspase-3 expression, senescence (Poulsen et al. 2014). 11β-HSD1, a GC enhancer, increased in the aged hippocampus and cortex and is in correlation with impaired spatial learning and inhibition of 11β-HSD1 had reversed the spatial memory impairments (Yau and Seckl 2012). Acute injection of mifepristone (GR antagonist) after stress has restored the stress-induced barrier implying the treatment of mood disorders by blocking the GC receptors (Mailliet et al. 2008).

The telomere shortening upon exposure to the stress in early life is related to the glucocorticoids exposure (Haussmann and Heidinger 2015). Offspring produced by the mothers who are reported to have experienced higher levels of stress has shorter telomeres at birth and in young adulthood. The humans with extended life time have less metabolic and cognitive disturbances, this probably explains the HPA axis is a potential candidate mechanism for the longevity (Jansen et al. 2015).

11.5.2.2 ACTH and CRH

Adrenocorticotropic hormone (ACTH) is also known as corticotropin. SCN plays an important role in the release of ACTH through vasopressin (Fig. 11.2). SCN relays temporal signals to the corticotropin-releasing hormone (CRH) neurons in the PVN, this in turn may modulate the rhythmic release of ACTH (Kalsbeek et al. 2012). The CRH mRNA in the PVN region has been shown to be rhythmic, with its peak expression at near lights on, and nadir expression close to lights off. Interestingly, the expression of *Per1*, *Per2* and *Bmal1* in the CRH-expressing PVN neurons are in anti-phase with the expression pattern in SCN. However, in the anterior pituitary and adrenal cortex, the clock genes expression are quite robust and are in synchronization with SCN (Girotti et al. 2009). ACTH stimulate the release of glucocorticoid hormones from adrenal cortex cells of zona fasciculata of adrenal glands through the ACTH receptors. The circulating glucocorticoid and ACTH with the robust circadian rhythms are the characteristic feature of adrenocortical system. ACTH follows a nocturnal rhythm in its expression, where the peak is at lights off and lowest is at lights on. However, the amplitude of ACTH rhythm is low and sometimes is not significant across 24 h period. Adrenal gland exhibits region specific circadian expression of clock genes. The circadian control of ACTH is regulated through *Per2* and *Cry1* (Oster et al. 2006). In turn, ACTH shows regulatory mechanism on clock genes (Table 11.2; Fig. 11.3).

11.5.3 Dehydroepiandrosterone (DHEA)

DHEA is produced by adrenal glands, brain, gonads and it is an intermediate for the synthesis of sex steroids (Labrie 2010). Cholesterol is the precursor molecule which is acted upon by P450 scc to convert to pregnenolone which then is converted to 17α-hydroxypregnenolone by Cytochrome P450 17A1 (CYP17A1) and then finally converted to DHEA. DHEA exhibits a rhythmic pattern with the peak at the night and is mediated by ACTH (Fig. 11.2). There is gender based difference in the levels of DHEA where in young adult women it is 10–30% lower than young males and shows a decline upon ageing. Interestingly, Caucasian women have higher DHEA levels than the Caucasian men during the day time but in the night hours the difference is diminished (Racchi et al. 2003). The flatter DHEA diurnal slope is associated with the longer telomere length (Dismukes et al. 2016).

DHEA in some regions is stated and sold as ‘super hormone’, ‘fountain of youth’, ‘hormone of youth’ and ‘anti-ageing agent’. A study in centenaries revealed that people with better functional status had higher levels of DHEA than in the individuals with poor functional status and is associated with mortality. The low levels of DHEA in ageing men are a consistent with predictor of death in smokers (Ohlsson et al. 2015).

The animals treated with DHEA appeared younger, with glossy hair and less grey hair than the controls. But DHEA is naturally not present in mice and therefore the authenticity of the results on animals is questionable. DHEA treatment has shown to improve the cognitive functions both physical and psychological conditions in men as well as women (Morales et al. 1998). DHEA replacement therapy decreased the insulin resistance and also reduced the inflammatory cytokines in ageing humans via peroxisome proliferator-activated receptor alpha (PPRα) receptors (Weiss et al. 2011). DHEA administration inhibited the IL-8, NF-kB, TNF-α and ROS (Altman et al. 2008). DHEA is a known reductant for several enzymes that generate oxygen-free radicals (Schwartz and Pashko 2004). DHEA inhibits ERK-1 phosphorylation and *c-fos* (Vanden Berghe et al. 2003). The physiological replacement of the DHEA in men and women improved sleep quality, mood, energy and stress-handling, decreased the fat body mass in men (Morales et al. 1998). On contrary, DHEA treatment for 23 months did not result in any significant change in physical performance, insulin sensitivity, or the physical and mental components of the quality of life (Nair et al. 2006). DHEA improved bone mineral density (BMD), skin hydration, epidermal thickness, sebum production and pigmentation in older women (Baulieu et al. 2000).

11.5.4 Growth Hormone (GH)

Growth hormone (GH) is also known as somatotropin. It is a peptide hormone that stimulates growth, cell reproduction, and cell regeneration in humans and other animals. GH is produced by specialized somatotroph cells which are present in the anterior pituitary gland. It is known to peak during the sleep irrespective of time of the sleep (Kim et al. 2015a, b). But still the role of GH in sleep is controversial. In young male, SCN is the site for the production of potent GH inhibitor, somatostatin. It displays a diurnal rhythm but is also reported to persist in DD condition. GH exhibits regulatory mechanism on clock genes (Table 11.2; Fig. 11.3). Overexpression of GH in pituitary gland of coho salmon has differential effect on clock genes (Kim et al. 2015a, b). Administration of Growth hormone-releasing peptide 6 (GHRP-6) has resulted in phase shift in the free running rhythms in mice (Zhou et al. 2014). In the early life, GH levels in the circulation is very high, this is associated with rapid somatic growth. In the course of ageing, the levels decline after the reproductive maturation and attainment of adult body size. As we age further, the levels diminish further (Bartke 2008). The age-related decline in the GH is consistent across different mammalian groups and is primarily attributed to the decreased levels of growth hormone releasing hormone (GHRH). This further decreases the insulin like growth factor-1 (IGF-I), a key regulator for GH (Maggio et al. 2006). The age-related decline in the activity of somatotropic axis is termed as 'somatopause'. It is through the interaction of IGF-I the GH exert its action to initiate the synthesis of DNA, RNA, and protein. The GH functions are mostly pronounced in the growth of long bone.

GH therapy has shown to inhibit age associated unwanted changes and improves cognitive functioning. In the GH deficiency patients, GH therapy has displayed beneficial effects. Long term treatment of GH in adult growth hormone deficiency patients resulted in improved quality of life and bone mineral density (Allo Miguel et al. 2016). Interestingly, treating the normal healthy elderly people with rhGH (recombinant human growth hormone) showed several undesirable effects such as arthralgias, edema, carpal tunnel syndrome, insulin resistance and possibly also diabetes besides small improvements in body composition. Children received GH treatment have increased risk of stroke (Poidvin et al. 2014). Rodents with the mutations in GH or GH resistance has greater lifespan than wild type. Dwarf rats treated with GH in the early age have improved longevity, but the deficiency of GH did not improve the lifespan (Sonntag et al. 2005).

11.5.5 Insulin-Like Growth Factor 1 (IGF-1)

IGF-1 shows pulsatile bursts from the pituitary gland. The expression is more pronounced in night time and is shown to be associated with slow-wave sleep. The secretion of IGF-1 is dependent on release of GHRH that releases GH (Fig. 11.2) and on somatostatin that inhibits its release (Sonntag et al. 2005). IGF-I is primarily involved in the skeletal and muscular development (Maggio et al. 2006). Age related decline of IGF-1 is highly consistent across the species and the sexes.

Administration of GHRP-6 to rats for one week has resulted in the increase of IGF-1 mRNA along with increase in Akt activation, inactivation of proapoptotic protein, associated death promoter (BAD) and increased B-cell lymphoma 2 (BCL-2) (Frago et al. 2002). IGF-1 plays important role in ischemia via hypoxia inducible factor 1 (HIF-1). It also improves the cognitive function especially coordinated motor functions along with the improvement in the spatial memory. Topical IGF-1 therapy improves the sudden deafness (Nakagawa et al. 2014). In the cerebellum of aged rats the reduced levels of IGF-1 is associated with increased cell death, proapoptotic enzymes caspase-3 and -9 and with administration of GHRP-6, the IGF-1 levels were restored and the cell death and caspase activation were decreased (Pañeda et al. 2003). Mice heterozygous for deletion of IGF-1 receptor allele had been shown to live 26% longer than the wild type (Holzenberger et al. 2003). IGF-1 administration can regulate the β-amyloid protein levels, where IGF-1 has shown to decrease amyloid beta (Ab) 1–40 protein levels in hippocampus and choroid plexus (Carro et al. 2002). Suppression of IGF-1 signalling mechanism can enhance the longevity only in females (Selman et al. 2008).

11.5.6 Thyroid Stimulatory Hormone (TSH)

TSH is secreted from pituitary gland to regulate the thyroid gland function (Mullur et al. 2014) (Fig. 11.2). TSH exhibits a diurnal pattern of expression along with thyroxine (T4) and triiodothyronine (T3) (Kalsbeek et al. 2000). TSH shows peak concentrations at middle of night and the minimum at mid-day (Aizawa et al. 2007). Pars tuberalis (PT) express TSHβ (Tsujino et al. 2013) and its mRNA is regulated by melatonin (Arai and Kameda 2004). Melatonin inhibits the expression of TSHβ and its heteromeric α-glycoprotein subunit (α-GSU). Melatonin is what appears to induce inhibition of TSH secretion at night, allowing the hormone to accumulate in thyrotropes, but only the next day the secretion is allowed until light-induced decrease of melatonin (Aizawa et al. 2007). TSH shows a sexual dimorphism in its releasing pattern, where in women it is more robustly produced and in males it is with lower amplitudes (Suzuki et al. 2012). SCN also has been reported for the presence of TSH in itself (Kalsbeek et al. 2000). SCN may signal thyroid gland via sympathetic nervous system.

Serum TSH concentration increases upon ageing (Surk and Hollowell 2007; Atzmon et al. 2009). Several reports suggest that hypothyroidism results in the extreme longevity in animal models (Edrey et al. 2011). It is consistent with the human studies where diminished thyroid function and longevity are in close association. In healthy centenarians, free T4 (FT4) and TSH are present in inverse correlation and this clearly suggest the reduced thyroid function and this might play important role in longevity. There is an association between low thyroid activity and exceptional familial longevity (Rozing et al. 2010). It is evident that the subclinical thyroid disturbances are more prevalent than the overt disorders (Yeap 2013). It is well understood that overt thyroid disorders affect the physical and cognitive functions of the elderly people. But the subclinical thyroid disorders are not manifested by any physical or cognitive function. The elderly people are prone to the thyroid nodules and thyroid neoplasms. Males are at the end of highest risk of cancer prevalence than the women (Rukhman and Silverberg 2011).

11.6 Conclusion

Understanding the interplay between circadian system and endocrine system at molecular level has opened a new beginning in chronoendocrinology. Circadian clock is a multifaceted regulatory system that synchronizes the endocrine system and in turn receives feedback signals in accordance to the persisting cue. The well-established tight interactions between these two systems are essential for systematic metabolism, growth and development and a healthy living. Ageing, an inevitable process, dampens the functionality of circadian clock and the endocrine system at several levels. With the existing knowledge, it is imperious that hormonal therapy can help in modulating the undesirable changes of ageing and enhancing the longevity. Though the development of anti-ageing drug is far from reach, the present status on chronoendocrinology has shed light on targeting the specific molecules responsible for pathophysiological conditions and paved path for future studies in developing therapeutic drugs. Understanding of age induced circadian dysfunction and desynchrony of hormones will be a step towards healthy ageing and longevity.

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Part IV
Brain, Immunity and Cytokinins

Chapter 12
Estrogens in Ageing

Mahendra K. Thakur and V. Paramanik

Abstract Estrogens are the primary female sex hormones and play important roles in both reproductive and non-reproductive organs. They are synthesized in non-reproductive tissues such as liver, heart, muscle, bone and brain, and tissue-specific estrogen synthesis is consistent with a diversity of estrogen actions. In this chapter, we discuss tissue and cell-specific estrogen synthesis and estrogen receptor signaling during ageing in reproductive system and non reproductive tissue especially in the brain and age related alteration and diseases. Estrogens act through two types of nuclear receptors, viz ERα, ERβ and cell-membrane receptors viz GPR30 and ER-X. These types of ERs are expressed in the reproductive organs and brain. For example, estrogens secreted from ovary play key roles in the reproductive system, such as puberty onset, fertility and the estrous cycle. Recent studies have shown that brain estrogens protect against insult-induced neuronal damages. Ageing is an inevitable process where brain undergoes structural, physiological and functional changes. During ageing, level of estrogen, ERs and binding of transcriptions factors to ER promoter decline with age. Moreover, the expression of coregulators and their interaction with ERs show variation during brain ageing. Further, the findings like identification of consensus CK2, PKC and N-myristoylation sites among ERβ interacting brain nuclear and mitochondrial proteins and deviation from LxxxLL motifs have opened new research avenues in the context of estrogen signaling. Such studies might be useful in exploring the therapeutic potential of estrogens in reproductive and neurological diseases. This warrants extensive research in the area of estrogen and ageing.

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Keywords Estrogen signaling · Estrogen receptors · Reproductive tissue · Ageing diseases · Brain

12.1 Introduction

Estrogens are a group of steroid molecules which are secreted mainly by the ovary, placenta and testes, and converted from peripheral aromatization of androgens. Their amount is higher in females as compared to males. They promote the development of female genital organs and features, growth of the endometrium, and inhibit the secretion of follicle stimulating hormone (FSH) by the pituitary. Thus, estrogen was primarily considered as a female reproductive hormone though it was known in 1930s that the developing testis was responsive to the "female" hormone (Wolff and Ginglinger 1935). Further, the developmental exposure to high doses of estrogens could induce malformation of the male reproductive tract (Arai et al. 1983). Thus, during the formative years of reproductive biology as a discipline it was suggested that estrogen might be important in the male; however, even in the early 1990s many scientists considered ERs presence in male reproductive tract to be a remnant from the indifferent sex stage of embryological differentiation (Greco et al. 1993). During 1970s, the prediction of an ER in testis and epididymis became a reality as estradiol binding was discovered (Danzo and Eller 1979). However, it was clear from subsequent publications that most scientists did not consider estrogen to be a major steroid hormone in the adult male reproductive tract (Toney and Danzo 1988). The potential importance of estrogen during development of the male reproductive system was made popular by the report that diethylstilbestrol (DES) treatment during pregnancy induced cryptorchidism and epididymal cysts in male mice (McLachlan et al. 1975). Furthermore, in 1980s, the identification of a transcriptionally active ER in the mammalian hippocampus (Tanapat et al. 1999) challenged the classical view and established the role of estrogen in the brain. Since then, the concept of estrogen and non-reproductive brain functions has been strengthened by (a) localization studies showing the expression of ER throughout the brain (Shughrue and Merchenthaler 2001), (b) behavioral studies showing sexual dimorphism (Fugger et al. 1998) as well as estrogen-dependent alterations in the performance of cognitive and memory tests (Markham et al. 2002), (c) anatomical studies showing the association of physiological fluctuation in estrogens or estrogen treatments with dramatic changes in the plasticity of hippocampal neurons (Woolley et al. 1990), and (d) availability of ERα and ERβ selective knockout mice showing the importance of ERβ in sustaining learning tasks and social activities (Rissman et al. 2002).

Both ERα and ERβ act as transcription factors following their binding to ligands and mediate the actions of estrogens (Thakur et al. 2005). ERα is expressed in several tissues including uterus, prostate (stroma), ovary, testes, bone, breast, white adipose tissue, liver, brain and muscle, whereas ERβ is expressed in colon, prostate (epithelium), testes, salivary gland, bone marrow, brain and vascular endothelium.

In addition to the development and maintenance of normal sexual and reproductive function in women, estrogens exert a wide variety of effects on many physiological systems in both sexes (Thakur and Sharma 2006). Estrogens mitigate post-injury disruption and inflammatory responses and play a protective role against damage, inflammation, oxidative stress and muscle injury (by its antioxidant and membrane-stabilizing properties). They also affect satellite cell activation and proliferation, thereby enhancing the growth and recovery potential of cells (Sivanandam and Thakur 2012).

The level of estrogens, their receptors, signaling mechanisms and functions undergo changes during ageing (Sharma and Thakur 2006), leading to many disorders. Therefore, proper maintenance of estrogen signaling is extremely important for healthy ageing. In the present article, we focus on current understanding of estrogen signaling during brain ageing and highlight the need of further research in this area.

12.2 Estrogen Receptors: A Historical Perspective

Prior to early 1990s, it was understood that estrogens exert main effects on reproductive tissues and sexual behavior (Arnold 2009; Engler-Chiurazzi et al. 2016a; Wilson and Davies 2007). These effects are mediated through ER, first discovered in uterine tissue (Gorski 1994; Jensen et al. 2010) and cloned by Greene et al. (1986). ERα was the maiden nuclear ER that demonstrated binding specificity for 17β-estradiol (E2) and was thought to be the sole ER with which all estrogens interacted. Later, an additional ER was discovered and named as ERβ (Gustafsson 1999). Like other members of the nuclear receptor superfamily, ERs have three functional domains, namely, transactivation domain (TAD), DNA binding domain (DBD) and ligand binding domain (LBD). TAD contains activation function (AF)1 and LBD contains AF2 (Kumar et al. 1987). The distribution of these ERs was mapped to several tissues such as uterus, ovary and brain including hypothalamus, cortex and hippocampus regions associated with cognitive functions (Handa et al. 1994; Miranda and Toran-Allerand 1992). The presence of ERs in extra-hypothalamic brain regions encouraged the study of effect of estrogen depletion and replacement on non-reproductive higher-order cognitive functions.

12.3 Mechanism of Estrogen Signaling

Estrogens regulate gene expression through genomic as well as non-genomic pathways (Acconcia and Kumar 2006). The classical estrogen action is mediated by genomic pathway which involves intracellular receptors, ERα (NR3A1) and ERβ (NR3A2). In the absence of ligand (estrogens), ERs are sequestered in a multiprotein inhibitory complex within the nuclei. The binding of ligand induces conformational changes in ERs such as homo and/or hetero-dimerization of receptors and high affinity binding to specific estrogen responsive elements (EREs) located as cis-acting enhancers within the regulatory regions of target genes. ERs can also modulate the expression of target genes that do not have ERE in their promoter regions. Such ERE independent pathway implies the interaction of liganded ERs with other transcription factors such as Fos and Jun proteins at AP1 and Sp1 binding sites in GC rich promoter sequences.

12.3.1 Genomic Pathways

ERE-dependent genomic pathway: In this mechanism, ERα and ERβ bind to the ligand (estrogen, phytoestrogen and selective ER modulator), and form homo and/or hetero-dimers. Then the ligand/ER complex moves to the nucleus and interacts with ERE *cis* element (AGGTCAxxxTGACCT) (Hall et al. 2001) present in the promoter of target genes (Fig. 12.1). The determination of consensus sequences of ERE and binding of ER help to recruit the transcriptional factors such as FOXA1 or GATA4, which will ensure the chromatin remodeling. The presence of two trans-activation domains AF1 and AF2 in ER will allow the sequential and cyclic recruitment of different cofactors of transcription. The DNA-bound receptors recruit general transcription apparatus either directly or indirectly via coregulators, cointegrators and other proteins having histone modification activities. Such involvement of coregulators and post-transcriptional regulation has increased the complexity of signaling mechanism. It is generally accepted that ER-coactivator interaction stabilizes the formation of transcription pre-initiation complex and facilitates the remodeling of chromatin at ERE (Fig. 12.2; Thakur and Paramanik 2009). Depending upon the cell and promoter context, the DNA-bound receptor exerts either positive or negative effects on the expression of downstream target genes.

ERE-independent genomic pathway: In this pathway, ERs interact with various transcriptional activator or repressor factors to regulate the transcription of estrogen-dependent genes that lack ERE. Indeed, many estrogen-dependent genes have no consensus ERE sequence in their regulatory regions. In this case, ER DBD does not bind to DNA, but participates in protein-protein interaction or recruitment of co-regulatory proteins to regulate the expression of genes. Many of ER/protein interactions, which occur in cells to regulate transcription of target genes, are composed of ER/AP-1 complex. In addition, ER and NF-kB compete for binding to the same transcriptional co-activators (p300/CBP and PCAF), and the pool of these coactivators is mobilized predominantly by ER at the expense of NF-kB in case of activation of transcription by 17β-estradiol (Ansari and Gandy 2007).

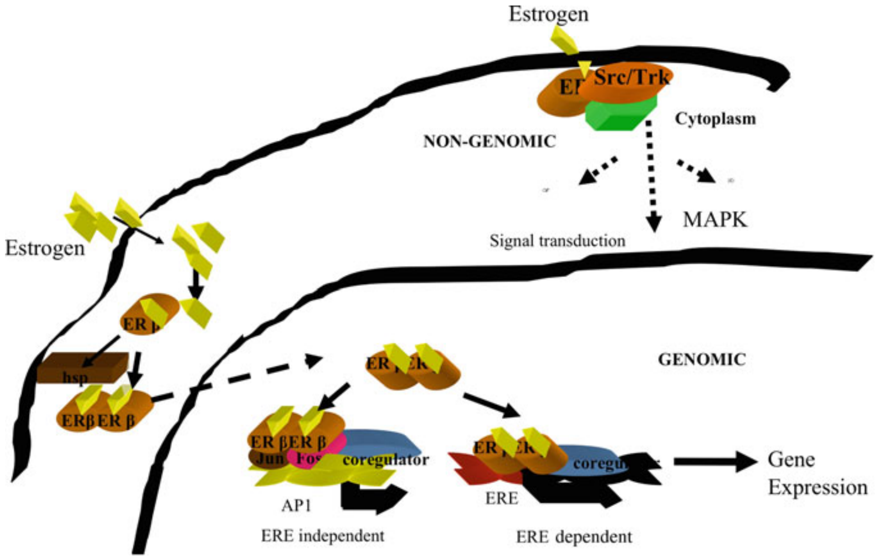

Fig. 12.1 Schematic presentation of estrogen signaling. Estrogen mediates its function through both genomic and non-genomic pathways

12.3.2 Non-genomic Pathway

Non-genomic pathway of estrogen signaling may be ligand dependent or independent. Ligand dependent pathway involves specific ligand and activation of many protein kinase cascades. Finally, it produces responses which depend on a number of conditions, such as signal transduction molecules and downstream targets present in the cell (Fig. 12.1). These responses include mobilization of intracellular calcium, stimulation of adenylate cyclase activity and cAMP production (Razandi et al. 1999; Revankar et al. 2005), which may lead to sequential activation of ras, raf, eNOS, NO, PI3 and mitogen activated protein kinase kinase (MAPKK). Non-genomic actions of estrogens are mediated by either a subpopulation of classical ERs, which are located at the plasma membrane (Manavathi and Kumar 2006) and exist as functional dimers when activated by estrogens (Razandi et al. 2004), or novel membrane ERs such as ER-X (McCarthy et al. 2009). In addition to such ligand mediated activation, ER functions can also be modulated by extracellular signals in the absence of ligand. These signals include peptide growth factors such as epidermal growth factor (EGF), insulin-like growth factor (IGF)1 and cAMP to activate ER and its target gene transcription (Smith et al. 1998). Specific domains of ERs are critical to ligand independent ER activation; cAMP dependent activities require AF2 while growth factor dependent activities require AF1. Growth factor dependent phosphorylation of ERα and ERβ occurs through MAPK signaling pathway (El-Tanani and Green 1997) and phosphorylated receptors interact with different coactivators for activation of target genes (Lannigan 2003).

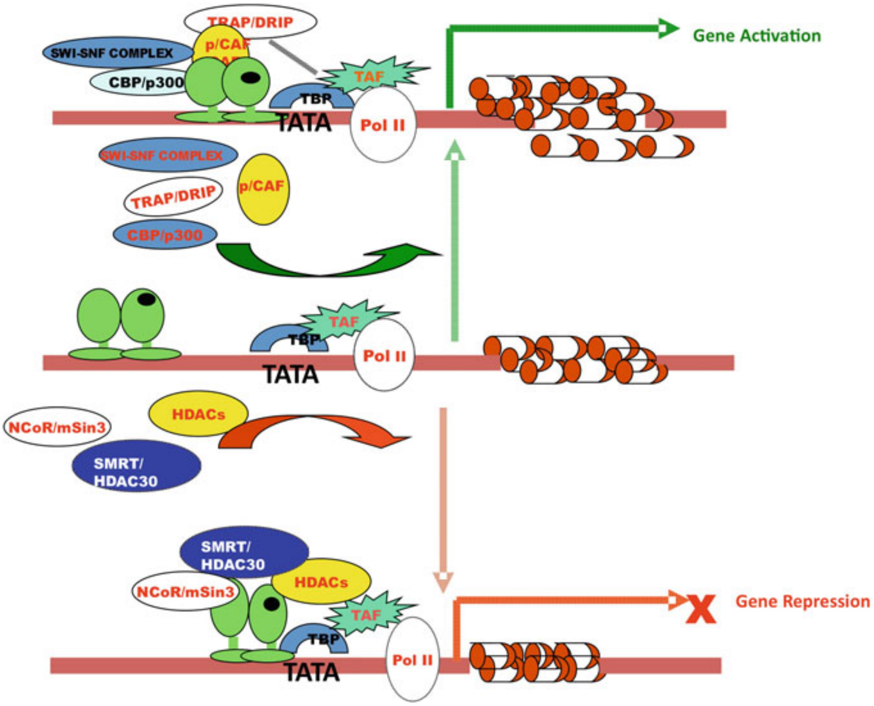

Fig. 12.2 Schematic presentation showing involvement of coregulators in estrogen signaling. Adopted from Thakur and Paramanik (2009)

ER signaling is also influenced by post-translational modifications of ERs. For instance, ER phosphorylation stimulates signaling; glycosylation is important for ER localization; acetylation enhances ER-DNA binding activity, hormone sensitivity and transcriptional activity; sumoylation favors ERα-dependent transcription; nitrosylation reduces DNA binding ability; ubiquitination promotes degradation; myristoylation and palmitoylation affect cross-talk of ERs with membrane proteins, trafficking, as well as signal transduction. Thus it is likely that the local differential post-translational modifications of ERs account for variations in ER-mediated effects under different experimental and clinical conditions.

12.4 Estrogen, Ageing and Reproduction

Estrogen and ERs have several functions in the reproduction system. The estrogens role in male reproduction has been reexamined especially after the clinical and biological analysis of aromatase-deficient men (Jenkins et al. 2015). Moreover from several epidemiological studies, decreased sperm counts and increased male reproductive tract disorders (cryptorchidism, hypospadia, testicular cancers) have been attributed to a deleterious effect of endocrine disruptors with either estrogenic or antiandrogenic actions (Jenkins et al. 2015).

A systematic review was performed for the effects of ageing on the male reproductive system using PubMed from 1980 to 2014 (Gunes et al. 2016). Both male and female reproductive capacities decline with age synergistic with estrogen. Compared to women, the decline in male reproductive capacity with age is less pronounced and men are considered to be able to have a child throughout their lifespan. In literature, the role and the importance of maternal age in fertilization has been extensively studied; however, the effect of the paternal age is poorly understood (Gunes et al. 2016). In developed countries, couples usually postpone having a child due to economic reasons, high standards of living, and career planning has substantially increased the significance of research for investigating the effects of ageing on reproductive systems (Gunes et al. 2016). In this chapter, we aim to discuss the changes in the male reproductive system in the course of ageing. Despite individual differences, testicular morphology is one of the effects of ageing on the male reproductive system. Mean testicular volume tends to increase between 11 and 30 years of age, remains constant between 30 and 60 years of age, and decreases gradually after age 60 (Yang et al. 2011). The mean testicular volume in men over 75 years is reported to be 31% less than in men between 18 and 40 years of age. This difference is associated with significantly higher mean serum levels of gonadotropins and lower serum free testosterone whereas in testicular disorders or failures, levels of serum gonadotropins increase (Mahmoud et al. 2003). Different age related changes in testicles including decrease in the number of Leydig cells act on feedback mechanisms and cause increased secretion of gonadotropins. Age-related increase in gonadotropins is mainly due to primary testicular failure. Testicular metabolism increases between 11 and 40 years of age, and it gradually decreases between the ages of 40 and 90 (Well et al. 2007; Zenzmaier et al. 2008).

Reproductive ageing was previously thought of as a state of estrogen deficiency but more recent studies have shown that estradiol concentrations in the follicular phase are the same or higher in women over 35 years of age with regular menstrual cycles compared with their younger counterparts (Welt et al. 1999, 2006). It is associated with an early follicular phase increase in FSH, that is accounted for a decrease in negative feedback from inhibin B (Santoro et al. 1999; Welt et al. 1999). The reciprocal changes in FSH and inhibin B mark the decrease in follicle number that also occurs at approximately 35 years of age (Gougeon et al. 1994). Thus, early in reproductive ageing, estrogen secretion is spared and even increased in the face of decreased follicle number as reflected by decreased inhibin B and increased FSH. Reproductive ageing is also associated with lower levels of androstenedione and testosterone. Approximately, half of circulating androstenedione, the precursor of testosterone, derives from the ovary and half from the adrenal gland (Judd 1976). Although there is a known decrease in adrenal androgen secretion that begins in women aged over 20 years (Labrie et al. 1997). Moreover, there is evidence that ovarian androgen secretion is decreased in older reproductive-aged women whereas androstenedione peaks at the mid-cycle in normal women (Adams et al. 2004). Other studies have reported that androstenedione levels are lower in the early- and mid-follicular phases of older compared with younger reproductive-aged women and fail to rise at mid-cycle (Davison et al. 2005). Thus, the contribution of ovarian androstenedione appears to decrease during reproductive ageing.

Androstenedione is converted to estrone (E1) by aromatase and is subsequently converted to E2 by 17β-hydroxysteroid dehydrogenase (HSD) (Brailly et al. 1981). Hence, the relative concentration of E1 and androstenedione can be used to approximate aromatase function. Decreased androstenedione in association with unchanged or increased estradiol concentrations in older compared with younger reproductive aged women suggests that ovarian aromatase activity increases during ageing. However, previous studies have not concomitantly examined E1, E2 and androstenedione concentrations across ageing in women with regular menstrual cycles (Welt et al. 2006). The effects of estradiol on a broad array of brain regions and the widespread distribution of ERs throughout the brain highlight the extraordinary integrative power of estradiol. In this interaction, brain controls estrogen release through the hypothalamus-pituitary-gonadal (HPG) axis as well as responds to estrogen. Neuroendocrine function initiates in the hypothalamus, but the circuits that respond to estrogens go well beyond the hypothalamus to include neocortex, hippocampus and brainstem (Welt et al. 2006).

In addition, estradiol plays a key role in the neurobiology of ageing, because endocrine and neural senescence overlap in time and are mechanistically intertwined in complex feedback loops. This aspect of ageing is fundamental as almost all women will experience a dramatic drop in circulating estrogens if they live long enough to reach the menopause transition (Welt et al. 2006). Interestingly, both life expectancy and the average age of onset of menopause for women has increased around the globe, although the average age of menopause remains in the early 50 s. In this perspective, basic neuroscience has a great deal to contribute to the clinical issues surrounding estrogen, menopause and the ageing brain (Welt et al. 2006). Over the past few decades, work is going on investigating the nature of neurologic changes during menopause. Several studies have revealed beneficial effects on cognition across multiple estrogen plus a progestin/estrogen alone regimens, as reviewed by Sherwin (2006). Simultaneously, a large number of studies on animal models suggest potential of estrogen to alter synaptic circuitry in hypothalamus, hippocampus and neocortex, as well as its capacity as neuroprotective (Bryant et al. 2006). In addition, animal studies have demonstrated positive effects of estrogen therapy on cognitive behavior, particularly in nonhuman primates (NHPs), although the extent and nature of cognitive enhancement varies with age (Lacreuse 2006). Thus, a consensus began to emerge (although not without controversy) that estrogen plus a progestin/estrogen alone at the time of the menopause transition and afterward could have beneficial effects on several neurological symptoms (Welt et al. 2006).

12.4.1 Estrogen, Ageing and Reproductive Diseases

In order to exert a biological role, testicular estrogens should interact with ER which in turn modulate the transcription of specific genes. Therefore considering the presence of both ERα and ERβ in testicular cells and in other parts of the genital tract, the physiological role of estrogens in mammalian testes and especially in human reproduction has been extensively reevaluated (Carreau et al. 2008). In sperm cells, the specific transcripts for ERs but only an ERα was found (Lambard et al. 2004). Several workers have described the presence of ERα and ERβ proteins in human ejaculated spermatozoa with some discrepancies on their respective localization. The decline in the estrogen level results into problems in spermatozoa mobility. The presence of several splice variants of ERβ in the human testicular cells and a putative relationship between two ERβ polymorphisms and man infertility have been suggested (Aschim et al. 2005). The effects of estrogens in human ejaculated spermatozoa are more obvious: besides the classical genomic effects, membrane ERs connected with numerous signal transduction pathways involving quick answers have been described (Luconi et al. 2004). Fraser et al. (2006) have demonstrated that genistein improves the capacitation and acrosome loss of human spermatozoa. In addition, the existence of ERs in mitochondria a cytoplasmic organelle very concentrated in the middle piece of spermatozoa (Yager et al. 2007) could be additionally relevant for a significant role of estrogens in male gamete motility. Together with the presence of ERs, new considerations about the role of estrogens all along the male genital tract and likely in sperm mobility and fertilizing ability have been discussed (Carreau et al. 2007). The observation decreased sperm motility in men with aromatase deficiency (Rochira et al. 2005) is a feature in common with the knockout models of mice (Jones et al. 2007). A significant decrease of aromatase expression in spermatozoa from infertile men likely suggests that aromatase is involved in the acquisition of sperm motility. In relation to the variations of aromatase ARNm with teratozoospermia, the another event in which aromatase could be implicated is spermiogenesis. The generation of ARKO mice has provided evidence for an estrogen dependent acrosome formation during spermiogenesis because the blockage of germ cell maturation at the spermatid stage leads to a 50% decrease of both the number of round and elongated spermatids and then non apoptotic round spermatids showed an acrosomal dysgenesis (O'Donnell et al. 2001). Besides the positive role for estrogens in male gamete quality, they have adverse effects in man testis. As a matter of fact, in leydigoma (Carpino et al. 2007) and in seminoma (Bouskine et al. 2008), estrogens (and/or xenoestrogens) are responsible for the abnormal cell proliferation and thus play a major role in these diseases.

The current trend toward delayed childbearing has increased our interest in the changes produced in the physiology of the human reproductive system with age. There is an evident decline in fecundity with age, clearly observed in populations in which contraception has not been employed (Menken et al. 1986). In such circumstances, fecundity decreases and infertility increases with age, suggesting that either the uterus, the ovary, or both are responsible for this impairment of fertility with age. When the ovary is separately analysed, the oocyte as well as the granulosa cells forming the follicle must be separated. There is little doubt that the quality of the egg is affected by age. Further, after the onset of menopause followed by decline in the level of estrogen, high serum FSH concentrations have been detected with age, these being most probably the consequence of variations in ovarian physiology that affect the secretory pattern of the gonadotrope. Furthermore, Chetkowski et al. (1991), using a mathematical model, were able to demonstrate a significant age-related drop in both embryo quality and uterine receptivity. In addition, due to low level of estrogen, ovaries stop releasing eggs (ova). The ovaries become less responsive to stimulation by FSH and luteinizing hormone (LH). To compensate for the decreased response, the body produces more of these ovary-stimulating hormones for a time and may result into cancer. Some other changes are also noticed during ageing in females like, changes occur in the woman's breast, frequency and urgency of urination, and increased risk of urinary tract infection. Taken together, it could be stated that estrogen has vital roles in reproductive system of both male and female.

12.5 Estrogen, Ageing and Brain

Estrogen biosynthesis begins in mitochondria because cholesterol is converted to pregnenolone, the precursor of all steroid hormones, by cytochrome P450 side-chain cleavage enzyme located on the inner mitochondrial membrane. Also, the electron transport chain (ETC) of mitochondria is involved in testosterone production in leydig cells and manipulation of this pathway has been shown to increase the production of testosterone and estrogen after aromatization.

Estrogens regulate various brain functions. It has become more complicated following the discovery of ERβ, different ER isoforms and new plasma membrane-associated receptors. One of the important estrogen functions in the brain is neuroprotection. However, this is not entirely dependent on the interaction of estrogen with ER as co-administration of tamoxifen only partially inhibits the estrogen dependent protection of SK-N-SH cells (Green et al. 1996). The phenolic structure of estrogen molecule (specifically the preservation of an intact phenolic A-ring and three rings of the steroid nucleus) is essential for the protection against oxidative stress and serum deprivation (Behl et al. 1997, Green et al. 1997). The A-ring modifications such as the addition of bulky alkyl groups at 2- and 4-carbon positions enhanced neuroprotection following a variety of in vitro insults as well as cerebral ischemia in vivo without stimulation of peripheral tissues. On the other hand, 3-O-conjugation of the phenolic A-ring abolished neuroprotective ability (Simpkins et al. 2004; Perez et al. 2005a, b).

The increasing evidences for beneficial effects of estrogen in several domains of neurological functions (Chakrabarti et al. 2014) facilitated the study of women's health initiative to evaluate estrogen-containing menopausal therapies for the prevention of a variety of age-related issues including stroke, coronary heart disease, hip fractures and cognitive decline (Manson et al. 2013). However, the null or even detrimental outcome of this and other clinical trials raised apprehension for clinicians and patients to use the exogenous estrogenic treatment (Gleason et al. 2015; Maki and Henderson 2012). Later, a number of hypotheses including the critical window for estrogenic intervention were proposed (Resnick and Henderson 2002), and now it is suggested that estrogen acts as a conditional neuroprotectant with a complex pattern of biological actions that are modulated by several interacting factors (Engler-Chiurazzi et al. 2016b).

Estrogens are involved consistently with morphological changes in neurons, increase in hippocampal pyramidal cell density and enhancement of working memory in Morris water maze task. This suggests that estrogen-dependent changes in hippocampal neuronal networks account for the hormonal effects on cognitive functions. Age related decline in circulating levels of gonadally produced estrogen has been well characterized. However, the estrogen level in brain can significantly differ from the circulating level due to presence of sex hormone binding globulin, steroid converting enzymes and neurosteroidogenesis (Thakur et al. 1993, 2005).

Estradiol is a powerful antioxidant at low concentration and protects against free radical damage of neurotransmitter function and other cells in the CNS. In females, after the onset of menopause, the probability of neurodegeneration is increased. Estrogen also regulates various genes which are involved in Alzheimer's disease (AD) etiology and brain development. The apolipoprotein (APO)E, amyloid precursor protein (APP), presenilin (PS)1 and PS2 genes show age and sex dependent expression in the brain (Singh and Thakur 2011; Thakur and Mani 2005; Ghosh and Thakur 2008a, b, Thakur and Ghosh 2007). The promoters of various genes involved in AD and brain development are modulated by estrogen. For example, APP mRNA expression is correlated with up-regulation of promoter activity, suggesting a major role of APP promoter in specific regulation of APP expression (Mani and Thakur 2006). APP promoter methylation is higher in females and regulated differentially by estrogen, suggesting a strong correlation between promoter methylation and transcriptional silencing of APP and a key role of methylation in the regulation of APP expression during ageing of mice (Mani and Thakur 2006).

12.5.1 Estrogen Action During Brain Ageing

Ageing affects estrogen action through alterations in the expression of ERβ and its coregulators such as growth factors, neuromodulators and neurotransmitters. For instance, young and adult rats responded to estrogen with an increased expression of BDNF, which is important for the maintenance of plasticity in ageing brain, whereas estrogen administration to senescent rats decreased BDNF expression in the olfactory bulb and basal forebrain, suggesting that there is a general decline in hormonal responsiveness of trophic receptors in reproductively senescent animals compared with younger animals (Cardona-Gomez et al. 2001). Estrogen treatment in aged rats fails to increase the spine numbers but has an impact on the molecular nature of CA1 axospinous synapse through enhancement of synaptic NR1A and NR2B expression. It suggests that estrogen can restore a partial youthful NMDA receptor profile in aged rats (Adams et al. 2001).

Estrogen influences the physiology of many brain regions. Aged female rats have decreased estradiol binding in preoptic area, but no difference was found in amygdala, medial basal hypothalamus and pituitary (Wise and Camp 1984). In contrast, another group reported decreased binding in hypothalamus, pituitary, preoptic area, ventromedial nucleus (VMN) and arcuate nucleus (ARC) of old female rats (Rubin et al. 1986; Brown et al. 1990). These results demonstrate discrepancy in the binding of estrogen in different brain regions, making interpretation difficult and highlighting the need of additional approach to understand the significance of age dependent changes in ER level. Further, in situ hybridization studies in ageing rats showed no effect of age on ERβ mRNA in periventricular preoptic nucleus, MPN or paraventricular nucleus (Wilson et al. 2002), though a significant decrease in ERβ mRNA level was noted in supraoptic nucleus. ERβ immunoreactivity also showed no change in the principal nucleus of the bed nucleus of stria terminalis (pBST), but it increased in AVPV of old rats (Chakraborty et al. 2003). Also the rate of ERβ transcript and level of ERβ RNA and protein declined in ageing mouse brain (Thakur and Sharma 2006, 2007). The difference in ER mRNA expression is regulated by DNA methylation of ER promoter. Ianov et al. (2016) examined ERα expression in CA1 and CA3 region of hippocampus of 3 months young and 18 months aged female F344 rats at two time points following ovariectomy to deplete circulating levels of E2, a short term period of 3 weeks and a long term period of 14 weeks. They observed higher ERα expression in CA3 as compared to CA1 and considerable variation in ERα promoter methylation across the 17 CpG sites, methylation of site 1 was increased relative to all other downstream sites.

The study of ER interacting proteins revealed that ERα LBD interacted with PELP1, RIP140, PGC1α, BAF60, ADA3 and beta tubulin (Ghosh and Thakur 2009a, b) and ERα TAD interacted with metastasis associated protein (MTA) 1 and p68 RNA helicase (Thakur and Ghosh 2009; Ghosh and Thakur 2009c) and these coregulators showed variation in their levels of interaction and expression during ageing of mouse brain. Further, the interaction of ERAP 140 with ERβ decreased, but its expression increased in old mouse brain as compared to young and adult (Paramanik and Thakur 2010). As ERAP 140 is involved in retinoic acid mediated neuronal differentiation in neuroblastoma-derived RTBM1 cells (Arai et al. 2008), high level of ERAP 140 interaction in young brain might be involved in neuronal growth and differentiation. Age-dependent decrease in the interaction of ERAP 140 may be due to reduced level of estrogen and change in its conformation similar to human lens protein (Levine and Stadtman 2001).

Higher level of interaction of AIB1 with ERβ TAD suggests higher neurogenesis in young male brain as compared to adult and old (Pekcec et al. 2008; Paramanik and Thakur 2011). The interaction of Src with ERβ LBD decreased with age in male, but it was significantly lower in adult and higher in old as compared to young females. The expression of Src increased with age in male, whereas it was high in young, decreased significantly in adult and then increased in old females (Thakur and Paramanik 2012). As Src is critical for the processes underlying physiological plasticity including learning and memory, and pathological plasticity such as pain and epilepsy (Sharp et al. 2008), its high level of interaction can be correlated with great plasticity and learning capacity. Trk A also showed decrease in interaction with age, and lower expression in adult as compared to young and old males, whereas female mice exhibited decline in both interaction and expression as a function of age (Thakur and Paramanik 2012). TrkA and Src are involved in many vital functions, viz. exocytosis, cell to cell communication, synaptic vesicle release and phosphorylation of proteins (Wightman and Haynes 2004).

With ERβ LBD, pCREB interacted in adult male only whereas young and old did not show interaction in the detectable range (Paramanik and Thakur 2013). On the other hand, in females, the interaction was higher in adult and lower in young than old. Further, the interaction of CREB, as compared to young, increased significantly in adult and decreased in old male. In females, the interaction was higher in young, but lower in adult than old. In males, the expression of pCREB was similar in young and old, but increased significantly in adult. However, in females, the expression was significantly higher in adult than both young and old. The expression of CREB decreased significantly in adult and old as compared to young male, but showed no significant change in females. The higher interaction of pCREB and CREB with ERβ in young can be correlated with higher neurogenesis, learning capacity and efficient signal transduction for the cell migration. The lower interaction and expression of pCREB and CREB with ERβ may be correlated with lower level of neurosteroids and estrogen in old mice. Thus, a number of ER coregulators have been identified in the brain and their age and sex dependent expression as well as interaction have been analyzed, but the modulation of estrogen dependent gene regulation by these coregulators is still to be investigated. In addition, the localization of ERβ in mitochondria (Yang et al. 2004) and identification of CK2, PKC and N-myristoylation sites among mitochondrial and nuclear interacting proteins (Paramanik and Thakur 2012) may be instrumental to target estrogen dependent diseases.

12.5.2 Estrogen in Age-Related Brain Disorders

Estrogen plays an important role in many age-related brain disorders. It ameliorates the memory decline that occurs with age and particularly after menopause. Working memory depends upon the excitation of neurons within the dorso-lateral prefrontal cortex (dlPFC) and is measured by observing the number and shape (straight, curved, or donut-shaped) of mitochondria (Carolyn 2008; Hara et al. 2014). Aged ovariectomized monkeys showed significantly impaired working memory and increase in presynaptic donut-shaped mitochondria as compared to control. Estradiol treatment reversed both the changes, suggesting that hormone replacement therapy may benefit cognitive ageing, in part by promoting mitochondrial and synaptic health in the dlPFC (Fig. 12.3).

Estrogen also affects main neurotransmitter systems such as acetylcholine, glutamate and gamma amino butyric acid (GABA). Choline acetyltransferase (CAT) that synthesizes acetylcholine is mainly reduced in the cortex and hippocampus of AD brain. Many biological mechanisms support the hypothesis that estrogens might protect against AD by influencing neurotransmitters, increasing cerebral blood flow, modulating growth proteins associated with axonal elongation and blunting the neurotoxic effects of β amyloid. In addition, estrogen also induces tau formation, a process that coincides with the enhanced growth of axons and dendrites. Tau helps the astrocytes to release trophic factors in damaged neurons and is involved in the compensatory restructuring of injured brain tissues. It also enhances gene expression, and exerts neuroprotective actions in in vitro and in vivo models of brain injury. Estrogen replacement therapy decreases ischemic injury in both sexes, and protects against ischemic injury in castrated male and female rats (Genazzani et al. 2007).

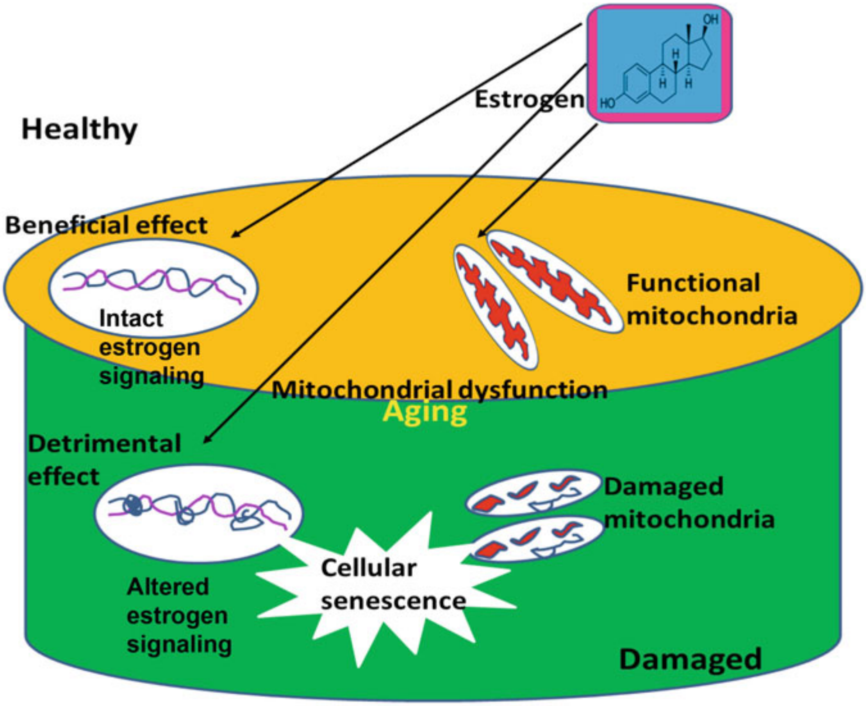

Fig. 12.3 Schematic presentation showing multifaceted roles of estrogen during healthy and ageing conditions

During ageing, the whole brain is affected but certain regions such as hippocampus, basal forebrain, entorhinal cortex and prefrontal cortex are exceptionally vulnerable. Age-related deterioration of these brain regions has been extensively documented in several mammalian species including humans, non-human primates, rats and mice. Of these brain regions, the hippocampus has been the primary focus of rodent studies examining the response to estrogen in ageing females. Estrogen treatment of middle-aged and/or aged females increases hippocampal levels of synaptophysin and nerve growth factor, augments dentate gyrus dendritic spine density, activates protein kinases, normalizes intracellular calcium homeostasis and phosphorylates NMDA receptors. Such estrogen-induced increase in synaptophysin protein levels in aged females is associated with improved spatial reference memory in Morris water maze test (Frick et al. 2002). The hippocampus of ageing rodents is also susceptible to long-term depression, and the fact that estrogen treatment can block induction of this phenomenon in middle-aged female rats suggests estrogen dependent memory improvement in ageing females.

A number of case reports and small scale clinical investigations suggest that estrogen therapies typically given for the management of disruptive menopausal symptoms could reduce AD risk and attenuate disease-associated cognitive deficits (Fillit et al. 1986). Estrogen imparts its beneficial actions in AD patients through its ability to impact the cholinergic system (Luine 1985), which is involved in learning and memory performance (Flicker et al. 1983). As the cholinergic system is dysregulated in AD, it is the therapeutic target of various pharmacological interventions available for the relief of dementia-related symptoms (Giacobini 1998). However, the involvement of estrogen in memory enhancement, its neurochemical basis for beneficial cognitive impacts, and therapeutic potential for the treatment of AD-like dementia, are not well understood.

12.6 Concluding Remarks

Roles of estrogens in reproductive organs are well understood. Recent studies have begun to demonstrate that localized estrogen production plays tissue-specific roles, with or without dependency on circulating estrogen, ERs and its coregulators. The cell and tissue specific actions of estrogen and ERs are directly involved in various age-related diseases in both reproductive and non reproductive organs. The complexity of estrogen mediated functions in almost all organs is enhanced due to the involvement of coregulators. Estrogen's role in reproductive as well as other organs is vital and needs in depth study in the context of various diseases. Further, identification of consensus CK2, PKC and N-myristoylation sites in ERβ interacting proteins of brain and mitochondria, and their deviation from LxxxLL motif needs to be studied in depth. Taken together, we believe that estrogens are no longer merely a sex hormone, but instead estrogen is important therapeutic targets for preventing diseases as disparate in reproductive organs and neurodegeneration as estrogen declines sharply during ageing.

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Chapter 13
Cytokines and Aging

Pramod C. Rath

Abstract Cytokines are secretory or membrane-bound protein families produced mainly by cells of the vertebrate immune system for immunity of the organisms against external challenges such as bacterial, viral and other pathogen infections. However, other cell types also secrete cytokines for many physiological functions. Cytokines are involved in cell proliferation, cell differentiation, cell death, immune function and many other physiologically important cellular responses. Generally, cytokines are produced in small quantities by cells in close proximity of the target cells and their actions on cells lead to cascades of cellular signal transduction pathways leading to physiological responses needed for cell survival. Under certain abnormal conditions, excessive or aberrant production and action of cytokines can lead to various pathological conditions in cells and tissues as well as diseases in mammals. It is now increasingly realized that during aging of mammalian cells and tissues, functions of many cytokines become abnormal and this leads to low-grade chronic inflammation causing various age-related pathogenesis and diseases. It is, therefore, important to understand first how cytokine-mediated functions become abnormal during aging and second which of these aspects may serve as control points to slow-down or block such abnormalities. Such topics are important for understanding biology of aging at cellular and organism levels as well as designing possible therapeutic strategies for age-related diseases. Here, we describe the relationship of cytokines and aging in mammals with reference to certain major tissues and organs as well as age-related diseases such as brain and neurodegenerative diseases; bone and osteoarthritis; kidney and chronic kidney disease; pancreas, adipose tissue and diabetes, obesity; eye and retinal degeneration; muscle and sarcopenia, liver and liver disease. Thus "cytokines and aging" is an important dimension of biogerontology and geriatrics.

Keywords Inflammation · Interleukins · Pathogens · Interferon · Tumour necrosis factor · TNF · Ligand · Apoptosis · Caspases · Eye · Kidney · Brain

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13.1 Introduction

Aging is a universal feature of all living organisms. Both unicellular and multicellular organisms start their journey of life from a single cell. Haploid (1× genome) and diploid (2× genome) cells or organisms, either through vegetative or sexual reproduction, mature into fully grown adults with capacity to reproduce their own kinds. This ensures genetic contribution to the gene pool and continuation of the species on an evolutionary scale. However, although the essential life span of an organism is biologically sufficient up to the reproductive age, almost all organisms continue living far beyond that. Extended parental care associated with offspring might be an exception to this concept. Higher organisms, like mammals have extended life span. Most organisms show that life span is a parameter closely associated with a species. If organisms live longer, their biological system at the levels of cells, tissues, organs and whole organism is ought to get slowly less and less efficient. This manifests in decline of all physiological functions after the attainment of adulthood and reproductive maturity as a fundamental expression of aging (Kanungo 1980, 1994). This results into increasing susceptibility of older individuals to various diseases. However, life beyond reproduction may become biologically less relevant but socially more relevant. Socially acceptable behavior of an organism may permit easier survival of an individual in its natural environment. It may be possible that during evolution the social characteristics of some species might have positively contributed to selective advantage of extending the life span, especially in those species, where the number of offspring produced by reproduction is limited possibly due to the need for extended parental care and social upbringing. For example, the human species has a well developed social environment for its existence, success and a theoretical lifespan of about 100 years, so also for elephants and whales. It has been proposed that due to an extended life span after the adulthood, the load of oxidative stress (Beckman and Ames 1998), mutations in DNA (Carnero 2013), negative changes in gene expression program (Kour and Rath 2016) and protein synthesis (Rattan 1996) as well as turnover (Cuervo and Wong 2014), damage to various cellular components (Kenyon 2010) increase in cells and tissues of aging organisms. The repeated sequences of telomeres at the ends of chromosomes decrease in number (Elsersawi 2010) and the stem cell capacity and reservoir in different tissues also decline (Gonzalez-Garza and Cruz-Vega 2017) in old age. As a result, the homeodynamic balance of cellular pathways and physiological processes are progressively affected in the organism (Toyama and Hetzer 2013). Mild stress like calorie restriction and regular exercise within a certain range of intensity and time period may help ameliorate such declining physiological status of old cells, tissues, organs and organisms. This is considered as “hormetic effect”, the mild stress as “hormetin” and the process “hormesis” (Rattan 2008). In this context, dietary restriction and regular exercise have been largely considered to contribute positive effects against the age-related decline of various functions at molecular, cellular, tissue, organ and organism levels (Mair and Dillin 2008; Goto 2015; Anderson and Weindruch 2007). Since cytokines, chemokines, growth factors and related cellular agents act as signaling molecules to elicit functional consequences and adaptive responses in cells to changes in both intracellular and extracellular environment (Alberts et al. 2002), they are not only essential for survival and physiological expressions of cells, their abnormal function(s) act as causative agents for aging and age-related diseases (Johnston-Carey et al. 2015). Since cytokines are major players in the immune system (Punt et al. 2013), defects in cytokine pathways are closely linked to negative changes in inflammation (Rathinam and Fitzgerald 2016) and immune response (Wang and Casolaro 2014). Due to cross-talk of various cytokine pathways (Michaud et al. 2013) in cells such abnormal changes exert profound effects and cause pathogenesis and diseases in many tissues and organs during aging. In the following sections we describe such cases with important examples linked to aging and age-related diseases in mammals.

13.2 Cytokines and Their Functions

Cellular responses to cytokines are generally described as signal transduction pathways elicited by cytokines (O'Shea et al. 2011). Usually, cytokines are secreted, soluble factors and their receptors are membrane-bound. However, under certain conditions, the ligand may be membrane-bound and the receptor may be soluble. A classical cytokine or growth factor receptor has at least three domains: an extracellular domain (ECD) to bind the ligand, a transmembrane domain (TMD) to allow it to move, rotate and alter its conformation in the membrane as well as multimerize, and an intracellular domain (ICD), which gets activated and interacts with other proteins in order to transduce the signal inside the cell (Cooper 2000). A general scheme for cytokine signaling is as follows. Specific receptors for cytokines existing on cell surface bind to cytokines as their cognate ligands. Both cytokines and their receptors have specific structural domains for conjugation, interaction and conformational alteration. This may involve dimerization or trimerization of the ligand-receptor complex. This is followed by activation of the receptor, its associated adaptor(s) and kinase(s), which leads to activation of specific transcription factor(s) either directly or through kinase/other enzyme(s) and second messenger(s). Common examples of second messengers, which activate kinases and transcription factors, are cyclic adenosine monophosphate (cAMP), calcium ion, phosphoinositides and diacylglycerol etc. (Lodish et al. 2000). Generally, second messengers activate many targets and amplify the signal transduced by the ligand. The transcription factor(s) gets translocated into nucleus and binds to promoters of genes and induce their transcription into mRNAs. Such gene expression program usually results into synthesis of new proteins, which perform the biochemical response(s) leading to cellular response(s). Cell proliferation, migration, inflammation, immunomodulation, cell-cell communication and cell death or apoptosis are common cellular responses elicited by cytokine signal transduction pathways (Kelso 1998). Such cytokine-mediated cellular signaling is common to both physiological and pathological conditions of cells and tissues, the latter condition being due to abnormal or uncontrolled signaling (Fig. 13.1). Cytokines are predominantly associated with cells of the immune system and their functions are related to various aspects of vertebrate immune responses. Various cytokines and their functions have been described (Dinarello 2007).

13.3 Cytokine Pathways in Cells

The most studied among the mammalian cytokines are the interferons (IFNs) (Wang et al. 2017) and tumor necrosis factor-alpha (TNF-α) (Rath and Aggarwal 1999) systems. IFNs were originally discovered as antiviral proteins, which protected mammalian cells against viral infections. They were considered as cell type specific and nonspecific for viruses. The cell growth inhibitory property of IFNs emerged as potential antineoplastic agent. Similarly, TNF-α was originally discovered as a protein factor, which could cause necrosis of tumor cells. Later it was shown as a physiological agent inducing the transcription factor, nuclear factor kappaB (NF-κB) needed for transcription of immunoglobulin genes. Subsequently, many functions of IFNs and TNF-α were discovered to be associated with normal physiology and pathological conditions of mammalian cells and tissues.

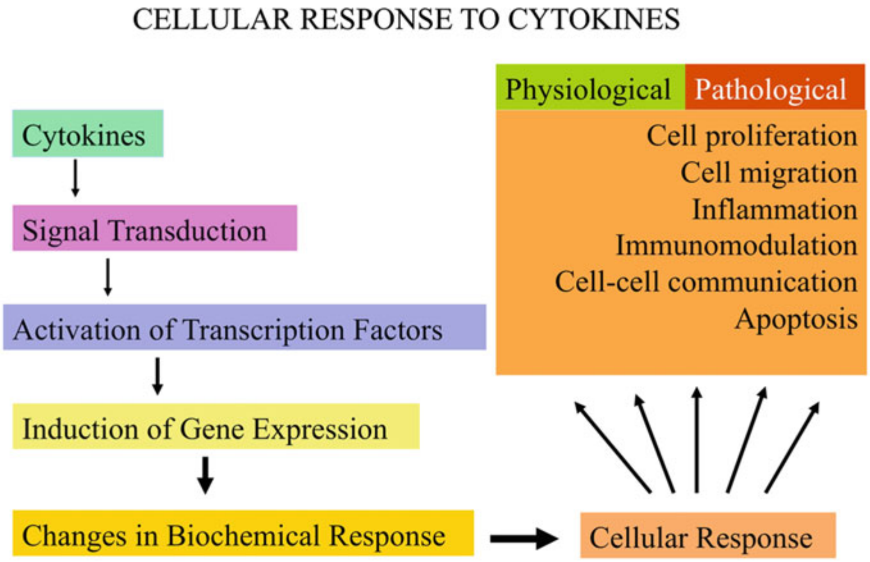

Fig. 13.1 A general scheme for cellular responses elicited by cytokines under physiological and pathological conditions

13.3.1 Interferon Pathway

IFNs are single polypeptide glycoproteins, involved in both innate and adaptive immune systems. They have three major types of actions: antiviral, antiproliferative and immunomodulatory functions. The type I (IFN-α, IFN-β) and type II (IFN-γ) IFNs bind to IFNA (alpha) R (type I) and IFNG (gamma) R (type II) receptors, respectively. IFN-α is commonly known as lymphoid IFN as it is mainly produced by lymphocytes. There are many subtypes of IFN-α encoded by different genes. Similarly, IFN-β is known as fibroblast IFN produced by fibroblasts and IFN-γ as immune IFN produced by cells of the immune system. IFN-β and IFN-γ each is single type and single gene encoded. There are also some other types of IFNs. IFNs are the first line defense against viruses and other pathogens in the innate immune system as well as they are essential components for the adaptive immune system, which results into antibody production for neutralization of infectious agents. Thus IFNs are essential for survival and maintenance of good health, both the aspects important for healthy aging and health span during life span. Besides this, IFNs are involved in bone marrow function, immunomodulation and also some aspects of reproductive physiology.

IFNs elicit cellular signal transductions by JAK (Janus Kinase)-STAT (Signal Transducer and Activator of Transcription) pathway (Stark and Darnell 2012) (Fig. 13.2). IFN-α and IFN-β bind to IFNAR and induce it to get dimerized. The ICD of IFNAR is associated with two janus kinases: JAK1 and Tyk2 as well as two transcription factors: STAT 1 and STAT 2. Dimerization of the IFN-α/β-IFNAR complex leads to self-activation and phosphorylation of JAK1 and Tyk2 at specific tyrosine (Y) residues. Phosphorylated JAK1 and Tyk2 become enzymatically active tyrosine kinases (TKs) and subsequently cross-phosphorylate STAT1 and STAT2 at specific tyrosines, which allows the STATs to dissociate from the receptor complex and reciprocally self-dimerize by virtue of binding of the specific phosphotyrosine phosphopeptide of STAT1 to the SH2 (src-homology domain 2) domain of STAT2 and vice versa. This represents a specific step of the pathway. This STAT1-STAT2 complex can now get translocated into the nucleus of the cell and bind to STAT-binding sequences in the promoters of IFN-stimulated genes (ISGs). ISGs have interferon stimulated response element (ISRE) or gamma activating sequence (GAS) in their promoters. ISRE binds with interferon stimulated gene factor (ISGF) transcription factor complex, the major component of which is STAT dimer. GAS binds dimeric STAT. There are hundreds of ISGs in the mammalian genome, which perform diverse cellular functions, thus explaining a variety of cellular and cell type-specific responses to IFNs. They include antiviral, antiproliferative, immunomodulatory, apoptotic, gene regulatory and protein synthesis inhibitory etc. genes and proteins (Pestka et al. 1987). Many cellular functions are linked to STATs through JAK-STAT pathway induced by cytokines and hormones such as IFN, interleukin-6 and prolactin etc. JAK-STAT pathway is also linked to embryonic development. In the subsequent section, it is described how IFNs impact aging and age-related diseases.

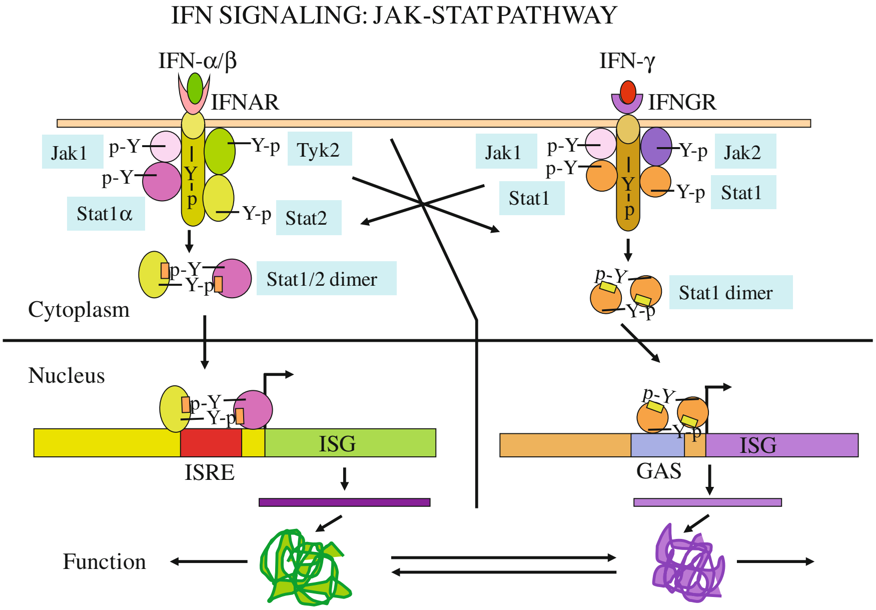

Fig. 13.2 Interferon (IFN)-mediated JAK-STAT signal transduction pathway in mammalian cells. Both type I and type II IFNs bind to their respective cell surface receptors and elicit Janus kinase (JAK)-Signal Transducer and Activator of Transcription (STAT) pathway to activate STATs and induce interferon stimulated genes (ISGs), which encode specific proteins and effect IFN-inducible cellular responses

13.3.2 Tumor Necrosis Factor-α Pathway

The human tumor necrosis factor-α (TNF-α) and its two receptors, TNF-receptors (TNFR1 and TNFR2) have other family members such as the death receptor (DR) family, which cause apoptosis of cells (Fig. 13.3). These ligand/receptor systems are as follows: Fas L/Fas (fibroblast associated), TWEAK (weak homologue of TNF)/DR3 (death receptor 3), TRAIL (TNF-related apoptosis inducing ligand)/DR4 (death receptor 4), DR5 (death receptor 5) and DR6 (death receptor 6) (Kiraz et al. 2016). The common aspect of all these receptors is the presence of a death domain (DD) in the ICD, except for the TNFR2. DD is an amphipathic alpha-helical region, which is responsible for protein-protein aggregation. This happens subsequent to ligand conjugation and activates the receptor. The most studied TNF-α/TNFR1 cell signaling system is shown in Fig. 13.3. The cellular TNF-receptor complex is a high molecular mass complex and it contains many proteins. TNF-α/TNFR1 is a trimeric complex, the DD-DD aggregation activates the receptor, which on the one arm (cell death or apoptosis arm) sequentially activates the adaptors, TRADD (TNF receptor associated death domain), FADD (Fas associated death domain) by DD-DD interaction as well as pro-caspase 8

(FLICE) by DD-DED (death effector domain) interaction. Pro-caspase 8 activates itself autoproteolytically to generate active caspase 8 (cystein protease acting after aspartic acid). Caspase 8 proteolytically activates pro-caspase 3 to activate Caspase 3, which in turn cleaves many cellular protein targets including PARP (poly-ADP ribosyl polymerase). Caspase 3 cleaves ICAD (inhibitor of caspase-activated deoxyribonuclease I) and CAD is released, which enters nucleus and degrades genomic DNA. Caspase 8 cleaves BID (a Bcl-2 family member) and activates it, which then binds and destabilizes the mitochondrial membrane bound Bcl-2/Bcl-XL complex resulting in leakage of the membrane and release of Cytochrome C from mitochondria into cytoplasm of the cell. Cytochrome C binds ATP, Caspase 9 and Apaf1 to form the apoptosome complex. This triggers further activation of Caspase 3 and also cleaves cellular protein targets. Thus the apoptosis arm of TNF-α/TNFR1 involves signal transduction from all four cellular compartments: cell membrane, cytoplasm, nucleus and mitochondria. This is a major cell death pathway, which is shared by other DRs also.

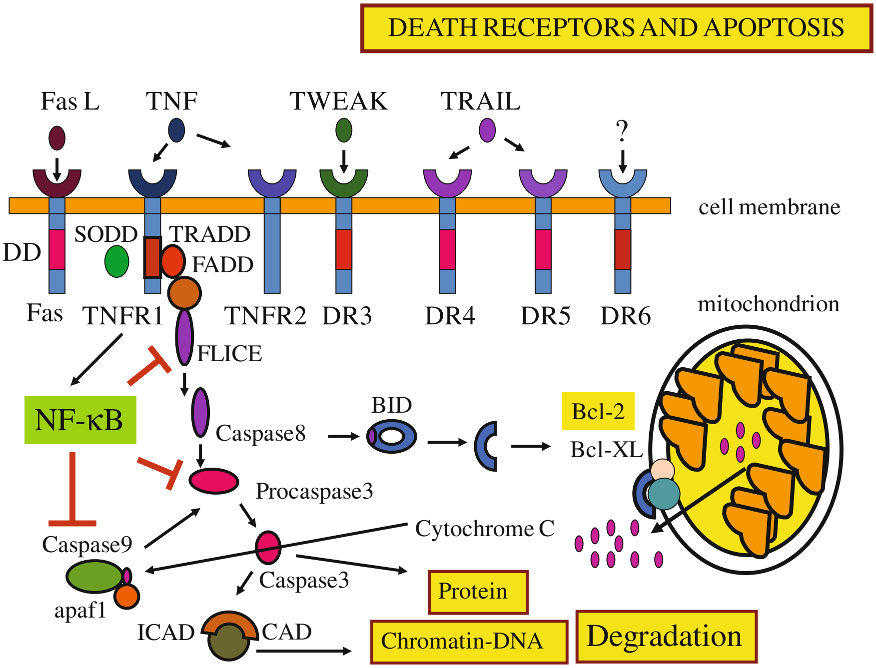

Fig. 13.3 Tumor necrosis factor-alpha (TNF-α) receptor (TNFR) and other death receptors (DRs) as well as apoptosis pathway caused by them in mammalian cells. Receptor (Fas) for Fas ligand (Fas L), TNF-receptors (TNFR1 and TNF2) and DRs (DR3, DR4/5, DR6) comprise the major apoptotic mechanisms of mammalian cells, which through caspase-pathway cause cell death through release of mitochondrial cytochrome C into cytoplasm and activation of degradation of cellular macromolecules (e.g., proteins and genomic DNA)

The second arm (NF-κB or cell survival arm) of TNF-α/TNFR1 signaling pathway constitutes activation of a series of serine/threonine kinases ending with activation of the transcription factor, nuclear factor kappa B (NF-κB) (Lenardo et al. 1989) (Fig. 13.4). It starts from phosphorylation of specific serine residue and activation of RIP (receptor interacting protein) kinase by TNFR1, RIP phosphorylates specific serine residue and activates TRAF-2 (TNF-receptor associated factor-2, also a polyubiquitin ligase) and NIK (NF-κB inducing kinase) and NIK phosphorylates specific serine residue and activates IKK (inhibitor kappa B kinase). IKK has three subunits, IKK-α, IKK-β and IKK-γ (NEMO). IKK-α phosphorylates specific serine residues (ser-32 and ser-36) of IκB-α, the inhibitory protein binding NF-κB subunits p65 and p50 and keeping it as a latent transcription factor in the cytoplasm of cells. Thus phosphorylated IκB-α is then ubiquitylated at lysine-21 and lysine-22 residues by specific ubiquitin ligases associated with the NF-κB complex. The ser-32, ser-36 phosphorylated and lysine-21, lysine-22 ubiquitylated IκB-α is then degraded by the 26 S proteasomal complex and the p65/p50 dimeric NF-κB complex is translocated into nucleus of the cell, by using the nuclear localization signal (NLS) of the p65, where it binds with the NF-κB binding sites in the promoter of hundreds of target genes and induces their transcription/expression. IκB-α is also a NF-κB target gene and it is transcriptionally upregulated by NF-κB following nuclear translocation of the latter due to degradation of IκB-α. Thus fresh synthesis of IκB-α due to NF-κB activation in cells downregulates NF-κB, thereby, negatively regulating excess NF-κB dependent gene expression under normal conditions. This presents an example of coordination of both positive and negative regulations of gene expression to control cellular signaling. Degradation of an inhibitory protein activates the transcription factor, which then upregulates the inhibitor for its own downregulation. NF-κB target genes positively regulate many normal physiological functions through cytokine, chemokine and growth factor production, cell proliferation, angiogenesis, inflammation and immune response as well as many pathological conditions such as antiapoptosis, tumorigenesis, metastasis and many other diseases. Dysregulated or hyperactive NF-κB induces upregulation of inhibitory proteins against caspases and block apoptosis in cancer cells. Excessive NF-κB activity produces excess cytokines, chemokines and causes persistant and chronic inflammation in cells and tissues, which causes degenerative diseases of the nervous, bone and muscle systems. This may be due to either overproduction of TNF-α, its receptor(s) and/or dysregulation(s) of NF-κB pathway. The more common effect of TNF-α/TNFR1 is activation of NF-κB system in almost all types of mammalian cells studied so far, it causes apoptosis under conditions, when protein synthesis is inhibited in cells. TNF-α/TNFR1 pathway also causes ceramide production through activation of spingomyelinase (SMase). Through TRAF-2, it also activates c-jun N-terminal kinase (JNK), which activates activator protein-1 (AP-1) transcription factor constituting c-jun/c-fos proteins. JNK functions as a cellular stress-inducible, serine/threonine kinase belonging to the mitogen-activated protein kinase or mapkinase (MAPK) family/pathway and it induces expression of AP-1-inducible genes and helps cell survival and proliferation. There are three levels of MAPKs downstream of many cytokine/growth factor receptors activating themselves as a cascade, i.e., MAP Kinase Kinase Kinase activating MAPKK activating MAPK in that order. TNFR2 activates TRAF-2 and similarly causes NF-κB activation but does not cause apoptosis in cells. NF-κB transcription factor is a major cellular system for various functions. The importance of TNF-α and NF-κB pathway(s) in the context of aging and age-related diseases is discussed below.

13.4 Changes in Cytokines and Cytokine Pathways During Aging

Cytokines causing inflammatory responses in mammalian cells (e.g., TNF-α, IL-1β, IL-6, TGF-β, IFN-β) may be over-produced in aging cells, their receptors may also be either over-expressed or become hyperactive in old age. Cytokine/growth factor-like proteins (e.g., collagen, aggrecan, *Klotho* and *Sirtuins*) may be expressed and produced at a lower level, therefore their positive effects on maintenance of tissues, organs and their functions may decline in aging cells. The above two conditions may give rise to a situation, where the normal physiological functions of

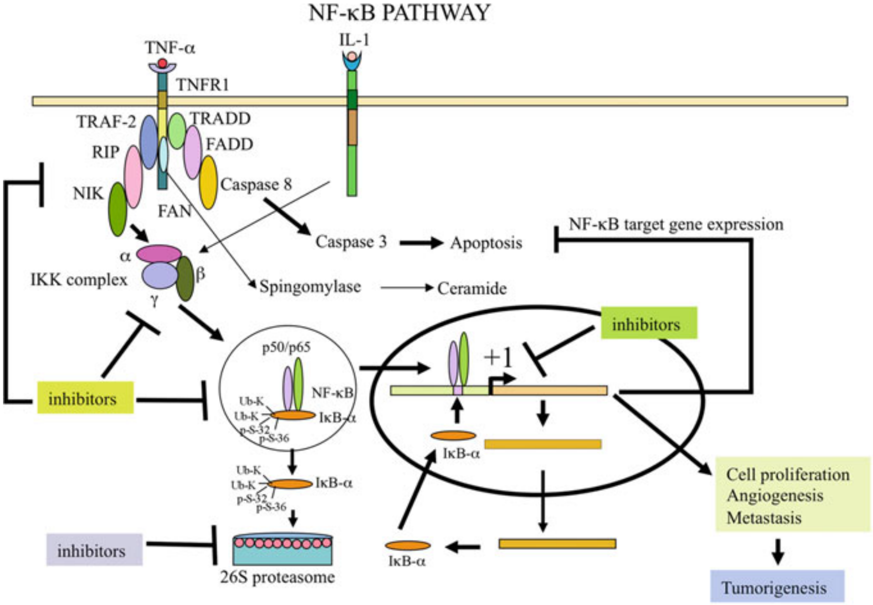

Fig. 13.4 TNF-α/TNF-receptor mediated activation of NF-κB pathway in mammalian cells. Nuclear Factor kappaB (NF-κB) is a major transcription factor complex induced by TNF for production of inflammatory cytokines, chemokines and growth factors. Controlled NF-κB activity is physiologically needed however excessive activity of NF-κB is linked to various diseases. NF-κB is responsible for anti-apoptosis

cells and tissues are reduced and their susceptibility to various diseases may increase. Persistent and chronic inflammation due to excessive pro-inflammatory cytokines (e.g., TNF-α, IL-1β, IFN-β, TGF-β) in cells and tissues of old organisms may increase the load of oxidative stress due to over-production of reactive oxygen species (ROS), this may cause chemical damage due to oxidation of proteins, lipids and nucleic acids as well as various sub-cellular structures containing these macromolecules. This effect may cause mutations in the DNA in both nuclear and mitochondrial compartments as well as crosslink proteins and RNAs affecting their functions. Since the efficiency of the cellular genomic repair machinery is reduced with advancing age, the load of DNA damage accumulates with aging. Moreover, since most of the mitochondrial proteins are synthesized by the nuclear genome, mutations in these proteins may contribute to disruption of mitochondrial functions. In addition to this, the mitochondrial mutations may add to various disease phenotypes in older organisms. Decrease in essential cytokines (e.g., IL-2, IL-4, IFN-γ) for functions of the immune system may lead to lower efficiency of mounting both cellular and humoral immune responses. The level of antibody production may also get reduced. Due to lack of cytokines and growth factors (e.g., colony stimulating factor or CSF) essential for maintenance and differentiation of the bone marrow stem cells, production of various types of functional cells of the blood and immune system may get affected and reduced. This would compromise both normal physiology of tissues and their defense system against disease causing infectious organisms and pathogens. Cytokines, growth factors and hormones are physiological agents of the body, which interconnect the cellular functions to changes in environmental conditions through various cell signaling pathways in gene expression-dependent and gene expression-independent manners. Therefore, alterations in these agents cause deficiencies/abnormality in both chromatin-dependent gene expression programs and protein-protein interaction-dependent sub-cellular structural and functional organizations. If such genes and proteins happen to be metabolically important enzymes, the metabolites produced by their actions are also altered, which may lead to reprogramming of the metabolic system of the cells during aging. In a negative sense, this will affect physiology of cells and tissues. Some metabolites are also gene regulatory in nature, therefore, their non-availability or over-production will affect expression of genes. Such effects may also affect RNA metabolism, such as synthesis, splicing and half-life regulation of RNAs and functions regulated by them, e.g., biogenesis of ribosomes, translation of mRNAs and regulation of protein functions. Taken together, the role of cytokines during aging and longevity depends on a balance between the two processes i.e., inflammaging and anti-inflammaging. It has been reported that a balance between the pro-inflammatory cytokines like IL-1, IL-6, IL-12, IL-15, IL-18, IL-22, IL-23, TNF-α etc. and the anti-inflammatory cytokines like IL-1 Ra, IL-4, IL-10, HSP (heat shock protein), Lipoxin A4 etc. would lead to adaptive aging delaying or escaping the diseases and resulting into healthy longevity, whereas a breakdown of this balance, due to over-production of the pro-inflammatory cytokines, would result in accelerated aging, frailty, age-related diseases and reduced life-expectancy (Minciullo et al. 2016).

13.5 Age-Related Changes in Cytokine Pathways and Diseases

13.5.1 Brain and Nervous System

During aging/in old age, the brain and nervous system experiences loss of cognition and memory, reduced alertness to respond to various external stimuli and in certain situations neurodegeneration. This is associated with lesser activity of functionally specialized groups of neurons present in different parts of the central (brain) and peripheral nervous systems. The interconnections of neurons through axons and dendrites also undergo structural and functional changes leading to lesser functional flexibility and plasticity of the system. Cytokine-induced inflammatory responses have been reported to be associated with these cellular and systemic malfunctioning of the nervous system. During aging, bacterial or viral infection, surgery and traumatic brain injury causes inflammation and immune challenge, which leads to impairment of memory. During such conditions, pro-inflammatory cytokines are produced by sensitized microglia, the macrophage-like cells representing the innate immune system of the brain. During aging, microglia undergoes a process of immunesenescence resulting into inflammation. Normal aging brain also shows such neuroinflammatory behavior of the microglia leading to impairment of brain function. Such primed/activated microglia express many immunological surface markers (e.g., MHC II, CD 11b, CD 86), which are not usually present in the resting microglia. Primed microglia can be further activated for neuroinflammation during subsequent challenge. Excess and long-lasting production of pro-inflammatory cytokines like IL-1β, TNF-α and IL-6 leads to decreased expression/production of brain-derived neurogenic factor (BDNF) and Arc proteins, which are essential for long term potentiation (LTP) of memory formation. This results in significant reduction of long-term contextual and spatial memory (Barrientos et al. 2015). Immune aging, dysmetabolism and neurological diseases have been linked. This leads to loss of neuroprotective role of microglia, activate them to cause inflammation and affect normal function of neuronal cells in the brain (Deleidl et al. 2015). A comparison between the age-related changes and the Alzheimer's disease-related changes in microglia communication has shown similarities in cytokines, complements and extracellular vesicles (EVs), which increase inflammation, decrease phagocytosis and motility of these cells. This results into neuronal impairments in synaptic plasticity and cognition (Udeochu et al. 2016). Cellular damage associated molecular patterns (DAMPs), environmental factors, e.g., elevated free fatty acids (FFA), pathogen associated molecular patterns (PAMPs) initiate activation of inflammasomes (e.g., NLRP3 and NLRC4) in astrocytes/microglia and release of inflammatory cytokines. This induces amyloidogenesis and neurofibrillary tangles in neurons. Subsequently, inflammatory cells like monocytes and lymphocytes cross the blood brain barrier (BBB) and release more diverse inflammatory molecules to promote the neurodegenerative disease phenotype in the brain (Liu and Chan 2014; Sochocka et al. 2016).

13.5.2 Muscle

During aging, wasting or loss of muscle mass occurs, which is described as sarcopenia. The skeletal muscle experiences decreased vascular communication, decreased blood flow regulation and decreased skeletal muscle function. Pro-inflammatory cytokine like TNF-α, advanced glycation products (AGEs), matrix metalloproteinases (MMPs), along with storage cells for inflammatory mediators (mast cells) cause a chronic low-grade inflammation state in aged cells and tissues. This can disrupt microvascular endothelium and impair blood flow leading to hypertension, diabetes, congestive heart failure and sarcopenia often observed in old age (Payne 2006).

13.5.3 Bone

Osteoarthritis (OA) is a whole joint disease of the bone. It occurs due to modification of the synovium and subchondral bone. Often it is associated with type 2 diabetes mellitus, obesity and metabolic syndrome. These disease conditions are often age-associated in human subjects. Diabetes-induced OA shows involvement of hyperglycemia in activation of local chondrocytes. Chondrocytes exposed to chronic high glucose concentrations produce advanced glycation end products (AGEs) in the cartilage. Interactions of AGEs with receptor of AGE (RAGE), Toll-like receptor 4 (TLR-4), through suppression of peroxisome proliferator-activated receptor gamma (PPAR-γ), induce generation of mitochondrial reactive oxygen species (mROS) and nitric oxide (NO) and release of cytokines (IL-1β, TNF-α) by the chondrocytes. Ages also contribute to stiffness and resistance in the cartilage. Matrix metalloprotease (MMP) expression is also induced by high glucose conditions (Courties and Sellam 2016). Cellular aging contributes to an increase in catabolic factors leading to increased cytokines and MMPs resulting into decreased levels of collagen type II and aggrecan synthesis and increased production of ROS. This leads to mitochondrial dysfunction and chondrocyte cell death thus causing OA in the bone joints (Li et al. 2016). Mechanical injury, loss of extra cellular matrix (ECM), loss of growth factors and generation of excessive ROS are reported to be responsible for cell death in chondrocytes in the cartilages of joints of the bones in OA. Excessive amounts of MMPs and aggrecanases destroy the cartilage matrix and cause chondrocyte cell death or apoptosis. Thus, during aging, dead chondrocytes accumulate in the articular cartilage. Mitochondrial ATP production decreases and endoplasmic reticulum (ER) stress increases in the chondrocytes, this further aggravates apoptosis of the cells and enhances OA (Komori 2016).

13.5.4 Eye

Age-related macular degeneration (AMD) is a common disease of eyes in old age in human subjects. AMD leads to degeneration of the retinal pigment epithelium (RPE) and death of photoreceptors resulting into loss of central vision. RPE cells are prone to oxidative stress and decreased levels of intracellular recycling and degradation due to attenuated heterophagy/autophagy in these cells during aging. Cell-associated and soluble pattern recognition receptors (PRRs) such as toll-like receptors (TLRs), inflammasome receptors, complement components recognize pathogen-associated molecular patterns (PAMPs) during infection and damage-associated molecular patterns (DAMPs) during tissue injury. TLRs have Leucine-Rich Repeat (LRR) in their ECD, which binds with their specific ligands and triggers dimerization and activation of the receptor. This triggers the Toll/IL-1 receptor (TIR) domain in their ICD, which in turn activates the two adaptor proteins: myeloid differentiation-primary response gene 88 (MyD88) and TIR-domain-containing adaptor inducing IFN-β (TRIF). MyD88 activates NF-κB and TRIF activates interferon regulatory factors (IRFs) by activation of respective kinases. These transcription factors induce expression of cytokine and chemokine genes. This triggers release of alarm-messengers like pro-inflammatory cytokines and chemokines (e.g., IL-6, TNF-α, IL-8, IL-1β, IL-12 and MCP-1).

These released inflammatory mediators cause activation of the endothelium of the blood vessels, up-regulated expression of cell adhesion molecules and increased vascular permeabilization. Integrins of the circulating leukocytes (neutrophils and monocytes) tightly interact with the endothelial cell adhesion molecules, leave circulation and travel to the inflamed target tissues along concentration gradients of chemokines released by the injured cells, where the monocytes differentiate into macrophages and dendritic cells depending on the local conditions (microenvironment) of the tissue. Thus cytokine-induced chronic inflammation is involved in AMD during aging (Kauppinen et al. 2016). Para-inflammation, an intermediate stage of basal and excessive inflammation has been described as a way to restore the damage in the tissues in order to gain normal functions. This has been suggested to be important for age-related eye diseases like glaucoma, diabetic retinopathy and AMD (Xu et al. 2009).

13.5.5 Kidney

Chronic kidney disease (CKD) is associated with aging and could be a fatal disease. Inflammatory cytokines like TWEAK, TNF-α, transforming growth factor-β (TGF-β) are either locally produced during kidney injury or they may be produced by inflammation in other organs and released into blood stream. Activation of NF-κB by TWEAK has dual actions in renal cells of the kidneys. It induces/upregulates expression of inflammation-related genes such as CCL2 encoding the chemokine monocyte chemoattractant protein 1 (MCP1) and inhibits/downregulates expression of genes encoding protective proteins such as Kl encoding Klotho and PPARGC-1A encoding peroxisome proliferator activated receptor γ coactivator-1α (PGC-1α). NF-κBiz, a NF-κB-related protein, antagonizes TWEAK-induced chemokine upregulation but promotes downregulation of Klotho. MCP1 causes chemotaxis of monocytes and promotes inflammation in tissues. PGC-1α is a transcription factor, which promotes biogenesis of mitochondria. Klotho is an anti-aging and nephroprotective protein, klotho level is decreased during aging. TGF-β activates the Smad-3 transcription factor. Thus production of inflammatory cytokines through activation of transcription factors like NF-κB, Smad-3 and epigenetic mechanisms like histone acetylation and methylation leading to downregulation of klotho gene expression is linked to CKD associated with aging (Ruiz-Andreas et al. 2016).

13.5.6 Liver

Virus infection, fatty liver disease, hepatic ischemia reperfusion and drug-induced liver injury cause apoptosis of hepatocytes. This involves mainly four mechanisms: both classical extrinsic (e.g., TNF-α/TNFR1) and intrinsic (e.g., release of cytochrome C from mitochondria into cytoplasm) apoptosis pathways, endoplasmic reticulum (ER) stress and oxidative stress-induced apoptosis. Excess release of the pro-inflammatory cytokine TNF-α causes apoptosis in liver cells and NF-κB protects it. Kuffer cells in the liver are equivalent of the macrophages and when activated, they release pro-inflammatory cytokines and inflammatory mediators. Excessive amounts of these cause chronic inflammation in liver and liver disease during aging (Cao et al. 2016).

13.5.7 Pancreas and Adipose Tissue

Pancreatic β-cells produce insulin and liver and muscle are the two major target tissues of insulin. Type 1 diabetes mellitus is associated with lack of insulin production by these cells, whereas type 2 diabetes mellitus is linked to insulin-resistance. Often diabetes is associated with life style and aging. Cytokine-induced chronic inflammation associated with aging affects normal lipid accumulation, functions of adipose tissue and mitochondria as well as causes endoplasmic reticulum (ER) stress, which in turn causes insulin-resistance. Chronic inflammation may also be promoted by insulin-resistance. It has been suggested that AKT-kinase signaling is reduced in insulin-sensitive target tissues like liver and muscle, whereas it is increased in non-metabolic organs like kidney and aorta, this may suggest a relationship between the two in the contexts of insulin-resistance and hyperinsulinemia. Obesity-induced insulin resistance is also linked to chronic inflammation. Obesity is determined by increasing the size and number of adipocytes. Increased adipogenesis causes addition of large number of new adipocytes (hyperplasia). This produces more adiponectin and less inflammatory adipokines. On the contrary, hypertrophied adipocytes produce less adiponectin and more inflammatory adipokines. Blood flow to adipose tissue is reduced by hypertrophied adipocytes. This leads to hypoxia and infiltration of macrophages. Then cytokines produced by these macrophages inhibit adipogenesis. This presents a reciprocal situation between hyperplasia and hypertrophy of adipocytes (Park et al. 2014).

13.5.8 Heart and Cardiovascular System

In the heart, ischemia induces production of IL-6, which through JAK/STAT pathway activates STAT-3 transcription factor. STAT-3 has cardioprotective function in ischemic pre- and post-conditioning. STAT-3 regulates expression of target genes and mitochondrial function, both contribute to cardioprotection. In STAT3 deficient (knock out) mice, reduction of the infarct size following cardiac infarction, is abrogated, this demonstrates cardioprotective role of STAT-3. In aged mice STAT-3 protein level is reduced (Boengler et al. 2008). In atherosclerosis, i.e., blockade of blood vessels in the heart, an inflammatory cytokine response similar to that described for the AMD (age-associated macular degeneration) eye-disease is mounted causing platelets to adhere to the endothelium and promote deposition of activated macrophages as the foam cells, which are filled with oxidized lipids.

13.5.9 DNA Damage and Immune System

Aging is often associated with an increase in inflammatory diseases in humans. Oxidative stress and exposure to radiation cause DNA damage and damage/attrition of telomeres at the ends of chromosomes in mammalian cells. Aging of mammalian cells is also associated with decrease in the number of telomeric repeat sequence (5′ TTAGGG3′). Depending on the extent of DNA damage and cell types, the DNA damage response (DDR) results into cell death or senescence. DDR-induced cell signaling activates ATM-p53 and ATM-IKK-α/β-IFN-β signaling pathways leading to elevated levels of expression of p53-tumor suppressor and IFN-inducible IFI16 genes. Damage of genomic DNA/telomere causes cell death and release of DNA from nucleus into cytoplasm of cells. This DNA induces IFI16 and STING-dependent activation of TBK1 kinase and interferon regulatory factor-3 (IRF-3) and production of IFN-β. This triggers IFI16 inflammasome and production/release of pro-inflammatory cytokines like IL-1β and IL-18. Increased expression of IFI16 protein stimulates p53- and pRB-mediated (cell cycle arrest), results in senescence and its reduced expression leads to cell proliferation in many cell types. This represents an example of association of DNA damage (genomic instability), transcription factors (p53, IRF-3), pro-inflammatory cytokines (IFN-β, IL-1β, IL-8) and cellular senescence as well as age-related human inflammatory diseases (Choubey and Panchanathan 2016).

During aging of mammals, both initiation and resolution of immune response are affected (immunosenescence) as well as chronic low-grade imflammation persists (inflammaging). This has been linked to occurrence of metabolic syndrome, atherosclerosis, cancer and neurodegenerative diseases in old age. Aging affects both the innate and adaptive immune system in mice and human subjects. The B cell response is reduced in old age. Production of high affinity, class-switched antibodies (IgG/E/A) is decreased due to reduced class-switch recombination (CSR), which is a DNA recombination event, regulated by activation-induced cytidine deaminase (AID), which opens the DNA at the switch regions of the immunoglobulin genes. AID also regulates somatic hypermutation in antibody genes and production of protective antibodies. AID levels are low in antigen-stimulated B cells from aged mice and human subjects. E47, a (helix-loop-helix) transcription factor essential for transcription of AID gene, is reduced due to decreased mRNA stability in aged B cells. Tristetraprolin (TTP), a negative regulator protein for mRNAs of E47 transcription factor and cytokines, is higher in the aged B cells because it is upregulated by higher levels of intrinsic TNF-α and the microRNAs, miR-155 and miR-16 in aged B cells. The endogenous levels of TNF-α and miRs of the unstimulated aged B cells are elevated by serum levels of pro-inflammatory cytokines, adipokines and infections. Due to this prior activation of the unstimulated aged B cells, exogenously given antigens, mitogens and vaccines do not induce them optimally, therefore, an effective immune response is not mounted in old age (Frasca and Blomberg 2016).

13.5.10 Interventions

Dietary restriction and regular exercise: It has been shown that dietary restriction (DR) and regular exercise can reduce inflammation by reducing both oxidative stress and production of pro-inflammatory cytokines in cells/tissues of mammals during aging (Sharma 2017). DR can also reduce age-related DNA-damage by promoting the DNA repair system in the genome.

Sirtuins: Silent information regulator-1 (SIRT-1), also known as sirtuin-1, is a member of the NAD+ dependent deacetylases family. It is involved in many important biological processes such as inflammation, mitochondrial biogenesis, cellular senescence and aging. SIRT-1 level is decreased at both transcriptional and post-transcriptional levels in aged cells. This is associated with attenuated mitochondrial biogenesis, which is often linked to many age-related diseases. SIRT-1 can activate many transcription factors, e.g., peroxisome proliferator activated receptor γ co-activator-1α (PGC-1α) and hypoxia inducing factor-1α (HIF-1α), this results into ameliorated mitochondrial biogenesis and extension of life span (Yuan et al. 2016).

Similarly, Sirtuin-6 (SIRT-6) also has multiple functions, in controlling homeostasis in organisms, lifespan and diseases. SIRT-6 improves longevity, similar to the Sir2 (silent information regulator 2) protein of yeast, and modulates genome stability, telomere integrity, transcription and DNA repair. SIRT-6 deficiency is linked to chronic inflammation, diabetes, cardiac hypertrophy, obesity, liver dysfunction, muscle/adipocyte disorders and cancer (Vitiello et al. 2016). Restoring SIRT-1 and SIRT-6 activities should suppress inflammation, aging and age-related diseases.

Resveratrol: Resveratrol has been shown to reduce oxidative stress, therefore it could decrease inflammation and reduce cellular aging (Gocmez et al. 2016).

Anti-inflammatory natural products: Many non-steroidal anti-inflammatory agents (NSAIDs) may be useful as supplements for reducing inflammation, therefore cellular aging (NSAIDs).

Klotho: Klotho, an anti-aging protein or a human aging-suppression molecule, provides protection to organs. Klotho-deficient mice showed accelerated aging phenotypes and over-expression of Klotho in mice extended lifespan. Klotho is a single-pass membrane protein mainly produced in kidney. The amino-terminal extracellular domain of Klotho is shedded and released into systemic circulation. Three forms of Klotho protein show distinct functions. Membrane-associated Klotho forms a complex with fibroblast growth factor (FGF) receptors and functions as an obligatory co-receptor for FGF-23, which is involved in aging and development of chronic diseases through regulation of Pi and Vitamin D metabolism. Secreted Klotho functions as a humoral factor with pleiotropic activities including regulation of oxidative stress, growth factor signaling, ion homeostasis and protection of organs. Intracellular Klotho suppresses inflammation-mediated cellular senescence and mineral metabolism.

Circulating levels of soluble Klotho is decreased in old age. Klotho gene is associated with increased risk of age-related diseases. Polymorphisms of Klotho gene are correlated with lifespan, coronary artery disease, atherosclerosis and osteoporosis in human subjects. Klotho is associated with severe calcinosis and stroke. Klotho deficiency is linked to acute and chronic diseases, cancers and salt-sensitive hypertension. Serum level of Klotho decreases with aging in humans. Both biological role of Klotho and how Klotho-deficiency contributes to age-related diseases are still far from being clearly understood. Restoration of Klotho protein and its functions should suppress inflammation, aging and age-related diseases (Kim et al. 2015).

Transplantation of autologous adult stem cells: Occurrence of chronic diseases like neurological, metabolic and cardiovascular degenerative diseases in human subjects increases with advancing age. In recent times, cell therapy is considered as an emerging possibility of treatment for these diseases. In this context, application of autologous stem cells is of particular interest because it eliminates post-transplantation immune rejection and ethical concerns about use of the cells. The regenerative capacity of the stem cells harvested from elderly persons may be a concern in this case. However, if self-renewal potential, differentiation capability and expression of stem-ness genes in the stem cells from the elderly people are up to satisfactory levels, then their application in clinical trials may be carefully examined for possible use of this approach in future (Gonzalez-Garza and Cruz-Vega 2017). Regenerative function of the immune system modulation of muscle stem cells may also help maintaining the health span during aging (Saini et al. 2016).

Telomere: Recently, the crosstalk of telomere dysfunction and inflammation through cell-free TERRA containing exosomes has been demonstrated (Wang and Lieberman 2016). Hence, attrition of telomere is not only an important hallmark of aging, maintenance of its normal structure and function is essential for health span.

Therefore, the intersection of cell death and inflammasome activation (Vince and Silke 2016) is an important dimension of aging and age-related diseases. An experimental approach for removal of senescent cells by inducible activation of caspase in these cells in an intact mouse has provided a step forward for both understanding the process of aging and extending lifespan as well as delaying some age-related diseases (Gil and Withers 2016; Backer et al. 2016).

13.6 Conclusions

Inflammatory cytokines like IL-1β, IL-6, TNF-α, IFN-β and TGF-β have been shown to be produced more in aged cells and tissues causing a state of low-grade, persistent and chronic inflammation, which leads to generation of excessive oxidative stress damaging cellular macromolecules, sub-cellular structures and disease phenotypes. This promotes degenerative diseases of the nervous, muscular, bone, liver and kidney tissues. Decreased levels of Klotho protein in old age cause failure of protection mechanisms of various tissues and organs. Dysregulated and hyperactive cytokine-mediated cell signaling mechanisms and transcription factors, e.g., NF-κB, IRF-3, Smad-3 and STAT-3 also contribute to aging and age-related diseases. Chronic inflammation and higher levels of oxidative stress may also contribute to lower levels of DNA repair mechanisms due to which increased DNA damage may lead to accumulation of mutations and genomic instability including attrition of telomeric repeats at the ends of chromosomes causing progressive aging and age-related diseases. Metabolic dysregulation in old age may also be associated with abnormal cytokine signaling. Dietary restriction and regular exercise, genetic composition for good health and healthy life style may ameliorate these harmful effects by reducing pro-inflammatory cytokines, immune mediators and inflammation during aging. Thus cytokines are closely related to aging and age-related diseases.

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Chapter 14
Plant Hormone Cytokinins for Modulating Human Aging and Age-Related Diseases

Jiří Voller, Barbara Maková, Alena Kadlecová, Gabriel Gonzalez and Miroslav Strnad

Abstract Cytokinins are phytohormones that regulate plant growth, development and senescence. Experiments both in vitro and in vivo demonstrate that they can also have diverse effects on animal cells and tissues. Particularly interesting is their ability to protect cells against various forms of stress and prevent some detrimental effects of cell aging. For example, human skin fibroblasts cultured in the presence of kinetin or *trans*-zeatin retain some characteristics of cells of lower passage. Kinetin is even able to increase the lifespan of invertebrates. In this chapter, we review protective effects of cytokinins in animals at molecular, cellular, tissue and organismal levels. We also discuss potential application of cytokinins for the treatment of age-related diseases, including neurodegenerations, inflammatory diseases and disorders caused by aberrant cell proliferation.

Keywords Cytokinin · Kinetin · Kinetin riboside · Zeatin · Benzyl adenine · Topolin · Skin · Anti-aging · Anti-inflammatory activity · Neuro protection

List of abbreviations

ADK	Adenosine kinase
$A_{2A}R$	A_{2A} adenosine receptor
A_3R	A_3R adenosine receptor
BA	N^6-benzyladenine
BAR	N^6-benzyladenosine
ENT	Equilibrative nucleoside transporter
*t*Z	*trans*-zeatin
*t*ZR	*trans*-zeatin riboside
iP	N^6-isopentenyladenine
iPR	N^6-isopentenyladenosine
ipt	Isopentenyltransferase

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K	Kinetin
KR	Kinetin riboside
KRTP	Kinetin riboside-5′-triphosphate
*o*TR	*ortho*-topolin riboside
*p*TR	*para*-topolin riboside
ROS	Reactive oxygen species

14.1 Introduction

Cytokinins are phytohormones identified originally as substances that promote plant cell division in the presence of another phytohormone, auxin (Skoog et al. 1965). They play important roles in the development and growth of both root and shoot systems. Processes regulated by cytokinins include water and nutrient mobilization, apical dominance, branching, flowering, breaking of bud dormancy and seed germination (Werner and Schmülling 2009). Cytokinins also delay leaf senescence—they prevent the degradation of chlorophyll and outflow of nutrients from the leaf. A good demonstration of this activity occurs in "evergreen" transgenic tobacco plants with the gene encoding ipt (isopentenyltransferase), the protein responsible for cytokinin biosynthesis under the control of a senescence-specific promoter (Gan and Amasino 1995).

Knowledge that cytokinins play key roles in the regulation of plant growth and development has stimulated studies into their potential utility for treating human diseases, especially those involving dysfunctional cell proliferation and/or differentiation. Cytokinin ribosides were even evaluated in patients with diverse malignancies as early as the 1970s (Mittelman et al. 1975). Later on, cytokinins inspired development of inhibitors of cyclin-dependent kinases olomoucine, bohemine, roscovitine and their analogues (Veselý et al. 1994; Havlíček et al. 1997; Vermeulen et al. 2002; Kryštof et al. 2002). The ability of cytokinins to prevent processes associated with plant senescence has attracted research focused on their ability to ameliorate aging traits in animals, including humans. In this article, we review the effects of cytokinins in animals at molecular, cellular, tissue and organismal levels with an emphasis on anti-aging and cytoprotective activity. We also discuss potential application of cytokinins in the treatment of inflammation and neurodegeneration as relevant to various age-related diseases. Finally, we include a section about the antiproliferative activity of cytokinins—they are cytotoxic for malignant cells of diverse histopathological origin but are also able to induce differentiation of some leukemia cells and keratinocytes.

14.2 Chemical Structure and Occurrence of Cytokinins

Regarding their chemical structure, plant cytokinins are adenine derivatives substituted at the N^6-position with either an isoprenoid or aromatic side chain. Synthetic compounds with cytokinin activity can have other scaffolds, phenylureas being the best known example. Isoprenoid cytokinins include *cis*- and *trans*-zeatin (*t*Z) and their analogues with a saturated side chain (dihydrozeatin) or without an hydroxyl group (N^6-isopentenyladenine, iP). Whereas isoprenoid cytokinins are present in all plants, aromatic cytokinins with N^6-benzyl substituents have only been found in certain taxa (Horgan et al. 1975; Strnad 1997). Besides N^6-benzyladenine (BA), its hydroxylated derivatives, i.e., topolins, have been described. Kinetin (K), the first identified cytokinin, has an N^6-furfuryl side chain. K was first recognized as the substance responsible for the cytokinin activity of autoclaved herring sperm, which was attributed to thermal DNA damage. Later it was reported to occur naturally in plant material (Ge et al. 2004), as well as human cells and urine (Barciszewski et al. 1996, 2000). However, its natural occurrence is probably very rare and rather mysterious. We have not even been able to unequivocally identify this compound in diverse plant material using sophisticated tandem mass spectrometry analysis. Barciszewski et al. (1997) proposed that endogenous K is a result of oxidative DNA damage. Both isoprenoid and aromatic cytokinins occur as free bases, ribosides, ribotides, N-glucosides and amino acid conjugates. Moreover cytokinins with hydroxylated isoprenoid side chains can also form O-glycosides. N^6-substituted adenine derivatives also occur in certain tRNA species of all organisms with the exception of Archea. For example, in mammals, an N^6-isopentenyladenosine (iPR) moiety forms part of tRNA[Ser]Sec. It facilitates codon-anticodon interactions and contributes to the efficiency of selenoprotein synthesis. Adenines and adenosines with N^6-substitution may be released into the cytosol, and subsequently into body fluids, as a result of tRNA turnover. iPR was detected in human urine many years ago (Chheda and Mittelman 1972) (Fig. 14.1).

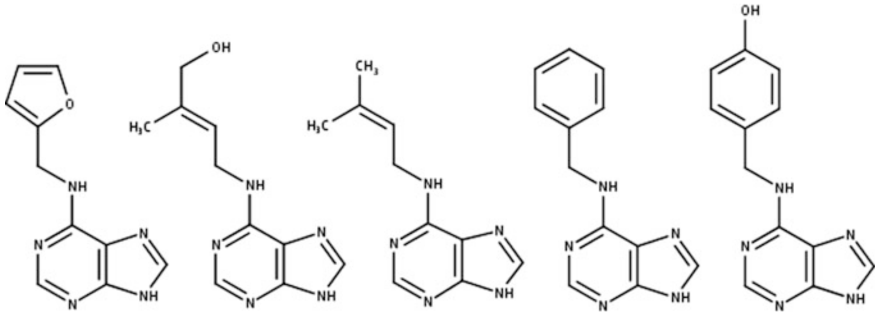

Fig. 14.1 Natural cytokinin bases studied as modulators of processes related to aging. From left to right—kinetin, *trans*-zeatin, N^6-isopentenyladenine, N^6-benzyladenine, *para*-topolin

14.3 Cytokinin Signaling in Plants

The cytokinin signal in plants is perceived by a His-Asp phosphorelay similar to the two-component systems of bacteria. After recognition of the cytokinin ligand by the extracellular domain of the transmembrane cytokinin receptor (AHK2, AHK3, or AHK4 in *Arabidopsis thaliana*), the intracellular portion of the receptor phosphorylates histidine phosphotransfer proteins (AHPs). These transmit the signal to nuclear response regulators (ARRs), which can activate or repress transcription of the response genes. Anti-senescence activity of cytokinins is mediated through the activation of AHK3, the type-B response regulator ARR2 and cytokinin response factor CRF6. Increased cell-wall invertase activity in response to cytokinins is both necessary and sufficient for the inhibition of senescence (Zwack and Rashotte 2013).

Although differences in the substrate specificity between individual cytokinin receptors exist, cytokinin bases are consistently the most active cytokinin form in both receptor assays and cytokinin biotests (Mok and Mok 2001; Spíchal et al. 2004). The intensity and duration of the signaling is dependent on the receptor and response regulator composition of the given cell/tissue and the availability of individual cytokinins. The rate-limiting step in cytokinin biosynthesis is catalyzed by isopentenyltransferases (IPTs), which synthesize either free cytokinin nucleotides (adenosine phosphate-IPTs) or modify adenosine in tRNA (tRNA-IPTs). Conversion of cytokinin 5′-monophosphates into their respective free bases is catalyzed by phosphoribohydrolase encoded by the gene LONELY GUY (LOG) (Kurakawa et al. 2007). An alternative pathway where dephosphorylation of riboside-5′-monophosphates precedes the cleavage of the glycoside bond also exists (Chen and Kristopeit 1981), but the genes responsible have not yet been characterized. Cytokinins are degraded by cytokinin oxidase/dehydrogenases (CKXs), which catalyze removal of the side chain. The cytokinin signal is also attenuated by conversion of free bases into less active (ribosides, ribotides) or inactive forms (glucosides, conjugates with alanine). With the exception of N7- and N9-glucosides, cytokinin conjugates can be converted back into free bases and are seen as transport/storage cytokinin forms. The uptake and efflux of cytokinins by cells is facilitated by members of the purine permease family (PUPs) of transmembrane channels (Gillissen et al. 2000) and by equilibrative nucleoside transporters (ENTs) (Hirose et al. 2008). Cytokinins are present in both phloem and xylem fluid and serve as both acropetal and basipetal messengers (Kudo et al. 2010). The first acropetal transporter was described recently (Zhang et al. 2014).

14.4 Effects of Cytokinins in Animal Systems

14.4.1 Cytoprotective and Anti-aging Activity of Cytokinins

Interest in the anti-aging activity of cytokinins started in 1994 when Rattan and Clark discovered positive effects of K on several characteristics related to aging in human skin fibroblasts during serial passage in vitro (Rattan and Clark 1994). The size and morphology of fibroblasts passaged in the presence of K resembled those of the cells at lower passage numbers. Treatment with K decreased the number of actin stress fibers and autofluorescence due to accumulated lipofuscin was also less intense. Similar effects of *t*Z on in vitro aging of a fibroblast population were reported more than 10 years later (Rattan and Sodagam 2005). Optimal anti-aging effects were observed at 80 µM concentration for both compounds. It is important to note that these long-term cultivation experiments were enabled by the remarkably low toxicity of the cytokinins tested. Beneficial effects of several other cytokinin bases on various parameters relevant for aging amelioration and/or age-related disease therapy were reported in the years following the original discovery, and we discuss them below. Active compounds include *para*-topolin, iP and a K derivative 6-furfurylamino-9-(tetrahydropyran-2-yl)-9*H*-purine (Pyratine-6, PRK-124) (Walla et al. 2010). K, *t*Z and Pyratine are the principal ingredients of several marketed cosmeceuticals.

Notably, K effects related to aging are not limited to cells and tissues as dietary K has been shown to increase the life span of *Zaprionus paravittiger* fruit flies. K prolonged the larval and pupal stages but also reduced the age-specific death rates throughout the adult lifespan (Sharma et al. 1995). The effect was accompanied by enhanced catalase activity (Sharma et al. 1997). We also recently discovered that K and several other cytokinin derivatives are able to increase the lifespan of *Caenorhabditis elegans* (our unpublished data).

14.4.2 Anti-oxidant Activity of Cytokinins

Since the discovery of anti-aging activity of K in human fibroblasts, research has primarily focused on its ability to protect macromolecules and cells against oxidative damage and stress. Barciszewski et al. (1997) hypothesized that endogenous K may arise as a consequence of oxidative damage of DNA, thus creating protection near to the site of damage. The proposed mechanism of K formation assumes that hydroxy radical attack at the 5′ carbon of the deoxyribose residue yields furfural. This aldehyde reacts with the amino group of adenine and, after intramolecular rearrangement, the resulting Schiff base is reduced into K (Barciszewski et al. 1997).

Using 8-oxo-2′-deoxyguanosine (8-oxo-dG) as a marker for oxidative damage of DNA, Olsen et al. (1999) showed that K significantly protects DNA against reactive oxygen species (ROS) generated by the Fenton reaction in vitro. The effect was dose dependent, with a maximum of about 50% protection observed at 100 μM K. K has also been shown to protect proteins against oxidative/glycoxidative damage more efficiently than adenine in several experimental systems in vitro (Olsen et al. 1999). Besides decreasing protein carbonylation in an iron/ascorbate system, it also prevented formation of advanced glycation end-product pentosidine and aggregation after incubation of proteins with sugars. Active K concentrations were in the range 50–200 μM (Verbeke et al. 2000). Recently, the antioxidant activities of K, BA, *para*-topolin and iP were evaluated by fluorimetric and spectrophotometric assays (Brizzolari et al. 2016). With the exception of BA, all the compounds showed significant activity in the oxygen radical absorbance capacity (ORAC) assay at 2.5 and 5 μM concentrations. In the Trolox equivalence antioxidant capacity (TEAC) assay, only *para*-topolin (0.5–5 μM) was active, probably due to the presence of a phenolic hydroxyl. All the compounds were able to react with hydroxyl radicals generated in the 2-deoxyribose degradation assay. A somewhat higher activity of iP was ascribed to the presence of the double bond in the N^6-side chain. Using electron spin resonance, Hsiao et al. (2003) showed that short pretreatment with K at concentrations of 70 and 150 μM effectively inhibited hydroxyl radical formation in collagen-activated platelets.

Direct comparison of K with a group of other established antioxidants, comprising L-ascorbic acid, DL-alpha-tocopherol, DL-alpha lipoic acid, ubiquinone and idebenone, has been carried out (McDaniel et al. 2005). K quenched radicals generated through the photochemical excitation of water molecules effectively only at a concentration of 1 μM. The effective concentrations of the other compounds, with the exception of DL-alpha lipoic acid, were one or two orders of magnitude lower. K (100 μM) completely prevented oxidation of low density lipoproteins by Cu^{2+}. The other compounds decreased the production of lipid hydroxyperoxides in equimolar concentrations less efficiently (idebenone: 80%, DL-alpha-tocopherol: 50%, ubiquinone: 26%, DL-alpha lipoic acid: 22%, L-ascorbic acid: 15%). However, this exceptional activity of K was not observed in the microsome oxidation (NADPH/ADP/Fe^{3+}) assay, which is considered to be a more realistic model of cell membrane peroxidation. Whereas the activity of K was comparable with that of ubiquinone and L-ascorbic acid (reduction of malonyldialdehyde equivalents by 24–27%), the other test compounds decreased MDA production by 47–55%.

Cytokinins may also form complexes with ions of metals with the ability to quench ROS. Superoxide dismutase-like activity of Cu^{2+} complexes of K and BA have been reported (Goldstein and Czapski 1991). The ability of Cu^{2+} complexes of BA derivatives to protect against oxidative damage in vivo (aloxan induced diabetes) has been observed (Štarha et al. 2009).

Besides their direct anti-oxidant activities, cytokinins also activate cellular anti-oxidant defense mechanisms. *tZ* has been shown to induce hydrogen peroxide decomposing enzymes in human skin fibroblasts. The treatment was also able to protect both proliferating and senescent cells against the hydrogen peroxide induced cell death (Rattan and Sodagam 2005). Induction of antioxidant enzymes may also contribute to the lifespan extension observed in flies after K treatment mentioned above (Sharma et al. 1997) because it enhances the catalase activity in this organism. K also has been demonstrated to exhibit protective effects in the D-galactose model of glycoxidative stress. In cultured rat astrocytes, K partially reversed a decrease in the activities of glutathione peroxidase and superoxide dismutase induced by D-galactose treatment. K treatment also decreased malonyldialdehyde concentration in the cell membranes and increased cell viability. The active concentrations were in the range 50–100 μM. In addition, K was shown to have beneficial effects in rats receiving daily subcutaneous injections of D-galactose at 125 mg/kg for 6 weeks. K (10, 20 and 40 mg/kg) was administered by gastric perfusion for the whole period of galactose exposition. However, no information was provided about K concentrations in plasma or brain tissue. A dose of 10 mg/kg was concluded to have the most promising effect because it was able to attenuate the negative effects of D-galactose on malonyldialdehyde concentration and activities of glutathione peroxidase and superoxide dismutase in brain tissue (Liu et al. 2011).

Although iPR and N^6-benzyladenosine (BAR) have been studied traditionally as anti-cancer agents, they possess cytoprotective activities as well. Dassano et al. (2014) showed that treatment of several cancer cell lines with iPR or BAR induced robust expression of genes regulated by transcription factor Nrf2. Moreover, the transcription of Nrf2 itself was upregulated. These expression changes were not a just a futile response to oxidative stress resulting from drug-induced damage. In fact, the treatment with cytokinin ribosides decreased the basal levels of ROS and increased the resistance of the cells to pro-oxidative insults. Our earlier unpublished observation that not only iPR but also other cytotoxic ribosides, including kinetin riboside (KR), in low micromolar concentrations induce Nrf2-dependent gene expression in multiple cell lines led us to speculate that the cytoprotective activity of K could be mediated by its metabolite KR and/or appropriate ribotides. The conversion of cytokinin bases into their respective riboside 5′-monophosphates by human cells has been reported (Mlejnek and Doležel 2005). However, treatment of several cell lines, including skin fibroblasts and immortalized keratinocytes, with 100 μM K did not have any significant effect on hemeoxygenase expression (unpublished data). Because tissues may differ in expression and/or activity of phosphoribosyltransferase, and thus in the rate of K conversion into its riboside, studies of a wider panel of cell lines could identify tissues where the proposed mechanism is relevant. Nevertheless, Hertz et al. (2013) recently demonstrated that conversion of K into its ribotides may be indeed important for its cytoprotective activity. Mitoprotective PTEN-induced putative kinase 1 (PINK1, PARK6) is able to use kinetin riboside 5′-triphosphate (KRTP) as a donor of a phosphate group more efficiently than ATP. Because of its unusual behavior, Hertz et al. (2013) designated KRTP as a PINK1 neo-substrate. They showed that K is converted into KRTP within the cells and that treatment with K accelerated Parkin recruitment to depolarized mitochondria and suppressed oxidative stress-induced apoptosis in human-derived neural cells in a PINK1-dependent manner.

14.4.3 Cytokinins in the Therapy of Skin Diseases and Cosmetics

Because the anti-aging activity of K, and later *tZ*, was demonstrated for the first time in skin fibroblasts, multiple studies evaluating the applicability of cytokinin bases in skin protection both in vitro and in vivo have been conducted. Positive effects include protection against UV-induced damage, improved wound healing and aquaporin induction. Cytokinins also modulate melanogenesis and keratinocyte differentiation. Several clinical trials have shown that they can improve multiple traits of photoaging skin and also some symptoms of acne rosacea.

McDaniel et al. (2005) compared the UV-protective activity of a group of antioxidants, i.e., K, L-ascorbic acid, DL-alpha-tocopherol, DL-alpha lipoic acid, ubiquinone and idebenone. K was shown to protect primary keratinocytes against UVB (single dose, 200 mJ/cm^2); it reduced the number of cells stained positively by immunohistochemistry with an antibody against thymine–dimer from 53 to 34%. Whereas idebenone, L-ascorbic acid and DL-alpha-tocopherol offered similar protection levels (29–35% of positive cells), ubiquinone and DL lipoic acid were not active. Subsequently, the UV-protective activity of the compounds was tested in patients, each compound on five subjects. The compounds were applied as ethanolic solutions (0.5% w/w) on mid-back regions once a day for 2 weeks. Sunburn cells (SBC) induced by a 1.5 × minimal erythema dose of UVB were quantified in biopsies obtained 20 h later. K reduced the number of SBC by 20%, compared to about 10% for ubiquinone and DL-lipoic. Whereas idebenone and DL-alpha-tocopherol were more active (reduction by 38 and 30%, respectively), ascorbic acid did not have any protective effect. Unfortunately, no UV protective effects of K were observed in pigs. Four days' application of 0.1% K cream or 0.5% K solution neither ameliorated UV-induced erythema nor reduced the number of SBC in pigs (Tournas et al. 2006) exposed to a one to five times minimal erythema dose of UV irradiation ($\sim$5 mW/cm^2 of UVB and $\sim$40 mW/cm^2 of UVA).

Cell culture experiments have suggested that *tZ* could improve skin hydration and wound healing, as well as prevent detrimental effects of photoaging on those processes (Ji et al. 2010). *tZ* at 40 and 80 μM concentration also induced expression of aquaporin 3 protein (AQP3) in spontaneously immortalized HaCaT keratinocytes. Treatment with *tZ* was also able to ameliorate a UV-induced decrease in AQP3 concentrations and membrane water permeability to a large extent. Experiments with pharmacological MAPK pathway inhibitors revealed that *tZ* inhibits UV-induced MEK/ERK activation. *tZ* has also been reported to promote wound healing in the scratch assay with either irradiated or non-irradiated cell cultures (Ji et al. 2010) and inhibit (*tZ* at 20–40 μM) UVB-induced MMP-1 expression in skin fibroblasts (Yang et al. 2009).

Cytokinin bases have been shown to promote differentiation of keratinocytes. K at 40–200 μM concentration induced growth arrest and changes of several markers of differentiation (keratin K10 and involucrin) in human keratinocytes in cell culture (Berge et al. 2006). The effect was augmented by the presence of Ca^{2+}

ions. Other markers of differentiation (*trans*-glutaminase) were unchanged, suggesting that K-induced differentiation might be mediated by pathways different from those activated by other differentiation inducing agents. In a subsequent study, Berge et al. (2008) reported that treatment with K improved the sensitivity of aging keratinocytes to the differentiating effects of Ca^{2+} ions. The authors mentioned unpublished data indicating that the effect was accompanied by induction of Hsp90, Hsp70 and heme-oxygenase-1. They suggested that the effects were mediated by stress-induced hormesis based on this observation.

A positive effect of K on filaggrin levels, another marker of keratinocyte differentiation, was observed in an in vitro reconstructed skin equivalent (Vičanová et al. 2006). In contrast to 2D culture, K promoted the growth of keratinocytes, as indicated by an increase in the number of Ki67-positive cells.

Both experimental systems mentioned above used human keratinocytes from healthy donors. However, it should be noted that models using keratinocytes from psoriatic lesions might be more appropriate for evaluation of the utility of cytokinins in the therapy of psoriasis.

Other dermatologic/cosmetic applications of cytokinins relate to the therapy of pigmentation disorders. Whereas K has been reported to decrease hyperpigmentation in dogs (Kimura and Doi 2004), BA is a stimulator of melanogenesis (Kim et al. 2009). At concentrations of 50 and 100 μM, it stimulated melanogenesis in B16 mouse melanoma cells through protein kinase A mediated induction of microphthalmia-associated transcription factor (MITF), tyrosinase and tyrosinase-related proteins 1 and 2 (TYRP1, TYRP2). In contrast to alpha-MSH, BA activated protein kinase A in a cAMP independent manner. Also, *para*-topolin induced tyrosinase expression and melanogenesis in B16 cells (our unpublished data).

14.4.4 Clinical Examination of Kinetin and Its Relatives

Topical K has been evaluated in multiple open-label single-arm clinical trials, in which effects were compared before and after treatment. K lotion 0.1% (Kinerase) applied twice daily was evaluated as a treatment for mildly to moderately photodamaged facial skin in 32 subjects. Twelve and twenty four week treatments were found to improve significantly skin texture, mottled hyperpigmentation and fine wrinkles according to the both patient's and physician's assessment. The treatment also improved transdermal water loss (McCullough and Weinstein 2002). The same regimen was later evaluated in patients ($N = 17$) with mild to moderate facial rosacea in a 12 week study (Wu et al. 2007). K treatment was found to reduce redness and had also a positive, yet statistically insignificant, effect on telangiectasia. The physician's rating of overall improvement of rosacea symptoms increased over the study period and by week 12, almost 60% of the subjects showed at least moderate improvement. K was generally well tolerated in those studies. However, in rare cases, acne or a rash was observed after 8 weeks' treatment. Wanitphakdeedecha et al. (2015) evaluated the effects of applying 0.1% K cream twice daily on the photoaging facial skin of 100 Thai subjects. Twelve weeks' treatment was accompanied by small but statistically significant improvements in overall skin condition, skin texture, color and wrinkles. K also improved ultraviolet spots and redness. Chiu et al. (2007) evaluated possible synergistic effects of using a combination of K (0.03%) and niacin (4%) in a double-blind clinical study on 52 Taiwanese subjects. Serum containing either K and niacin (27 subjects) or niacin alone (25 subjects) was applied to one half of the face and the vehicle to the other twice daily for 12 weeks. Although the combination of K and niacin had a larger positive effect on most of the evaluated parameters, including corneal hydration and erythema index, than niacin alone when compared to the baseline, the differences between the treatments were not statistically significant. Also, vehicle alone had a positive effect on multiple parameters, yet the effects of K were typically larger.

To the best of our knowledge, no reports of clinical trials with *tZ* have been published. Several open-label, single-arm clinical trials have evaluated the K derivative 6-furfurylamino-9-(tetrahydropyran-2-yl)-9*H*-purine (Pyratine-6, PRK-124), which originated from our laboratory (Laboratory of Growth Regulators, Olomouc, Czech Republic) and then was developed by companies Senetek PLC and Pyratine PLC, USA. In a trial evaluating its effects on aging and photodamaged skin of 40 subjects (34 finished the study), Pyratine (0.1%) was reported to improve skin moisturization, roughness, mottled hyperpigmentation, fine wrinkles and facial erythema in comparison with the baseline within 4 weeks (McCullough et al. 2008). Application of the compound (0.125%) twice daily was also found to improve symptoms of acne rosacea compared to the baseline in a 12 week study (Ortiz et al. 2009), with some beneficial effects observed as early as week 4. In a later study, 18 subjects were followed over an extended 48 week trial (Tremaine et al. 2010). A mean 44% reduction in erythema severity and 89% reduction in inflammatory lesions was observed at week 48. The treatment also had a positive effect on telangiectasias, transepidermal water loss and skin dryness. No treatment-induced skin irritation was observed.

14.5 Neuroprotective Activity of Cytokinins

Multiple reports of cytokinin activities relevant to the treatment of neurodegeneration exist. Notably, such effects are not limited to cytokinin bases only, but cytokinin ribosides have also been shown to possess neuroprotective activity. As discussed above, K protects rat astrocytes in vitro as well as rat brain against glycoxidative damage in the D-galactose model via promotion of anti-oxidant defense (Liu et al. 2011). KRTP increases kinase activity of PTEN-induced putative kinase 1 (PINK1). PINK1 is critical for mitochondrial quality control—it accumulates in the outer membrane of impaired mitochondria and, through the recruitment of Parkin protein, targets them for autophagy. Both PINK1 and Parkin are mutated in the familial forms of recessive Parkinson's disease. Treatment with K protected SH-SY5Y5 neuroblastoma cells against proteasomal and oxidative stress in PINK1-dependent manner. K is therefore an interesting drug candidate for the treatment of familial form of Parkinson's disease and possibly also therapy of other diseases with mitochondrial dysfunction. It would be interesting to identify other N^6-substitued adenines with similar type of activity. This may prove difficult because both base and riboside require not only multistep metabolic activation, but they also have to mimic the shape and behavior of ATP in the PINK1 binding pocket (Hertz et al. 2013).

K is also a candidate drug for neurodegenerative disease familial dysautonomia. A clinical study (NCT02274051) is in the process of recruiting patients at the time of writing this text according to web page www.clinicaltrials.gov. Both in vitro and in vivo experiments have demonstrated that K is able to correct aberrant splicing of pre-mRNA originating from the IKBKAP gene (inhibitor of kappa light polypeptide gene enhancer in B-cells, kinase complex-associated protein). The mechanism of K's action is unknown. However, the very limited number of transcripts with splicing influenced by the treatment suggests that K interacts with regulators or components of specific spliceosome sub-species.

tZ also exhibits multiple activities relevant for the therapy of diseases of the central nervous system, in particular Alzheimer's disease. It was identified as the substance responsible for inhibition of rat acetylcholinesterase contained in an extract from the traditional Korean edible plant *Fiatoua villosa* (IC_{50} 1.09×10^{-4} M) (Heo et al. 2002). Follow-up studies showed that *tZ* protects rat pheocytohroma cells PC12 against toxic effects of an amyloid beta fragment comprising amino acids 25–35. *tZ* treatment had a positive effect on both ROS production and cell viability (Choi et al. 2009). Also reported was a beneficial effect of long-term treatment with *tZ* on scopolamine-induced amnesia. Scopolamine is an antagonist of muscarinic acetylcholine receptors, but its administration also influences other neurotransmitter systems. For example, repeated treatment of scopolamine has been shown to decrease levels of monoamines (noradrenaline, dopamine and serotonin) and also increase oxidative stress in brain (Haider et al. 2016). In Choi et al.'s study (2009), animals had ad libitum access to either normal drinking water (control and scopolamine control groups) or *tZ* solution (0.002, 0.004, and 0.008% w/v) for 21 days. More specific information about dosing (drinking volume by the individual animals) and *tZ* concentrations in plasma and brain tissues was not reported. After the treatment period, temporal amnesia was induced by a single s.c. injection of scopolamine (1 mg/kg) and behavioral tests were started 30 min later. All three tested concentrations markedly attenuated negative effects of scopolamine on passive avoidance and spontaneous alternation behaviors in the Y-maze test. After finishing the tests, the animals were sacrificed and acetylcholinesterase activity in brain lysates was measured. The two highest *tZ* concentrations were able to reduce the effects of scopolamine on acetylcholinesterase activity. Similar effects on behavior were observed when mice were provided food mixed with *tZ* ad libitum for 4 weeks (Choi et al. 2009). However, the mechanism of the observed *tZ* activity is unclear. The authors provided no rationale for the treatment regime length—much shorter regimes and possibly single *tZ* injection may have been more appropriate for evaluation of acetylcholinesterase inhibition or direct antioxidant capacity. It would be interesting to assess the effect of *t*Z on the level of oxidative stress and level/activity of the antioxidant defense system in brain before and after scopolamine treatment.

KR and *trans*-zeatin ribosides (tZRs) have been shown to have cytoprotective activities. Both of them, but not BAR, can protect PC12 cells against serum starvation induced apoptosis (Lee et al. 2012). The corresponding free bases, tZ and K were inactive in this model. The protective effects of KR were apparent at concentrations as low as 1 μM, whereas tZR did not show any significant activity at this concentration. However, at a concentration of 100 μM, tZR was more effective than equimolar KR and was therefore selected for follow-up experiments. Its protective activity in a serum deprivation model was mediated by activation of the A_{2A} adenosine receptor (A_{2A}R) and the downstream protein kinase A (PKA) as co-treatment with inhibitors of those proteins abolished the protective effect. A_{2A}R has been suggested as a potential therapeutic target in Huntington's disease because it is highly expressed in the striatum, where mutant huntingtin causes early damage. Moreover, A_{2A}R-selective agonists effectively ameliorate several symptoms of Huntington's disease in both cell cultures and animal models (Chou et al. 2005; Chiu et al. 2015). Therefore, tZR's ability to prevent huntingtin aggregation was tested in PC12 cells overexpressing the mutant protein. Indeed, tZR treatment prevented both the mutant huntingtin aggregation and subsequent proteasome dysfunction in an A_{2A}R and PKA dependent manner.

Para-Topolin riboside (*p*TR, 6-(4-hydroxybenzylamino)-9*H*-purine riboside) is an A_{2A}R agonist as well. It was isolated as a neuroprotective substance from the Chinese medicinal plant Gastrodia elata (Huang et al. 2011a, b). *p*TR (designated as T1-11 in the study) has been shown to interact with not only A_{2A}R but also equilibrative nucleotide transporter ENT1. ENT1 inhibition can increase the amount of adenosine in extracellular space and possibly contribute to modulation of adenosinergic signaling in synapses where both proteins are present. Treatment of R6/2 mice with *p*TR administered using a subcutaneous Alzet minipump for 48 h improved motor deterioration. *p*TR administered in drinking water (0.05 mg/ml ad libitum, plasmatic or brain concentration unknown) improved the rotarod performance already after 2 weeks and the effect persisted until the end of the experiment after 7 weeks. Analysis of the brains showed a lower accumulation of huntingtin, improved activity of proteasome and higher levels of mRNA for brain-derived neurotrophic factor (BDNF). Finally, *p*TR also binds to A_3R, another adenosine receptor with a known role in CNS physiology (Borea et al. 2014). For example, agonists of A_3R have been shown to have neuroprotective effects in subarachnoid hemorrhage-induced brain damage (Maria Pugliese et al. 2007). Nevertheless, the therapeutic utility of targeting the brain is somewhat controversial because prolonged A_3R activation may be neurotoxic (Luo et al. 2010). Recently, A_3R agonistic activity of *i*PR has been reported (Blad et al. 2011). However, to the best of our knowledge, its effects in models of neurological diseases have not yet been studied.

14.6 Immunomodulatory Activities of Cytokinins

In the previous section, we discussed interaction of cytokinin ribosides with the adenosine receptor A_{2A} in relation to their neuroprotective activities. However, A_{2A} is also expressed in various immune system cells (T-lymphocytes, macrophages, monocytes and polymorphonuclear leukocytes) and its activation plays an important role in the regulation of both innate and adaptive immunity (Milne and Palmer 2011). Selective A_{2A} receptor agonists have been suggested as drugs for treatment of graft-versus-host disease, colitis and T-lymphocyte mediated ischemia-reperfusion injury (Chhabra et al. 2012; Jones and Kang 2015). The immunomodulatory activity of *t*ZR was demonstrated for the first time by Lappas (2015). It was shown to inhibit production of interferon (IFN)-γ, IL-2 and TNF-α in either CD3+CD4+ or CD3+CD8+ T-lymphocytes incubated with anti-CD3 antibody. In the case of the CD3+CD4+ population, *t*ZR also inhibited production of IL-4. EC50 values were in the range 10–100 μM. Furthermore, *t*ZR inhibited upregulation of CD25, CD69 and CD40L. The inhibitory effects in the latter assays were strongly attenuated by co-treatment with 10 μM of the selective A_{2A} receptor antagonist ZM241385, thereby confirming its involvement. In mice, *t*ZR decreased the number of white-blood cells in intraperitoneal infiltrate in thioglycollate-induced peritonitis. *t*ZR was administered as a 1 mg/kg i.p. bolus at times 0, 1.5 and 3 h after the thioglycollate injection. Overall, the data showed that *t*ZR is not a very potent immunomodulator in terms of the active concentration. However, the absence of cytotoxic effects up to 1000 μM suggest that the low activity could be possibly compensated by the administration of higher doses.

As discussed above, iPR and BAR activate the Nrf2 pathway and protect cells against oxidative stress (Dassano et al. 2014). The employed models also included 12-O-tetradecanoylphorbol-13-acetate (TPA)-induced superoxide production by HL-60 cells differentiated along the neutrophilic lineage. Further, the authors tested the activity of the cytokinins in a mouse ear inflammation model where topical TPA-induced oxidative stress stimulates an inflammatory response. Pretreatment with iPR and BAR attenuated the inflammation and reduced the number of infiltrating neutrophils. The exact mechanism of the protective effect is unclear and other processes besides Nrf2 activation could be involved. The authors discussed possible involvement of glucocorticoid receptor signaling (iPR induced several transcripts in this pathway) and adenosine receptor A_3R activation. The A_3R agonistic activity of iPR and tZR was reported recently (Blad et al. 2011). A_3R regulates various aspects of inflammation, including neutrophile chemotaxis and superoxide production (van der Hoeven et al. 2010). The anti-inflammatory effects of A_3R activation are mediated through the inhibition of NF-κB dependent production of cytokines, including TNF-α. A_3R agonists have been shown to have robust anti-inflammatory activity in animal models of inflammatory bowel disease, systemic toxemia, pulmonary and liver inflammation as well as rheumatoid arthritis (Borea et al. 2014). Nanomolar A_3R agonist CF101 (IB-MECA) structurally related to aromatic cytokinins is currently being evaluated as an antirheumatic and antipsoriatic agent in clinical trials. Recently, promising activity in patients with moderate to severe plaque psoriasis was reported (David et al. 2016).

Another explanation of the anti-inflammatory activity of iPR was offered by studies of its effects on natural killer cells (NK cells) (Ciaglia et al. 2014). iPR at 10 μM concentration inhibited the cytotoxicity of NK cells against leukemia K562 cells. The treatment prevented ERK/MAPK and STAT5 activation in IL-2-activated NK cells, downregulated the expression of activating receptors NKp44 and NKG2D and decreased secretion of cyto/chemokines (RANTES, MIP-1α, TNF-α and IFN-γ). Topical application of iPR significantly reduced ear edema in a mouse model of croton oil-induced ear dermatitis with a potency comparable to that of indomethacin. Histology analysis showed lower leukocyte infiltration and decreased staining of the natural cytotoxicity receptor NKp46, whose expression is typical for NK cells, in irritated mid and papillary dermis.

Finally, cytokinin bases may also possess anti-inflammatory activity. K and Pyratine have been shown to have beneficial effects on acne rosacea, a skin condition with an inflammatory component (Wu et al. 2007; Tremaine et al. 2010).

14.7 Antiproliferative Effects of Cytokinins and Cytokinin Analogues

Natural cytokinin ribosides iPR, KR, BAR, *ortho*-topolin riboside (oTR) and N^6-(2-hydroxy-3-methoxybenzyl)adenosine (but not their respective bases) have been reported to exhibit strong cytotoxic effects against a range of human cell lines derived from both hematological malignancies and solid tumors (Doležal et al. 2007; Voller et al. 2010, 2017). Numerous studies with various experimental designs (assay principle, endpoint, length of treatment) have demonstrated that some cytokinin ribosides are active at submicromolar (against some leukemia) or micromolar concentrations (against other leukemia, adherent cells). The toxicity of *t*ZR and *cis*-zeatin riboside, other natural cytokinins that differ from iPR by hydroxylation of the isoprenoid side-chain, is very limited (Ishii et al. 2002; Rattan and Sodagam 2005; Voller et al. 2010). Further, isomers of oTR with a hydroxy group in either the *meta*- or *para*-position of the phenyl ring do not show significant toxicity (Voller et al. 2010).

In leukemia cell lines, cytokinin ribosides induce rapid apoptosis (Mlejnek and Kuglík 2000; Ishii et al. 2002; Voller et al. 2017). Cell death is preceded by ATP depletion. An exception is N^6-(2-hydroxy-3-methoxybenzyl)adenosine, where apoptosis ensues without marked effects on cell energy levels (Voller et al. 2017). It has recently been demonstrated that KR may be a potential drug for the treatment of multiple myelomas. In several cell lines, KR has been found to suppress cyclin D1 and D2 transcription, followed by arrest of the cell-cycle and selective apoptosis in tumor cells (Tiedemann et al. 2008). KR may be also effective against leukemia stem cells (McDermott et al. 2012).

Cytotoxic effects of iPR and KR against mammalian cell lines derived from solid tumors have been reported many times (Meisel et al. 1998; Griffaut et al. 2004; Laezza et al. 2006, 2009, 2010; Spinola et al. 2007; Cheong et al. 2009; Cabello et al. 2009; Colombo et al. 2009; Rajabi et al. 2012; Wang et al. 2012; Ciaglia et al. 2014, 2016). Depending on the cell line and cytokinin used, the treatment resulted in apoptosis, G1 or G2/M block. The spectrum of the effects induced by cytokinin ribosides in the tested cell lines included ATP depletion, genotoxic stress (Cabello et al. 2009), JNK activation (Laezza et al. 2009), inhibition of farnesyl-protein transferase activity (Laezza et al. 2006), inhibition of EGFR signaling (Ciaglia et al. 2016) and changes in levels of mitochondrial proteins (Cheong et al. 2009). Recently, microarray analysis of the effects of iPR (100 µM) on MCF7 and A549 cell lines was published. iPR induced a set of genes involved in stress induced cell cycle arrest, e.g., PPP1R15A, DNAJB9, DDIT3, and HBP1 (Colombo et al. 2009). Cytokinin ribosides may also interfere with neoangiogenesis (Pisanti et al. 2014). Further, cytokinin riboside-5′-monophosphates have been identified as inhibitors of putative oncogene RCL1 (Amiable et al. 2013; Voller et al. 2017).

In vivo anticancer activity of iPR, KR, oTR and BAR has been demonstrated using several animal and xenograft models of cancer (Griffaut et al. 2004; Laezza et al. 2006; Tiedemann et al. 2008; Voller et al. 2010). iPR and BAR have been shown to exhibit limited activity against a diverse range of cancers in a small clinical trial (Mittelman et al. 1975). Micromolar concentrations of both cytokinin ribosides and cytokinin bases can also induce cell death in plant cell cultures, with some traits typical for apoptosis (activation of caspase-like proteases and fragmentation of DNA) (Mlejnek and Procházka 2002; Mlejnek and Doležel 2005). This cell death is preceded by depletion of ATP and the production of ROS.

In contrast to their hormonal activity in plants, which requires interaction with specific membrane-bound receptors, intracellular conversion of cytokinins to 5′-monophosphates is necessary for their cytotoxic effect. The concentrations of cytokinins required to produce cytotoxic effects are higher than those found endogenously in plant tissues, but they do fall within the range used in plant bioassays (Carimi et al. 2003; Mlejnek and Doležel 2005). Phosphorylation of cytokinin ribosides by adenosine kinase (ADK) is a requirement for the cytotoxic effect of cytokinin ribosides in both animal (Mlejnek and Doležel 2005; Voller et al. 2010) and plant cells (Mlejnek and Procházka 2002). Low affinity to ADK (and possibly other nucleoside kinases) may explain the lack of activity of other cytokinin ribosides (Mlejnek and Doležel 2005) and possibly also their analogues with ribose replaced by acyclic polyols (Colombo et al. 2009; Ottria et al. 2009). Contrary to other nucleoside analogues that are converted to nucleoside triphosphates, the dominant metabolites of cytokinin ribosides are their respective riboside monophosphates (Mlejnek and Doležel 2005). This observation suggests that cytokinin ribosides have a different mechanism of action than classical antimetabolites which, after phosphorylation, directly interfere with the synthesis of nucleic acids. In cultured plant cells, the cytoxicity of cytokinin bases and their corresponding ribosides are comparable because, in contrast to human cell lines, plant cells are able to convert both metabolic forms efficiently into riboside-5′-monophosphates (Mlejnek and Procházka 2002; Mlejnek and Doležel. 2005).

A characteristic trait of leukemia cells is blockade of their differentiation into functional mature cells. Various chemicals acting by diverse mechanisms are able to force malignant cells to undergo terminal differentiation. Such differentiation therapy could be much safer than regimens based on cytotoxic effects. Cytokinin bases K, iP, BA and, to lesser degree, also *tZ* have been shown to induce granulocytic differentiation of human myeloid leukemia cell line HL-60 (Ishii et al. 2002) derived from the peripheral blood leukocytes of a patient with acute myeloid leukemia. The activity was mediated by phosphorylation of ERK1/2, expression of CEBPD and S100P (Ishii et al. 2005a, b). Whereas cytokinin bases induced differentiation at rather high concentrations (25–100 μM), their ribosides caused rapid apoptosis at low micromolar levels. Treatment with caspase inhibitors shifted the activity of iPR in HL-60 from pro-apoptotic to growth inhibitory and differentiating activity (Ishii et al. 2002). Cytokinins are also able to promote differentiation of keratinocytes, as discussed above.

14.8 Conclusions

Cytokinins regulate a wide range of plant processes, including growth, differentiation and organ senescence. This fascinating activity together with reports that some cytokinins occur in animals, including humans, has inspired interest in studying their effects in mammalian cell cultures and animal models. However, current knowledge of cytokinin signaling in plants suggests that the diverse effects of cytokinins in animal systems are relayed by mechanisms and pathways different from those that mediate hormonal activity in plants.

In plants, cytokinins are recognized by receptor histidine kinases, which are part of the His-Asp phosphorelay resembling the two-component environmental sensors of bacteria. No signaling system with similar organization exists in animals. In addition, plant leaf senescence, an active and highly regulated process, is a phenomenon completely different from animal aging (stochastic accumulation of damage) and cell senescence (irreversible growth arrest that protects the body against tumor development).

Nevertheless, studies of cytokinins in mammalian cell cultures and animals have led to discoveries of a range of responsive pathways and processes, as well as numerous prospective therapeutic applications. The propensity of cytokinins, which are adenine and adenosine derivatives, to influence diverse processes in animal cells is probably a consequence of their ability to interact with various components of the animal purinome.

The anti-proliferative activity of cytokinin ribosides (through induction of cell cycle block or/and cell death) and bases (through induction of cell differentiation) has prompted studies into their potential utility for the therapy of proliferative diseases like leukemias, cancers and psoriasis. Although anti-cancer cytokinin ribosides were evaluated in patients with diverse malignancies as early as the 1970s (Mittelman et al. 1975), limited activity and problems with stability led to a loss of interest in further development at that time. Recent interest in cytotoxic anti-cancer ribosides has been stimulated by molecular studies of their mechanism of action, e.g., by microarray analyses (Colombo et al. 2009) and discovery of the high anticancer activity of BARs hydroxylated on the phenyl ring (Doležal et al. 2007; Voller et al. 2010). Further, inhibitors of cyclin-dependent kinases olomoucine, bohemine, roscovitine, developed in our laboratory, were inspired by cytokinins (for a review see Jorda et al. 2012).

Another fertile research area was spurred on by the finding that the cytokinin bases K and *t*Z delay the onset of several characteristics related to aging in human skin fibroblasts during serial passage in vitro (Rattan and Clark 1994; Rattan and Sodagam 2005). Some cytokinin bases even extend the lifespan of invertebrates such as drosophila (Sharma et al. 1995) and *C. elegans* (our unpublished data). Even today, when *t*Z, K and its analogue Pyratine are the principal components of cosmetics used for the treatment of aging skin, their mechanism of action is still not fully understood. Since the original discovery, research has mainly focused on the ability of cytokinins to protect macromolecules and cells against oxidative damage and stress. Numerous studies have reported the ability of K to quench radicals directly and this activity has also been observed for several other cytokinin bases. However, K and *t*Z also induce cellular antioxidant defense by an as yet unidentified mechanism. Moreover, K modulates splicing of certain pre-RNAs (Slaugenhaupt et al. 2004). Therefore, it is tempting to speculate that it may also modulate splicing of transcripts of some genes related to cytoprotection.

Recently, Hertz et al. (2013) described an unusual mechanism of cytoprotective activity of K, including its intracellular conversion into respective ribotides. KRTP increases the activity of the mitoprotective kinase PINK1 by acting as a more active ATP analogue (neosubstrate). Other mechanisms of action including the ribosylation of cytokinin bases cannot be ruled out either. Cytokinin ribosides induce Nrf2-dependent transcription (Dassano et al. 2014, our unpublished data), inhibit proteosynthesis (as indicated by a decrease of activating phosphorylation of p70 S6 kinase and S6 protein) and activate autophagy (increase in levels of LC3-II) (our unpublished data). All these pathways have been implicated in cytoprotection and aging prevention (Bruns et al. 2015; Madeo et al. 2015). If cytokinin bases are metabolized into respective ribosides/ribotides in quantities sufficient to activate those stress response pathways, they could act as hormetin precursors, i.e., prohormetins.

In the last few years, cytokinin ribosides have been studied in connection with their anti-inflammatory and neuroprotective activities. Activation of A_{2A} or A_3 adenosine receptors has been implicated in the effect. Because much more active agonists have already reached clinical trials, purinergic activity alone may not warrant future development of such compounds into drugs. However, other pathways could possibly be involved in immunomodulatory activity, including activation of an Nrf2 response and inhibition of farnesyl diphosphate synthase. Notably, inhibition of protein farnesylation by iPR mediates not only its anti-cancer and immunomodulatory activities (Ciaglia et al. 2014; Laezza et al. 2006) but also its ability to decrease the accumulation of abnormal lamin A (progerin) in the nuclear envelope of cells originating from patients with Hutchinson-Gilford progeria (Bifulco et al. 2013). Progerin resulting from aberrant processing of wild type lamin A transcripts accumulates in tissues including skin of elderly and was therefore suggested as a biomarker of aging (McClintock et al. 2007). Hence, topical application of iPR (or possibly iP) could have multiple benefits.

A major obstacle in the development of cytokinin ribosides into anticancer drugs has been their pharmacokinetics. Clinical trials in the 1970s showed that they have a short plasmatic half-life (Mittelman et al. 1975). Our studies indicate that cytokinin ribosides may have problems with crossing biological barriers (Voller et al. 2010). However, studies in rodents reviewed above suggest that some cytokinin ribosides can be absorbed from the gut and even reach the brain, whereas others are able to penetrate the skin, as indicated by their therapeutic effects. Unfortunatelly, no information about plasma and/or tissue concentration has been reported. Such observations do not guarantee similar behavior in humans because of differences in physiology. For example, human skin has not only less hair follicles but also a thicker epidermis and dermis, which pose a much higher barrier to the penetration of drugs, especially hydrophilic ones. Prodrugs may be necessary to allow the active compound to reach target deep layers of (intact) skin. Another option could be administration of cytokinin bases that could be converted into active ribosides within the tissue, as discussed above. Once a cytokinin riboside is present in target skin layers, its pharmacokinetic properties may be seen as an advantage as they will prevent it from reaching the systemic circulation, and thus limit possible side effects (e.g., vasodilatation in the case of A_2AR agonists).

K, *tZ* and Pyratine are principle components of cosmetics products (Kinerase and Pyratine-6 lines). Clinical evaluation reports have been published for K and Pyratine. Both compounds showed beneficial effects on photoaging skin and also ameliorated some symptoms of acne rosacea. Most of the studies were open-label and single-arm and effects were compared to the state before the treatment. The reported positive results together with multiyear experience of their safe usage in cosmetics could encourage testing of these compounds in clinical trials with a better design that may eventually support their approval in acne rosacea or possibly other inflammatory skin conditions. Recent discoveries of the unique activities of K relevant for the treatment of Parkinson's disease (PINK1 activation) or familial dysautonomia (correction of aberrant splicing of IKBKAP transcripts) may result in clinical trials in those indications. Recruitment of patients for a study of familial dysautonomia is currently underway. Because the therapy would require chronic administration of K, we may also learn about its anti-aging activities not yet described from those studies.

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