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Tesis doctoral:

CORTISOL AND THE AGING BRAIN: Hypothalamic-pituitary-adrenal axis activity and cognitive performance in older people.

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A mis padres y a mis hermanos

Hay hombres que de su ciencia
tienen la cabeza llena;
hay sabios de todas menas,
mas digo sin ser muy ducho -
es mejor que aprender mucho
el aprender cosas buenas.

Martín Fierro.

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Por último, quiero agradecer a las personas más importantes de mi vida. Mamá y Papá, no tengo palabras para agradecerles todo lo que han hecho y hacen por mí. Este doctorado no hubiese sido posible sin ustedes. Gracias por demostrarme que siempre hay que pelearla hasta el final, por apoyarme y ayudarme siempre en todas las decisiones que tomé, por su sacrificio y por un amor sin fronteras. A mis hermanos, Carolina y Sebastián, mis modelos a seguir y apoyo incondicional. A mi abuelo Juan Pedro, por siempre preocuparte por mí y por ser un ejemplo de que las aventuras valen la pena.

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Matías M. Pulópulos
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Dissertation outline

One of the greatest achievements in modern society is the increase in life expectancy. This increment is so great that children born after 2011 have a one in three chance of reaching their 100th birthday, and specifically in Europe, a quarter of the population will be over 60 years of age by 2020 (European Commission, 2014). This is an important change in society that is occurring for the first time in human history and producing new challenges that need to be addressed, especially those concerning age-related health problems. In this regard, one of the main aims of new research programs, such as Horizon 2020 in Europe, is to increase the possibilities of keeping older people healthy and independent as long as possible. To do so, it is critical to understand what processes can provoke, or at least contribute to, an increase in the vulnerability of older people because this will help to develop new strategies of prevention and intervention.

Conserving good cognitive functioning is especially important for the maintenance of independent life. Therefore, it is essential to identify those mechanisms that can affect cognitive performance in older people and their adaptation to the environment. Along these lines, changes in the activity of the hypothalamus-pituitary-adrenal axis (HPA-axis) have been related to changes in the cognitive performance of both young and older people, and it has become a research target that can shed light on the changes that can be observed in aging. This dissertation presents the results of a research study focused on investigating the relationship between cognitive performance and the activity of the hypothalamus-pituitary-adrenal axis (HPA-axis) in older people.

The first chapter of this dissertation provides a description of the HPA-axis, its main functions and the biological samples commonly used in research to measure the hormone cortisol, the end product of this neuroendocrine axis. Additionally, this chapter explains the age-related changes that can be observed in the circadian activity of the HPA-axis and in the stress-induced cortisol response. The last part of this chapter presents a short overview of the studies that have investigated the effects of acute stress and cortisol on memory performance in young and older people, and the rela-

tionship between the basal activity of the HPA-axis and cognitive performance in older people.

The second and third chapters present two studies designed to investigate the effect of acute stress on memory in older men and women. The first empirical study of this dissertation investigated the effects of the cortisol response to stress on long-term memory retrieval. In the second study, two experiments were performed to investigate the effects of acute stress on the memory span and the executive component of the working memory.

The fourth chapter describes the third study, designed to investigate whether differences in the long-term endogenous cortisol exposure and in the diurnal regulation of the HPA-axis are related to inter-individual differences in the cognitive performance of older people. In this study, a new technique was used to assess cortisol levels in hair samples, making it possible to measure cortisol exposure during the three months prior to the cognitive performance assessment.

The fifth chapter includes a study designed to investigate whether the cortisol awakening response, a discrete component of the diurnal HPA-axis, was related to walking speed, a commonly used measure of physical activity that has been closely related to cognitive performance in older people. Also focused on the CAR, the sixth chapter describes the fifth study. It investigated whether the CAR is different in normotensive and hypertensive older people. Additionally, the relationship between the cortisol awakening response and cognitive performance was studied in these two groups.

Each empirical study contains a short introduction describing the most important related literature and a discussion of the main findings.

The seventh chapter contains a general discussion, the clinical implications of the findings, study limitations, and future considerations drawn from the results of the previous empirical studies. Finally, the eighth chapter includes a summary of the main conclusions of this doctoral dissertation.

Abbreviations

AUCg = Area under the curve with respect to the ground

AUCi = Area under the curve with respect to the increase

BMI = Body mass index

CAR = Cortisol awakening response

GR = Glucocorticoid Receptors

HCC = Hair cortisol concentrations

HPA-axis = Hypothalamic-Pituitary-Adrenal axis

IAPS = International Affective Picture System

LNS = Letter number-sequencing

MR = Mineralocorticoid receptors

PFC = Prefrontal cortex

RAVLT = Rey Auditory Verbal Learning Test

sAA = Salivary alpha-amylase

SAM = Self-Assessment Manikin

SES = Subjective socioeconomic status

SNS = Sympathetic Nervous System

TMT-A = Trail Making Test form A

TMT-B = Trail Making Test form B

TSST = Trier Social Stress Test

WS = Walking speed

WM = Working memory

Chapter 1

HPA-axis activity, cortisol and cognitive performance

Part of the information included in this chapter is being prepared as a review article to be submitted to a journal: Pulopulos, M.M., Hidalgo, V., Almela, M., Salvador, A. The cortisol awakening response and cognitive performance in older people: a systematic review.

1. The HPA-axis activity and glucocorticoids.

1.1. *The HPA-axis* and general function of glucocorticoids.

The Hypothalamic-Pituitary-Adrenal axis (HPA-axis) is a highly complex neuroendocrine system that has a pivotal role in the control of resting and stress-related homeostasis. The activity of the HPA-axis follows a circadian rhythm, but it is especially activated in conditions of acute psychological and/or physical stress (Ulrich-lai and Herman, 2009).

The HPA-axis comprises three discrete components located in the hypothalamus, the pituitary gland and the adrenal cortex. The paraventricular nucleus of the hypothalamus is the first component of the HPA-axis. When neurons of this nucleus are stimulated (for instance in a stressful situation), corticotropin-releasing hormone and arginine vasopresine are synthesized and secreted into the hypophysial portal system, reaching the anterior lobe of the pituitary. Corticotropin-releasing hormone and arginine vasopresine then trigger the synthesis and secretion of the adrenocorticotropine hormone into the bloodstream by the corticotroph cells of the anterior pituitary gland. Finally, when the adrenocorticotropine hormone reaches its receptors located on the adrenal gland cortex, it produces the release of glucocorticoids into the bloodstream (Jacobson et al., 2005).

The primary function of the glucocorticoids is to prepare the organism for action in response to physical or psychological threats, and to re-establish the homeostasis in the body when the threats are over (Ulrich-lai and Herman, 2009). Given its crucial role for the survival of individuals, it is not surprising that glucocorticoids may affect the activity of an important number of functions in the body. Thus, glucocorticoids may have excitatory effects, such as the secretion and mobilization of glucose and fatty acids to increase the availability of energy, or increases in blood pressure and breathing to ensure a rapid distribution of glucose and oxygen to key structures for survival (Sapolsky et al., 2000). In addition, they also shut down functions that are not essential to dealing with the stressful or threaten situation, for example, sexual behaviors (Sapolsky et al., 2000). Glucocorticoids can produce these excitatory and inhibitory

effects due to a large number of well distributed receptors located on most cells of the human body. These receptors allow the HPA-axis to regulate various systems, such as cardiovascular, immune and metabolic mechanisms, to promote homeostasis (McEwen, 1998). Additionally, and what is most important for this doctoral dissertation, glucocorticoids are liposoluble. Due to this property, they can cross the blood-brain barrier and reach their receptors located in various regions of the brain related to emotion and cognition (de Kloet et al., 2005).

1.2. Glucocorticoid receptors in the brain and mechanism of actions.

Glucocorticoids play at least three functions in the brain: (i) They regulate energy availability during the stress response (as in the rest of the body); (ii) they promote behavioral adaptation (including changes in cognitive performance); and (iii) they regulate the activity of the HPA-axis. Glucocorticoids exert these effects by binding to two different types of receptors: Mineralocorticoid receptors (MR or Type I) and Glucocorticoid Receptors (GR or Type II). Glucocorticoids are able to bind to both types of receptors, but there are some differences between them. One important characteristic is that MR show a 10-fold higher affinity for glucocorticoids than GR do (Reul and de Kloet, 1985). This difference in affinity means that the binding pattern of glucocorticoids to these receptors may differ depending on the levels of this hormone. Under conditions of basal glucocorticoids levels, MR occupation is approximately 70-80%, whereas GR show a low occupation. Under conditions of high glucocorticoid levels, for example in stress situations, MR is saturated, whereas there is a GR occupation of approximately 70% (Reul and de Kloet 1985; de Kloet et al., 2005). Another important difference between the receptors is their distribution in the brain. MR are especially located in the limbic system, with a preferential presence in the hippocampus, while GR can be found in the whole brain (including both subcortical and cortical structures), especially in the prefrontal cortex, hippocampus and amygdala (Patel et al., 2000; Jöels et al., 2009) (see Figure 1).

Two different mechanisms of glucocorticoid action have been described so far. The first mechanism is through a rapid non-genomic pathway. This non-genomic (or non-classical) mechanism is a fast route that allows glucocorticoids to exert their ef-

fects in a matter of seconds to minutes. This fast pathway changes the excitability and activation of neurons, and it is especially involved in the rapid negative feedback-inhibition of the HPA-axis (Groeneweg et al., 2011). The mechanisms underlying this rapid non-genomic effect are still not well understood, but it has been suggested that this effect occurs through the membrane actions of the nuclear receptors (GR and MR) (Ulrich-Lai and Herman, 2009; Groeneweg et al., 2011).

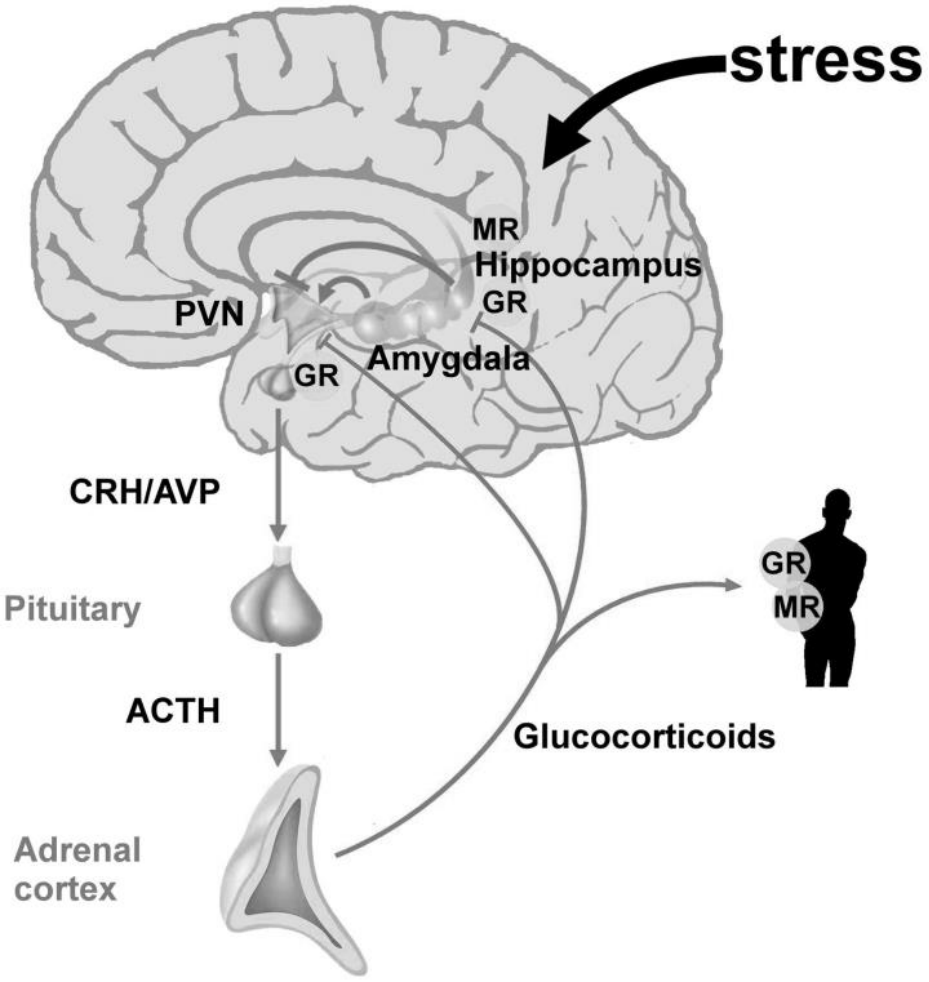

Figure 1. The hypothalamic-pituitary-adrenal axis responds to different inputs, including circadian signals from the suprachiasmatic nucleus and physical and psychological stressors. Glucocorticoids (cortisol in humans) affect body and central nervous system functioning through glucocorticoid and mineralocorticoid receptors located in most cells of the human body. Adapted and reprinted with permission from Raabe and Spengler (2013). Frontiers in Psychiatry, 4:80.

The second mechanism of glucocorticoid action is the slow genomic (or classical) pathway. In this case, the effects of glucocorticoids occur after minutes to hours, and the main characteristic of this mechanism is that it is mediated by GR-driven gene transcription. After glucocorticoids bind to MR and GR near the cellular membrane,

they are translocated to mitochondria and the nucleus of the cell, where they regulate the expression of genes that are mainly involved in synaptic plasticity and neuronal remodeling (Ulrich-Lai and Herman, 2009; Du et al., 2009).

1.3. Regulation of the HPA-axis.

It is worth noting that prolonged exposure to high levels of glucocorticoids may have negative effects on several systems, such as the cardiovascular and/or the immune system (Sapolsky et al., 2000; McEwen, 2008). Additionally, and as discussed in the following points, it is well known that long-term exposure to unhealthy high levels of glucocorticoids is damaging to brain tissues (Lupien et al., 2005). Due to this negative effect of glucocorticoids, the activity of the HPA-axis and the secretion of glucocorticoids are steadily regulated by a negative feedback system that maintains glucocorticoids within tolerable levels (Keller-Wood and Dallman, 1984). Both type of receptors (MR and GR) are involved in this negative feedback, and the affinity and location of these receptors have important implications for the regulation of the HPA-axis. Because MR are nearly always activated (under both low and high glucocorticoid levels), they seem to be involved in the regulation of glucocorticoid levels in basal conditions (Jöels et al., 2008). Instead, GR are especially occupied in conditions of high glucocorticoid levels, and so these receptors are particularly involved in the regulation of the HPA-axis when glucocorticoid levels are high (e.g., in stressful situations) (Jöels et al., 2008).

The first control of glucocorticoid levels is produced by the same subcortical structures that trigger their secretion. An important number of GR can be found in neurons of the paraventricular nucleus of the hypothalamus and the nucleus of the pituitary gland. When these structures detect a considerable increase in glucocorticoids in the bloodstream, they inhibit the secretion of corticotropin-releasing hormone and arginine vasopresine (in the paraventricular nucleus) and adrenocorticotropine hormone (in the pituitary), inhibiting the production of glucocorticoids by the adrenal cortex (Jankord and Herman, 2008). An additional inhibitory pathway of glucocorticoid secretion is performed in the frontal cortex and hippocampus. As mentioned above, the frontal cortex and hippocampus have a large number of MR and GR. When high

levels of glucocorticoids are received by these structures, they send inhibitory inputs to the paraventricular nucleus through an indirect pathway that blocks the synthesis and secretion of corticotropin-releasing hormone and arginine vasopresine (Sapolsky et al., 2000; Sullivan and Gratton, 2002).

1.4. Acute response and circadian activity of the HPA-axis.

The main glucocorticoids produced by the HPA-axis are cortisol, cortisone and corticosterone. This dissertation will focus on the effects of cortisol, the most important glucocorticoid in humans.

1.4.1. Acute HPA-axis response.

As indicated above, the main function of glucocorticoids (and cortisol) is to prepare the organism to respond to physical or psychological stressors and reestablish homeostasis once the stressor has ended. Mason (1968) indicated that the endocrine response to stress can be triggered by any physical or psychological situation (or stimulus) that is perceived as novel, unpredictable, uncontrollable or ambivalent, or when negative organismic or psychological consequences can be anticipated.

The increase in cortisol levels in stressful situations has many complex functions that can be classified as permissive, stimulating, suppressive and preparative (Sapolsky et al., 2000). The first two kinds of functions (permissive and stimulating) are responsible for reinforcing the first defense mechanism through which individuals respond to stress (e.g., enhancement of energy through increases in glucose levels). The suppressive function of glucocorticoids controls the stress response and propitiates the return to homeostasis before the stress response can harm the body (e.g., after a few minutes, suppression of the rapid activation of the immune system in response to stress). Finally, the preparative action of cortisol modulates our response to a future stressor. An example of this function is the effect of glucocorticoids on memory and learning, which allows individuals to avoid similar future threatening situations (this function will be discussed in further sections). Thus, in stressful situations, cortisol will affect the body's response to the stressor, but it will also affect the response to future potential stressors (Sapolsky et al., 2000).

In human research, laboratory-based stress tasks have been developed to investigate the acute response of the HPA-axis. These stress tasks are based on cognitive tasks (e.g., vigilance-reaction time tasks), emotion-induced procedures (e.g., watching videos with negative emotion content), physical stressors (e.g., cold pressor test) or psychosocial stressors (e.g., Trier Social Stress Test). Although differences in the magnitude of the cortisol response can be observed, an increase in cortisol levels is expected when using most of these laboratory-based stress tasks (Dikerson and Kemeny, 2004). This increase in cortisol levels will reach its peak approximately 10 to 20 minutes after the onset of the stressor, and the return to basal levels may occur after one hour or even later (Ulrich-Lai and Herman, 2009; Dickerson and Kemeny, 2004). In addition, sex may moderate this cortisol response because it has been shown that the cortisol increases in laboratory-based psychosocial stress tasks are up to twice as high in men as in women (for review see: Kudielka et al., 2009).

It should be noted that, in addition to the activation of the HPA-axis in response to stress, acute cortisol increases can also be observed in activating and stimulating situations that, at first glance, would seem to be positive for the individuals. For instance, moderate physical exercise and/or cognitively challenging activities have shown a moderate acute increase in cortisol levels, even though they may not be perceived as stressful by the individuals (Cadore et al., 2008; Kukolja et al., 2008). Thus, both negative and positive situations can trigger acute increases in cortisol.

However, the acute increase in cortisol levels is not the only physiological response to stress. Another important system that is acutely activated in stress, and that shows a faster response than the HPA-axis, is the Sympathetic Nervous System (SNS) (Iversen et al., 2000). As occurs with the HPA-axis, the stress response of the SNS begins with the integration of the information about the potential stressor that converges in the paraventricular nucleus of the hypothalamus and activates both the SNS and the HPA-axis. When the SNS is activated, a large amount of noradrenaline is secreted in sympathetic nerves ending in tissues and glands across the body, and both adrenaline and noradrenaline are secreted in the adrenal medulla (Iversen et al., 2000). Unlike the HPA-axis, noradrenaline and adrenaline levels show a rapid increase in the

response to stress that occurs after a few seconds and returns after minutes to basal levels (Ulrich-Lai and Herman, 2009).

1.4.2. Circadian rhythm of the HPA-axis.

When individuals are not in stressful situations, the activity of the HPA-axis follows a circadian rhythm, showing important differences in the levels of cortisol depending on the moment of the day. The circadian activity of the HPA-axis involves three discrete components: (i) the cortisol awakening response (CAR), (ii) a steep decrease in the secretion of cortisol during the rest of the day, and (iii) an increase in cortisol levels from the second half of the night until waking (see figure 2) (Elder et al., 2014). The CAR is a rapid 50% to 160% increase in cortisol levels that typically peaks between 30 and 45min after morning awakening and that can be observable in approximately 70%-80% of healthy adult people (for reviews about the CAR see: Clow et al., 2010a; 2010b; Fries et al., 2009; Elder et al., 2014). Pruessner et al. (1997) described the CAR for the first time and indicated that it can be considered a good index of adrenocortical activity. Later research suggested that the CAR is independent from the cortisol release the rest of the day, and a growing number of studies have highlighted the importance of investigating the effect of a specific dysregulation of the CAR (Chida and Steptoe, 2009). In fact, evidence suggests that the CAR is regulated differently from the cortisol secretion the rest of the day because the suprachiasmatic nucleus of the hypothalamus seems to play a more important role in its regulation (Clow et al., 2004; Hucklebridge et al., 2005). This nucleus exerts its function through two possible routes: (i) via input to the paraventricular nucleus of the hypothalamus and the HPA-axis cascade (corticotropin-releasing hormone and adrenocorticotropine hormone) and (ii) via its direct sympathetic innervations to the adrenal gland by the splanchnic nerve (Fries et al., 2009).

The main function of the CAR is not well understood. However, given that the hypothalamic suprachiasmatic nucleus is considered to be the light-sensitive endogenous biological clock, several researchers suggest that the CAR may have a close relationship with the process of sleep and waking (Fries et al., 2009; Elder et al., 2014). In this vein, it has been proposed that the CAR may be involved in the transition from sleep to awakening and in the synchronization of the sleep-wake and light-dark cycle

(Elder et al., 2014). In fact, the CAR seems to be affected by the time of awakening, with individuals who wake up later showing lower CAR (Thorn et al., 2006; Stalder et al., 2009). Moreover, the CAR might play a part in preparing the organism to meet the physical and mental demands of the coming day (Fries et al., 2009). Although more research is needed to elucidate the function and control of the CAR, over the past decade associations between the CAR and a wide range of variables in the psychosocial and health domains have been reported (Stetler et al., 2005; Wessa et al., 2006; Chida and Steptoe, 2009; Jabben et al., 2011).

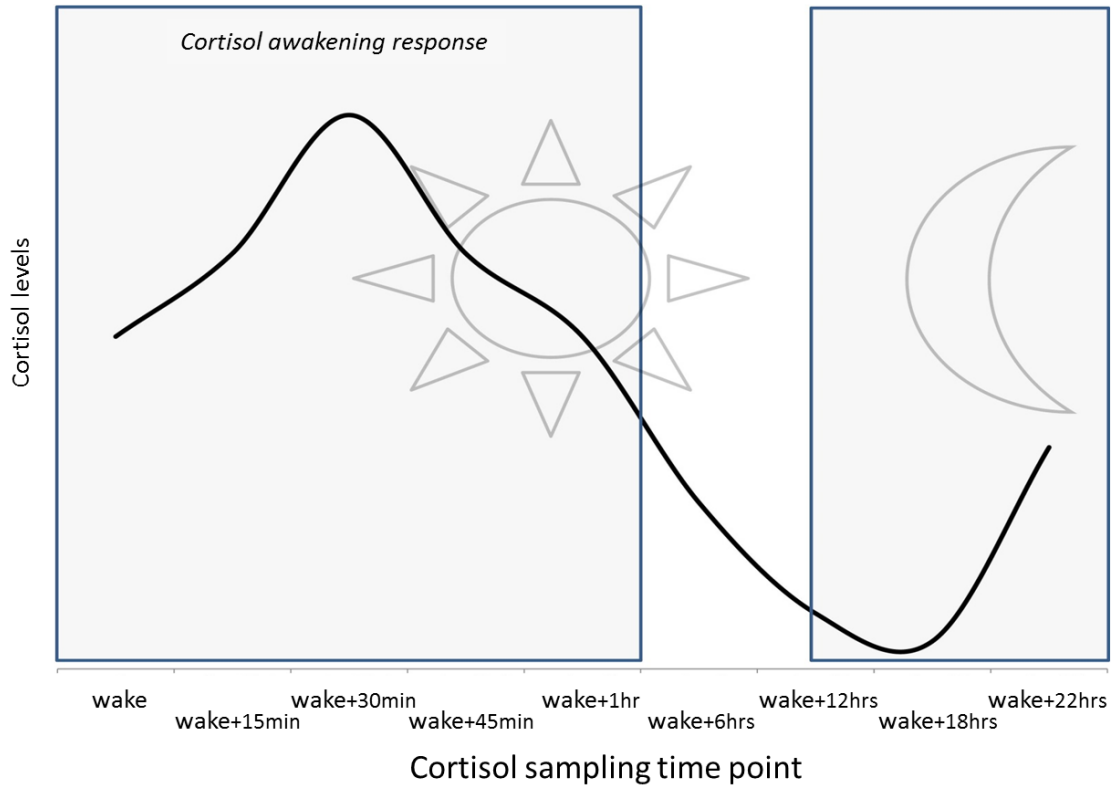

Figure 2. A typical diurnal cortisol profile over a twenty-four hour period in healthy individuals. Adapted and reprinted from Sleep Medicine Reviews, 18, Elder et al., 2014, The cortisol awakening response–Applications and implications for sleep medicine, 215-224, Copyright (2014), with permission from Elsevier.

It has been proposed that cortical structures, especially the frontal cortex and hippocampus, participate in the regulation of the CAR; however, their role is still not well understood. A clear understanding of the direction of causality is not possible yet, but it has been shown that the dynamic of the CAR closely parallels that of the reactivation of the prefrontal cortex, which would be related to the attainment of full alertness (Clow et al., 2010a). In fact, it has been shown that a larger CAR is related to high-

er state arousal and lower fatigue (Thorn et al., 2004, 2009; Adam et al., 2006), and changes in the CAR have been related to prefrontal cortex functioning (Bäumler et al., 2014a). With regard to the hippocampus, it seems that this structure also has a central role in the regulation of CAR. Evidence comes from studies showing a positive association between hippocampal volume and the CAR (Bruehl et al., 2009; Pruessner et al., 2007), and from research showing the existence of anatomical and functional connectivity between the hippocampus and the suprachiasmatic nucleus of the hypothalamus (Krout et al., 2002; Pace-Schott and Hobson, 2002; Stranahan et al., 2008).

After cortisol reaches its peak in the CAR, a steeper decrease is expected in the levels of this hormone, reaching its minimum level during the first half of the sleep period (Kirschbaum and Hellhammer, 1989; Pruessner et al., 1997). Finally, cortisol levels remain low during sleep and then rise again in the middle of the night until morning awakening (Elder et al., 2014). As described above, the hippocampus and frontal cortex are part of the negative feedback in these HPA-axis components.

1.5. Measurements of cortisol.

As the main product of the HPA-axis, cortisol levels are commonly measured as an outcome of HPA-axis activity. Cortisol levels in humans can be measured from different type of biological samples, with blood, urine and saliva samples being commonly used. Among them, saliva samples have been the most widely utilized in human research in the last decade, given that, compared to blood and urine, they are easier to obtain (e.g., using small cotton pieces) and less invasive, and they offer good control of the time of sampling (Golden et al., 2011).

An important difference between blood, urinary and salivary samples is that both salivary and blood samples provide a measure of cortisol levels at a single point in time, while urine samples reflect integrated cortisol secretion over a longer period of time, but not usually exceeding 24hs. Because of this time-related characteristic, urinary samples are usually used to measured cortisol exposure in one day or one night, while salivary and blood samples are used to measure both acute stress-induced and circadian rhythm-related changes in cortisol levels. To do so, several samples of blood

or saliva are obtained to assess cortisol levels at various time points, making it possible to represent the pattern of the cortisol change.

Given that these samples provide a relatively short-term measure of cortisol exposure (especially blood and salivary samples), they have contributed greatly to research focused on investigating changes in the regulation of the HPA-axis rhythmicity (e.g., the diurnal cortisol pattern or specifically the CAR). This is possible due to the fact that blood and salivary samples at several points in the day make it possible to observe whether an individual's cortisol levels follow the expected increase after awakening and the steeper decrease during the day or, in contrast, they show a dysregulated pattern (e.g., lower levels in the morning and higher levels than expected in the evening). In spite of this, these kinds of samples have some limitations when measures of long-term cortisol exposure are needed. Due to time-related restrictions, a large number of samples are required if we need to measure cortisol exposure over an interval of months. Additionally, cortisol levels measured in saliva, blood or urine are highly variable, as they can be affected by several factors that may occur shortly before sampling, such as acute stress, food intake, exercising, among others (Stalder and Kirschbaum, 2012). Therefore, blood, urine and saliva samples are not suitable for this purpose.

Recently, the analysis of cortisol in hair samples has been proposed as a valid and easily obtainable measure of cortisol exposure over periods of up to several months (for reviews on this topic see: Gow et al., 2010; Meyer and Novak, 2012; Russell et al., 2011; Stalder and Kirschbaum, 2012; Staufenbiel et al., 2012; Wosu et al., 2013). Hair grows at an average of 1 centimeter per month on some parts of the head (e.g., posterior vertex) (Wennig et al., 2010), and it has been suggested that the assessment of cortisol in hair can reflect the individual's total cortisol exposure in the previous months (e.g., 3cm of hair would represent approximately 3 months).

The physiological mechanism through which cortisol gets into hair is not well known yet, but the most widely accepted explanation is that cortisol passively diffuses from blood capillaries into the growing hair cells. Importantly, after cortisol is secreted by the adrenal glands into the bloodstream, most of the cortisol is rapidly bound to

some carriers, such as corticosteroid-binding globuling (CGB) or albumin, and only a small part of the cortisol released (approximately 5%) remains unbound (Lewis et al., 2005). Only the unbound cortisol would be responsible for the cortisol effects on the body and the brain because only it is free to diffuse across cell membranes and bind intracellular GR and MR (Henley and Lightman 2011). In this regard, it has been proposed that if cortisol passively diffuses from blood capillaries into the growing hair cells, it implies that only the unbound, free hormone fraction is incorporated into the hair. Thus, assessments of cortisol in hair indicate the levels of the free unbound cortisol fraction, as observed in salivary samples (Stalder and Kirschbaum, 2012). It has been proposed that other mechanisms for cortisol incorporation into the hair might be through sweat, from external sources and/or from a small synthesis of cortisol from an HPA-like system in hair follicles (Stalder and Kirschbaum, 2012; Sauve et al., 2007; Sharpley et al., 2012). However, the current evidence does not support these later explanations, and the bloodstream seems to be the main contributor of cortisol to hair (Stalder and Kirschbaum, 2012; Meyer and Novak, 2012; Grass et al., 2015).

Several studies have shown the general validity of hair cortisol in young and older people (e.g., Davenport et al., 2006; Kirschbaum et al., 2009; Thomson et al.,2010; D'Anna-Hernandez et al., 2011; Manenschijn et al., 2011) and high test-retest reliability (Stalder et al., 2012a). From a methodological point of view, it is particularly noteworthy that hair cortisol measurement is a noninvasive method that is not affected by intra- and inter-day variation (e.g., diurnal rhythmicity) (Meyer and Novak, 2012). Additionally, hair samples are collected retrospectively and do not affect the normal activity of the individuals. Thus, with hair samples we can assess cortisol without restricting the daily activity of the participants, offering an ecological measure.

Furthermore, it should be noted that, unlike saliva or blood samples, hair cortisol cannot be used to investigate changes in the daily regulation of the circadian rhythms of the HPA-axis (e.g., changes in CAR, changes in cortisol levels in the evening) because hair samples only offer an output of total cortisol exposure for a long period of time (e.g., 1 month or more). Therefore, although complementary, hair cortisol offers a different kind of information about HPA-axis activity from what can be obtained from

saliva, blood or urine samples. This means that different results may be observed in studies using salivary (or blood) samples to measure the circadian HPA-axis activity and hair samples to determine long-term cortisol exposure in the same participants (e.g., Steudte et al., 2013; Vanaelst et al., 2012) So far, only a few studies (compared to research using blood or salivary samples) have used hair cortisol, especially in older people. However, the promising results of previous studies encourage the inclusion of hair cortisol in psychoneuroendocrinology research.

Finally, it is pertinent to comment that two different indexes are usually used to specifically assess the CAR. The CAR is usually measured using two or more salivary samples immediately after awakening and at different moments during the first hour after awakening (e.g., immediately after awakening and 30min, 45min and 60min after awakening). Using these samples, researchers can calculate the total cortisol secretion during the first hour after awakening, and/or they can specifically calculate the cortisol increase from awakening until 30min to 60min later. While the former index indicates the overall morning cortisol exposure, the latter index is actually considered to be a measure of the CAR because it reflects the dynamic of the post-awakening cortisol increase (Clow et al., 2010a).

2. Changes in HPA-axis activity in older people.

Evidence indicates that HPA-axis activity changes with age, and it has been proposed that this age-related change is, in fact, due to the cumulative exposure of the brain to cortisol throughout life. Sapolsky (1986) proposed the glucocorticoid cascade hypothesis, based on the idea that excessive exposure to cortisol across the lifespan produces a progressive down-regulation of the glucocorticoid receptors in the hippocampus. A reduction in glucocorticoid receptors would result in a dysregulation of the negative feedback loop of the HPA-axis, leading to higher levels of glucocorticoids and a gradual dysregulation of the HPA-axis. Due to the neurotoxic properties of glucocorticoids, in the long run they would lead to brain atrophy. Similar effects of cortisol would be observed in the prefrontal cortex (Lowy et al., 1995). Later, the glucocorticoid cascade hypothesis was renamed as the Neurotoxicity hypothesis (Gilbertson et al., 2002) because it was proposed that prolonged exposure to stress hormones reduc-

es the ability of neurons to resist insults, increasing the rate at which they are damaged by other toxic challenges. The harm to these structures by other toxic events would also contribute to a reduction in cortical volume, also leading to a decrease in the number of glucocorticoid receptors. However, not everybody will show the same dysregulation of the HPA-axis, and important inter-individual differences can be observed (Lupien et al., 1996; Seeman et al., 1997). This variability has been explained by vulnerability factors, such as genetics, personality or life adversity, which would predispose some individuals to show higher age-related dysregulation of the HPA-axis and higher glucocorticoid-related brain damage (Lupien et al., 2009)

Along these lines, most studies have shown that there is a reduction in the density and sensitivity of the cortisol receptors with age, especially in some areas of the prefrontal cortex and hippocampus (Reul et al., 1991; Newcomer et al., 1995; Bhatnagar et al., 1997; Heuser et al., 2000; Heffelfinger and Newcomer, 2001; Nichols et al., 2001; Gupta and Morley 2014; Giordano et al., 2005; Perlman et al., 2007; Mizoguchi et al., 2009; Wang et al., 2013). These age-related changes may provoke a dysregulation of the HPA-axis activity that can be observed in both (i) the acute response of the HPA-axis to stress and (ii) the circadian rhythm of the cortisol levels.

With regard to the acute HPA-axis activation in stressful conditions, it has been proposed that an age-related decrease in the density and sensitivity of cortisol receptors would contribute to a decrease in the sensitivity of the hippocampus and frontal cortex to detecting cortisol (Newcomer et al., 1995; Heffelfinger and Newcomer, 2001). Due to the critical role of MR and GR on the down-regulation of high cortisol levels after stress, the primary defect of a reduction in cortisol receptors in older people seems to be a prolonged response to stressful conditions. This idea is supported by studies showing that older people have a reduced inhibition of the HPA-axis activity after the infusion of dexamethasone or cortisol (Kudielka et al., 1999; Otte et al., 2005), and by studies showing higher cortisol response to acute stress in older people than in young adults (Seeman et al., 2001; Kudielka et al., 2004; Traustadóttir et al., 2005; Strahler et al., 2010a; Almela et al., 2011b; but see Nicolson et al., 1997 and Rohleder et al., 2002). Along these lines, it has been proposed that aging is character-

ized by a gradual loss of the ability of the body and the brain to maintain homeostasis and adapt to changing conditions, such as stressful situations (Bloss et al., 2010; Pardon et al., 2007).

With regard to the impact of age on the basal HPA-axis activity, studies using blood and saliva samples have indicated that the circadian rhythm is flattened in older people, as they show lower levels in the morning and higher levels in the evening than those observed in young people (Dmitriva et al., 2013; Veldhuis et al., 2013; Van Cauter et al., 1996; Ferrari et al., 2001; Kumari et al., 2010; Milcu et al., 1978). As observed in acute stress, an impaired negative feedback in the HPA-axis seems to underlie this dysregulation of the daily basal HPA-axis (Oxenkrug et al., 1983; O'Brien et al., 1994; Gupta and Morley 2014).

Furthermore, given that blood and salivary samples are measured at several time points during the day, this flattened diurnal cortisol slope (i.e., dysregulated HPA-axis) results in higher cortisol output (e.g., mean cortisol levels on the sampling days) in older people (Weller et al., 2004). In line with this, several studies have observed that higher mean diurnal cortisol levels can be observed in the older population (Deuschle et al., 1997; Touitou and Haus 2000; Larsson et al., 2009). Additionally, recent studies assessing cortisol in hair samples have also observed higher long-term endogenous cortisol exposure in older people (Dettenborn et al., 2012; Feller et al., 2014). However, it is important to note that the magnitude of the increase in diurnal cortisol levels among older people is rather variable because some individuals show a marked increase in mean cortisol levels, whereas others show only moderate cortisol increases (Lupien et al., 1996; Seeman et al., 1997).

Unlike the general effect of age on the overall diurnal HPA-axis activity, studies investigating the specific impact of age on the CAR are not conclusive. While some studies have shown no effect of age on the CAR (Pruessner et al., 1997; Wust et al., 2000), some studies have observed a lower CAR in older people (Kudielka and Kirschbaum, 2003), and also a positive relationship between age and the CAR (Almeida et al., 2009; Kumari et al., 2010). Therefore, it is difficult to make generalizations about the impact of age on the CAR in healthy older people. Remarkably, a growing number of

studies are showing that the magnitude of the CAR may be associated with age-related psychological and physical health problems. For example, a dysregulation of the CAR has been observed in older people with depression (Rhebergen et al., 2015; Fiocco et al., 2006), central obesity (Lasikiewicz et al., 2008) or type II Diabetes (Bruehl et al., 2009).

In this dissertation we will focus on physical performance and systemic hypertension. The time needed to walk a few meters is commonly used as a measure of physical performance (Guralnki et al., 1994). Poor performance by older people on this test has been related to several age-related health problems, such as dementia, cardiovascular disease and subsequent risk of fractures (Cooper et al., 2011), and slower walking speed has been considered a marker of health status in older people (Fritz and Lusardi, 2009; Sustakoski et al., 2015). With regard to the HPA-axis, several studies have shown that individuals with less diurnal cortisol variability (lower levels in the morning and higher levels in the evening) have worse performance on this test (Gardner et al., 2011, 2013; Johar et al., 2011), and it was proposed that a dysregulation of the HPA-axis affects physical performance through a reduction in muscle mass and strength. In contrast to the evidence about the diurnal activity of the HPA-axis, the specific relationship between the CAR and physical performance is less understood, and conflicting results have been observed. A previous study showed that individuals with a higher CAR tended to walk slower (Kumari et al., 2010). By contrast, a weak association between slower walking speed and a lower CAR was observed by Gardner et al. (2011), and an individual participant meta-analysis that included data from six middle-aged and older adult cohorts of population-based studies showed that lower CAR and morning cortisol levels were related to slower walking speed (Gardner et al., 2013). Recently, a lack of relationship between CAR and walking speed has also been reported (Johar et al., 2014). Together, these results suggest that the CAR and walking speed may be related, but more research is needed to better understand this relationship.

Hypertension is the most common cardiovascular risk factor and one of the most common health problems in older people (Virdis et al., 2011). It is well known that ex-

tremely high levels of cortisol can provoke secondary hypertension (al'Abasi and Arnett, 2000), but it has also been proposed that a dysregulation of the circadian rhythm of the HPA-axis might contribute to the development of systemic hypertension (Gold et al., 2005; Rosmond and Björntorp, 2000; Matuszek and Boutcher, 2008). Brain structures involved in the HPA-axis activity, such as the limbic system and the hypothalamus, also play a role in controlling blood pressure (Sapolsky et al., 2000). Additionally, cortisol has a direct effect on blood pressure through MR and GR located in the heart and in the vascular smooth muscle of the resistance vessels (Kenyon and Fraser, 1992), where cortisol acts directly to maintain vascular tone and modify vascular inflammatory responses to injury (Walker et al., 2007). Moreover, chronic high blood pressure damages several cortical areas, especially the frontal cortex, which are involved in the negative feedback of the HPA-axis (Tzourio et al., 2014). Along these lines, previous studies have shown that people with systemic hypertension show a reduced inhibition of the HPA-axis activity compared to older people without a diagnosis of hypertension (Gold et al., 2005; Wirtz et al., 2007).

The specific relationship between the CAR and systemic hypertension is not completely understood, but there is evidence indicating that cortisol secretion immediately post-awakening can be altered in older people with hypertension. A research carried out by Wirtz and colleagues (Wirtz et al., 2007) showed that middle-aged people with systemic hypertension have an attenuated CAR and lower cortisol exposure immediately after awakening. Similar results were observed in Kuehl et al (2015), which studied whether several parameters of the metabolic syndrome were related to hair cortisol and/or the cortisol secretion immediately after awakening. Their results showed that higher hair cortisol was related to higher parameters of the metabolic syndrome and that lower morning cortisol levels were related to higher blood pressure. Moreover, lower morning cortisol levels have been observed in middle-aged people with metabolic syndrome (DeSantis et al., 2011) and cardiovascular risk factor (Rosmond and Björntorp, 2000), including high BP. Importantly, in Kuehl et al (2015), DeSantis et al. (2011) and Rosmond and Björntorp, (2000), the authors did not specifically measure the magnitude of the cortisol increases after awakening (i.e., the CAR). Instead, they used an index that represents the overall morning cortisol secretion (in-

cluding both the CAR and the total cortisol exposure after it) (Kuehl et al., 2015) or a single measure minutes after awakening (DeSantis et al., 2011; Rosmond and Björntorp, 2000). In these latter studies it is not clear whether the effect of high blood pressure is specifically on the CAR or on the total morning cortisol levels, independently from the CAR. Additionally, the results are not unequivocal, since no differences in CAR were also observed (Kejantie et al., 2003; Strahler et al., 2010b). Together, these findings suggest that differences in the cortisol secretion immediately after awakening may be observed in hypertensive and normotensive older people; however, more research is clearly needed to better understand whether the CAR may be affected in older people with systemic hypertension.

3. Effects of cortisol on cognitive performance.

In addition to the key role of the frontal cortex, hippocampus and amygdala in the regulation of the HPA-axis, these brain structures are also involved in several cognitive functions (e.g., executive function, memory, attention). Previous studies have shown that acute and long-term changes in the HPA-axis activity and in cortisol levels may affect cognitive function, an effect exerted through the high number of glucocorticoid receptors located in these structures. Additional effects of cortisol on cognition occur through changes in dopamine and glucose levels and blood flow (de Leon et al., 1997; de Quervain et al., 2003). In the following sections, we will describe some of the effects of both stress-induced cortisol increases and changes in the circadian HPA-axis activity on cognition in young and older people.

3.1. Stress-induced cortisol increases and memory performance.

3.1.1. Research in young adults.

From an evolutionary perspective, the stress-induced physiological and behavioral response has developed to accomplish a key goal: to increase survival possibilities by enhancing our ability to deal with threats and by inducing long-term adaptive responses that increase the possibilities of preventing similar dangerous situations in the future (McEwen, 1998; Farmer et al., 2014). One mechanism through which the cortisol response to stress can increase our chances of survival is by affecting our cognitive abilities before, during and after a threatening or stressful situation occurs. Several

studies have been performed to explore the specific effects of acute stress-induced cortisol increases on cognitive performance, with memory being the most studied cognitive ability. These studies were carried out mainly in young people, and they have focused especially on declarative and episodic short- and long-term memory and working memory. Results have shown that the effects of stress and cortisol on memory depend on several factors, such as the memory phase tested, the type of memory task used, and the sex of the individuals, among others.

In long-term memory, we can distinguish distinct memory phases: encoding/learning, consolidation and retrieval. Initially, the to-be-remembered material is encoded, followed by a consolidation process. If the information is successfully consolidated, a memory trace can be retrieved hours to days to years later. Consolidation and memory retrieval mainly depend on the activity of the hippocampus and prefrontal cortex (Takashima et al., 2006), and several studies performed in young people have consistently shown that stress- or pharmacologically-induced increases in cortisol levels enhance consolidation (e.g., Buchanan and Lovallo, 2001; Cahill et al., 2003; Smeets et al., 2008), but they impair memory retrieval (e.g., de Quervain et al., 2000; Kuhlmann et al., 2005a, 2005b; Buchanan and Tranel, 2008; Smeets et al., 2008, 2011). While sex-related differences in cortisol effects on consolidation have been reported, such differences do not seem to occur in memory retrieval (Wolf, 2009).

Regarding the effect of cortisol on working memory, the pharmacological approach in humans and rodents has mainly shown a detrimental effect of an acute glucocorticoid increase on working memory (e.g. Wolf et al., 2001; Roozendaal et al., 2004; Terfehr et al., 2011). Similarly, most of the previous research has shown that a stress-induced cortisol increase impairs working memory (Roozendaal et al., 2004; Elzinga and Roelofs, 2005; Oei et al., 2006; Schoofs et al., 2008; Duncko et al., 2009; Luethi et al., 2009; Schoofs et al., 2009; Schoofs et al., 2013), but there is also evidence of enhancing effects (Weerda et al., 2010; Duncko et al., 2009; Cornelisse et al., 2011; Stauble et al., 2013;) or no effects (Smeets et al., 2006). The working memory is a PFC-dependent ability that includes both (i) the active maintenance of a limited amount of information (i.e., memory span component of the working memory) and

(ii) the executive function of manipulating this information (i.e., executive component of the working memory) (D'Esposito, 2007). Studies in young people have shown that working memory tasks requiring executive functions (e.g. Digit Span Backward, n-back, O-Span, Letter-Number Sequencing and Sternberg paradigm) are more prone to being affected by cortisol than tasks assessing only the memory span component of working memory (e.g. Digit Span Forward) (Schoofs et al., 2009). Furthermore, impairing effects of cortisol on WM were observed in both men and women (Elzinga and Roelofs, 2005; Oei et al., 2006; Schoofs et al., 2008; Duncko et al., 2009; Luethi et al., 2009; Schoofs et al., 2009; Schoofs et al., 2013), but enhancing effects have only been reported in men (Weerda et al., 2010; Cornelisse et al., 2011; Schoofs et al., 2013).

Moreover, a noradrenergic activation of the basolateral complex of the amygdala (due to a stress-induced SNS activation) has been shown to be necessary in order to observe cortisol effects on both hippocampus and prefrontal cortex-dependent memory performance (for a review see Roozendaal et al., 2009). Thus, the blockade of the noradrenergic activation of the amygdala blocks the effects of cortisol increases on long-term memory and working memory (Roozendaal et al., 2004; Schwabe et al., 2009).

Taken together, these studies in young people suggest that cortisol increases after acute stress impair working memory (although this effect depends on several factors), enhance consolidation, and impair long-term memory retrieval. Roozendaal (2002) proposed that a possible explanation for these conflicting effects of cortisol on memory may be related to the adaptive function of stress and cortisol. Stress would block some memory processes (e.g., long-term memory retrieval) to facilitate others (e.g., learning, consolidation). The author indicates that a temporary disruption of memory retrieval during stressful conditions may diminish retroactive interference (of material previously learned), thereby facilitating the consolidation of the stressful experience and allowing the brain to learn new important information to be used in the future. As an example of this adaptive mechanism, we can imagine a young adult who is walking in the middle of the night through an unfamiliar neighborhood and, at some point in his walk, a group of individuals attack him and steal his wallet. This stressful

situation will trigger the cortisol response, which will reach its peak 15-20min later. Thanks to the effects of cortisol on memory, this young person will have strong memories of this event and the characteristics of the unfamiliar neighborhood where he/she was walking (location, name, etc.). Additionally, thanks to the blockade of memory retrieval, cortisol will diminish a possible effect of interference caused by similar neighborhoods previously known (blockade of the retroactive interference) when consolidating this new information. Together, these effects will allow the individual to recognize this neighborhood and, for example, avoid it in the future. From this perspective, cortisol-induced memory impairments are not always detrimental; they also have an important adaptive value, and this effect can be viewed as logical and salutary (Sapolsky et al., 2000; Roozendaal, 2002; Roozendaal et al., 2009).

3.1.2. Research in older people.

Surprisingly, while a large number of studies have investigated the effects of acute stress on memory in young people, evidence in older people is scarce. As indicated previously, important changes in the activity of the HPA-axis can be observed in older people and a reduction in the density and sensitivity of glucocorticoid receptors occurs with age. Whether the effects of cortisol on memory seen in young people can also be observed in healthy older people unclear because only a few studies have been performed, and the results are not conclusive.

Using pharmacological approaches, previous studies suggest that the effect of cortisol on memory in older people is not as strong as what is observed in young adults. Wolf et al. (2001) studied whether an injection of cortisol may affect the performance on different kinds of memory tasks in healthy young and older men. They showed that, as observed in young men, cortisol impaired the recall of a word-list studied 75min before the cortisol administration in older men. However, due to the short delay between word-list learning and recall in this study, it was difficult for the authors to isolate whether high cortisol levels affected consolidation, retrieval or even both. Additionally, some other declarative memory tasks were used minutes before the authors tested the effects of cortisol on memory retrieval, and so an effect of interference from the material previously learned cannot be ruled out. However, in con-

trast to young men, Wolf et al. (2001) did not observe an effect of cortisol on working memory in older men. This lack of effect on working memory in healthy older men was also observed in two later studies (Porter et al., 2002; Yehuda et al., 2007). These authors indicated that a reduction in the sensitivity of the prefrontal cortex to elevated cortisol levels could explain these results for working memory.

With regard to research using stress-induced cortisol increases, previous studies in older people have only investigated the effects of acute stress prior to learning (without distinguishing between encoding, consolidation and retrieval). Two studies did not observe any effect on learning (Bohnen et al., 1990; Domes et al., 2002). However, a recent study found that acute stress prior-learning enhanced word span only in older women, but it also impaired retroactive interference (Almela et al. 2011a), an effect that was not observed in young people (Hidalgo et al., 2014). The cortisol response to stress was related to retroactive interference, but not to changes in memory span. Similarly, Lupien et al. (1997) observed that acute stress prior-learning impaired declarative memory performance. Given that in this study the memory task was measured shortly after another similar memory task was performed, it is possible that, as observed in Almela et al. (2011a), the effect of stress was due to a cortisol-induced impairment in retroactive interference.

In summary, it has been suggested that stress impairs long-term memory retrieval as a mechanism to block retroactive interference and facilitate consolidation. However, only a few studies have investigated the effects of stress on memory in healthy older people, and so it is not known whether this effect may be observed in this population. Generally, the evidence might suggest that older people are less sensitive to the effects of cortisol on memory retrieval. With this in mind, **the first research objective** of this dissertation was: *to investigate whether increases in acute stress-induced cortisol affect long-term memory retrieval in older people.* Additionally, given that retroactive interference is related to working memory and prefrontal cortex activity, **the second research objective** was: *to investigate whether increases in acute stress-induced cortisol affect working memory in older people*.

3.2. Long-term HPA-axis activity and cognitive performance.

As people age, a decrease in various cognitive functions may be observed (Silver et al., 2012). However, there are large differences among older individuals and the pattern and magnitude of this age-related cognitive change is highly variable. As an explanation for this variability, several authors have proposed that changes in the circadian HPA-axis activity in older people may contribute to the differences in cognitive performance. This idea is based on studies showing that prolonged high glucocorticoid levels suppress neurogenesis in the dente gyrus and provoke structural alterations in the hippocampus and frontal cortex, such as dendritic atrophy and synaptic and spine loss (Magariños et al., 1996; Gould and Tanapat, 1999; Wellman et al., 2001; Cerqueira et al., 2005). Similarly, chronic low cortisol levels produce a reduction in synaptic transmission through long-term potentiation, loss of neuronal integrity, and apoptosis of dentate granule cells (Sloviter et al., 1993; Stienstra et al., 1998; Wossink et al., 2001). Thus, the inter-individual variability in HPA-axis activity in older people might be related to inter-individual variability in cognitive performance.

Several studies have investigated the relationship between basal HPA-axis activity and cognitive performance in healthy older people. Most of these studies have shown that HPA-axis dysregulation (especially, higher cortisol release) is related to worse cognitive performance (e.g. Hodgson et al. 2004; Karlamangla et al., 2005; MacLullich et al., 2005; Li et al. 2006; Kuningas et al. 2007; Lee et al 2007, 2008; Beluche et al., 2010; Comijs et al. 2010; Evans et al., 2011; Franz et al., 2011; Gerritsen et al. 2011; Johansson et al. 2011), although the evidence is not unequivocal (Singh-Mamoux et al., 2014). Given that all these studies were performed using salivary, blood or urine samples to measure HPA-axis activity, they indicate that a dysregulation of the daily HPA-axis activity is related to worse cognitive performance. However, due to the time limitation of these kinds of samples, these studies were not able to explore whether differences in long-term endogenous cortisol exposure may be related to differences in cognitive performance in healthy older people.

Cortisol measurements in hair samples offer a good opportunity to investigate this issue because they make it possible to assess differences in endogenous cortisol

exposure in the previous months in healthy individuals. Few studies have investigated the relationship between hair cortisol and cognitive performance in humans, and none of them have been performed in healthy older people. Recently, patients with coronary artery disease were shown to have high cortisol levels during the three months prior (measured in 3cm hair samples) to the beginning of the coronary disease (Pereg et al., 2011), as well as an increased rate of decline in verbal memory (Vinkers et al., 2005). In addition, those patients with higher cortisol in hair at the beginning of the coronary artery disease showed less recovery of verbal memory performance after one year of cardiac rehabilitation (Saleem et al., 2013). Cognitive impairment has also been observed in patients with both unhealthy high (e.g., Cushing syndrome) and low (e.g., Addisson's disease) long-term endogenous cortisol secretion (Klement et al., 2010; Henry and Thomas et al., 2014; Resmini et al., 2011; Starkman et al., 1992). Based on these results in clinical populations, **the third research objective** of this dissertation was: *to investigate whether differences in endogenous long-term cortisol exposure (measured in hair samples) may be related to differences in cognitive performance in healthy older people.*

A growing number of studies indicate that a dysregulation of the CAR may be related to worse cognitive performance, especially in older people. Given that the frontal cortex and the hippocampus are involved in its regulation, and that the CAR may have characteristics unrelated to the cortisol secretion the rest of the day (Fries et al., 2009), previous research suggested that the CAR may make a specific contribution to cognitive performance in older people. Along these lines, a higher increase in cortisol levels after awakening (i.e., higher CAR) was associated with better executive function performance in healthy older people (Evans et al., 2012). Almela et al (2012) showed that a higher CAR was related to worse performance on declarative memory and, especially in older men, to better performance on working memory. Additionally, studies in healthy young children have shown an association between a higher CAR and better prospective memory functioning (a frontal cortex- and hippocampus-related memory ability) (Bäumer et al., 2014a, 2014b). Not all the studies reported this association (Franz et al., 2011; Singx-Manoux et al., 2014); however, in contrast to previous research in older people, these later studies only included two salivary samples to assess

the CAR (i.e., at awakening and 30min later), which could affect the reliability of the CAR measurement. In summary, evidence suggests that a higher CAR may be related to cognitive performance and, especially, to better frontal cortex-related cognitive functioning.

There is an important gap in the knowledge about whether a dysregulation of the CAR in age-related diseases may be associated with differences in cognitive performance. In this dissertation we will focus on the relationship between the CAR and physical performance and between the CAR and the cognitive performance of hypertensive older people. Worse physical performance, and especially slower walking speed, has been consistently related to worse cognitive performance on frontal cortex-related tasks in older people and to age-related health problems such as cardiovascular risk factors (Herman et al., 2010; Cooper et al., 2011). Therefore, given that both walking speed and the CAR are associated with similar age-related changes, especially in cognitive performance, **the fourth research objective** was: *to investigate whether a dysregulation of the CAR was related to slower walking speed in older people.*

Finally, older people with systemic hypertension have been shown to have worse performance on frontal cortex-functioning (especially executive function and processing speed) (Tzourio et al., 2014), but at the same time, evidence indicates that hypertensive older people may show a dysregulation of the CAR (Wirtz et al., 2007). Based on these findings, **the fifth research objective** was: *to investigate differences in the CAR and morning cortisol secretion between hypertensive and normotensive older people. Additionally, this study investigated whether a dysregulation of the CAR may be related to worse cognitive performance on frontal cortex-related cognitive tasks in both hypertensive and normotensive older people*.

4. Aims and hypotheses.

Overall, the literature described in this chapter shows that acute and diurnal activity of the HPA-axis affects cognitive performance; however, there are still important questions that need to be addressed to better understand the interaction between HPA-axis activity and cognition in older people. The present doctoral dissertation in-

vestigates whether acute and basal activity of the HPA-axis may be related to cognitive performance in older people. Five studies with the following aims and hypothesis were performed:

Study 1: The aim of the first study was to investigate the effects of acute stress on long-term memory retrieval in older people. No studies have investigated the effects of acute stress on long-term memory in older people. Based on previous research performed in young adults, we expected that stress would impair memory retrieval.

Study 2: In the second study, two independent experiments explored whether acute stress affects working memory performance in older people. Two different predictions about the expected results were made in this study. Based on previous studies showing no effect of a pharmacological cortisol increase on a working memory task in older men, the absence of a stress and cortisol effect on working memory was expected, at least in men. However, based on previous results showing psychosocial stress effects on memory span and retroactive interference in older women, enhanced memory span and an impaired executive component of working memory after stress were expected, at least in women.

Study 3: The aim of the third study was to investigate the relationship between cognitive performance and the levels of endogenous cortisol exposure during the previous three months in healthy older people. Additionally, the results from hair samples were compared to the results using salivary diurnal cortisol levels. Based on previous studies with salivary, blood and urine samples, it was expected that higher cortisol levels in hair and diurnal salivary cortisol would be associated with worse cognitive performance.

Study 4: The fourth study investigated the relationship between the CAR and walking speed (a measure of physical performance that has been associated with frontal cortex-related functioning). Based on previous studies, those participants that walked slower were expected to show a lower CAR.

Study 5: The aim of the fifth study was to investigate differences in CAR and overall morning cortisol secretion in hypertensive and normotensive older people. Ad-

ditionally, this study aimed to investigate whether the CAR was associated with cognitive performance, with a special focus on frontal-cortex related cognitive tasks. Based on previous studies, an attenuated CAR, lower overall morning cortisol exposure, and worse frontal cortex-dependent task performance was expected in hypertensive participants. Furthermore, a positive relationship between the CAR and frontal cortex-dependent task performance in normotensive and hypertensive individuals was hypothesized.

Chapter 2

Study 1: Acute stress and long-term memory retrieval in older people

This study has been published as: Pulopulos, M.M., Almela, M., Hidalgo, V., Villada, C., Puig-Perez S., Salvador, A. (2013). Acute stress does not impair long-term memory retrieval in older people. Neurobiology of Learning and Memory, 104, 16–24.

1. Introduction.

Aging involves important changes in cognitive performance, especially memory. Although individual differences exist, elderly people usually perform worse on delayed recall and recognition tasks than younger people (Davis et al., 2003; Huh et al., 2006). These memory deficits due to increasing age have been related to structural and functional changes in the prefrontal cortex, hippocampus and amygdala (Hedden and Gabrieli, 2004). Interestingly, these same brain regions are closely associated with important processes related to stress. In fact, a large number of studies have shown that exposure to stress can modulate memory performance through the activity of the prefrontal cortex, hippocampus and amygdala (for reviews see: Lupien et al., 2007; Wolf, 2009). However, most of these studies have been performed in young people, and more research is needed to find out whether the same effects occur in older people.

Stressful situations provoke the activation of both the Hypothalamus-Pituitary-Adrenal axis (HPA-axis) and the Sympathetic Nervous System (SNS), resulting in the release of glucocorticoids (cortisol in humans) and several SNS biomarkers (e.g. catecholamines, salivary Alpha-Amylase) (Sapolsky et al., 2001). It has been suggested that acute stress would affect memory processes through both the influence of cortisol on the hippocampus, prefrontal cortex and amygdala (Wolf, 2009) and the noradrenergic activation of the amygdala (McGaugh and Roozendaal, 2002). Additionally, studies performed mainly in young people have shown that the impact of stress on memory depends on several factors, such as the phase of the memory tested (i.e. learning, consolidation or retrieval) and the emotional valence of the material to be remembered (i.e. positive, negative or neutral) (Lupien et al., 2005).

Most studies performed in young people have revealed that stress-induced or pharmacologically-induced increases in cortisol levels usually enhance consolidation (Buchanan and Lovallo, 2001; Cahill et al., 2003; Smeets et al., 2008), but they impair memory retrieval (e.g., de Quervain et al., 2000; Kuhlmann et al., 2005a, 2005b ; Buchanan and Tranel, 2008; Smeets et al., 2008, Smeets, 2011). This effect has been explained as a blocking effect of cortisol on retrieval processes, in favor of consolidation processes, in order to allow the brain to consolidate new important information to be

used in the future (Roozendaal, 2002). Furthermore, noradrenergic activation of the amygdala and amygdala-hippocampal interactions have been shown to be necessary in order to observe cortisol effects on hippocampus-dependent memory performance (for a review see: Roozendaal et al., 2009).

However, it is not clear whether these effects of stress on memory processes occur in older populations as well, because only a few studies are available, and most of them investigated the effects of stress without distinguishing between the different memory phases (i.e. learning, consolidation and retrieval). Moreover, their results have not been consistent, as two studies observed that stress impaired learning (Lupien et al., 1997; Almela et al., 2011a), while two studies found no effect (Bohnen et al., 1990; Domes et al., 2002). To the best of our knowledge, only one study has investigated the effects of cortisol on memory retrieval in older people (Wolf et al., 2001). In this study, cortisol (0.5 mg/kg of hydrocortisone sodium succinate) was injected into young (from 19 to 30 years old) and older (from 59 to 76 years old) men 75min after they had learned a list of neutral words. The authors found that hydrocortisone impaired memory retrieval in both age groups. However, there are major neuroendocrine differences between pharmacologically-induced glucocorticoid elevations and stress-induced glucocorticoid elevations (for more details see: Raison and Miller, 2003). Obviously, stress is not equal to glucocorticoid increases; many other psychological and physiological changes occur in stress that are not present with exogenous glucocorticoid administration, including mood changes or SNS activation, which also play a role in memory modulation.

In this context, it is important to study the effects of exposure to an acute psychosocial stressor on long-term memory retrieval in older men and women. Furthermore, despite the lack of studies investigating this matter, several findings suggest that the relationship between stress and memory retrieval could be affected by some age-related changes in the hippocampus and amygdala. Thus, older people may be less sensitive to the effects of cortisol on memory, due to (i) an age-related reduction in cortisol receptor density and sensitivity in the hippocampus (Newcomer et al., 1995; Bhatnagar et al., 1997; Heffelfinger and Newcomer, 2001; Nichols et al., 2001; Mizogu-

chi et al., 2009) and (ii) reduced functional interconnectivity between the amygdala and hippocampus in memory processes (Mather, 2006; St. Jacques et al., 2009; Murty et al., 2010). Nevertheless, it is not currently known whether these age-related changes in the brain can affect the relationship between stress and memory retrieval in older people.

The main goal of the present study was to investigate the effects of stress on hippocampus-dependent memory retrieval in older people. To this end, older men and women learned a series of pictures, words and stories. Then, one day later, they were exposed to an acute psychosocial stressor (or a control task) before recovery of the material learned the previous day. Additionally, to investigate whether stress has different acute effects on memory retrieval for emotional or neutral material, the pictures presented on the learning day were neutral, positive and negative. According to previous studies performed with young people, we expected that stress would impair memory retrieval.

2. Methods.

2.1. Participants.

The sample was composed of 76 participants (38 men and 38 women) ranging in age from 56 to 76 years (Men: *M*=64.63, SD=4.57; Women: *M*=63.74, SD=3.67). Most of them had an educational level beyond high school (84.2%), and their subjective socioeconomic status was medium-high (subjective SES scale: Adler et al., 2000). Participants were randomly assigned to a stress (19 men and 18 women) or control group (19 men and 20 women). There were no significant differences between the stress and control groups in age, Body Mass Index (BMI), SES and educational level (all $p>0.163$). Men and Women had similar ages, SES and educational levels ($p=0.168$), but men had higher BMI (Men, $M=27.83$, $SD=3.34$; Women=25.99, $SD=3.67$; $p=0.026$). All of the female participants were postmenopausal and had had their last menstrual period more than 3 years before the testing time. None of the participants scored less than 28 on the MEC (Spanish version of the Mini-Mental Status Examination; Lobo et al., 1999), indicating the absence of cognitive impairment.

Participants belonged to a study program at the University of Valencia for people over 55 years of age. Exclusion criteria were: smoking more than 10 cigarettes a day, alcohol or other drug abuse, visual or hearing problems, diabetes, presence of an HPA-axis, neurological or psychiatric disease, using any medication directly related to emotional or cognitive functioning or able to influence hormonal levels, such as glucocorticoids, psychotropic substances or sleep medications, having been under general anesthesia once or more than once in the past year, and the presence of a stressful life event during the past year. Because hypertension is a common problem in the older population (Virdis et al., 2011), we decided not to exclude participants who were taking anti-hypertensive medication (men-stress=7; women-stress=5; men-control=4; women-control=8). Nevertheless, the statistical results and conclusions of this study do not change if we exclude those participants taking anti-hypertensive medication.

2.2. Memory assessment.

2.2.1. Picture recall.

Participants were shown 30 color pictures (10 negative, 10 positive and 10 neutral) chosen from the International Affective Picture System (IAPS; Lang et al., 2005). Pictures were presented individually for 5s on a computer screen, and then separated by a black screen that appeared for 15s. Participants were told to look at the stimuli for the entire 5s and, when the black screen was displayed, rate the emotional valence (from 1=*very negative* to 9=*very positive*) and arousal (from 1=*low arousal* to 9=*high arousal*) of the pictures with the Self-Assessment Manikin (SAM; Lang, 1980). Ratings of the pictures showed that negative pictures (M=1.21, SEM=0.08) were rated lower on emotional valence than neutral (M=4.26, SEM=0.22) and positive pictures (M=7.16, SEM=0.12) (for all p<0.001). Neutral pictures were rated lower on valence than positive pictures (for all p<0.001). There were no significant differences between groups or sex (all p>0.434). Positive (M=4.30, SEM=0.18), and negative pictures (M=7.94, SEM=0.13) were rated as more arousing than neutral pictures (M=3.62, SEM=0.13; all p<0.004). Women rated all the pictures as more arousing than men did (Women: M=5.53, SEM=0.20; Men: M=5.03, SEM=0.21; p=0.003), and there were no differences

between the control and stress groups (Control: *M*=5.27, SEM=0.21; Stress: *M*=5.31, SEM=0.21; *p*=0.738).

The following day, participants were instructed to try to recollect as many pictures as possible from the set they had seen the previous day. They had 10min to write a short detailed description of the pictures. Two independent judges, blind to the group to which each participant belonged, determined which picture (if any) was described by each description. Agreement between judges was 93%, and discrepancies were resolved by consensus. One man in the control group was removed from the free picture-recall analysis because his descriptions could not be matched to any pictures, as they were too vague. After that, participants performed a recognition test. The 30 originally-viewed pictures and 30 new pictures (10 negative, 10 positive and 10 neutral) were presented individually on a computer screen. Participants were asked to determine whether the picture was new or had been presented the previous day. D-prime (*d'*) was used for the recognition analysis (MacMillan and Creelman, 1991).

2.2.2. Rey Auditory Verbal Learning Test (RAVLT).

To measure declarative memory, the Spanish version of the RAVLT (Miranda and Valencia, 1997) was used as described previously (Almela et al., 2012). Briefly, participants had to learn a target list of 15 neutral words repeated five times (trials 1–5: Total Learning). Then, participants had to repeat an interference list presented only once, followed by the recovery of the target list. After a delay of 20min, they had to recall the target list again (20-min delayed recall). One day later, participants performed a delayed free recall task.

2.2.3. Rivermead stories subtest.

The Story Recall subtest from the Spanish version of the Rivermead Behavioral Memory Test (Wilson et al., 1985) was used to obtain ecologically valid measures of verbal memory (Lezak et al., 2004). Participants had to repeat two short stories immediately after their oral presentation, after a 20-min delay, and 1 day later. They had to recall as many memory units or ''ideas'' as possible. The sum of the correctly recalled "ideas" from the two stories was calculated for the (i) immediate, (ii) 20-min delayed recall, and (iii) 1-day delayed recall. Participants' responses were recorded and subse-

quently corrected by an experimenter who was blind to the sex and group of the participant. The maximum score possible in each recall trial was 42.

2.3. Procedure.

Participants attended two individual sessions that took place on two consecutive days. Before each session, participants were asked to maintain their general habits, sleep as long as usual, refrain from heavy physical activity the day before the session, and not consume alcohol since the night before the first session. Additionally, they were instructed to drink only water, and not eat, smoke, take any stimulants (such as, coffee, cola, caffeine, tea or chocolate), or brush their teeth at least 1hr. prior to the first session and 2hr. prior to the second session. All participants provided written informed consent to participate in the study, which was conducted in accordance with the Declaration of Helsinki. The protocol was approved by the Research Ethics Committee of the University of Valencia.

The first session (acquisition session) was carried out between 10:00hr. and 12:00hr. in a laboratory at the Faculty of Psychology. In this session, participants performed the MEC, the picture-encoding task, the RAVLT, and the Rivermead Story subtest. They were not told that the next day they would be asked to recall the pictures, the RAVLT words and the Rivermead stories. Additionally, participants in both groups provided two saliva samples (pre and post memory assessment) to measure the cortisol levels during the acquisition session.

The next day, participants returned to the laboratory between 16:00hr. and 18:00hr. to perform the second session (retrieval session) (see Figure 1). Participants in the stress group were exposed to the Trier Social Stress Test (TSST; Kirschbaum et al., 1993; for a detail description of the TSST see: Almela et al. 2011b), and participants in the control group performed a control task that consisted of 5min of talking aloud about a recent non-emotional experience, and 5min counting by 5 aloud. This kind of control task has been used in previous studies (Almela et al., 2011b; Hidalgo et al., 2012), and it was designed to be similar to the stress task in mental workload and global physical activity, but without a stressful component. After the stress/control

task, participants answered four questions (5-point Likert scale; not at all=1, to extremely=5) about their perceptions of both tasks (situational appraisal), based on the following aspects: stress, difficulty, frustration and effort (e.g. How much effort did the task require?). Fifteen min after they finished the stress/control task, they completed the free recall and the recognition test of the pictures they had seen the previous day. After that, they performed the free recall task with the RAVLT words and the Rivermead stories.

During the retrieval session, participants in both groups provided six saliva samples to measure cortisol and sAA levels: 15min before the TSST/Control task (-15min); between the free speech/speaking aloud and arithmetic tasks (+5min); immediately after the TSST/Control task (+10min); before the free recall of pictures (+25min); before the recognition of pictures (+35min); between the RAVLT and the Rivermead recall task (+45min); and, finally, after the Rivermead recall task (+50min)(see Figure 1).

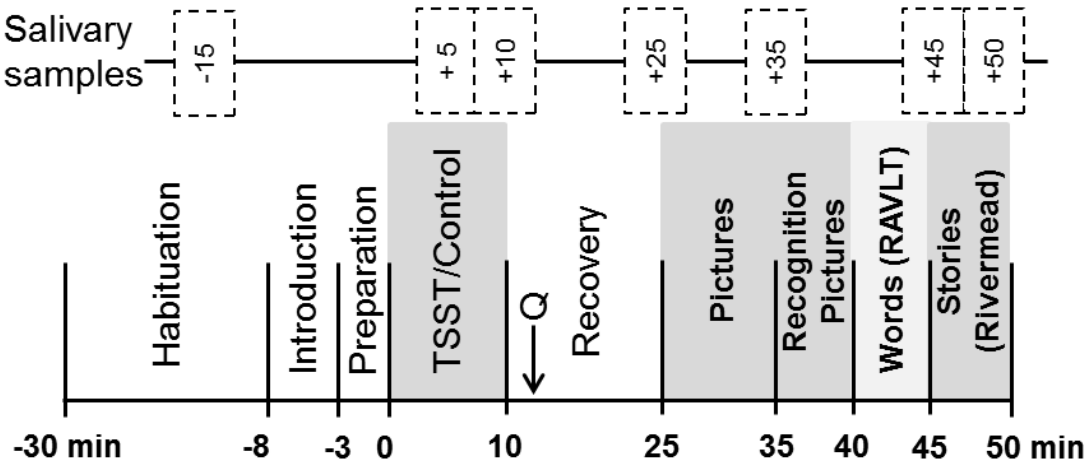

Figure 1. Timeline of the second day for the stress and control group. Square with dotted lines depicts the time of collection of saliva samples.
TSST = Trier Social Stress Test. Q = Situational appraisal.

2.4. Biochemical analyses.

We measured the activity of the HPA-axis and the SNS by analyzing the salivary cortisol and alpha-amylase (sAA) levels, respectively. Participants provided saliva samples by using salivettes (Sarstedt, Nümbrecht, Germany). They were instructed to keep the cotton swab in their mouths for exactly 2min, not chew the cotton, and move the swab around in a circular pattern to collect saliva from all salivary glands. The samples

were centrifuged at 3000 rpm for 5min, resulting in a clear supernatant of low viscosity that was stored at -80ºC until the analyses were performed in the Central Research Unit (Unidad Central de Investigación) of the Faculty of Medicine, University of Valencia (Spain). Both the salivary cortisol and sAA levels were measured in duplicate and each participant's samples were analyzed in the same trial.

For the salivary cortisol levels, the samples were analyzed by a competitive solid phase radioimmunoassay (tube coated), using the commercial kit Spectria Cortisol RIA (cat. Nu 06119) from Orion Diagnostica (Espoo, Finland). Assay sensitivity was 0.8 nmol/L, and the within- and inter-assay variation coefficients were all below 8%.

The sAA concentration was measured by using an enzyme kinetic method with the commercial salivary α-amylase assay kit (cat. nº 1-1902, 1-1902-5) from Salimetrics (USA). Assay sensitivity was 0.4 U/mL. Inter- and intra-assay variation coefficients were all below 10%.

2.5. Statistical analysis and data management.

Data were tested for normal distribution and homogeneity of variance using Shapiro-Wilk and Levene's tests before the statistical procedures were applied. These analyses revealed significant deviations in cortisol and sAA outcome values; therefore, they were square root transformed. We used two-way ANOVAs to investigate sex and group differences on demographic and anthropometric measures, situational appraisal, and valence and arousal of pictures. Cortisol and sAA responses in the retrieval session were assessed using ANOVAs for repeated measures with Group (stress vs. control) and Sex as between-subject factors, and Time (-15, +5, +10, +25, +35, +45 and +50) as a within-subject factor. Two outliers in the cortisol data (one woman and one man in the control group) and one outlier in the sAA data (one man in the stress group) were removed from the cortisol and sAA analyses because their concentrations differed by more than 3 S.D. from the total sample mean.

To investigate whether there were basal differences in learning and memory performance between the control and stress groups, we performed ANOVAs with Sex and Group as between-subject factor. As dependent variables, we used the following out-

comes from the (i) RAVLT: Total Learning, and 20-min Delayed recall; and (ii) Rivermead: Immediate recall and 20-min Delayed recall.

In order to investigate the effects of stress on delayed recall of pictures, data were analyzed using an ANOVA, with Sex, Group (Stress vs. Control) and Valence (Positive, Negative and Neutral pictures) as between-subject factors. Moreover, to study the effect of stress on recognition, the same analysis was performed, but with the recognition test scores (*d'*) for Positive, Negative and Neutral pictures as the dependent variable. Additionally, to study the effects of stress on word and story memory test outcomes, we performed ANOVAs with Sex and Group as between-subject factors, and the percentage of 1-day correct delayed recall (relative to the 20-min delayed recall) of both the RAVLT and Rivermead as dependent variables.

Finally, the area under the total response curve with respect to the ground (AUCg) and with respect to the increase (AUCi) for stress-induced cortisol release was computed using all the salivary samples. The trapezoid formulas specified in Pruessner et al., (2003) were used to calculate these two indexes. AUCi and AUCg give important information about HPA-axis activity and help to simplify the statistical analyses: (i) AUCi was employed as a measure of change in cortisol levels; and (ii) AUCg was employed as a measure of overall cortisol secretion. Correlation analyses were used to investigate the relationship between these two indexes and memory performance in the stress group.

We used Greenhouse–Geisser when the requirement of sphericity in the ANOVA for repeated measures was violated. *Post-hoc* planned comparisons were performed using Bonferroni adjustments for the *p* values. The level of significance was fixed at <0.05. When not otherwise specified, the results shown are means ±SEM. We used SPSS 19.0 to perform the statistical analyses. To facilitate their interpretation, the values in the figures represent raw values, and not square-root-transformed values. Error bars represent standard error of mean (S.E.).

3. Results.

3.1. Situational appraisal.

The stress task was perceived as more stressful ($F(1,73)=48.906$; $p<0.001$), frustrating ($F(1,73)=43.115$; $p<0.001$), difficult ($F(1,73)=64.004$; $p<0.001$), and requiring more effort ($F(1,73)=38.613$; $p<0.001$) than the control task. Women perceived the stress task as requiring more effort than men (Group × Sex: $F(1,73)=4.562$; $p=0.036$; women vs. men: $p=0.025$); however, there were no sex differences in the perception of stressfulness, frustration and difficulty of the stress task ($p>0.108$).

3.2. Salivary cortisol and alpha-amylase response.

3.2.1. Salivary cortisol.

Acquisition session. Figure 2A shows the mean cortisol values for the stress and control groups during the acquisition session. ANOVAs for repeated measures with Time (pre and post acquisition) as a within-subject factor and Group (stress vs. control) and Sex as between-subject factors showed that, following the cortisol circadian rhythm, cortisol levels decreased from the beginning to the end of the acquisition session (Time: Pre vs. Post $F(1,80)=24.794$; $p<0.001$). The factor Group was not significant ($p>0.6$), and the factor Sex was marginally significant ($F(1,80)=3.300$; $p=0.073$), showing that men had slightly higher cortisol levels than women. There were no interactions among the three factors (all $p>0.250$).

Retrieval session. Figure 2B shows the mean cortisol values for the stress and control groups during the retrieval session. The repeated-measures ANOVA showed the main effects of Group ($F(1,71)=26.508$; $p<0.001$), Time ($F(2.06,146.61)=22.33$; $p<0.001$), and Sex ($F(1,71)=10.312$; $p=0.001$), and the interaction among the three factors ($F(2.06,146.61)=4.84$; $p=0.039$).

In the stress and control groups, men and women showed similar baseline cortisol levels (both $p<0.139$). In the stress group, men showed higher cortisol levels than baseline immediately after the speech (-15 vs. +5: $p=0.007$). Then cortisol levels continued to increase until reaching peak levels 25min after the onset of the stress task. Afterwards, cortisol levels decreased, without reaching baseline levels in the last saliva

sample (-15 vs. +45: p<0.001). Cortisol levels of women in the stress group were higher than baseline immediately after the arithmetic task (-15 vs. +10: p=0.018), reached their peak level 25min after the onset of the stress task, and started to decrease afterwards, reaching baseline levels before the story recall (-15 vs. +45: p=0.417). In addition, in the stress group, men showed higher cortisol levels than women immediately after the speech and in the other consecutive samples (for all p<0.021). In the control group, neither men nor women showed a significant increase or decrease in their cortisol levels compared to baseline (all p>0.99), and men and women had similar cortisol concentrations in all samples (all p>0.099). Finally, cortisol levels were higher in the stress group than in the control group, from the +5 sample in men and the +10 sample in women until the end of the study (all p<0.022).

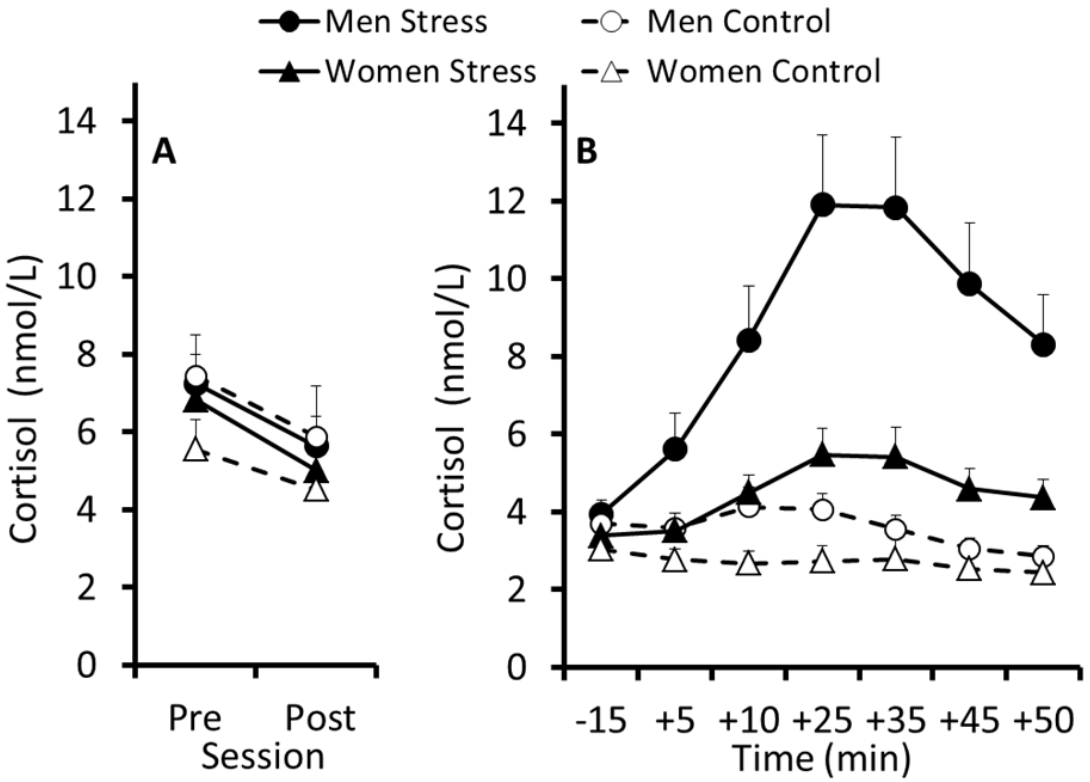

Figure 2. (**A**) Salivary cortisol concentrations in the stress and control groups for the acquisition session (*Pre-Post session*). In both samples, there were no significant differences in the cortisol levels between men in stress and control groups (both p>0.827) and between women in stress and control groups (both p>0.234). (**B**) Salivary cortisol concentrations in the stress and control groups for the retrieval session (*-15, +5 , +10, +25, +35, +45, +50*). Cortisol levels were higher in the stress group than in the control group from the +5 sample until the end of the study (all p<0.027).

3.2.2. Salivary alpha-amylase.

Figure 3 shows the mean sAA levels for the stress and control groups in the retrieval session. The repeated-measures ANOVA with sAA as dependent variable showed that the factors Group and Sex were not significant, nor was the interaction between Sex and the other factors (for all $p>0.579$). However, results showed a main effect of Time ($F(4.82,347.632)=19.576$, $p<0.001$) and a significant interaction between Time and Group ($F(4.82,347.632)=2.281$, $p=0.048$). There were no baseline differences between the stress and control groups ($p=0.430$). In both groups, sAA levels increased above baseline 5min after the onset of the task (-15 vs. +5: control group, $p=0.028$; stress group, $p<0.001$). Only in the control group, participants recovered baseline levels 10min after the onset of the task (-15 vs. +10: control group, $p>0.999$; stress group, $p=0.001$); however, in the stress group, baseline levels were recovered later, 25min after the onset of the stress task (-15 vs. +25: stress and control group, both $p>0.999$). There were no differences between the stress and control groups in sAA concentrations in any sample (all $p>0.245$).

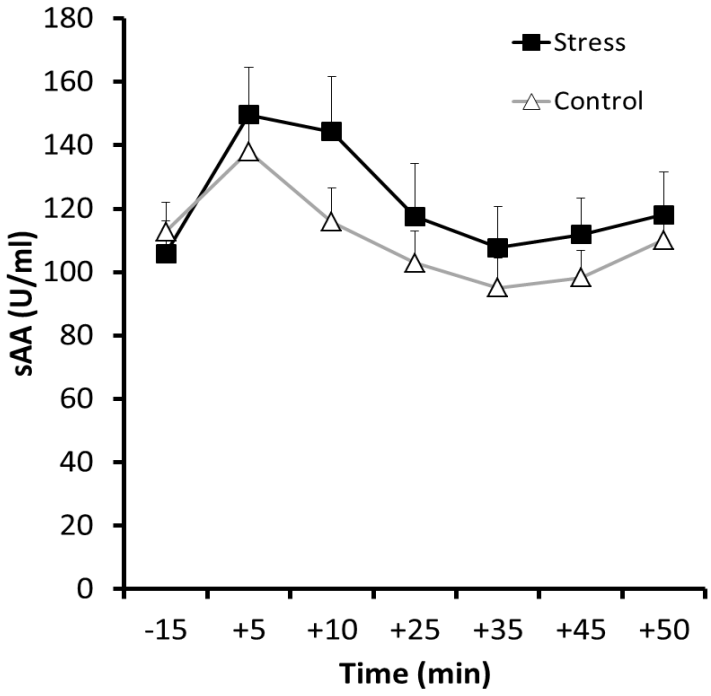

Figure 3. Salivary alpha-amylase concentrations in the stress and control groups for the retrieval session. Control and stress groups show similar sAA levels in all the samples ($p=0.245$), but the control group recovered baseline levels 10min after the onset of the task (-15 vs. +10: control group, $p>0.999$; stress group, $p=0.001$), while the stress group recovered baseline levels 25min after the onset of the stress task (-15 vs. +25: stress and control group, both $p>0.999$).

3.3. Memory performance.

3.3.1. Acquisition session.

RAVLT. The performance of the stress and control groups was similar (all $p>0.630$; See table 1). There were no differences between men and women on Total Learning ($F(1,72)=1.892$, $p=0.173$), but women recalled more words than men in the 20-min delayed recall trial ($F(1,72)=4.372$, $p=0.040$). The interaction between group and sex was not significant (all $p>0.725$).

Rivermead stories subtest. The performance was similar between the stress and control groups (all $p>0.616$; See table 1). The factor Sex was significant in both immediate recall ($F(1,72)=11.867$, $p=0.001$) and 20-min delayed recall ($F(1,72)=7.353$, $p=0.008$), showing that men recalled more "ideas" in these trials than women. The interaction between sex and group was not significant (all $p>0.215$).

Table 1. Memory performance in the acquisition session (Mean scores ± SEM).

		Stress	Control	*F*(1,72)	*p*
RVLT	Total Learning[a]	49.46 (1.26)	50.39 (1.23)	0.005	0.944
	Recall 20-min after learning	10.15 (0.37)	10.42 (0.36)	0.233	0.630
Rivermead	Immediate recall[b]	16.97 (0.72)	16.49 (0.70)	0.253	0.616
	Recall 20-min after learning[b]	16.94 (0.74)	16.56 (0.72)	0.086	0.770

[a]The sum of the words recalled in the first five trials.
[b]The sum of the "ideas" recalled from the two stories.

3.3.2. Stress effects on memory retrieval.

Pictures recall. Figure 4 shows the means of the free recall picture outcomes. There was a main effect of Valence ($F(2,213)=50.447$, $p<0.001$) because negative pictures were recalled more than positive ($p<0.001$) and neutral pictures ($p<0.001$), and positive pictures were recalled more than neutral pictures ($p<0.001$). However, there were no differences between the performances of the stress and control groups ($F(1,213)=0.537$, $p=0.465$) or between men and women ($F(1,213)=0.188$, $p=0.665$). Furthermore, there were no interactions among Group, Sex and Valence (all $p>0.380$).

ANOVAs with the picture recognition outcome (d') as the dependent variable revealed that there were no main effects of Group ($F(1,216)=2.282$, $p=0.132$), Valence ($F(2,216)=0.208$, $p=0.812$) or Sex ($F(1,216)=2.640$, $p=0.106$), nor were the interactions among these factors significant (all $p>0.615$).

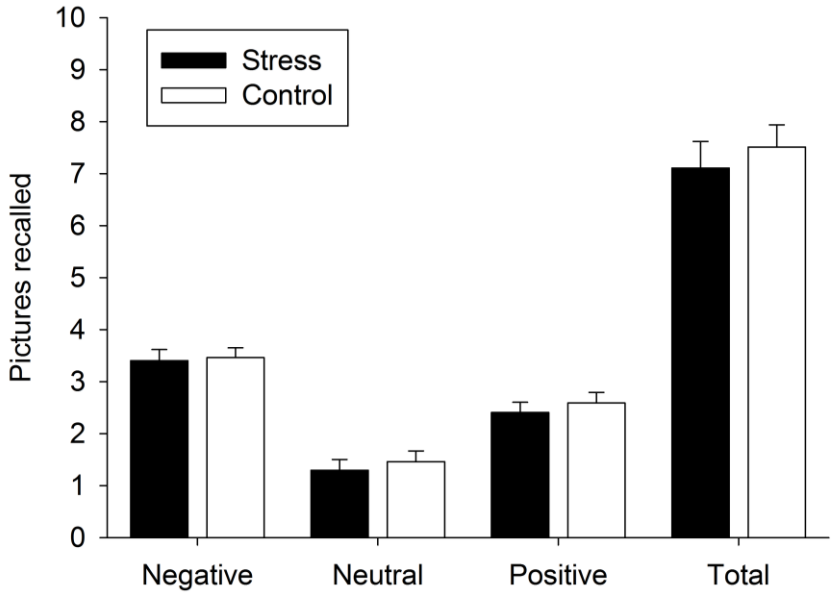

Figure 4. Number of negative, neutral, positive and total pictures recalled for the stress and control groups. Stress had no effect on memory retrieval of negative, positive or neutral pictures.

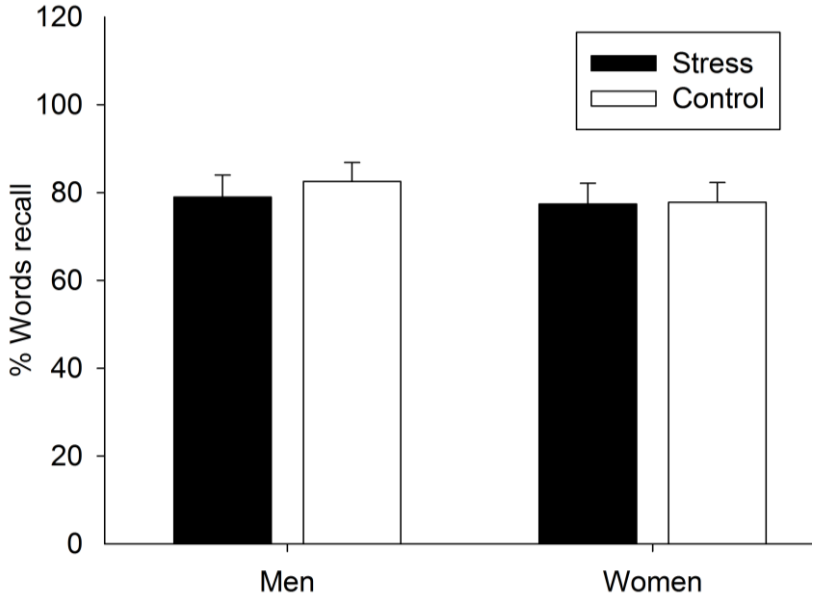

Figure 5. Percentage of words recalled for the stress and control groups during the retrieval session with respect to the recall 20min after learning in the acquisition session. Stress had no effect on memory retrieval of words.

RAVLT. Figure 5 shows the percentage of free recall of words. There were no significant differences between the stress and control groups ($F(1,72)=0.179$, $p=0.673$) or between men and women ($F(1,72)=0.460$, $p=0.500$). The interaction between these two factors was not significant ($F(1,72)=0.116$, $p=0.735$).

Rivermead stories subtest. Figure 6 shows the percentage of free story recall. There were no main effects of Group ($F(1,72)=1.262$, $p=0.265$) or Sex ($F(1,72)=0.002$, $p=0.965$), and the interaction between these two factors was not significant ($F(1,72)=1.065$, $p=0.306$).

3.3.3. Cortisol response and memory performance in the retrieval session.

In the stress group, there were no significant associations between cortisol indexes (AUCi and AUCg) and free recall of positive (AUCi: $r=0.128$, $p=0.450$; AUCg: $r=0.115$, $p=0.447$), negative (AUCi: $r=0.217$, $p=0.196$; AUCg: $r=0.190$, $p=0.260$) or neutral pictures (AUCi: $r=0.168$, $p=0.320$; AUCg: $r=0.151$, $p=0.371$). The associations between cortisol response and free recall performance on the RAVLT (AUCi: $r=0.035$ $p=0.835$; AUCg: $r=0.167$, $p=0.323$) and Rivermead Stories (AUCi: $r=0.056$, $p=0.741$; AUCg: $r=0.026$, $p=0.878$) were not significant either.

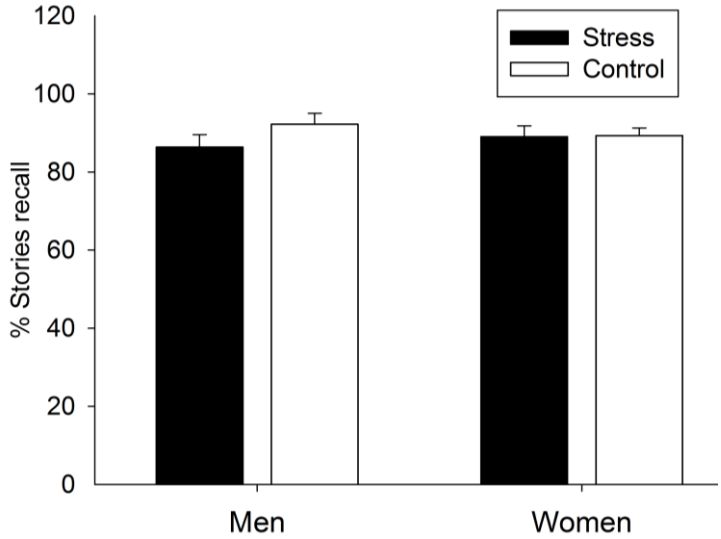

Figure 6. Percentage of "ideas" recalled from the stories for the stress and control groups during the retrieval session with respect to the recall 20min after learning in the acquisition session. Stress had no effect on memory retrieval of stories.

4. Discussion.

This is the first study to investigate the effects of acute stress on long-term memory retrieval in older people. To this end, we tested 1-day delayed memory retrieval for different kinds of material (pictures, words and stories) after stress induction or a control situation. No significant effect of stress on memory retrieval was observed for pictures, words or stories. Additionally, no association was observed between stress-induced cortisol response and memory retrieval.

The stress task was more stressful, frustrating and difficult, and required more effort, than the control task. In addition, the TSST provoked a greater cortisol and sAA release than the control task. However, although the TSST was effective in triggering a stress response, it did not significantly affect recall in the stress group, for pictures (positive, negative or neutral), words or stories. Therefore, our results show that a stress-induced cortisol increase does not produce any effect on memory retrieval in older people. It is important to note that our findings cannot be explained by basal differences in cortisol levels or memory performance between the stress and control groups.

Our findings do not agree with those observed by Wolf et al. (2001), who found impairment in memory retrieval of words in young and older men after an injection of hydrocortisone. However, in the same study, Wolf et al. found that cortisol affected working memory in young men, but not in older men. Wolf et al. (2001) explained this age-related difference as a reduced sensitivity in older people to cortisol effects in the prefrontal cortex, but not in the hippocampus. Based on the results of our study, the lack of stress-induced cortisol effects on recall of pictures, words and stories suggests that older people may also be less sensitive to cortisol effects on hippocampus-dependent memory retrieval. There are at least two possible explanations for the discrepancy with Wolf et al. First, the cortisol increase in Wolf et al. was four times higher than in our study. Therefore, it is possible that memory retrieval in older people is only affected by large cortisol increases. Second, Wolf *et al.* injected hydrocortisone approximately 75min after the participants had learned the word list, and then they measured cortisol effects on memory retrieval 30min later. Therefore, as the authors

discuss, it is likely that they observed an effect of cortisol on memory consolidation and not on memory retrieval (McGaugh, 2000; Wang and Morris, 2010). In contrast, we found no effects of stress on long-term memory retrieval in older people.

The current findings are supported by previous animal studies suggesting that older individuals may be less sensitive than younger individuals to cortisol-induced memory effects (Newcomer et al., 1995; Heffelfinger and Newcomer, 2001; Nichols et al., 2001). Along these lines, the current results show that, contrary to what has been observed in young people, stress does not impair long-term memory retrieval in older men and women (de Quervain et al., 2000; Kuhlmann et al., 2005a, 2005b; Buchanan and Tranel, 2008; Smeets et al., 2008). Basically, this age-difference in stress (or cortisol) effects cannot be explained by differences in cortisol concentrations, as in our study the increase in cortisol in response to the TSST was similar to that observed in studies performed with young participants (e.g. Kuhlmann et al., 2005b; Buchanan et al., 2006; Smeets, 2011). Moreover, this discrepancy cannot be explained by the type of memory tested or by the emotional valence of the material, as no effects were found for the recall of pictures, words and stories, and no effects were found for emotional and neutral material.

At least two different age-related changes in the central nervous system could underlie this decrease in stress-induced cortisol effects on memory retrieval in older people: (i) a reduction in hippocampal glucocorticoid receptors (GR) density and sensitivity (Bhatnagar et al., 1997; Mizoguchi et al., 2009) (ii) and a reduction in hippocampal activity and in the interconnectivity between the amygdala and hippocampus (Mather, 2006; St. Jacques et al., 2009; Murty et al., 2010). With age, (i) there is a reduction in GR density and sensitivity, especially in the hippocampus (Bhatnagar et al., 1997; Mizoguchi et al., 2009). Both kinds of cortisol receptors, the mineralocorticoid receptors and the GR, are located throughout the forebrain, especially in important areas for memory performance, such as the hippocampus, amygdala and prefrontal cortex (de Kloet et al., 1999). In these areas, stress effects on long-term memory performance have been associated with a greater occupation of GR (Oitzl et al., 1997; Cahill and McGaugh, 1998; de Kloet et al., 1999). In fact, Rimmele et al. (2013) have

shown that GR are necessary to observe the detrimental effect of high cortisol levels on memory retrieval, since high cortisol levels do not impair long-term memory retrieval after administration of mifepristone (a blocker of GR). Therefore, an age-related reduction in GR density and sensitivity would reduce the stress-induced cortisol effects on memory performance in older people (Newcomer et al., 1995; Heffelfinger and Newcomer, 2001; Nichols et al., 2001). This explanation is supported by studies performed in patients with major depression disorder, which seems to be characterized by a reduction in GR sensitivity (Holsboer, 2000; Webster et al., 2002). Thus, several studies have observed that an acute cortisol increase does not impair memory performance in patients with major depression, due to the altered GR functioning (Bremmer et al., 2004; Scholesser et al., 2010; Therfehr et al., 2011a, 2011b).

Moreover, (ii) a reduced activity of the hippocampus and in the interconnectivity between the amygdala and the hippocampus could explain the lack of effects of stress on memory retrieval in older people. Previous studies have found that noradrenergic activation of the amygdala and its interactions with the hippocampus is necessary in order to observe cortisol effects on memory retrieval (Roozendaal et al., 2009). Thus, it has been observed that an administration of a β-adrenoceptor blocks the cortisol-induced effect on memory retrieval (de Quervain et al., 2007; Schwabe et al., 2009). Interestingly, fMRI studies have shown that healthy aging is associated with reduced functional interconnectivity between amygdala and hippocampus in memory processes (Mather, 2006; St. Jacques et al., 2009; Murty et al., 2010), which may reduce the stress-induced cortisol effects on memory retrieval in older people. Certainly, further studies are needed to test these possible explanations.

Similar to previous studies in young and older people, in the stress group, women showed a lower cortisol response than men (Kudielka et al., 2004; Almela et al., 2011a; Hidalgo et al., 2012). Thus, it is possible that the lack of stress effect on memory retrieval observed in women may be due to this low cortisol reactivity. Nevertheless, it has been suggested that, contrary to what has been observed in learning or fear conditioning, cortisol affects memory retrieval in young men and women similarly (Wolf, 2008; Smeets et al., 2008). Therefore, in our opinion, it is likely that women in the

stress group would have shown also no effect of stress on memory retrieval even if they had shown a similar cortisol response than men. Future research may help to clarify this matter by showing if in older people there is a lack of effect of stress on memory retrieval when women have a similar cortisol response than men.

It is worth noting that some participants were taking anti-hypertensive medication, which has been observed to affect noradrenaline levels (Wenzel et al., 2000). Thus, this medication might affect the noradrenergic activation of the amygdala and, thus, the relationship between stress and memory (de Quervain et al., 2007; Schwabe et al., 2009). However, no stress effect on memory retrieval was observed, even when we excluded from the analyses those participants taking anti-hypertensive medication (total sample: 52 subjects; men-stress=12; women-stress=13; men-control=15; women-control=12). Therefore, the current absence of memory impairment seems to be due to age-related changes in the central nervous system and not to a possible effect of this medication.

With regard to the design of the current study, memory retrieval was tested in the afternoon, when, following its circadian rhythmicity, baseline cortisol levels are low (Rosmond et al., 1998), which could affect the relationship between cortisol and memory performance, since it has been proposed that this relationship has an inverted-U shaped and depends on the time of day when memory is tested (Lupien and McEwen, 1997; Lupien et al., 2002). Thus, as stress was applied when cortisol levels were low, it could also promote performance. However, although our data were able to show memory enhancement for pictures or words (but not for story recall, given that our participants showed a high performance on this task, and we observed a possible ceiling effect), this positive effect on performance was not observed in the current data. Along these lines, it is possible that we did not observe memory enhancement since, as has been proposed in previous research, cortisol-related retrieval impairment would not be due to absolute cortisol concentrations, but instead to cortisol reactivity (Smeets, 2011). In fact, a meta-analysis performed by Het, Ramlow and Wolf (2005) suggests that, while time of day is an important modulator of acute cortisol effects on learning, the impairing effect of high cortisol levels on retrieval seems to be

independent of time of day. Accordingly, several studies performed in young people have demonstrated that an acute cortisol increase impairs memory retrieval in the afternoon (Kuhlmann et al., 2005b; Buchanan et al., 2006; Buchanan and Tranel, 2008; Smeets et al. 2008, Smeets, 2011), as well as in the morning (Wolf et al., 2001; Kuhlmann et al., 2005a; Smeets, 2011).

Related to this methodological issue, our results might be affected (at least partially) by the time when learning and retrieval took place, as acquisition took place in the morning (when basal cortisol levels were high), whereas retrieval occurred in the afternoon (when basal cortisol levels were low). Thus, differences in cortisol levels at the moment of the acquisition and retrieval might affect our results. However, previous studies in young people have observed detrimental stress/cortisol effects on retrieval, even when learning took place in the morning and retrieval in the afternoon (e.g. Kuhlmann et al., 2005b; Kuhlmann and Wolf, 2005; Smeets, 2011). Taken together, these studies suggest that the lack of a stress effect on retrieval in the current study would also be observed if the retrieval session took place in the morning. However, further studies are needed to investigate whether the time of day might affect the relationship between stress and retrieval, specifically in older people.

A limitation of the present study is that it cannot be concluded that stress-induced cortisol levels did not have any effect on story recall in women, because they had recovered baseline levels before story recall was assessed. However, their cortisol levels were higher than those of the women in the control group, and still no differences were found in their memory performance on this memory test. Furthermore, although cortisol levels in men in the stress group remained high when they performed the story recall, we did not find any effects compared to the control group (men and women), or to women in the stress group. Therefore, we would expect women in the stress group to not show cortisol effects on memory retrieval of stories, as observed for picture and word recall. Another limitation is that we did not counterbalance the order of the three memory tasks. Thus, differences in cortisol levels across the three memory tests might affect the relationship between stress and retrieval. Further stud-

ies should counterbalance the order of the memory tests to control for this possible effect.

In sum, results of our study show for the first time that acute social stress does not affect long-term memory retrieval in older people. Moreover, this lack of stress-induced cortisol effects was observed consistently for pictures, words and stories, and for neutral and emotional material. Therefore, our findings provide empirical evidence showing that, as suggested previously, older people are less sensitive to cortisol effects on memory retrieval (Newcomer et al., 1995; Heffelfinger and Newcomer, 2001; Nichols et al., 2001). An age-related decrease in cortisol receptors and functional changes in the amygdala and hippocampus could underlie the differences observed with studies performed with young people.

Chapter 3

Study 2: Acute stress and working memory in older people

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1. Introduction.

Acute stress can modulate memory performance through the action of cortisol on glucocorticoid and mineralocorticoid receptors (GR and MR, respectively), especially those located in the prefrontal cortex (PFC), hippocampus and amygdala (Roozendaal et al., 2009). Most studies in young adults have shown that acute stress impairs working memory (WM) (e.g., Oei et al., 2006; Schoofs et al., 2008, 2009; Duncko et al., 2009; Luethi et al., 2009), but there is also evidence to the contrary (e.g., Smeets et al. 2006; Cornelisse et al. 2011; Stauble et al., 2013). Memory enhancing effects have only been reported in men (see Schoofs et al., 2013). WM is a PFC-dependent ability that includes both a memory span component (maintenance of a limited amount of information) and an executive component (manipulation of this information) (D'Esposito, 2007). Along these lines, the executive component seems more prone to being affected by acute stress than the memory span component (Schoofs et al., 2009). Additionally, an study with young men have shown that stress may enhance the initial encoding of information in the WM, a cognitive function common to all WM tasks (Stauble et al., 2013).

However, few studies have investigated the effects of cortisol on WM in older[1] people. Using pharmacological approaches, previous findings showed no cortisol effects on WM in older men (Wolf et al., 2001; Porter et al., 2002; Yehuda et al., 2007). These results coincide with the idea that older people may be less sensitive to acute effects of cortisol on memory due to a loss and/or dysfunction of glucocorticoid receptors in the aging brain (Heffelfinger and Newcomer, 2001; Porter et al., 2002; Giordano et al., 2005; Perlman et al., 2007). However, the effects of acute psychosocial stress on WM tasks are still unknown. Using a task designed to assess declarative memory, we

[1] Previous studies that investigated the effect of acute stress and/or cortisol increases on memory in older people have included samples with a mean age of more than 60 years old and an age range from 52 to 83 years old (e.g., an age range from 54 to 72 in Almela et al. (2011a); from 52 to 81 in Yehuda et al. (2007); and from 59 to 76 in Wolf et al. (2001)). To be consistent with the terms used in most of these studies, in this article we refer to the participants as *older* people; however, it should be noted that in the present study and previous studies some participants are in the second half of the age range called *middle-aged* people (from 50 to 60 years old); therefore, the results and conclusions should also be applicable to them.

found that stress improved memory span and impaired retroactive interference (a PFC-dependent executive ability) in women from 54 to 72 years old (Almela et al., 2011a), but not in young adults (Hidalgo et al., 2014), suggesting that stress may have a sex-dependent effect on WM in older people.

We present the results of two experiments designed to investigate whether acute stress affects memory span (Experiment 1) and the executive component of WM (Experiment 1 and Experiment 2) in older people. Based on pharmacological studies, we did not expect stress and cortisol to affect memory span (Experiment 1) or the executive component of WM (Experiment 1 and Experiment 2), at least in men. However, based on our previous results using a psychosocial stressor, we expected that, in women, stress would enhance memory span (Experiment 1) but impair the executive component of WM (Experiment 1 and Experiment 2). Thus, sex differences in acute stress effects on specific components of WM are expected.

2. Material and Methods.

2.1. Participants.

Participants of both experiments belonged to a study program at the University of Valencia for people over 55 years old. There was not overlap between participants in Experiment 1 and those in Experiment 2. Exclusion criteria were: smoking more than 10 cigarettes a day, alcohol or other drug abuse, visual or hearing problems, diabetes, presence of an HPA-axis, neurological or psychiatric disease, and using any medication directly related to emotional or cognitive functioning or able to influence hormonal levels, such as glucocorticoids, psychotropic substances or sleep medications. Use of anti-hypertensive medications was allowed (Experiment 1: men=9, women=5; Experiment 2: men-stress=7, women-stress=5, men-control=4, women-control=8), but including these participants did not change the statistical conclusions of this study. None of the participants had been under general anesthesia in the past year. Only postmenopausal women who had had their last menstrual period more than one year prior to the study were allowed to participate.

Experiment 1. The sample in the first experiment was composed of 63 participants (30 men and 33 women) ranging from 55 to 77 years old (*M*=63.40, *SD*=4.42). Their subjective socioeconomic status (SES scale; Adler et al., 2000) was medium-high, and over half of the participants (60.30%) had an educational level beyond high school. There were no significant differences in age between men and women ($t(61)=-0.561$; $p=0.577$). Men had a higher body mass index (BMI; Men, *M*=27.80, *SD*=3.96; Women: *M*=25.57, *SD*=3.55; $t(61)=2.352$; $p=0.022$), a higher SES ($t(61)=2.013$; $p=0.049$), and a slightly higher educational level than women ($U=363.5$; $p=0.059$).

***Experiment 2*.** The sample in the second experiment was composed of 76 participants (38 men and 38 women) ranging from 56 to 76 years old (*M*=64.26, SD=4.10). Their SES was medium-high, and most of them (84.20%) had an educational level beyond high school. In Experiment 2, participants were randomly assigned to a stress (19 men and 18 women) or control condition (19 men and 20 women). There were no significant differences between men and women in age, SES or educational level ($p>0.168$), but men had higher BMI (Men, *M*=27.83, *SD*=3.34; Women, *M*=25.99, *SD*=3.67; $F(1,72)=3.727$, $p=0.026$). There were no significant differences between the stress and control groups in age, BMI, SES or educational level (all $p>0.163$).

2.2. Procedure.

When they arrived at the laboratory, participants' weight and height were measured, and the experimenter checked to see whether they had followed the instructions given to them previously: the day before the session they had to maintain their general habits, sleep as long as usual, and refrain from heavy physical activity; they could not consume alcohol the night before, and two hours prior to the session they could not drink (except water), eat, smoke or take any stimulants, such as coffee, cola, caffeine, tea or chocolate. The sessions were carried out individually and started between 16:00 and 18:00h in a laboratory at the Faculty of Psychology. The study was conducted in accordance with the Declaration of Helsinki, and the protocol was approved by the Research Ethics Committee of the University of Valencia. All participants provided written informed consent to participate in the study.

Experiment 1. In this experiment we measure WM of the participants before and after they performed a stress task. The procedure began with a habituation phase of 15min to allow participants to adapt to the laboratory setting. After the habituation phase, participants completed the State Anxiety Inventory (STAI-S; Spielberger et al. 1970) to measure baseline anxiety scores (STAI-pre), and they performed the first version of the Digit Span. Following the Digit Span, participants were exposed to the stress task. After this, they completed the STAI-S for the second time (STAI-post), and after a recovery phase of 10min, participants performed the second version of the Digit Span (the timeline for the session is represented in Figure 1).

Experiment 2. The procedure was similar to the one described for the first experiment, but in Experiment 2 we included a control condition, and we measured the performance on the WM task after the stress or control tasks. Upon arrival at the laboratory, participants began a habituation phase of 15min. After the habituation phase, the participants completed the STAI-pre and then, they remained seated until they were introduced to the stress or control task. Immediately after the stress or control task, participants completed the STAI-post, and after a recovery phase of 10min, they performed the Letter-Number Sequencing (LNS) (the timeline for the session is represented in Figure 4). After the LNS, and as part of a larger study performed to investigate the effect of acute stress on different memory processes in older people, participants in Experiment 2 performed three more memory tasks to measure long-term memory retrieval, and they provided three more salivary samples (results not included here, but shown in Pulopulos et al., 2013).

2.3. Stress task.

Experiment 1. The Trier Social Stress Test (TSST; Kirschbaum et al., 1993) was used to provoke acute stress. After an introduction phase (5min) in which participants were informed about the procedure for the stress task in front of a committee, participants had 5min to prepare for the task. After this phase, participants carried out a 5min free speech task and a 5min arithmetic task, standing at a distance of 1.5m from the committee. The participants were informed that the speech and arithmetic tasks would be

filmed with a video camera and a microphone, which were clearly visible. The committee was composed of a man and a woman, and interactions with participants were always performed by the committee member of the opposite sex.

Experiment 2. The stress task was the same as the one described for Experiment 1, but with a preparation phase of 3min instead of 5min (Kudielka et al., 2007). The control task consisted of 5min of talking aloud about a recent non-emotional experience, and 5min counting by 5 aloud. The control task was performed in the same room as the stress task, but none of the stressful elements (video camera, microphone and committee) were present.

2.4. Working memory task.

Experiment 1. Both the Digit Span Forward and Digit Span Backward subtests of the Wechsler Memory Scale III (Wechsler, 1997) were applied before and after the stress task. These tests require participants to listen to a series of numbers of increasing lengths (ranging from 0 to 9). Participants have to repeat the numbers in the same order (Digit Span Forward) or the reverse order (Digit Span Backward) in which they were presented. Each set length was tested twice, and for each correctly repeated digit set, the number of digits was added up. The maximum score possible in each test condition is 16. Two parallel versions of the test were administered. The order of presentation was counterbalanced, and performance on the two versions of each subtest was similar (p>0.112). The Digit Span Forward is a task used to measure the memory span component of WM and attentional processes, and the Digit Span Backward is used to measure the executive component of WM (Conklin et al., 2000).

Experiment 2. The LNS from the Wechsler Memory Scale III (Wechsler, 1997) was used to assess WM performance. This test requires participants to listen to a sequence of alternating digits (ranging from 0 to 9) and letters (from A to Z) of increasing length. Then, they have to repeat the digits and letters from the sequence, beginning with the digits in numerical order, followed by the letters in alphabetical order. The LNS test requires participants to categorize alternating letters and numbers into separate classes and re-order the stimuli within each class. The maximum score possible is 16. Acti-

vation of the orbital frontal lobe, dorsolateral prefrontal cortex, and posterior parietal cortex has been observed during this test (Haut et al., 2000).

2.5. Saliva samples and biochemical analyses.

Experiment 1. Participants provided two saliva samples by depositing 3 ml of saliva in plastic vials in order to measure cortisol levels immediately before the first Digit Span assessment (-15min) and immediately after the second Digit Span assessment (+25min).

Experiment 2. In Experiment 2, we measured both salivary cortisol and sAA levels (using salivettes; Sarstedt, Nümbrecht, Germany). Given that the increase in sAA after the onset of exposure to the stressor is faster than the increase in cortisol, as is recovery to baseline (Nater et al., 2005; Almela et al., 2011b), in Experiment 2 we included one salivary sample immediately after the speech task and another one immediately after the arithmetic task, in order to have a more complete picture of the sAA response. The samples were provided 15min before the TSST (-15min), between the speech and arithmetic tasks of the TSST (+5min), immediately after the TSST (+10min), and immediately after the LNS (+25min).

Salivary samples in both studies were analyzed to measure cortisol levels in duplicate through a competitive solid phase radioimmunoassay (tube coated), using the commercial kit Spectria Cortisol RIA (cat. Nu 06119) from Orion Diagnostica (Espoo, Finland). Assay sensitivity was 0.8 nmol/L, and the intra- and inter-assay variation coefficients were all below 8%. The sAA concentration in Experiment 2 was measured through an enzyme kinetic method using the commercial salivary α-amylase assay kit (cat. no 1-1902, 1-1902-5) from Salimetrics (USA). Assay sensitivity was 0.4 U/mL. Inter- and intra-assay variation coefficients were all below 10%.

2.6. Statistical analysis and data management.

Because cortisol (Experiment 1 and Experiment 2) and sAA (Experiment 2) did not show normal distributions, they were log transformed. Stress response (Experiment 1: Cortisol and STAI-S; Experiment 2: Cortisol, sAA, and STAI-S) and stress effects

on the Digit Span Forward and Digit Span Backward (Experiment 1) were assessed using repeated-measures ANOVAs with Sex as a between-subject factor and Time (Experiment 1: for Cortisol= -15min and +25min; for STAI-S and Digit Span= pre and post; Experiment 2: for Cortisol and sAA= -15min, +5min, +10min, and +25min; for STAI-S= pre and post) as a within-subject factor. We used Greenhouse–Geisser when the requirement of sphericity in the repeated-measures ANOVA was violated. Two-way ANOVAs were performed to test the effect of stress on LNS performance (Experiment 2), with Group (stress vs. control) and Sex as between-subject factors. *Post-hoc* planned comparisons were performed using Bonferroni adjustments for the *p* values. Partial eta squared (partial η^2) is reported as a measure of effect sizes for ANOVAs (Cohen 1973). Partial correlations with SES[2] as covariate were used to investigate the relationship between cortisol (Experiment 1 and Experiment 2), sAA (Experiment 2) and WM (Experiment 1: Digit Span Forward and Digit Span Backward; Experiment 2: LNS) in men and women.

Observed power was calculated using G*Power (Faul et al., 2007). Previous studies in young adults have shown medium to large effect sizes of stress on WM (Oei et al., 2006; Porcelli et al., 2008; Schoofs et al., 2009; Schoofs et al., 2013). Thus, with a medium-large effect size (Cohen's *f*=0.33), the power of our studies were 0.84 and 0.81 for Experiment 1 and Experiment 2, respectively. The power of both studies was sufficient to detect an effect like the one observed in young adults, if present in the data. Two outliers in Experiment 1 (cortisol: one woman and one man) and three outli-

[2] We performed two-ways ANOVAs with Study (Study 1 vs. Study 2) and Sex as between-subject factors for Age, BMI, SES, and educational level to explore differences in the demographic characteristics between participants in the two studies. Participants in study 1 reported higher SES than participants in Study 2 ($F(1,94)=9.098$; $p=0.003$). No significant differences were observed for Age, BMI and educational level ($p>0.189$). Overall, men showed higher SES, BMI and educational level ($p<0.051$) than women. None of the interactions between the factors Study and Sex were significant ($p>0.282$). Additionally, we performed correlation analyses for men and women (participants in both studies together) to explore whether these variables were related to the cortisol response to stress. Only in men, there was a significant negative association between cortisol response and SES ($r=-0.321$; $p=0.026$). None of the other associations for men and women were significant ($p>0.247$). Thus, to compare the relationship between cortisol and WM across the two studies, we included SES as a covariate in the correlation analyses of both studies to control for its effect on the cortisol response to stress.

ers in Experiment 2 (cortisol: one woman and one man in the control group; sAA: one man in the stress group) were removed from the cortisol and sAA analyses because their concentrations differed by more than 3 SD from the total sample mean. When not otherwise specified, the results shown are means ±SEM.

3. Results.

3.1. Results of Experiment 1.

3.1.1. Stress Response.

Anxiety. The repeated-measures ANOVA revealed significant effects of Time ($F(1,61)=18.550$, $p<0.001$, partial $\eta^2=0.233$) and Sex ($F(1,61)=11.753$, $p=0.001$, partial $\eta^2=0.150$) and the interaction between Time and Sex ($F(1,61)=10.768$, $p=0.002$, partial $\eta^2=0.162$). Men and women had similar anxiety scores before the stress task ($p=0.182$, partial $\eta^2=0.029$). Women increased their anxiety scores after the TSST ($p<0.001$, partial $\eta^2=0.331$), but men did not ($p=0.481$, partial $\eta^2=0.008$). Therefore, after the stress task, anxiety was higher in women than in men ($p<0.001$, partial $\eta^2=0.225$).

Cortisol. *T*he repeated-measures ANOVA showed the main effects of Time ($F(1,59)=19.990$, $p<0.001$, partial $\eta^2=0.253$). After the stress task, cortisol levels were higher than baseline levels in both men and women. The factor Sex ($F(1,59)=2.358$, $p=0.130$, partial $\eta^2=0.038$) and the interaction between Time and Sex were not significant ($F(1,59)=0.588$, $p=0.446$, partial $\eta^2=0.010$) (Figure 1).

3.1.2. Stress effects on Digit Span Forward and Backward.

Figure 2 shows the performance on the Digit Span Forward and Digit Span Backward before and after the stress task.

For the Digit Span Forward, there was a significant effect of Time ($F(1,61)=5.929$, $p=0.018$, partial $\eta^2=0.089$). Neither the effect of Sex ($F(1,61)=3.611$, $p=0.062$, partial $\eta^2=0.056$) nor the interaction between Time and Sex ($F(1,62)=3.685$, $p=0.085$, partial $\eta^2=0.048$) reached statistical significance. *Post Hoc* exploration of the interaction between Time and Sex showed that men had a similar performance before and after the stress task ($p=0.639$, partial $\eta^2=0.004$), while women improved their performance after the stress task ($p=0.004$, partial $\eta^2=0.131$).

For the Digit Span Backward, results showed a main effect of Sex (F(1,61)=6.728, p=0.012, partial η^2=0.099). Overall, men performed better on this test than women. Neither the factor Time (F(1,61)=0.137, p=0.713, partial η^2=0.002) nor the interactions between Time and Sex were significant (F(1,61)=0.992, p=0.323, partial η^2=0.016).

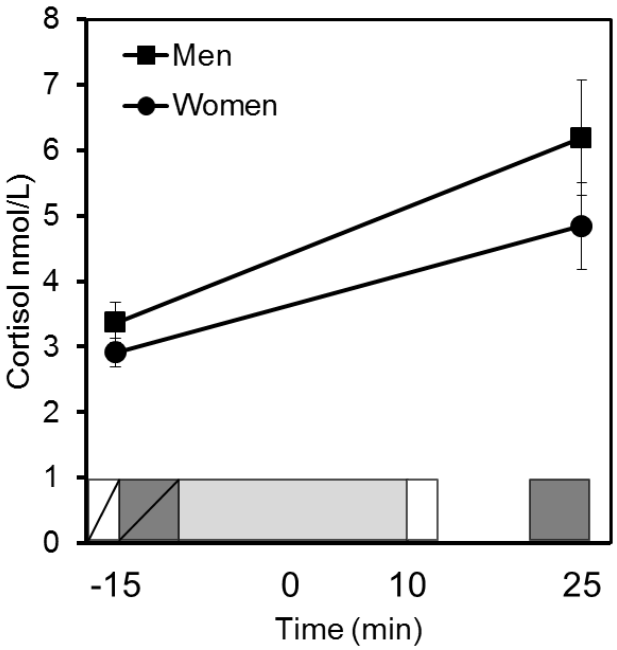

Figure 1. *Experiment 1:* salivary cortisol concentrations for men and women. After a 15min habituation phase (not represented in the figure), participants completed the STAI-pre (white rectangle with diagonal line). Afterwards, they performed the pre-stress Digit Span (dark gray rectangle with diagonal line). Next, they performed the TSST (light gray rectangle): (i) they were introduced to the task, (ii) they prepared the free speech and (iii) they performed the free speech and arithmetic tasks. Immediately after that, they completed the STAI-post (white rectangle), and after 10min recovery, they performed the post-stress Digit Span (dark grey rectangle). The 0min time point was fixed at the beginning of the free speech task.

3.1.3. Relationship between cortisol and Digit Span Forward and Backward.

Using partial correlation analyses, we analyzed: (i) the relationship between pre-stress cortisol and pre-stress Digit Span; (ii) the relationship between post-stress cortisol and post-stress Digit Span; and (iii) the relationship between cortisol response (change in cortisol levels) and the change in Digit Span performance. Cortisol response was calculated by saving the unstandardized residual scores from the regression analyses, using pre-stress cortisol as a predictor and post-stress cortisol as the dependent variable for men and women separately (Mehta et al., 2008; van der Meij et al., 2012). The same method was used to calculate the change in Digit Span Forward and Digit

Span Backward performance. We used residual scores to control for the influence of baseline values on the magnitude of possible change (e.g., smaller increases in cortisol levels and Digit Span in participants with higher baseline scores) (Cohen et al., 2000).

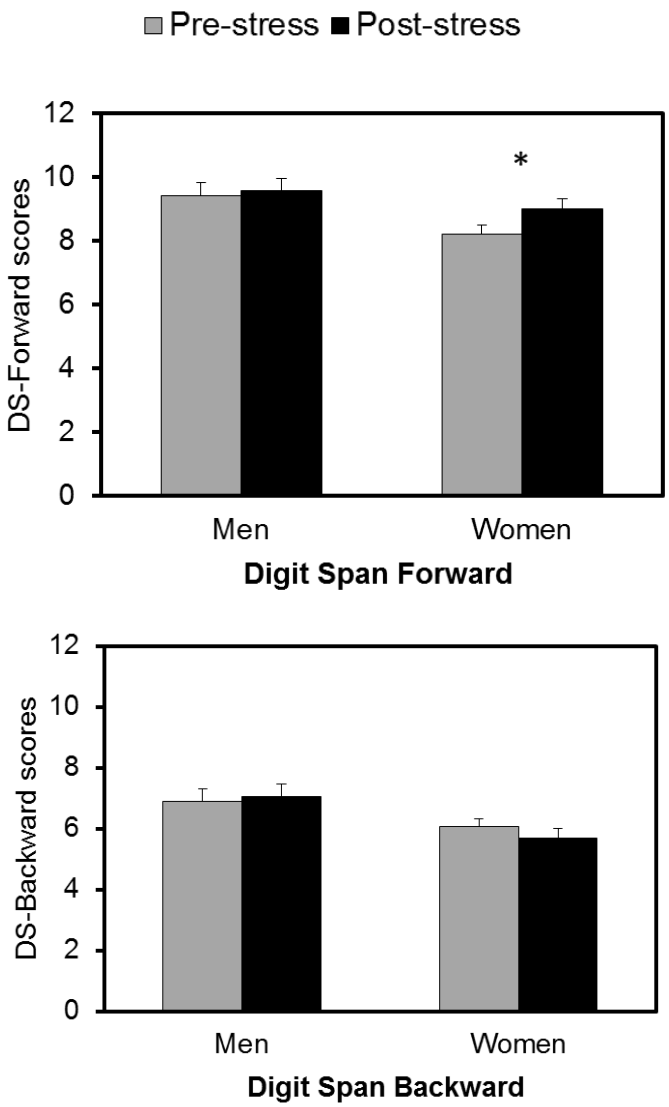

Figure 2. *Experiment 1:* performance on Digit Span Forward (up panel) and Digit Span Backward (down panel) before (gray) and after (black) the stress task. *Women improved their performance on Digit Span Forward after the stress task (p=0.004).

In men, none of the partial correlation analyses showed significant results (all p>0.210). In women, there was a positive relationship (approaching significance) between pre-stress cortisol levels and pre-stress Digit Span Forward (r=0.349, p=0.054; Figure 3A), and a positive significant association between post-stress cortisol levels and post-stress Digit Span Forward (r=0.443, p=0.013; Figure 3B). The relationship between cortisol response and change in Digit Span Forward performance was not significant

(*r*=0.301, *p*=0.099). Results for Digit Span Backward showed a positive significant relationship between pre-stress cortisol levels and pre-stress Digit Span Backward (*r*=0.526, *p*=0.002; Figure 3C), and between post-stress cortisol levels and post-stress Digit Span Backward (*r*=0.369, *p*=0.041; Figure 3D). No significant associations were observed between cortisol response and change in Digit Span Backward performance (*r*=0.289, *p*=0.144).

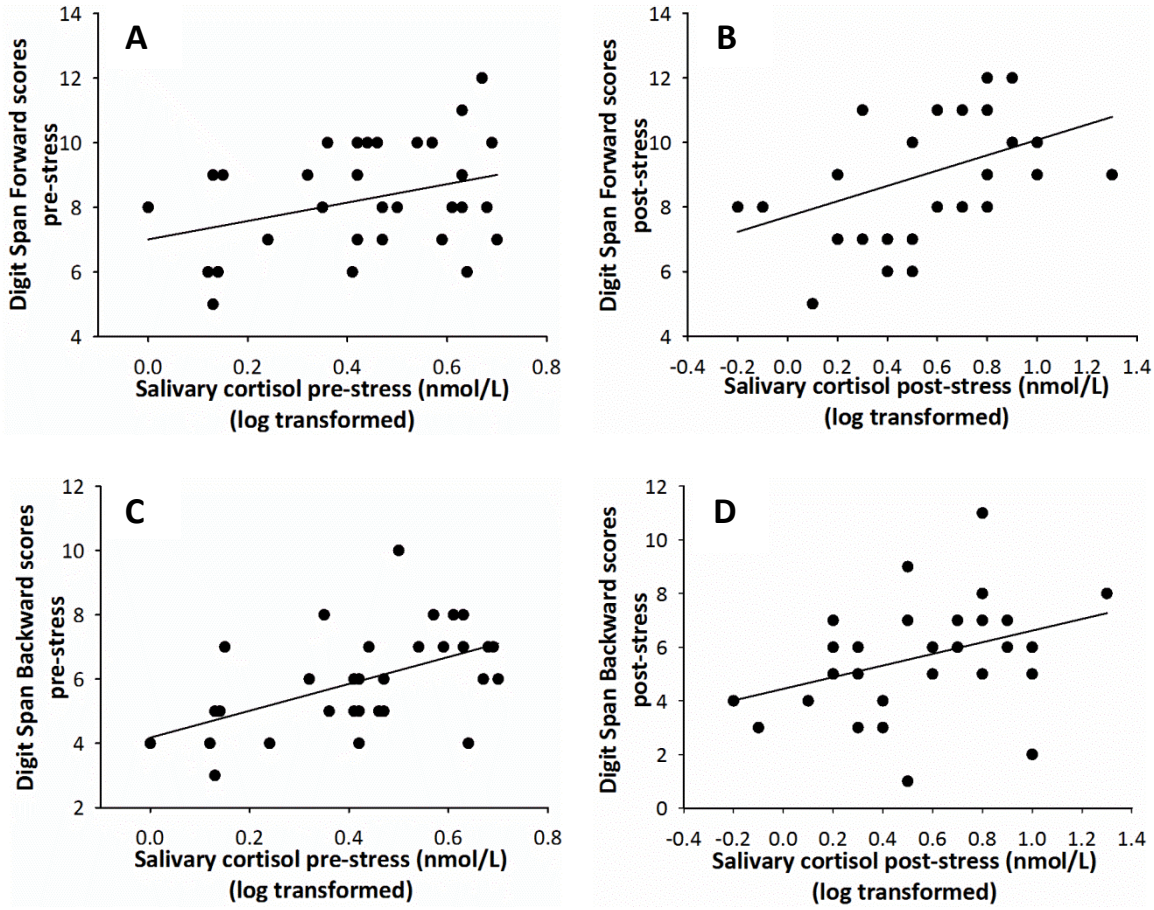

Figure 3. *Experiment 1:* scatter plots for the association between acute cortisol levels and Digit Span performance in women. (A) Relationship between acute cortisol levels and Digit Span Forward pre-stress (*r*=0.349, *p*=0.054). (B) Relationship between acute cortisol levels and Digit Span Forward post-stress (*r*=0.443, *p*=0.013). (C) Relationship between acute cortisol levels and Digit Span Backward pre-stress (*r*=0.526, *p*=0.002); this latter relationship remained significant after controlling for Digit Span Forward (*r*=0.462, *p*=0.010). (D) Relationship between acute cortisol levels and Digit Span Backward post-stress (*r*=0.369, *p*=0.041); this latter relationship did not remain significant after controlling for Digit Span Forward (*r*=0.216, *p*=0.252).

Additionally, given that Digit Span Backward is a task that contains both a memory span component (i.e. participants have to keep the numbers in mind for a short period of time) and an executive component (i.e. participants have to mentally

change the order of the numbers presented), we explored the association between cortisol and Digit Span Backward more thoroughly. To find out whether the relationship between cortisol and Digit Span Backward might be due to the effect of cortisol on memory span (as observed for Digit Span Forward), we included pre-stress Digit Span Forward, post-stress Digit Span Forward and change in Digit Span Forward outcomes as covariates, respectively, in the partial correlations. With these analyses, we controlled for the effect of cortisol on memory span, thus focusing only on the executive function of manipulating numbers in memory. In men, all the associations remained non-significant (p>0.408). In women, the results showed that the association between pre-stress cortisol levels and pre-stress Digit Span Backward remained significant (r=0.462, p=0.010). However, neither the association between post-stress cortisol and post-stress Digit Span Backward (r=0.216, p=0.252) nor the association between cortisol response and change in Digit Span Backward (r=0.073, p=0.700) was significant when controlling for Digit Span Forward outcomes.

3.2. Results of Experiment 2.

Experiment 1 showed that higher levels of cortisol after the stress task improved memory span only in older women; however, they did not affect the executive component of WM in older men or women. This lack of association between post-stress cortisol levels and/or cortisol response and WM executive processes may be due to the low sensitivity of the Digit Span Backward test to these effects in older people. Therefore, in a second experiment we investigated whether stress affects executive processes in older people by using a task that places more demands on the executive component of WM. In this second experiment, we used the LNS, a test that is more cognitively demanding than the Digit Span Backward test, and it is considered a measure of the executive component of both verbal and visual WM (Crowe, 2000; Haut et al., 2000). Furthermore, previous studies in young adults found effects of stress on the executive component of WM when comparing the performance of participants exposed to the TSST to the performance of participants exposed to a control condition (e.g., Oei et al., 2006; Duncko et al., 2009; Schoofs et al., 2009, Schoofs et al., 2013). Therefore, in Experiment 2 the procedure was similar to the one described for the first

experiment, but in this case the participants were exposed to either the TSST or a control task, and we compared the WM performance of the stress and control groups. Based on the results of Experiment 1, we do not expect to find an effect of stress on LNS performance.

Furthermore, in addition to the activation of the HPA-axis, previous studies have shown that stress-induced activation of the sympathetic nervous system (SNS) is necessary in order to observe stress effects on memory performance (Roozendaal et al., 2004; Elzinga and Roelofs, 2005; Schwabe et al., 2009). Thus, to explore whether the stress task provoked an activation of the SNS in our participants, during the session we measured the levels of salivary alpha-amylase (sAA), an oral cavity enzyme that is considered a sensitive biomarker of sympathetic-adrenal-medullary system activity (i.e., higher sAA levels indicate higher SNS activity; for reviews see: Nater and Rohleder, 2009). Higher basal levels of sAA have been observed in older people and sex differences are not expected for the TSST-induced sAA response (Almela et al., 2011b).

3.2.1. Stress Response.

Anxiety. The repeated-measures ANOVA showed the main effects of Time ($F(1,71)=22.689$, $p<0.001$, partial $\eta^2=0.242$), Sex ($F(1,71)=11.315$, $p=0.001$, partial $\eta^2=0.137$) and Group ($F(1,71)=6.852$, $p=0.011$, partial $\eta^2=0.088$), and the interaction between Time and Group ($F(1,71)=14.904$, $p<0.001$, partial $\eta^2=0.174$). Overall, women showed higher anxiety scores than men ($p=0.001$). The stress and control groups had similar anxiety scores before the stress task ($p=0.659$, partial $\eta^2=0.003$). The stress group increased their anxiety after the TSST ($p<0.001$, partial $\eta^2=0.341$), but the control group did not ($p=0.522$, partial $\eta^2=0.006$).

Cortisol. Figure 4 shows the mean cortisol values for men and women in the stress and control groups. The results showed the main effects of Time ($F(1.678,117.433)=17.074$, $p<0.001$, partial $\eta^2=0.196$), Sex ($F(1,70)=11.132$, $p=0.001$, partial $\eta^2=0.137$), Group ($F(1,70)=14.516$, $p<0.001$, partial $\eta^2=0.172$), and the interaction between Time and Group ($F(1.678,117.433)=21.305$, $p<0.001$, partial $\eta^2=0.233$), and between Time and Sex ($F(1.678,117.433)=6.971$, $p=0.003$, partial $\eta^2=0.091$). The interaction between

Time, Group and Sex was not significant (F(1.678,117.433)=0.228, p=0.877, partial η^2=0.003).

The stress and control groups had similar cortisol levels in the first saliva sample (p=0.138). In the stress group, cortisol levels were higher than baseline in the two samples provided after the stress task (all p<0.001). In the control group, cortisol levels did not change in any sample provided (all p>0.999). Cortisol levels were higher in the stress group than in the control group in samples +5min, +10min and +25min (all p<0.011). Finally, considering the stress and control groups together, men had higher cortisol levels than women (all p<0.045).

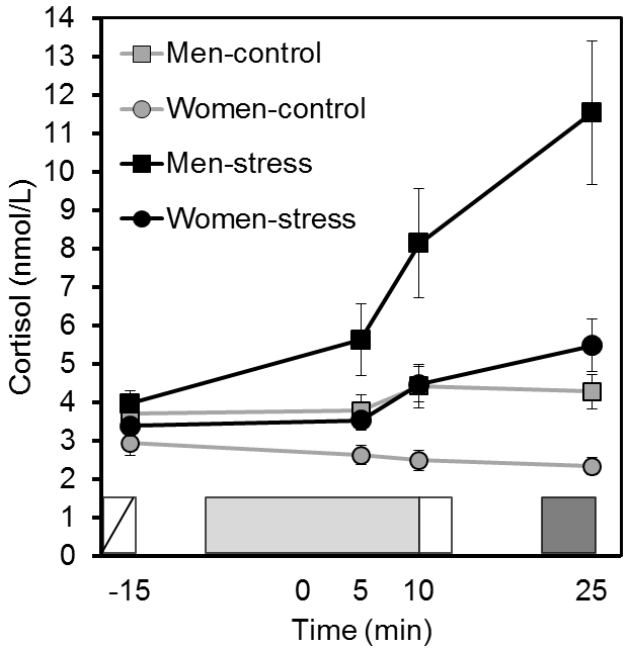

Figure 4. *Experiment 2:* salivary cortisol concentrations for men and women in the stress and control groups. After a habituation of 15min (not represented in the figure), they completed the STAI-pre (white rectangle with diagonal line). Next, they were exposed to the TSST or control task (light grey rectangle): (i) they were introduced to the task, (ii) they prepared the free speech and (iii) they performed the free speech and arithmetic/counting tasks. Immediately after that, they completed the STAI-post (white rectangle) and after a recovery of 10min, they performed the LNS test (dark gray rectangle). The 0-min time point was fixed at the beginning of the free speech. Cortisol levels were higher in the stress group than in the control group in samples +5 min, +10 min and +25 min (all p<0.011).

sAA. Repeated measures ANOVA indicated a main effect of Time (F(2.167,153.860) =16.945, p<0.001, partial η^2=0.193). The factors Sex (F(1,71)=1.524, p=0.221, partial η^2=0.021) and Group (F(1,71)=0.099, p=0.754, partial η^2=0.001) and the interactions

between these factors (all p>0.176, partial η^2<0.024) were not significant. In both groups, sAA levels were above baseline 5min after the onset of the task (-15 vs. +5: p<0.001), and then participants recovered baseline levels 10min after the onset of the task (-15 vs. +10: p=0.904). Although the interaction between group and time was non-significant, a one-way ANOVA with Group (stress vs. control) and Sex as between-subject factors showed that the increase in sAA levels, computed as the total response curve with respect to the increase (AUCi, Pruessner et al., 2003), was higher in the stress group than in the control group (F(1,72)=4.327, p=0.041, partial η^2=0.057). Neither the factor Sex nor the interaction between Group and Sex was significant (F(1,72)>0.027, p>0.716, partial η^2<0.002). These results confirmed that the TSST was able to provoke a stronger sympathetic-adrenal-medullary system response than the control task, and that there were no differences between men and women.

3.2.2. Stress effects on Letter-Number Sequencing.

Figure 5 shows performance on LNS after the stress or control task. There was a significant effect of Sex (F(1,72)=6.323, p=0.014, partial η^2=0.081), as men showed better performance than women. Neither the factor Group (F(1,72)=0.006, p>0.937, partial η^2<0.001) nor the interaction between Group and Sex was significant (F(1,72)=0.0087, p>0.769, partial η^2=0.001).

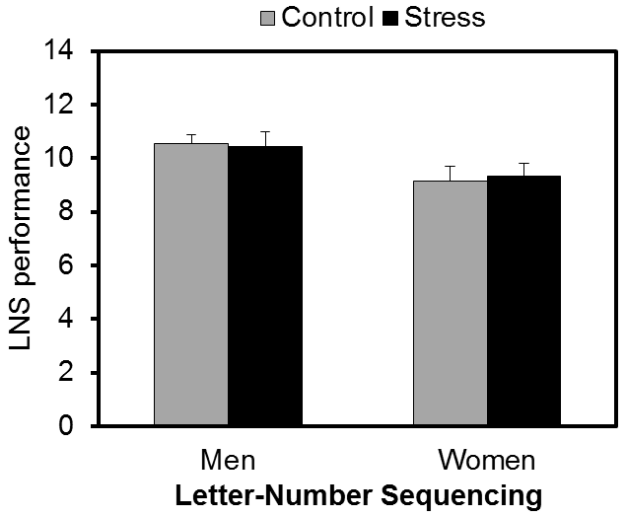

Figure 5. *Experiment 2:* performance on Letter–Number Sequencing for the stress and control groups.

3.2.3. Relationship between cortisol, sAA and Letter-Number Sequencing.

For participants in the stress group, partial correlations were used to explore (i) the relationship between post-stress cortisol (+25min) and post-stress LNS performance and (ii) the relationship between cortisol response (unstandardized residual scores using cortisol levels at -15min as pre-stress outcome and cortisol levels at +25min as post-stress outcome) and post-stress LNS performance. Additionally, to explore whether sAA is related to LNS, the same partial correlation analyses were performed for sAA. Because the peak of the stress-induced sAA increase in our participants occurred immediately after the speech task (+5min), we used the sAA levels at the +5min sampling point for the partial correlation analyses (instead of the +25min sampling point used for the cortisol data)[3]. Thus, the partial correlation analyses for sAA were (i) sAA levels immediately after the free speech task (+5min) and LNS, and (ii) between the sAA response (unstandardized residual scores using sAA levels at -15min as pre-stress outcome and sAA levels at +5min as post-stress outcome) and LNS.

These analyses showed that there were no significant relationships between cortisol levels after the stress task (+25min) and LNS performance in men (r=-0.156, p=0.536) or women (r=0.159, p=0.542). Cortisol response to stress was not related to LNS performance in men (r=-0.153, p=0.543) or women (r=0.074, p=0.778). Partial correlation analyses with sAA showed that LNS was not related to sAA post-stress (men: r=-0.49, p=0.851; women: r=0.249, p=0.336) or the sAA response (men: r=0.019, p=0.943; women: r=0.229, p=0.376).

4. Discussion.

We investigated whether acute stress affects WM performance in men and women between 55 and 77 years of age. To this end, we performed two studies, using two different memory tests to assess WM. In Experiment 1 we observed that older women, but not men, improved their performance on the Digit Span Forward (a measure of the memory span component of WM) after the stress task. The correlation analyses showed a positive association between Digit Span Forward and cortisol levels

[3] The statistical conclusions of the partial correlations for sAA are the same if we perform these analyses using sAA levels at +10min or +25min.

at the moment of testing, but not between cortisol response and change in Digit Span Forward. Thus, although cortisol seems to be a main contributor to this finding, stress itself would not affect memory span and might not be necessary to achieve the effects observed. These results coincide with a previous study by our group that showed an enhanced memory span in older women after a stress task. As in the present study, this effect was not related to the stress-induced cortisol increase (Almela et al., 2011a). Given that cortisol may increase dopamine's actions in the PFC (a catecholamine that influences WM) (Arnsten, 2009), it is possible that, regardless of stress, changes in cortisol levels interact with dopamine levels, affecting WM. However, it is also possible that the memory span enhancement observed is related to other changes that occur with stress (e.g., increase in general arousal), but are not measured in our study. Thus, further research is needed to examine whether changes in memory span would be observed in older women after a pharmacologically-induced cortisol increase, without other effects of stress. Additionally, we performed these studies in the late afternoon, when endogenous cortisol levels are low. Thus, given that the relationship between cortisol and memory may follow an inverted U-shaped pattern (e.g., Lupien et al., 2002; Schilling et al., 2013; de Veld et al., 2014), further studies could also examine whether stress and cortisol can impair memory span in the morning, when endogenous cortisol levels are higher, and a greater GR and MR occupation is observed.

Our results show that cortisol affected memory span in women, but not in men. Sex-differences in cortisol receptor expression might explain these results. Although no differences in MR and GR between young males and females have been reported in the PFC (Peijie et al., 2004; Elaković et al., 2011), estrogen has been shown to down-regulate corticosteroid receptor expression in the brain (Bangasser et al., 2013). Thus, after a drastic reduction in estrogen levels due to menopause, older women might show slightly higher cortisol receptor expression than older men, which might affect the relationship between circulating cortisol levels and WM. Another possible explanation would be the higher number of dopamine receptors in the PFC in women (Shansky and Lipps 2013), which would make them more sensitive to the effects of cortisol levels on WM.

Results of Studies 1 and 2 showed, in men and women, that stress and the cortisol response to stress did not affect the executive component of the WM (Digit Span Backward and LNS), and that sAA was not related to LNS. These results agree with prior research in healthy older men showing that pharmacologically-induced cortisol increases did not affect performance on the Digit Span Backward (Porter et al., 2002; Wolf et al., 2001) or the LNS (Yehuda et al., 2007). Therefore, contrary to what has been observed in most studies with young adults (e.g., Oei et al., 2006; Duncko et al., 2009; Luethi et al., 2009; Schoofs et al., 2009), neither a stress-induced cortisol and SNS response nor high circulating cortisol levels after stress affect the executive component of WM in healthy older people.

Our findings support the idea that older people are less sensitive than young adults to the acute effects of stress and cortisol on memory processes. In contrast to the clear effects observed in young adults (Wolf, 2009), previous studies in older animals and humans did not show any acute effects on spatial memory (Buechel et al., 2014), learning, short-term declarative or non-declarative memory (Porter et al., 2002), or long-term memory retrieval of pictures, words and stories (Pulopulos et al., 2013). However, we have observed a specific effect of stress-induced cortisol response on retroactive interference (i.e., impairment in memory due to the interference of previously-learned material) in middle-aged people (Almela et al., 2011a), but not in young adults (Hidalgo et al., 2014). Similarly, Lupien et al. (1997) and Wolf et al. (2001) found an impairing effect of cortisol increase on short-term word list recall. In these studies, word-list recall was measured after other memory tasks were performed (e.g. a different word-list recall); therefore, the weakening of word-list recall could also be due to the enhancement of retroactive interference. Therefore, most of the memory processes might be unaffected by stress in older people.

One possible explanation for these age-related differences would be a loss and/or dysfunction of MR and GR in the aging brain (Lupien et al., 2002; Giordano et al., 2005; Perlman et al., 2007; Mizoguchi et al., 2009), which could affect HPA axis regulation (Garrido et al., 2012) and the acute effects of cortisol response on memory performance. Along these lines, our results showed that, in older women, the execu-

tive component of the Digit Span Backward was related to acute cortisol levels at baseline, when there is only a moderate MR occupation; however, this association was not observed after the stress task, when cortisol levels are high and a greater occupation of MR and GR is needed to observe stress effects on memory (Oitz et al., 1997). Supporting the idea of low sensitivity, patients with major depression disorder show both a reduction in GR sensitivity and no effects of acute cortisol increases on declarative memory and WM (Terfehr et al., 2011a, 2011b).

Interestingly, these age-related changes may have negative consequences for older individuals' adaptation. Roozendaal (2002) proposed that the effect of stress on memory observed in young individuals is an adaptive mechanism that blocks some memory processes (e.g., long-term memory retrieval) to facilitate others (e.g., consolidation). It has been suggested that this mechanism would diminish retroactive interference, allowing the brain to learn new important information to be used in the future (e.g., dangerous places in animals) (Jöels et al., 2006). Following this line of thinking, if older individuals are less sensitive to the effects of stress on several memory processes, and at the same time stress might increase retroactive interference, this condition would make them more vulnerable to the environment, since they would not benefit from learning necessary information to avoid potential problems.

Stress response in men in Experiment 2, and in women in both studies, is in accordance with previous studies in young and older people (Almela et al., 2011b; Kudielka et al., 2009). By contrast, men in Experiment 1 showed a moderated cortisol response compared to men in Experiment 2. One possible explanation for these difference across-studies would be that men in Experiment 1 reported higher SES than men in Experiment 2. Thus, factors related to low SES (e.g., fewer psychological resources in stressful social interactions) might affect the stress response (Derry et al., 2013). This effect would especially be observed in men because they reported higher SES than women. Most importantly, our results indicate that the lack of stress and cortisol response effects on WM is independent from the magnitude of the cortisol response.

One limitation of Experiment 1 is that we did not include a stress-free control condition. Thus, other factors not controlled for may have affected the increase in Digit Span Forward performance in women. However, the habituation phase was long enough to consider the first cortisol and WM assessments as a baseline measure to be compared to scores after stress, and the results observed coincide with previous studies (Wolf et al., 2001; Porter et al., 2002; Almela et al., 2011a). In addition, the focus of the present studies was to investigate the effects of stress-induced cortisol increases on WM. Therefore WM was measured 10min after the TSST, when cortisol levels were high; however, SNS activity had returned to basal levels at that moment. Previous research in young adults has shown cortisol effects on WM, even when the control and stress groups did not differ on SNS activity (e.g., Cornelisse et al., 2011; Schoofs et al., 2013). However, there is also evidence that the effects of cortisol response on WM require the concurrent activation of the SNS (Elzinga and Roelofs, 2005). Future studies should investigate whether stress can affect WM in older people when high cortisol levels and SNS activity coincide.

In conclusion, our results showed that older women, but not men, increased their memory span after stress. This effect was related to acute circulating cortisol levels, but not to the magnitude of the stress-induced cortisol increase. In addition, in both older men and women, cortisol and stress response did not affect the executive component of the WM. Together, our findings provide empirical support for the idea that healthy older people may be less sensitive than younger people to the acute effects of stress-induced cortisol increases on several types of memory processes.

Chapter 4

Study 3: Relationship between long-term and diurnal cortisol and cognitive performance in older people

This study has been published as: Pulopulos, M.M., Hidalgo, V., Almela, M., Puig-Perez S., Villada, C., Salvador, A. (2014). Hair cortisol and cognitive performance in healthy older people. Psychoneuroendocrinology, 44, 100-111.

1. Introduction.

Aging is associated with a decrease in many cognitive functions (Silver et al., 2012). However, the pattern and magnitude of this decline are highly variable and depend on several factors. Stress and cortisol, the end product of the hypothalamic-pituitary-adrenal axis (HPA-axis), have been proposed as potential mediators of this age-related cognitive change. Cortisol can affect cognitive performance acutely through the activation of receptors located in the prefrontal cortex, hippocampus and amygdala (for review see: Lupien et al., 2007). But more interestingly, HPA-axis activity has been linked to cognitive performance in older people, since a marked increase in basal cortisol levels with age has been associated with cognitive decline and a reduction in hippocampal volume (Lupien et al., 1998; Li et al., 2006). In addition, previous studies have shown that patients with Alzheimer's disease and Mild Cognitive Impairment have heightened basal cortisol levels (Arsenault-Lapierre et al., 2010; Venero et al., 2013).

Similarly, most of the studies that have investigated the cross-sectional relationship between basal HPA-axis activity and cognitive performance in healthy older people have shown that HPA-axis dysregulation (particularly, higher cortisol release) is related to worse cognitive performance (e.g. Hodgson et al., 2004; Karlamangla et al., 2005; MacLullich et al., 2005; Li et al. 2006; Kuningas et al. 2007; Lee et al 2007, 2008; Beluche et al., 2010; Comijs et al. 2010; Evans et al., 2011; Franz et al., 2011; Gerritsen et al., 2011; Johansson, et al. 2011), although other studies have not found any relationship between cortisol and cognition (Peavy et al., 2009; Köhler et al., 2010; Schrijvers et al., 2011). In all of these studies, salivary, blood or urinary samples have been used to measure HPA-axis activity. These biological samples are useful to obtain information about HPA-axis dynamics, as repeated samples allow researchers to measure variations in cortisol levels across a given period by comparing different points, and they can also be used to determine day-to-day variations in cortisol levels.

These biological samples reflect point measures (plasma or saliva) or integral cortisol levels over a few hours (urine). Therefore, a large number of samples would be required to measure cortisol exposure over an interval of months. Additionally, cortisol

levels measured in saliva, blood or urine may be highly variable, as they are likely to be affected by several factors that may occur shortly before sampling (Stalder and Kirschbaum, 2012). Thus, although these measures have contributed greatly to understanding the short-term relationship between HPA-axis activity and cognitive performance, much more research is needed to explore the relationship between long-term endogenous cortisol exposure (months) and cognitive performance.

The measurement of cortisol levels in hair, a recently developed and more stable way to measure basal cortisol exposure over months than salivary, blood or urine samples, appears to be a good candidate for use in this context. Hair cortisol concentrations (HCC) have been considered an integrated measure of cortisol exposure over a period of up to several months (for more details, see: Russell et al., 2011; Stalder and Kirschbaum, 2012). Previous studies have shown that HCC might be unaffected by circadian rhythmicity and situational context and have a high degree of intra-individual stability (Skoluda et al., 2012; Stalder et al., 2012a; but see also Sharpley et al., 2012). However, as it is a relatively new technique, some questions still remain unanswered, such as the physiological mechanisms through which cortisol gets into hair (Meyer and Novak, 2012). At the moment, several studies support the idea that HCC may be a useful technique in investigating long-term exposure to cortisol in young and older people. These studies have shown, for example, higher HCC in individuals with diseases or conditions that typically show higher cortisol levels, such as Cushing's syndrome (Thomson et al., 2010), coronary artery disease (Pereg et al., 2011), chronic pain (Van Uum et al., 2008), diabetes mellitus in older people (Feller et al., 2014) and hydrocortisone replacement therapy in young and older people (Gow et al., 2011). Moreover, higher HCC have been found in endurance athletes (Skoluda et al., 2012) and long-term unemployed individuals (Dettenborn et al., 2010).

The aim of the present study was to investigate the relationship between cognitive performance in healthy older people and cortisol exposure in the previous months determined by measuring cortisol in hair. We carried out a neuropsychological assessment (learning, short- and long-term memory, attention and executive function) of healthy older people, and we took samples of their hair to measure total cortisol expo-

sure in the previous three months. Additionally, diurnal cortisol levels were measured using salivary samples, in order to compare the results with the findings in hair samples. Based on previous studies with salivary, blood and urine cortisol samples, we expected higher HCC to be associated with worse cognitive performance.

2. Method.

2.1. *Participants.*

As a part of a larger study designed to investigate the effects of stress and cortisol on cognitive performance in older people (Mneme Project), we recruited a healthy subgroup of older people to participate in this study. Participants belonged to a study program at the University of Valencia for people older than 55 years of age. We recruited subjects to participate in the present study in the classes of this study program. Two hundred twenty-two individuals volunteered to participate, and these volunteers were interviewed telephonically to determine whether they met the study prerequisites. In order to avoid a large number of potentially confounding factors that could interfere with the study, we selected a homogeneous healthy sample. The exclusion criteria and the number of volunteers excluded for these reasons were the following: smoking more than 10 cigarettes a day (n=2), alcohol abuse (we asked the participants how many glasses and what kind of alcoholic beverages they drank per week; following the UK National Health Service definitions, only lower-risk drinkers were allowed to participate; www.nhs.uk/livewell/alcohol) or other drug abuse (n=1), visual or hearing problems (except wearing glasses) (n=2), presence of an endocrine (n=9), neurological (n=6) or psychiatric (n=6) disease, using any medication directly related to emotional or cognitive function or medication that was able to influence hormonal levels, such as glucocorticoids, anti-diabetic medication, antidepressants, anticoagulants, β-blockers, benzodiazepines or psychotropic substances (n=33) (Vitamins and sporadic use of painkillers were allowed), having been under general anesthesia once or more in the past year (n=9), and the presence of a stressful life event during the last year (volunteers were asked about the occurrence of any important event considered stressful that changed their life in a negative way; e.g. widowhood) (n=8). After this interview, 63 volunteers decided not to enroll in the study due to the demands of the study pro-

tocol (neuropsychological assessment and two days of salivary sampling at home). In addition, 26 volunteers had to be excluded because they did not have enough hair for biochemical cortisol analyses (3cm).

Finally, 57 participants (14 men and 43 women), from 56 to 77 years old, met the requisites to participate in this study. All the participants scored more than twenty-eight on the MEC (Spanish version of the Mini-Mental Status Examination; Lobo et al., 1999), indicating the absence of cognitive impairment, and none of the participants met the criteria for dementia, as defined by the NINCDS-ADRDA criteria for Alzheimer's disease, or the criteria for Mild Cognitive Impairment, as defined by the European Consortium on Alzheimer's Disease (Portet et al., 2006). All female participants were postmenopausal; they had had their last menstrual period more than 2 years before the testing time, and none of them were taking estrogen replacement therapy. Thirteen participants (4 men and 9 women) were taking anti-hypertensive medication (none of them were taking β-blockers). Nevertheless, the inclusion of these participants did not change the statistical results and conclusions of this study. None of the participants had any other kind of cardiovascular disease.

2.2. Procedure and neuropsychological assessment.

Participants were asked to attend a neuropsychological assessment between 1000hr. and 1200hr. in a laboratory at the Faculty of Psychology. Previously, they were asked to maintain their general habits, sleep as long as usual, refrain from heavy physical activity the day before the session, and not consume alcohol since the night before the session. Additionally, they were instructed to drink only water, and not eat, smoke, take any stimulants (such as coffee, cola, caffeine, tea or chocolate), or brush their teeth at least 1hr. prior to the session. All participants provided written informed consent for their participation in the study, which was conducted in accordance with the Declaration of Helsinki, and the protocol was approved by the Research Ethics Committee of the University of Valencia.

Upon arrival, the height and weight of the participants were measured to calculate the Body Mass Index (BMI). In order to avoid the possible effects of fatigue and/or

a testing-related stress effect (Goldstein and McNeil, 2004), the neuropsychological assessment was designed to last no longer than 1.5hr. Moreover, the selection of the cognitive tasks was based on previous studies by our group and others that have investigated the relationship between cortisol and cognitive performance (MacLullich et al., 2005; Lee et al., 2007; Evans et al., 2011; Almela et al., 2012). The Spanish version of the Rey Auditory Verbal Learning Test (RAVLT; Miranda and Valencia, 1997) and the Story Recall subtest of the Spanish version of the Rivermead Behavioral Memory Test (Wilson et al., 1999) were used to measure learning and verbal memory. On the RAVLT, the experimenter reads out a list of 15 words (list A) that the participant is asked to reproduce, and this procedure is repeated five times. In trial six, participants were asked to reproduce a new 15-word list (interference list); after that, participants had to recall list A without hearing it again. Three outcomes were used in subsequent analyses: (i) first trial: total number of words recalled on the first trial; (ii) total learning: total number of words recalled on the first five trials; (iii) immediate recall: total number of words recalled after the interference trial. On the Story recall subtest of the Rivermead, participants had to recall as many ''ideas'' as possible from two brief stories read aloud by the experimenter. Recall was scored according to the manual. From this test, two outcomes were used in the subsequent analyses: (i) immediate story recall: total number of ''ideas'' recalled from the two narratives immediately after having heard them; (ii) delayed story recall: percentage of the total number of ''ideas'' recalled from the two narratives after 20min, compared to the number of ideas recalled on the immediate recall trial. The Trail Making Test form A (TMT-A) was used to assess general psychomotor speed and attention, and the Trail Making Test form B (TMT-B) was used to assess executive function (Reitan, 1992); the outcome of each part was the time (seconds) needed to perform the test. Finally, the Digit Span Forward Subtest of the Wechsler Memory Scale III (Wechsler, 1997) was used as a task related to attentional processes, and the Digit Span Backward subtest was used to assess working memory (Conklin et al., 2000).

2.3. Cortisol measurements.

2.3.1. Hair cortisol.

At the end of the neuropsychological session, 3-cm hair samples (~3 mm diameter) were carefully cut with fine scissors as close as possible to the scalp from a posterior vertex position. Based on a hair growth rate of 1 cm/month (Wennig, 2000), these segments are assumed to reflect hair grown over the three-month period prior to the respective sampling points. Hair samples were prepared and analyzed in the laboratory of Prof. Kirschbaum (Department of Psychology, Technische Universität Dresden, Germany), following the laboratory protocol described in detail in Kirschbaum et al. (2009). Hair samples were incubated in 1800 µl methanol for 18hr. at 45ºC (see Stalder et al., 2012a, for a more detailed description), and then analyzed by liquid chromatography mass spectrometry/MS.

2.3.2. Salivary cortisol.

Participants provided one salivary sample at the beginning (pre) and one at the end (post) of the neuropsychological session, by using salivettes (Sarstedt, Nümbrecht, Germany). Two indices were calculated from the salivary cortisol samples during the session: (i) $Mean_{CortNeuro}$ (mean cortisol levels during the neuropsychological assessment) and (ii) cortisol change ($Change_{CortNeuro}$: cortisol levels pre-session minus cortisol levels post-session).

In order to measure diurnal cortisol levels, at home participants collected 3 saliva samples per day for 2 consecutive days using salivettes. There was a mean of 9 days (±1.5) between the neuropsychological assessment and the measurement of the diurnal cortisol. No samples were provided over the weekend, and participants were instructed to drink only water, and not eat, smoke or brush their teeth, at least 1h prior to each saliva sample. The samples were provided immediately after waking, 30min post-awakening and at 2300hr. In order to objectively verify participant adherence, salivettes were stored in MEMS T TrackCap containers (MEMS 6 TrackCap Monitor, Aardex Ltd., Switzerland), and participants wrote down the exact sampling times in a diary. There were no differences between the salivary cortisol levels at home across days (p=0.297) (Figure 1). Two indexes were calculated from these salivary cortisol samples: (i) $Mean_{CortDay}$: Mean of cortisol levels in the three samples and (ii) $AUC_{CortDay}$: Averaged area-under-the-curve with respect to the ground (Pruessner et al., 2003).

Salivary samples provided during the session and at home were stored and analyzed as described in detail in Almela et al. (2012).

2.4. Statistical analysis and data management.

Because hair and salivary cortisol values did not show normal distributions, they were square root transformed (Sqrt). We performed regression analyses to investigate the relationship between cortisol outcomes (HCC, $Mean_{CortDay}$ and $AUC_{CortDay}$) and cognitive performance, and several covariates were included to control for possible confounder effects. Age and body mass index (BMI) were included because of their effects on both cognitive performance and HPA-axis activity (Cournot et al., 2006; Dettenborn et al., 2012; Silver et al., 2012; Stalder and Kirschbaum, 2012). Subjective Socio-Economic Status (SES, measured using the MacArthur Scale of Subjective Social Status; see Adler et al., 2000) was also included as covariate because it is related to HPA-axis activity (Wright and Steptoe, 2005; Cohen et al., 2006) and health (Adler et al., 2000; Singh-Manoux et al., 2005; Demakakos et al., 2008). We included SES but not educational level as covariate, given that SES is related to educational level (r=0.320, p=0.015) and has a stronger relationship with HPA-axis activity than educational level (Wright and Steptoe, 2005; Cohen et al., 2006)[1]. $Mean_{CortNeuro}$ and $Change_{CortNeuro}$ were also included as covariates to control for the possible acute effect of cortisol on cognition (Almela et al., 2011; Hidalgo et al., 2012, 2014) and the stressfulness of the testing situation (Sindi et al., 2013).

In the regression analyses testing the relationship between $AUC_{CortDay}$ and cognitive performance, we also included as covariate the mean of the cortisol levels in the first saliva sample (immediately after awakening) to control for differences in the awakening time that could produce differences in cortisol concentrations (Clow et al., 2010a).

Moreover, for the Digit Span Backward and TMT-B, we included the Digit Span Forward and TMT-A, respectively, as covariates, in order to specifically pinpoint the

[1] The inclusion of educational level instead of SES as a covariate in the regression analyses does not change the statistical conclusion of the analyses performed with HCC and $MEAN_{CortDay}$, but it reduces the significance of the relationship between $AUC_{CortDay}$ and immediate story recall, approaching a non-significant association (β=-0.267, *p*=0.124).

executive function measure of these two tasks (executive component of the working memory for Digit Span Backward and set-shifting ability for TMT-B).

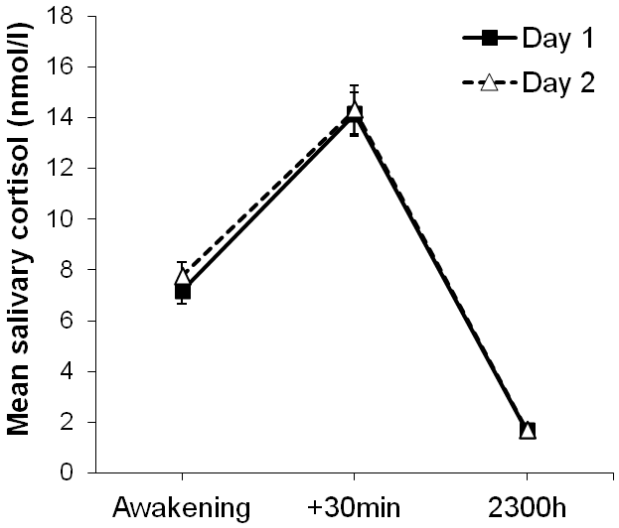

Figure 1. Means of diurnal salivary cortisol levels during both days of sampling.

The design of the regression analyses was as follows: in step 1, we included the covariates and Sex (0=Women; 1=Men). In step 2, we added HCC or $MEAN_{CortDay}$ or $AUC_{CortDay}$. Based on Aiken and West (1991), we performed a moderator regression analysis to investigate whether sex was a moderator. Therefore, in step 3, we included the interaction between HCC or $Mean_{CortDay}$ or AUC_{CorDay} and Sex. Correlation analyses revealed that there were no significant associations among the covariates included (all *p>0.*118). Effect sizes (f^2) were reported for the regression analyses (Cohen 1988). When not otherwise specified, values are mean ± standard error of mean (SEM).

3. Results.

3.1. Description of the sample.

Fifty-seven participants were assessed in this study; however, the number of participants used for each analysis varies, given that some of the participants were not included in some of the analyses for the following reasons: (i) 1 woman did not provide a large enough salivary sample in the neuropsychological session; (ii) 2 women and 1 man did not provide a large enough salivary sample at home; (iii) 2 women and 1 man were outliers for salivary cortisol data at home (+3SD); (iv) 2 women were outliers for HCC (+3DS). Thus, the sample available for the analyses was composed of: HCC = 54

participants (14 men and 40 women; reasons for participants' exclusion i and iv); Salivary cortisol data = 50 participants (12 men and 38 women; reasons for participants' exclusion i, ii and iii). Finally, specifically for the TMT, four participants (2 men and 2 women) did not perform this test because they were not wearing their glasses, and they needed them to take the test (HCC: n=50; Salivary data: n=46).

The mean age of the sample was 64.75 years (from 56 to 77 years old). Half of the participants (52.6%) had an educational level beyond high school and their SES was medium (M=5.48, SD=0.972). Student t-tests showed that there were no sex differences in age (men: M=64.50, SD=5.34; women: M=64.84, SD=3.78), body mass index (men: M=27.02, SD=2.69; women: M=25.99, SD=3.72), SES, educational level, $Mean_{CortNeuro}$ or $Change_{CortNeuro}$ (all p>0.262). Men showed higher HCC than women (t(53)=2.06, p=0.044; Men: M=3.37, SD=2.48; Women: M=2.07, SD=2.07), but there were no sex differences in $Mean_{CortDay}$, t(49)=1.40, p=0.165; Men: M=8.69, SD=2.41; Women: M=7.50, SD=2.59) or the $AUC_{CortDay}$ (t(49)=1.32, p=0.192; Men: M=44.60, SD=41.59; Women: M=41.59, SD=7.01).

3.2. Unadjusted correlation analyses.

Correlation analyses with HCC and separate cortisol measures show that associations between HCC and +30min and evening cortisol were not significant (p>0.113), and with the awakening sample, the association was r=0.273 (p=0.057).

Table 1 shows unadjusted correlations among all variables included in the regression analyses. HHC was associated with BMI (r=0.389, p=0.003), unadjusted Digit Span Backward (r=0.305, p=0.024) (Figure 2A), first trial of the RAVLT (r=0.275, p=0.042) (Figure 2B), immediate story recall (r=0.274, p=0.043), and delayed story recall (r=0.290, p=0.033) (Figure 2C). $MEAN_{CortDay}$ was associated with $AUC_{CortDay}$ (r=0.878, p<0.001). Additionally, there were significant intercorrelations among cognitive test outcomes. These significant associations range from r=0.343 between the first trial and immediate recall on the RAVLT (p=0.009), to r=0.675 between the first trial and total learning on the RAVLT (p<0.001).

Table 1 Unadjusted correlation analyses between the variables included in the regression analyses.

	Cortisol					Age	BMI	SES[a]	TMT-A	TMT-B[b]	DSF	DSB[c]	First trial RAVLT	Total learn. RAVLT	Imm. recall RAVLT	Rivermead	
	HCC	M_{CD}	AUC_{CD}	M_{CN}	C_{CN}											Imm. recall	%Del. recall
HCC																	
$Mean_{CortDay}$	**0.27#**																
$AUC_{CortDay}$	-0.14	**0.87****															
$Mean_{CortNeuro}$	0.12	**0.23#**	0.19														
$Change_{CortNeuro}$	**0.23#**	0.18	0.19	0.10													
Age	0.07	-0.05	-0.05	-0.10	-0.18												
BMI	**0.38***	-0.12	-0.17	-0.11	-0.15	0.121											
SES[a]	0.02	0.01	-0.06	-0.17	0.05	**-0.22#**	-0.02										
TMT-A	-0.01	-0.04	0.07	-0.12	0.02	**0.33***	-0.17	-0.06									
TMT-B[b]	-0.20	-0.10	-0.009	0.22	-0.12	0.22	0.003	-0.84	**0.44***								
DSF	0.18	-0.13	-0.07	-0.10	0.13	**-0.30***	-0.02	-0.11	-0.21	**-0.24#**							
DSB[c]	**0.30***	-0.07	-0.02	-0.18	0.03	-0.07	-0.10	-0.12	-0.16	**-0.51****	**0.54****						
1st T. RAVLT	**0.27***	0.05	0.08	-0.15	**-0.26#**	0.04	0.06	0.12	0.19	-0.00	-0.05	0.09					
Learn. RAVLT	**0.26#**	-0.07	-0.04	**-0.25#**	**-0.32***	-0.03	0.56	0.10	0.07	-0.13	0.04	0.09	**0.67****				
Imm. RAVLT	0.14	-0.16	-0.12	-0.19	-0.15	0.08	0.05	0.00	-0.11	-0.03	-0.18	0.07	**0.34***	**0.64****			
Imm. Rivermead	**0.27***	0.06	-0.11	-0.14	-0.10	-0.04	-0.04	-0.08	-0.13	-0.22	**0.42***	**0.44****	0.14	0.18	0.04		
%Delayed recall Rivermead	**0.29***	0.12	<0.001	-0.15	-0.004	-0.06	0.04	0.11	-0.22	-0.21	0.04	**0.34***	0.16	0.20	0.17	**0.36***	

M_{CD}: $Mean_{CortDay}$; AUC_{CD}: $AUC_{CortDay}$; M_{CN}: $Mean_{CortNeuro}$; C_{CN}: $Change_{CortNeuro}$; BMI: Body Mass Index; SES: Subjetive socio-economic status; TMT: Trail Making Test; DSF: Digit Span Forward; DSB: Digit Span Backward; RAVLT: Rey Auditory Verbal Learning Test.

[a] Unadjusted correlation analyses for educational level show a significant association with SES (r = 0.320, p = 0.015).

[b] Adjusted correlations for TMT-B show a significant association with Digit Span Backward (r = -0.474, p < 0.001), and the association with MeanCortNeuro was r = 0.289 (p = 0.058).

[c] Adjusted correlations for Digit Span Backward also show a significant association with delayed story recall (r = 0.302, p = 0.046). None of the other correlation analyses for educational level, adjusted TMT-B, or adjusted Digit Span Backward were significant (p > 0.108).

#p < 0.10; *p < 0.05; **p < 0.001

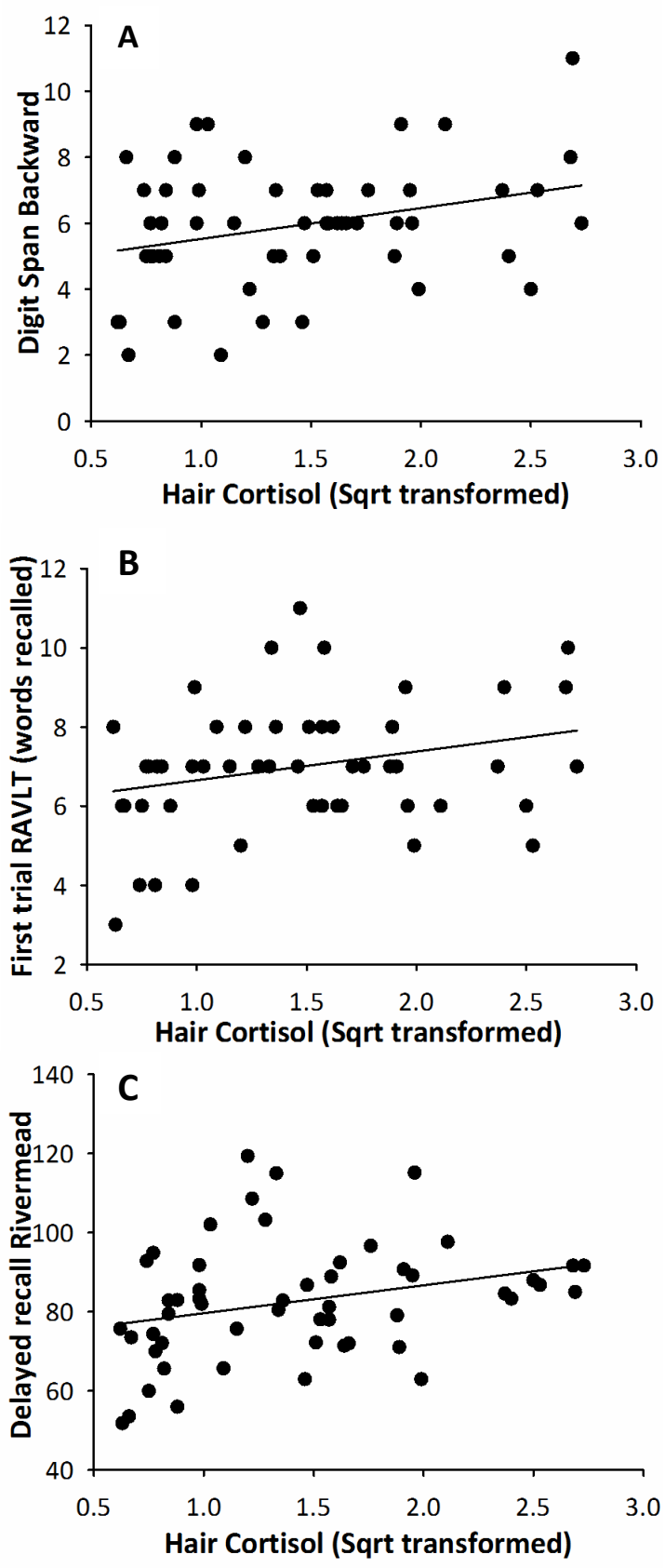

Figure 2. Scatter plots for the correlations between hair cortisol and (A) unadjusted Digit Span Backward (r=0.305, p=0.024), (B) First trial on the RAVLT (r=0.275, p=0.042), and (C) Delayed recall on the Rivermead (r=0.290, p=0.033).

3.3. Regression analyses.

3.3.1. Relationship between cortisol outcomes (HCC, $MEAN_{CortDay}$ and $AUCg_{CortDay}$) and cognitive performance.

In the first step of the regression analyses, we included as covariates: age, BMI, SES, $Mean_{CortNeuro}$, $Change_{CortNeuro}$, Sex (0=Women; 1=Men), cortisol levels in the first saliva sample at home (for analyses with $AUC_{CortDay}$), TMT-A scores (for analyses with TMT-B), and Digit Span Forward (for analyses with Digit Span Backward). Table 2 shows the second step of the regression analyses with HCC, $MEAN_{CortDay}$ and $AUCg_{CortDay}$ as predictors, and cognitive test outcomes as dependent variables.

The results show that higher HCC were related to better performance on the adjusted Digit Span Backward (β=0.271; *p*=0.050), the first trial of the RAVLT (β=0.514; *p*=0.001), total learning on the RAVLT (β=0.591; *p<0.*001), Immediate recall on the RAVLT (β=0.489; *p*=0.001), and delayed story recall (β=0.390; *p*=0.022). In addition, higher $Mean_{CortDay}$ was related to worse performance on the Digit Span Forward (β=-0.250; *p*=0.031), and higher $AUC_{CortDay}$ was related to worse performance on Immediate story recall (β=-0.430; *p*=0.008).

3.3.2. Relationship between the ratio of diurnal salivary cortisol over HCC and cognitive performance.

Given that regression analyses showed an opposite relationship between salivary cortisol and HCC and some cognitive outcomes, we performed a set of analyses to explore whether the relationship between diurnal salivary data and cognitive performance can be interpreted in relation to baseline long-term cortisol levels (i.e. HCC). To do so, we performed the same regression analyses, but using the ratios of diurnal salivary cortisol over HCC ($MEAN_{CortDay}$/HCC and $AUCg_{CortDay}$/HCC) as a predictor.

Table 3 shows the regression analyses with $MEAN_{CortDay}$/HCC and $AUCg_{CortDay}$/HCC as predictors. The results show that a higher $MEAN_{CortDay}$/HCC ratio was related to worse performance on the adjusted Digit Span Backward (β=-0.308; *p*=0.029), the first trial of the RAVLT (β=-0.538; *p*<0.001), total learning on the RAVLT (β=-0.506; *p*=0.001), Immediate recall on the RAVLT (β=-0.384; *p*=0.012), and delayed story recall (β=-0.367; *p*=0.032). Importantly, $MEAN_{CortDay}$/HCC shows stronger associations with the adjusted Digit Span Backward and the first trial of the RAVLT than the association between the

performance on these cognitive tasks and each of these two cortisol outcomes considered separately.

Table 2. *Step 2* of the regression analyses with cortisol outcomes (HCC, $Mean_{CortDay}$ and $AUC_{CortDay}$) as predictors.

Step 2		**First Trial RAVLT[a]**	**Total Learning RAVLT[a]**	**Immediate recall RAVLT[a]**	**Immediate recall Rivermead**	**% Delayed recall Rivermead**
HCC	Adj[c] R^2	0.20	0.37	0.28	0.16	0.03
	β	0.51	0.59	0.48	0.25	0.39
	P	**0.001**	**<.001**	**0.001**	ns	**0.02**
	f^2	0.26	0.44	0.26	0.05	0.12
$Mean_{CortDay}$	Adj[c] R^2	0.03	0.01	0.07	0.05	0.04
	β	0.13	0.05	-0.05	-0.01	0.16
	P	ns	ns	ns	ns	ns
	f^2	0.01	0.001	0.005	< 0.001	0.01
$AUC_{CortDay}$	Adj[c] R^2	0.01	-0.04	0.03	0.23	-0.09
	β	0.17	0.07	-0.01	-0.43	-0.16
	p	ns	ns	ns	**0.008**	ns
	f^2	0.02	0.003	0.001	0.19	0.01

Step 2		**TMT-A[b]**	**TMT-B[b] (Adjusted)**	**Digit span Forward**	**Digit span Backward (adjusted)**
HCC	Adj[c] R^2	0.01	0.28	0.45	0.33
	β	-0.03	-0.26	0.06	0.27
	p	ns	**0.07**	ns	**0.05**
	f^2	0.001	0.08	0.006	0.08
$Mean_{CortDay}$	Adj[c] R^2	-0.001	0.17	0.46	0.22
	β	0.03	-0.06	-0.25	-0.06
	p	ns	ns	**0.03**	ns
	f^2	0.002	0.005	0.11	0.004
$AUC_{CortDay}$	Adj[c] R^2	0.02	0.16	0.44	0.20
	β	0.25	0.06	-0.12	-0.05
	p	ns	ns	ns	ns
	f^2	0.05	0.004	0.02	0.002

Table 2. [a]RAVLT=Rey Auditory Verbal Learning Test. [b]TMT=Trail Making Test.
Step 1 (covariates)**=**In the first step of the regression analyses, we included as covariates: age, BMI, SES, $Mean_{CortNeuro}$, $Change_{CortNeuro}$, Sex (0=Women; 1=Men), mean cortisol levels in the first saliva sample at home (for analyses with $AUC_{CortDay}$), TMT-A scores (for analyses with TMT-B) and Digit span forward (for analyses with Digit span backward). *Step 3* (Sex Interactions)=Digit Span Forward: HCC×Sex, *p*=0.088; *Post Hoc:* Men, β=0.313, *p*=0.096; Women, β=-0.086, *p*=0.565. Immediate recall Rivermead: $Mean_{CortDay}$×Sex, *p*=0.086; *Post Hoc:* Men, β=-0.584, *p*=0.107; Women, β=0.100, *p*=0.534. None of the other sex interactions were significant (p>0.100).

Additionally, a higher $AUCg_{CortDay}$/HCC ratio was related to worse performance on the first trial of the RAVLT (β=-0.469; *p*=0.003), total learning on the RAVLT (β=-0.463; *p*=0.003), immediate recall on the RAVLT (β=-0.348; *p*=0.025), immediate story recall (β=-0.348; *p*=0.024) and delayed story recall (β=-0.379; *p*=0.030). None of these associations were stronger than the one observed between the performance on these cognitive tasks and each of these cortisol outcomes considered separately.

Table 3. Regression analyses with ratio outcomes ($Mean_{CortDay}$/HCC and $AUC_{CortDay}$/HCC) as predictors.

Step 2		**First Trial RAVLT**[a]	**Total Learning RAVLT**[a]	**Immediate recall RAVLT**[a]	**Immediate recall Rivermead**	**Delayed recall Rivermead**
$Mean_{CortDay}$/ HCC	Adj R^2	0.27	0.22	0.22	0.13	-0.05
	β	-0.53	-0.50	-0.38	-0.29	-0.36
	P	**<0.001**	**0.001**	**0.012**	**0.06**	**0.03**
	f^2	0.36	0.30	0.17	0.09	0.12
$AUCg_{CortDay}$/ HCC	Adj R^2	0.21	0.18	0.18	0.20	0.009
	β	-0.46	-0.46	-0.34	-0.34	-0.37
	P	**0.003**	**0.003**	**0.02**	**0.02**	**0.03**
	f^2	0.26	0.24	0.13	0.14	0.13

		TMT-A[b]	**TMT-B**[b] **(adjusted)**	**Digit span Forward**	**Digit span Backward (adjusted)**
$Mean_{CortDay}$/ HCC	Adj R^2	0.006	0.22	0.40	0.32
	β	0.01	0.23	0.01	-0.30
	p	ns	ns	ns	**0.02**
	f^2	<.001	0.06	<0.001	0.13
$AUCg_{CortDay}$/ HCC	Adj R^2	-0.01	0.20	0.43	0.28
	β	0.08	0.22	0.00	-0.26
	p	ns	ns	ns	**0.06**
	f^2	0.006	0.05	<0.001	0.09

Table 3. [a]RAVLT=Rey Auditory Verbal Learning Test. [b]TMT=Trail Making Test.
Step 1 (covariates)**=**In the first step of the regression analyses, we included as covariates: age, BMI, SES, $Mean_{CortNeuro}$, $Change_{CortNeuro}$, Sex (0=Women; 1=Men), cortisol levels in the first saliva sample at home (for analyses with $AUC_{CortDay}$), TMT-A scores (for analyses with TMT-B) and Digit span forward (for analyses with Digit span backward). *Step 3* (Sex Interactions)=None of the sex interactions were significant (*p*>0.098).

4. Discussion.

We investigated the relationship between HCC and diurnal cortisol secretion and cognitive performance in healthy older people. We observed that lower long-term cor-

tisol exposure (i.e. the previous three months), measured in scalp hair, was consistently related to worse performance on: working memory (Digit Span Backward), learning (total learning on the RAVLT), short-term verbal memory (first trial and immediate recall on the RAVLT), and long-term verbal memory (delayed story recall). Additionally, higher diurnal salivary cortisol was related to worse performance on attention (association between $MEAN_{CortDay}$ and Digit Span Forward) and short-term verbal memory (association between $AUCg_{CortDay}$ and Immediate story recall). Finally, the $MEAN_{CortDay}$/HCC ratio showed a stronger negative relationship with working memory (Digit Span Backward) and short-term verbal memory (first trial of the RAVLT) than the association observed between the performance on these cognitive tasks and each cortisol outcome considered separately.

It is important to emphasize that, given the correlational nature of this study, we cannot endorse causal relationships. However, our findings are noteworthy, as they provide consistent evidence for an association between low long-term cortisol exposure, measured in scalp hair, and worse executive function (working memory) and verbal memory (learning and short and long-term verbal memory). This association is at odds with our hypothesis, because it has the opposite direction from the one observed previously with salivary, blood or urine data (e.g. Lee et al., 2007; Comijs et al., 2010; Evans et al., 2011; Franz et al., 2011). In our study, higher salivary cortisol output was also related to worse cognitive performance, but this relationship was only found for two cognitive tasks (Digit Span forward and Immediate story recall). It is likely that the inclusion of only three samples, although on two consecutive days, would account for the weaker results. Nevertheless, our results for acute (salivary) cortisol measurements are in the same direction as previous studies that associated higher daily cortisol output with worse cognitive performance.

In our opinion, the apparently contradictory results observed with HCC and salivary cortisol can be explained by differences in the information provided by hair and salivary samples. While hair cortisol measurement serves as a biomarker of integrated HPA activity over months, salivary samples measure cortisol levels at certain times of

the day and, therefore, provide information about the regulation of HPA-axis circadian rhythmicity (Meyer and Novak, 2012).

Consistent with our results for HCC, animal studies have shown that low long-term cortisol exposure may have a negative effect on cognition, an effect that has been related to a low occupation of the mineralocorticoid receptors in the central nervous system (Sloviter et al., 1993; Stienstra et al., 1998; Wossink et al., 2001; Berger et al., 2006). These receptors are located especially in the hippocampus and prefrontal cortex and, under healthy basal cortisol levels, are almost saturated (cortisol occupation of approximately 70-80%). These results suggest that a low cortisol occupation would be detrimental to these brain structures (for a review, see: de Kloet et al., 1999).

It is possible that, in our healthy sample, higher HCC in those participants with better cognitive performance may reflect the exposure to more activating and stimulating situations every day. Along this line, it has been shown that activities such as physical exercise, cognitively challenging activities, and social interactions can trigger intermittent hormonal activation during the day (Cadore et al., 2008; Kukolja et al., 2008; van der Meij et al., 2010). The repetition of these types of activities every day would reflect, in the long run, higher long-term cortisol exposure, which might produce a higher occupation of mineralocorticoid receptors and, thus, the maintenance of brain structures involved in better cognitive performance. Supporting this idea, several studies have shown that living in an enriched environment increases basal cortisol levels and enhances the cognitive performance of young and aging animals (e.g. Kempermann et al., 2002; Marashi et al., 2003; Moncek et al., 2004; Sampedro-Piquero et al., 2013). Of course, the direction of this relationship could also be the opposite. That is, healthy older people with better cognitive performance might be more interested in highly stimulating activities that would provoke higher long-term cortisol levels. The direction of these relationships should be addressed in future research. In any case, individuals' lifestyles and long-term activities would have an impact on cortisol concentrations measured in hair, but much less in salivary, blood or urine samples.

In the case of salivary samples, higher daily cortisol output would reflect a dysregulation of the diurnal rhythm of the HPA-axis, which would be related to worse cognitive performance. It is likely that a flatter salivary cortisol slope will result in higher total salivary cortisol output on the sampling days (Beluche et al., 2010). Both factors, i.e. having a flatter daily cortisol slope and higher daily cortisol output, have been consistently related to cognitive impairment in older people (e.g. Lee et al., 2007; Evans et al., 2011; Franz et al., 2011; Stawski et al., 2011) and to health problems such as cancer and chronic fatigue (Abercrombie et al., 2004; Nater et al., 2008). A dysregulation in HPA-axis rhythmicity can be measured with salivary, blood or urine samples, but not with HCC. This translates into a low association between HCC and salivary cortisol levels in our study and in others (Meyer and Novak, 2012).

Interestingly, we observed that the MEANCortDay/HCC ratio had a stronger negative relationship (lower *p* value and higher effect size) with working memory (Digit Span Backward) and immediate verbal memory (first trial of the RAVLT) than the association of these tasks with each of these two cortisol outcomes considered separately. This result was also observed for the immediate story recall task, although this relationship did not reach statistical significance (p=0.061). These are interesting findings, as they show a link between our results in HCC and salivary samples, indicating that those individuals with higher long-term basal cortisol levels (which might represent a higher cortisol occupation of the mineralocorticoid receptors) would be less vulnerable to the detrimental effect of a dysregulation of the HPA-axis on cognition. This effect was observed specifically in working memory and short-term verbal memory; however, further studies using more salivary samples might reveal whether the same associations could be observed with other cognitive tasks. Additionally, these results support Walton et al. (2013), who, investigating patients with acute trauma, suggested that hair-normalized salivary cortisol might be a better biomarker of HPA-axis activity than salivary cortisol alone. Future studies could benefit from using this kind of analysis.

The findings reported in this study are noteworthy, as they contribute to the knowledge about the relationship between HCC and cognition in healthy older people. To exclude unknown interactions between HCC and possible confounds, we used very

restrictive exclusion criteria, and our sample consisted of healthy individuals with cortisol levels in the normal range. As a result, our sample was small; therefore, future studies with larger samples should confirm our findings, especially in men. Furthermore, the exclusion criteria for our study can affect the generalizability of our results to older people with some age-related diseases. For example, patients with coronary artery disease have shown both high HCC and memory impairments (Vinkers et al., 2005; Pereg et al., 2011; Saleem et al., 2013), suggesting that higher HCC levels than those observed in our study, which may be reflecting unhealthy levels, can also be related to worse cognitive performance. In the same sense, the use of a homogeneous sample could affect our results, as it could cause insufficient variance in cognitive performance, reducing the number of significant associations (e.g., the relationship between HCC and immediate story recall or TMT-B). Future studies may benefit from including older people with age-related diseases.

A limitation of the study is that we did not control the frequency of hair washing and hair treatments, which have been observed to affect HCC in some studies (Sauve et al., 2007; Manenschijn et al., 2011), but not in others (Dowlati et al., 2010; Dettenborn et al., 2012; Stalder et al., 2012b). Furthermore, due to characteristics of the technique, bald people and/or people with hair shorter than 3 cm could not participate in the study. Results of previous studies suggest the HCC can be used in the older population (Gow et al., 2011; Pereg et al., 2011; Feller et al., 2014); however, the interpretation of timing in HCC (three months) should be considered with caution, as the rate of hair growth might be reduced in some older individuals (Van Neste, 2004). Finally, it has been proposed that a small quantity of local cortisol can be synthesized in hair follicles and might provoke transitory changes in HCC (see Sharpley et al., 2012). However, direct and indirect validation studies support the notion that the bloodstream would be the principal source of the cortisol concentrations in hair (for reviews see: Gow et al., 2010; Meyer and Noval, 2012; Russell et al., 2012; Sharpley et al., 2012; Stalder and Kirschbaum, 2012; Staufenbiel et al., 2013; Wosu et al., 2013). Nevertheless, future research can explore whether this *peripheral* cortisol might also contribute to the observed relationship between HCC and cognition.

In summary, the current study presents the first evidence of a relationship between low long-term cortisol exposure, measured in scalp hair, and worse cognitive performance (working memory, learning, and short and long-term verbal memory) in healthy older people. We also replicated, at least in part, results of previous studies showing that higher daily salivary cortisol output is related to worse cognitive performance. Additionally, we observed that those individuals with lower long-term cortisol exposure would be more vulnerable to the negative effect of HPA-axis dysregulation on cognition. Taken together, our results suggest that differences in cognitive performance in normal aging are related to inter-individual variability in both long-term cortisol exposure and cortisol variation during the day.

Chapter 5

Study 4: Cortisol awakening response and walking speed in older people

The main results of this study are under review for publication: Pulopulos, M.M., Puig-Perez, S., Hidalgo, V., Villada, C., Salvador, A. Cortisol awakening response and walking speed in older people.

1. Introduction

The cortisol awakening response (CAR) is a discrete component of the hypothalamic-pituitary-adrenal axis (HPA-axis) activity. It consists of a rapid increase of 50 to 160% in cortisol concentration after awakening that typically peaks between 30 and 45min later (Clow et al., 2010a). A dysregulation of the CAR, independently from the rest of the diurnal HPA-axis activity, has been related to several health problems, such as chronic stress, cardiovascular disease, sleep disorders (see Fries et al., 2009) and worse cognitive performance in older people (Almela et al., 2012; Evans et al., 2012).

Walking speed (WS) is an objective and commonly used measure of physical performance in older people. Slower WS has also been related to worse cognitive performance and other health problems (e.g., cardiovascular disease) in older people (Cooper et al., 2011). Importantly, recent studies have shown that individuals with less diurnal HPA-axis activity variability walk slower (e.g., Kumari et al., 2010; Gardner et al., 2011, 2013; Johar et al., 2014), and that a HPA-axis dysregulation might affect physical performance, including WS (Gardner et al., 2011). However, the specific relationship between the CAR and WS is not fully understood. A lower CAR has shown an association with slower WS (Gardner et al., 2011), faster WS (Kumari et al., 2010), or even no association with WS (Johar et al., 2014). Moreover, an individual-participant meta-analysis that included data from six middle-aged and older adult cohorts of population-based studies (containing participants included in Kumari et al., 2010 and Gardner et al., 2011) concluded that a lower CAR was related to slower WS (Gardner et al., 2013). Thus, conflicting evidence has been reported, and more research is needed to better understand this relationship.

Although all these previous studies used large sample sizes and had high statistical power, some methodological issues could contribute to the mixed results. For example, they used only two salivary samples (awakening and 30min later) to measure the CAR, an issue that could affect the reliability of the CAR measurements (Clow et al., 2010a). Additionally, cortisol levels on awakening (an indicator of pre-awakening cortisol secretion) can affect the magnitude of the CAR (Clow et al., 2010a), and so it is im-

portant to investigate whether the possible relationship between the CAR and WS is independent from cortisol levels on awakening.

Therefore, we aimed to investigate whether the CAR (measured with six saliva samples on two consecutive days) was related to WS in older people, and whether this relationship was independent from cortisol levels on awakening. Additionally, we controlled for the possible effects of several possible confounders. Based on the results of Gardner et al. (2013) (the study with the largest sample size and that included participants from previous studies), we expected a lower CAR to be related to slower WS.

2. Method

2.1. Participants and procedure

Eighty-eight participants who could ambulate household distances independently were recruited for this study. Two women were removed from the analyses because they were outliers for cortisol concentration (+3 S.D). Thus, the final sample was composed of 86 participants from 56 to 72 years old (men=41; women=45) (see Table 1 for sample characteristics). The exclusion criteria were: alcohol or other drug abuse, endocrine diseases that affect HPA-axis activity (e.g., Cushing's syndrome), psychiatric illness, using medication that was able to influence hormonal levels (e.g., glucocorticoids or antidepressants), and having been under general anesthesia once or more in the past year. All the female participants were postmenopausal, had had their last menstrual period more than 2 years before the testing time, and were not receiving treatment for hormone replacement. Results of the Spanish version of the Mini-Mental Status Examination (Lobo et al., 1999) indicated the absence of cognitive impairment.

Participants performed the WS test at the Faculty of Psychology. They were asked to walk 10m on a line at their usual speed, turn around, and walk back as fast as possible without running. The time taken to walk the six central meters in both directions was measured. After the WS test, the participants collected a total of 6 saliva samples at home on two consecutive weekdays. Finally, the participants filled out a questionnaire on demographic and health-related characteristics (e.g., age, smoking status, physical activity) and completed the Perceived Stress Scale (PSS; Cohen et al.,

1993) to assess overall perceived stress in the past month. The Ethics Committee of the University of Valencia approved the protocol, and all the participants provided informed consent.

Table 1. Characteristics of the sample

	Mean±SEM
Age	64.42±0.42
SES	5.51±0.13
BMI	27.17±0.36
Physical activity	1.79±0.08
PSS	16.17±0.69
Walking speed test (sec.)	8.00±0.11
Sleep hours (hh:mm)	06:41±00:06
Wake-up time (hh:mm)	07:07±00:05
CAR	239.34±16.36
Cortisol Awakening (nmol/L)	7.11±0.32
Cortisol 30min (nmol/L)	15.12±0.70
Cortisol 45min (nmol/L)	14.98±0.71

SES: Subjective socioeconomic status; BMI: Body Mass Index; PSS: Perceived Stress Scale; CAR: Cortisol awakening response.

2.2. Cortisol measurements.

Saliva samples were collected to measure the CAR (using salivettes Sarstedt, Nümbrecht, Germany) immediately after waking (cortisol awakening) and 30min and 45min post-awakening. Additionally, participants logged the time of each saliva collection, their sleep duration the nights before the CAR measurements, and their awakening time. To objectively verify participant adherence to the saliva sampling, salivettes were stored in MEMS TrackCap containers (MEMS 6 TrackCap Monitor, Aardex Ltd., Switzerland). There was a mean of 12 days (±1.31) between the WS test and the measurement of the cortisol levels at home. The protocol for the cortisol analyses and the instructions given to participants for the CAR were the same as in previous studies (See Almela et al., 2012 for a detailed description).

2.3. Statistical analysis and data management.

Cortisol values were square root transformed. As an index of the CAR, we calculated the area under the curve with respect to the increase using cortisol levels on awakening, +30min and +45min (see Pruessner et al., 2003).

It has been proposed that, in most individuals, a negative CAR may be caused by a delay in performing the first saliva sample (Thorn et al., 2006). To control for this possible effect, we identified those participants who showed a negative CAR on one or both days. Twenty-five participants (men=16; women=9) showed a positive CAR on only one day. None of the participants showed a negative CAR on both days. Given that a negative CAR on only one day might be caused by a delay in taking the sample immediately post-awakening, for participants showing a positive CAR on only one day, only the data for the positive CAR day were used in the analyses. For participants showing a positive CAR on both days, the CAR data for the two days were averaged.

Correlation analyses were used to investigate whether the CAR and cortisol awakening were related to WS. Additionally, we performed regression analyses to explore whether these relationships were still observed after controlling for several confounders (e.g., Clow et al., 2010a; Fries et al., 2009). As covariates, in the first step we included age, body mass index, subjective Socio-Economic Status (measured using the MacArthur Scale of Subjective Social Status; Adler et al., 2000), Sex (0=Women; 1=Men), time of awakening, mean sleep time, smoking status (0=No; 1=Yes), PSS and physical activity (0=None; 1=Low; 2=Moderate; 3=High). In step 2, we added the CAR or cortisol awakening. Kumari et al. (2010) showed that the relationship between the CAR and WS was especially observed in men. Therefore, in step 3 we included the interaction between the CAR or cortisol awakening and Sex to investigate possible sex-related differences.

3. Results

3.1. Correlation analyses

Results showed that cortisol awakening was not related to WS (r=-0.140; p=0.198). Instead, a lower CAR was related to slower WS (r=-0.223; p=0.039).

3.2. Regression analyses

Regression analyses (see Table 2) indicated that, even after controlling for several factors, cortisol awakening was not related to WS (p=0.414). Instead, a lower CAR

was still related to slower WS (p=0.022). To explore whether this latter relationship was independent from the cortisol levels at awakening, we included cortisol on awakening in step 1 as a covariate. This analysis showed that a lower CAR was still related to slower WS, regardless of the cortisol awakening concentrations (p=0.031). None of these relationships were moderated by sex (p>0.775).

As in previous studies (e.g., Thorn et al., 2006; Almela et al., 2012), we explored whether these results would be observed if we excluded participants who showed a negative CAR on one day. Results showed that excluding these participants reduced the significance of the relationship between the CAR and WS, approaching a marginally significant relationship for the CAR and WS (p=0.069) and a non-significant association for the CAR and WS controlled for awakening cortisol (p=0.151). Importantly, the reduction in the significance of the results is probably due to a reduction in the sample size because the values of the partial correlation from step 2, previously reported as significant, were minimally affected (CAR and walking speed: complete sample partial r=-0.26, 2day CAR partial r=-0.25; CAR and walking speed controlling for waking cortisol: complete sample partial r=-0.24, 2day CAR partial r=-0.20).

Table 2. *Step 2* of the regression analyses with cortisol awakening and CAR as predictors and walking speed as dependent variable.

	Adj. R^2	β	*p*
Cortisol awakening	0.28	-0.08	0.414
CAR	0.32	-0.21	**0.022**
CAR controlled for cortisol awakening	0.31	-0.21	**0.031**

Adj: Adjusted. CAR: Cortisol awakening response. *Step 1 (covariates)* = In the first step of the regression analyses, we included as covariates: age, BMI, SES, Sex (0=Women; 1=Men), smoking status (0=No; 1=Yes), physical activity (0=none; 1=low; 2=moderate; 3=high), perceived stress scale, time of waking and mean sleep time, and cortisol awakening (for analyses with CAR controlled for cortisol awakening). *Step 2 (sex interactions)* = none of the sex interactions were significant (p>0.775).

4. Discussion

We observed that a lower CAR was related to worse performance on a WS test, and that this relationship was independent from the cortisol levels on awakening. This result coincides with Gardner et al. (2011) and Gardner et al. (2013). The latter study observed an association between slower WS and a lower CAR in a sample of participants from six different population-based studies. However, our result does not coincide with two other studies (Kumari et al., 2010; Johar et al., 2014). However, it is worth noting that our participants provided more salivary samples on two consecutive weekdays in order to obtain a more reliable measure of the CAR, and that we controlled for possible non-adherence to the protocol. Furthermore, our results were still significant after controlling for important confounders (Fries et al., 2009; Clow et al., 2010a). Thus, these methodological differences could explain the different results.

Previous studies have shown that less diurnal variability in cortisol levels is related to slower WS (Kumari et al., 2010; Gardner et al., 2011, 2013; Johar et al., 2014). It has been suggested that a dysregulation of the HPA-axis activity may be a potential mechanism for sarcopenia (i.e., loss of muscle mass and strength), affecting physical performance and WS (Gardner et al., 2011). Thus, a less pronounced CAR, along with higher night cortisol levels, could lead to less dynamic HPA-axis activity and less diurnal variability in cortisol levels, contributing to sarcopenia.

Two other explanations can be considered. It is possible that the CAR affects physical performance, independently from the rest of the HPA-axis, because the CAR is considered a discrete component of HPA-axis activity that may make a separate contribution to a person's health condition (Fries et al., 2009). Along these lines, a lower CAR has been related to worse executive function performance (Almela et al., 2012; Evans et al., 2012), a cognitive ability that seems to be critical in walking performance (Yogev-Seligmann et al., 2008). To date, no causality has been established between the CAR and cognitive performance, but it is possible that a dysregulation of the CAR might affect executive function, leading to worse walking performance. Another possible explanation is that the observed relationship between the CAR and WS would mean that both measures can function as markers for similar age-related changes and health

conditions. In fact, both a lower CAR and slow WS have been related to worse cognitive performance and cardiovascular disorders (Cooper et al., 2011; Almela et al., 2012; Evans et al., 2012).

One limitation of this study is that, due to the cross-sectional design, no conclusions can be drawn about causality. Additionally, although we controlled for a possible delay in performing the awakening salivary sample, further studies may benefit from the implementation of electronic devices to objectively verify the awakening time and saliva sampling, in order to improve CAR measurements (Clow et al., 2010a).

In conclusion, a lower CAR in older people is related to slower WS. This might imply that the CAR contributes to physical performance independently from the rest of the HPA-axis, and that it can be considered as a separate intervention target to improve physical functioning in older people. Another possibility is that the CAR and WS could be considered as markers for similar age-related changes in health condition and/or cognitive performance.

Chapter 6

Study 5: Cortisol awakening response and cognitive performance in hypertensive and normotensive older people

The main results of this study are under review for publication: Pulopulos, M.M., Hidalgo, V., Puig-Perez, S., Salvador, A. Cortisol awakening response and cognitive performance in hypertensive and normotensive older people.

1. Introduction.

Cardiovascular risk factors and cognitive functioning problems have a strong tendency to increase with age. However, it is still not fully understood whether (and how) they might be interlinked. Hypertension, the most common risk factor for cardiovascular diseases, has been related to cognitive decline in older people, especially to worse frontal cortex functioning (see Tzourio et al., 2014). It has been indicated that high blood pressure (BP) may affect cognitive performance through vascular brain injury (Verhaaren et al., 2013); however, the activity of the hypothalamic-pituitary-adrenal axis (HPA-axis) might also play a role in the relationship between hypertension and changes in cognitive performance in older people. The HPA-axis affects cognitive function and BP through the effects of cortisol, the main hormone secreted by the HPA-axis in humans, on receptors that are especially located in the frontal cortex and the limbic system, as well as in cardiovascular tissues (Ku, 2006; Lupien et al., 2007). In this regard, previous studies have shown that worse HPA-axis regulation is related to worse cognitive performance (Lupien et al., 2007) and systemic hypertension in older people (Gold et al., 2005). In spite of this evidence, research investigating whether the HPA-axis may be associated with cognitive performance in hypertensive and normotensive older people is sparse, and more studies are needed.

The circadian rhythm of the HPA-axis involves three discrete components: (i) the cortisol awakening response (CAR), a rapid increase in cortisol levels that peaks between 30 and 45min following morning awakening; (ii) a sharp decrease in the secretion of cortisol during the rest of the day; and (iii) an increase in cortisol levels from the second half of the night until waking (Fries et al., 2009). While previous studies have shown a relationship between higher overall diurnal cortisol secretion (without distinguishing between the CAR and the rest of the diurnal cortisol secretion) and worse cognitive performance in older people (e.g., Franz et al., 2011; Lee et al., 2007; MacLullich et al., 2005; Pulopulos et al., 2014; but see Singh-Manoux et al., 2014), few studies have examined the specific effect of a CAR dysregulation on cognition in healthy older people. Additionally, the effect of a dysregulation of the CAR on cognitive performance in older people with hypertension has not been studied.

The CAR has characteristics that are unrelated to the cortisol secretion during the rest of the day, and the frontal cortex and hippocampus have been found to be especially involved in its regulation (Clow et al., 2010a; Fries et al., 2009). Along these lines, an increasing number of studies have highlighted the importance of investigating the CAR's contribution to cognitive performance in older people. We recently showed that a higher increase in cortisol levels after awakening (i.e., higher CAR) was related to worse performance on a hippocampus-dependent memory task and, especially in older men, to better performance on a frontal cortex-dependent working memory task (Almela et al., 2012). Similarly, Evans et al. (2012) observed that a higher CAR was associated with better performance on executive functioning (i.e., a frontal cortex-dependent ability) in healthy older people. Additionally, a lower CAR has been observed in young children with worse performance on prospective memory (i.e., a frontal and hippocampal-dependent memory task) (Bäumler et al., 2014b) and in young adults with frontal and hippocampal-related amnesia (Buchanan et al., 2004; Wolf et al., 2005). However, some studies did not observe any relationship (Franz et al., 2011; Singh-Manoux et al., 2014). Together, these results suggest that the magnitude of the CAR may be related to cognitive performance, and especially to better frontal cortex functioning.

In addition, systemic hypertension and other cardiovascular risk factors have been associated with a dysregulation of the cortisol secretion immediately after awakening (DeSantis et al., 2011; Kuehl et al., 2015; Rosmond and Björntorp, 2000; Wirtz et al., 2007). Wirtz et al. (2007) showed an attenuated CAR and HPA-axis feedback sensitivity in hypertensive middle-aged people. Similarly, Kuehl et al. (2015) observed a negative relationship between BP and overall cortisol secretion post-awakening (i.e., a measure that included both the CAR and the total cortisol exposure the first hour after awakening). However, differences in the magnitude of the CAR have not been always observed (Strahler et al., 2010b). Previous studies have suggested that hypertensive individuals show worse performance and a more rapid decline in executive function and processing speed (i.e., two cognitive functions related to frontal cortex activity) (for a review see Tzourio et al., 2014). Thus, it is possible that these changes in CAR might contribute to changes in frontal cortex-related cognitive performance observed in hyper-

tension. Along these lines, Gold et al. (2005) showed that reduced HPA-axis feedback sensitivity was associated with frontal lobe atrophy in hypertension. The authors also showed worse performance on executive function in hypertensive participants, but no association was observed between overall night cortisol secretion and cognitive performance. In their study, the CAR was not measured, and the question of whether morning cortisol response may be related to frontal cortex-related functioning in hypertension has not yet been studied.

With this in mind, the purpose of the present study was to investigate the differences in CAR and overall morning cortisol secretion in hypertensive and normotensive older people. Additionally, we aimed to study whether the CAR was associated with their cognitive performance. To do so, participants provided ambulatory saliva samples to measure morning cortisol levels, and they performed a neuropsychological session focused especially on frontal cortex-dependent tasks (working memory, attention, switch tasks, processing speed and inhibition). Based on previous studies, we expected to find an attenuated CAR, lower overall morning cortisol exposure, and worse frontal cortex-dependent task performance in hypertensive participants. Furthermore, a positive relationship was expected between the CAR and frontal cortex-dependent tasks performance in normotensive and hypertensive individuals.

2. Method.

2.1. Participants.

Sixty participants from 56 to 78 years old were recruited from a study program for people over 55 years old. The hypertensive group was composed of 30 participants (17 men and 13 women). Following the WHO/ISH definition, participants were included in the hypertensive group if they showed a systolic blood pressure (SBP) of 140mmHg or higher, a diastolic blood pressure (DBP) of 90mmHg or higher, and/or treatment for a previous diagnosis of essential hypertension (Kjeldsen et al., 2002). All the participants in this group had been taking antihypertensive medication for more than one year (*M*=8.69 years, ±1.41; range from 1.33 to 31.5 years). Twenty-three participants were taking angiotensin II receptor antagonists, five participants were taking calcium antag-

onists, four participants were taking angiotensin-converting-enzyme inhibitors, three participants were taking beta blockers, and one participant was taking renin inhibitors. Four participants were undergoing treatment with more than one antihypertensive. The control group was composed of 30 age- and educational level-matched participants (14 men and 16 women) without a previous diagnosis of hypertension and considered normotensives after SBP and DBP measurements (< 140/90 mmHg) at the beginning of the neuropsychological session.

In order to avoid potentially confounding factors, we recruited participants who, except for the diagnosis of hypertension, reported being in good mental and physical condition. The exclusion criteria were: alcohol or other drug abuse, smoking more than 10 cigarettes a day, presence of an endocrine, neurological or psychiatric disease, and using any medication directly related to emotional or cognitive function or medication that was able to influence hormonal levels (e.g., glucocorticoids, anti-diabetic medication, antidepressants, and psychotropic substances). Having been under general anesthesia once or more in the past year, the presence of a stressful life event during the last year, and a diagnosis of secondary hypertension were also reasons for exclusion. All female participants were postmenopausal, and they had had their last menstrual period more than 2 years before the testing time. None of them were taking estrogen replacement therapy.

Results on the Spanish version of the Mini-Mental Status Examination (Lobo et al., 1999) indicated the absence of cognitive impairment (all the participants scored 28 or more on this test). None of the participants met the criteria for dementia, as defined by the NINCDS-ADRDA criteria for Alzheimer's disease, or the criteria for Mild Cognitive Impairment, as defined by the European Consortium on Alzheimer's Disease (Portet et al., 2006).

2.2. Procedure and neuropsychological assessment.

Before the neuropsychological assessment, participants were asked to refrain from heavy physical activity the day before the session, sleep as long as usual, and not consume alcohol since the night before the session. Additionally, they were instructed to

drink only water, and not eat, smoke, take any stimulants (e.g., coffee, cola, caffeine, tea, chocolate), or brush their teeth at least 1h prior to the session. The neuropsychological assessments started between 1000h and 1200h. Upon arrival to the laboratory and after a 15min rest, three seated BP measurements were obtained using an automated sphygmomanometry device (Omron M6 HEM-7223-E, Omron Healthcare Europe B.V. Hoofddorp, The Netherlands). The average of the three BP measurements was calculated. Next, the participants performed the MMSE and completed the Beck Depression Inventory (BDI; Beck et al., 1996).

To measure cognitive performance, the following cognitive tests were used in the neuropsychological session:

Stroop Color-Word Interference Test: Golden's version of the Stroop Color-Word Interference Test was used (Golden, 1978). This version of the STROOP contains three different pages with 100 color words printed in black ink (Words page), 100 "Xs" printed in color (red, green, and blue) (Colors page), and 100 words from the first page printed in colors from the second page (the color and the word do not match) (Color-Word page), respectively. Participants were asked to read the words on the first page and name the ink color on the second and third pages as fast and precisely as possible for 45 seconds. Two outcomes from this test were used: (i) Word-Color naming: the score on the word and color pages were z-transformed and averaged, and this outcome was used as a measure of processing speed; (ii) Interference Stroop: The interference index (calculated as indicated in Chafetz and Matthews, 2004) was used as a measure of the ability to inhibit automatic responses (i.e., higher scores indicate better performance).

Trail Making Test A and B (Reitan, 1992): The Trail Making Test form A (TMT-A) was used to assess general psychomotor speed and attention, and the Trail Making Test form B (TMT-B) assesses the efficiency of attention-switching performance. The outcome of each form was the time (seconds) needed to perform the test (i.e., lower time means better performance). To specifically pinpoint the executive function measured by the TMT-B (i.e., set-shifting ability), we included the TMT-A as a covariate in all the analyses performed with the TMT-B.

Digit Span: The Digit Span Forward subtest of the Wechsler Memory Scale III (Wechsler, 1997) was used as a measure of the attention and memory span component of working memory, and the Digit Span Backward subtest was used as a measure of the executive component of working memory (Conklin et al., 2000). To specifically pinpoint the executive function measured by the Digit Span Backward (i.e., executive component of working memory), we included the Digit Span Forward as a covariate in all the analyses performed with the Digit Span Backward.

Rey Auditory Verbal Learning Test: The Rey Auditory Verbal Learning Test (RAVLT; Rey 1958) was used to measure learning and verbal memory. Three indexes were used in subsequent analyses: (i) Total learning: total number of words recalled on the first five trials (trial I to V); (ii) Immediate recall: total number of words recalled after the interference trial (trial VI); (iii) Delayed recall: percentage of the total number of words recalled after 20min (trial VII), compared to the number of words recalled on the immediate recall trial.

2.3. Cortisol measurements.

All the saliva samples were collected using salivettes (Sarstedt, Nümbrecht, Germany). Salivary samples to measure the CAR were collected at home on two consecutive weekdays. The saliva samples were provided immediately after waking (cortisol awakening) and 30min (+30min) and 45min (+45min) post-awakening. Additionally, to control whether possible differences between groups could be explained by differences in cortisol levels the previous night, participants provided one saliva sample immediately before they decided to go to sleep on the nights prior to the two CAR measurements (cortisol night). Participants were thoroughly instructed about how to provide saliva samples, and they were given written instructions. They were instructed to drink only water and not eat, smoke or brush their teeth at least 1h prior to each saliva sample. To objectively verify participant adherence to the saliva sampling time at home, salivettes were stored in MEMS TrackCap containers (MEMS 6 TrackCap Monitor, Aardex Ltd., Switzerland). Additionally, participants recorded on a log the time of each saliva collection, the time they went to bed, their sleep duration the nights before the CAR measurements, and their awakening time. There was a mean of 11 days (±2.15)

between the neuropsychological assessment and the measurement of the cortisol levels at home. To control for the possible effect of acute cortisol levels at the moment of neuropsychological testing, participants provided one saliva sample at the beginning and at the end of the neuropsychological session. Saliva samples provided during the session and at home were stored and analyzed as described in detail in Almela et al. (2012).

2.4. Statistical analysis and data management.

Cortisol values did not show normal distributions; therefore, they were log transformed. Two indexes were calculated using cortisol levels on awakening, +30min and +45min: (i) the area under the curve with respect to the increase, used as a measure of the CAR (i.e., the dynamic of the cortisol increase after awakening); and (ii) the area under the curve with respect to the ground (AUCg), used as a measure of the overall morning cortisol secretion (see Pruessner et al., 2003 for the formula).

Student's *t*-test, *U*-Mann Whitney (for educational level) and ANCOVAS (for TMT-B and Digit Span Backward) were used to compute differences in subjects' characteristics, cortisol outputs (cortisol night, cortisol awakening, CAR, and AUCg) and cognitive performance. Correlation analyses were used to study relationships among cortisol outputs (cortisol night, cortisol awakening, CAR, and AUCg), sleep parameters (time they went to bed, time of awakening, mean sleep time) and time being treated for hypertension.

Finally, we performed regression analyses to investigate whether the CAR was related to cognitive performance. In the regression analyses, we included as covariates age, body mass index (BMI), subjective Socio-Economic Status (SES, measured using the MacArthur Scale of Subjective Social Status; see Adler et al., 2000), Group (0=Hypertensive; 1=Normotensive), Sex (0=Women; 1=Men), the mean cortisol levels during the neuropsychological assessment, the cortisol change during the neuropsychological assessment (cortisol levels pre-session minus cortisol levels post-session), time of awakening and mean sleep time, to control for their possible effects on cognitive performance and CAR (e.g., Clow et al., 2010a; Cournot et al., 2006; Sindi et al.,

2013; Wright and Steptoe, 2005). The design of the regression analyses was as follows: In step 1, we included the covariates, and in step 2, we added CAR. Based on Aiken and West (1991), we performed a moderator regression analysis to investigate whether these relationship were different for the hypertensive and normotensive groups. Therefore, in step 3, we included the interaction between the CAR and hypertension.

Four participants were removed from the analyses because their cortisol concentration at home differed by more than 3 S.D from the total sample mean (one hypertensive man and one normotensive woman), due to lack of information about sleep parameters and the time of saliva collection (on the log and MEMOTRACK) (one hypertensive man), and due to sleep problems the days of cortisol measurement at home (one hypertensive man). Additionally, two participants (one hypertensive man and one normotensive man) did not perform the Stroop test because of problems in differentiating the ink colors. Therefore, the final sample was composed of 27 hypertensive subjects and 29 normotensive subjects (26 hypertensive and 28 normotensive subjects for the Stroop test data analyses).

3. Results.

3.1. Preliminary analyses.

Previous studies have shown that a delay in the first saliva sample results in higher cortisol awakening values and affects the reliability of the CAR measurement (Griefahn and Robens, 2011; Smyth et al., 2013). Thus, it has been proposed that a flat or negative CAR in most of the individuals may be caused by a delay in performing the first saliva sample (Thorn et al., 2006). To control for this possible effect, and as done in previous research (Almela et al., 2012; Thorn et al., 2006), we identified those participants who showed a positive CAR on both days (i.e., cortisol AUCi>0) and those who showed a negative CAR on one or both days. Thirty-nine participants (hypertense=17; normotense=22) showed a positive CAR on both days (2 Day-CAR subgroup) and 17 participants (hypertense=10; normotense=7) showed a positive CAR on only one day (1 Day-CAR subgroup). None of the participants showed a negative CAR on both days. There was no significant difference in the number of hypertensive and normotensive

participants who showed a positive CAR on two days or only one day (p=0.294). The 2 Day-CAR and the 1 Day-CAR subgroups did not differ in age, educational level, SES, BDI scores, time they went to bed, mean sleep time, time of awakening and time taking antihypertensive medication (all p>0.191). Participants in the 1 Day-CAR subgroup showed a slightly higher BMI than the participants in the 2 Day-CAR subgroup (p=0.083).

An ANOVA for repeated measures with Day and Time (cortisol awakening; +30min and +45min) as a within-subject factor was used to investigate differences in cortisol levels across days between the 2 Day-CAR and 1 Day-CAR subgroups. Greenhouse-Geisser was used because the requirement of sphericity for the ANOVA for repeated measures was violated (p<0.001). These analyses showed that the factors Day, Subgroups and the interaction between Day and Subgroups were not significant (*all* p>0.118). The factor Time (F(1.64,78.73)=89.072, p<0.001) and the interaction between Time, Day and Subgroups were significant (F(1.45,78.73)=16.141, p<0.001). *Post Hoc* planned comparisons (using Bonferroni adjustments for p values) indicated that the 2 Day-CAR subgroup did not show significant differences in cortisol awakening, +30min and +45min across days (p>0.473), and that cortisol levels increase from awakening to +30min on both days (p<0.001). For the 1 Day-CAR subgroup, on the day with negative CAR, cortisol levels were higher at awakening than on the day with positive CAR, and lower at +30min and +45min (p<0.002). Cortisol levels increase from awakening to +30min on the day with positive CAR (p<0.001), but cortisol levels decrease from awakening to +30min and +45min (p<0.015) on the day with negative CAR.

Therefore, given that there were no differences between the cortisol levels at home across days in the 2 Day-CAR participants, the data for the two days of the study were averaged for these participants in order to compute the cortisol outcomes and sleep parameters. For the 1 Day-CAR participants, given that higher cortisol on awakening on the day with negative CAR may indicate a delay in performing the first salivary sample, only the data for the day that showed a positive CAR were used in the analyses.

Following Thorn et al. (2006), the analyses performed in this study were repeated, excluding the participants with 1 Day-CAR. In the text, we indicate the analyses that are

still significant when performed only with participants who showed a positive CAR on both days of sampling (2Day-CAR subgroup).

Table 1. Participants' characteristics, sleep parameters and cognitive performance

	Hypertensive	Normotensive	*t,U,F*	*p*
Age	65.89±0.90	65.55±0.78	0.28	0.778
Educ. Level	2.56±0.20	2.59±0.20	3.34	0.501
SES	5.18±0.31	5.48±0.24	-0.75	0.454
BMI	29.31±0.84	27.04±0.52	2.32	**0.024**
BDI	4.74±0.76	4.41±0.68	0.32	0.751
Systolic BP	136.66±2.72	122.84±2.11	4.03	**<0.001**
Dystolic BP	85.82±1.55	78.59±0.96	3.95	**<0.001**
Bedtime (hh:mm)	00:14±00:06	00:25±00:09	-1.04	0.302
Sleep hours (hh:mm)	06:27±00:10	06:53±00:09	-1.89	**0.065**
Wake time (hh:mm)	06:41±00:10	07:18±00:09	-2.76	**0.008**
MMSE	29.56±0.11	29.45±0.14	0.58	0.564
W-C naming	0.01±0.20	0.00±0.20	-0.05	0.963
Stroop Interf.	6.73±1.66	5.07±1.36	0.78	0.441
TMT A	38.30±2.45	35.66±1.75	0.89	0.386
TMT B	94.74±6.73	85.66±4.83	1.11	0.273
Digit Span Forward	9.59±0.46	8.76±0.35	1.44	0.155
Digit Span Backward	6.11±0.40	6.07±0.31	0.08	0.935
RAVLT Learn	50.59±1.90	50.82±1.23	-0.10	0.916
RAVLT Immed.	9.93±0.57	10.72±0.36	-1.19	0.246
RAVLT Delayed	104.85±3.24	99.70±2.16	1.34	0.187

Educ Level: Educational level; SES: Subjetive socioeconomic status; BMI: Body Mass Index; BDI: Beck Depression Inventory; BP: Blood Pressure; Awak: Awakening; MMSE: Mini-Mental Status Examination; W-C naming: Word and Color task of the Stroop test; TMT: Trail Making Test; DS: Digit Span; RAVLT: Rey Auditory Verbal Learning Test.

3.2. Subjects' characteristics and differences in cortisol levels and cognitive performance.

Table 1 shows the characteristics of the sample and differences in sleep parameters and cognitive performance for the total sample (2Day-CAR and 1Day-CAR). Hypertensive participants showed a higher BMI (p=0.024), SBP (p<0.001) and DBP (p<0.001), they woke up earlier (p=0.008), and they slept slightly fewer hours than the normotensive group (p=0.065). The hypertensive group showed lower cortisol awakening (t= -3.05, p=0.004) and AUCg (hypertensives: 460.37 ±35.48, normotensives: 598.67 ±41.71; t=-2.66, p=0.010) (Figure 1). No significant differences in cortisol night (t=0.08,

p=0.938) and CAR were observed (hypertensives: 197.26 ±24.37, normotensives: 242.06 ±26.37; *t*=0.80, *p*=0.425).

If the analyses are performed with the 2Day-CAR subgroup only, the differences between hypertensives and normotensives on SBP (*p*<0.001), DBP (*p*=0.006), time of awakening (*p*=0.023), AUCg (*p*=0.023) and cortisol awakening (*p*=0.017) are still observed.

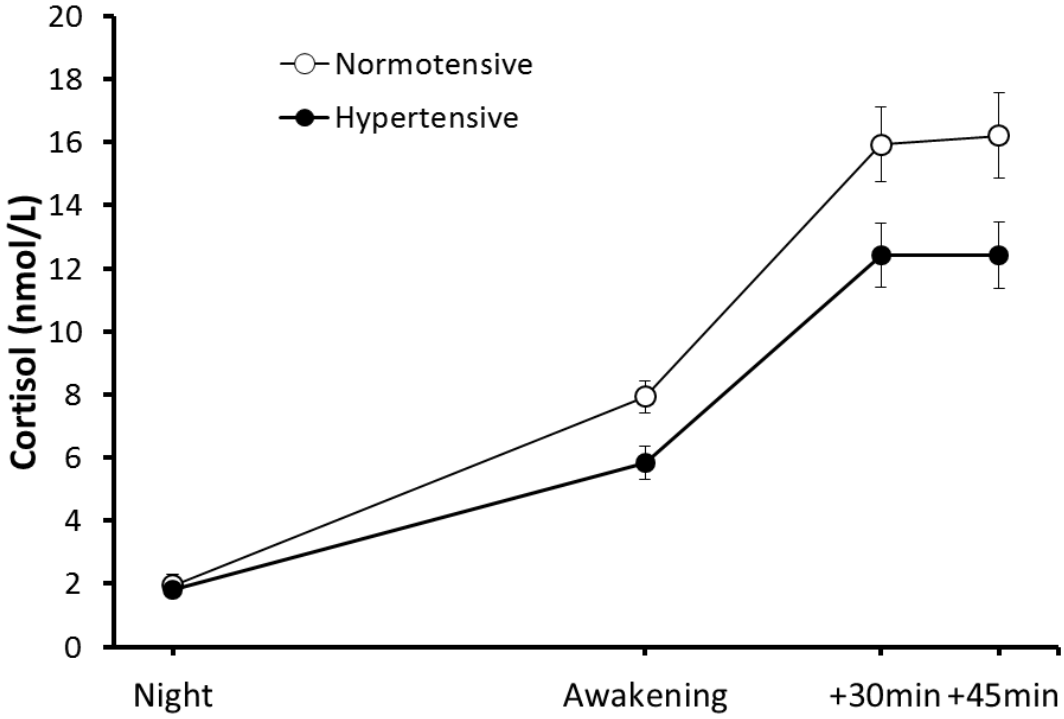

Figure 1. Mean cortisol levels (±SEM) for hypertensive and normotensive participants. No differences were observed in cortisol levels at night and CAR (*p*>0.425), but hypertensive participants showed lower cortisol levels at awakening, +30min, +45min and AUCg (*p*<0.033).

3.3. Relationships among cortisol levels, time of awakening, mean sleep time and time receiving treatment for hypertension.

Table 2 shows the partial correlation analyses for the total sample (2Day-CAR and 1Day-CAR). Age and IMC were included as covariates to control for their possible effect on these associations. In the hypertensive group, higher cortisol night was related to a lower amount of mean sleep time (*r*=-0.55, *p*=0.004) and an earlier waking time (*r*=-0.54, *p*=0.005). The participants who went to bed later slept les hours (*r*=-0.44, *p*=0.024). Higher cortisol awakening was related to higher AUCg (*r*=0.84, *p*<0.001). The

participants taking antihypertensives for a longer time showed a higher CAR (r=0.40, p=0.047; Figure 2). If one-tailed partial correlation analyses are performed to confirm

these results with the 2Day-CAR subgroup only, associations between cortisol night and a lower amount of mean sleep time (r=-0.50, p=0.028), and between cortisol awakening and AUCg (r=0.88, p<0.002) are still observed. Moreover, the participants taking antihypertensives for a longer time showed a higher CAR (r=0.50, p=0.028).

In the normotensive group, the participants who went to bed later slept les hours (r= -0.48, p=0.010) and woke up later (r=0.56, p=0.002). Higher cortisol awakening was related to higher AUCg (r=0.88, p<0.001). For the 2Day-CAR subgroup, all these analyses remained significant (association between bedtime and wake-up time: r=0.62, p=0.002; association between bedtime and wake-up time: r=-0.42, p=0.031; association between cortisol awakening and AUCg: r=0.92, p<0.001).

3.4. Relationship between CAR and cognitive performance.

Table 3 shows the results of the regression analyses with CAR as a predictor and cognitive performance as dependent variables for the complete sample (2Day-CAR and 1Day-CAR). Higher CAR was related to better performance on the TMT-B (β=-0.25, p=0.026) and Word-Color naming (β=0.47, p=0.001). None of the interactions between CAR and Group were significant, indicating that there were no differences between hypertensive ad normotensive participants in the relationship between CAR and cognitive performance in (p>0.106).

If the analyses are performed with the 2Day-CAR subgroup only, the same results are observed. Higher CAR was related to better performance on the TMT-B (β=-0.46, p=0.003) and Word-Color naming (β=0.49, p=0.002). None of the other analyses were significant for this subgroup (p>0.117).

Table 2. Correlation analyses for cortisol data, sleep parameters and time taking antihypertensives for hypertensive (top of the table) and normotensive (bottom of the table) participants.

	Bed Time	Mean sleep time	Wake-up time	Cortisol night	Cortisol awakening	CAR	AUCg
				Hypertensive group			
Time Medication	*r*=-0.08 *p*=0.680	*r*=0.01 *p*=0.680	*r*=-0.05 *p*=0.807	***r*=0.37** ***p*=0.063**	*r*=0.03 *p*=0.870	***r*=0.40** ***p*=0.047**	*r*=0.25 *p*=0.228
Bedtime		***r*=-0.44** ***p*=0.024**	*r*=0.17 *p*=0.397	*r*=0.09 *p*=0.670	*r*=-0.06 *p*=0.758	*r*=-0.26 *p*=0.202	*r*=-0.20 *p*=0.324
Mean sleep time	***r*=-0.48** ***p*=0.010**		***r*=0.80** ***p*<0.001**	***r*=-0.55** ***p*=0.004**	*r*=0.20 *p*=0.334	*r*=0.24 *p*=0.237	*r*=0.32 *p*=0.110
Wake-up time	***r*=0.53** ***p*=0.002**	***r*=0.49** ***p*=0.019**		***r*=-0.54** ***p*=0.005**	*r*=0.17 *p*=0.392	*r*=0.09 *p*=0.658	*r*=0.22 *p*=0.284
Cort. night	*r*=0.22 *p*=0.266	*r*=0.03 *p*=0.860	*r*=0.26 *p*=0.189		*r*=-0.08 *p*=0.683	*r*=-0.03 *p*=0.864	*r*=-0.10 *p*=0.626
Cort. awakening	*r*=0.22 *p*=0.250	*r*=0.13 *p*=0.519	***r*=0.35** ***p*=0.068**	*r*=0.25 *p*=0.198		*r*=-0.29 *p*=0.293	***r*=-0.84** ***p*<0.001**
CAR	*r*=-0.05 *p*=0.789	*r*=-0.23 *p*=0.240	*r*=-0.27 *p*=0.163	*r*=0.12 *p*=0.525	*r*=-0.12 *p*=0.544		*r*=0.33 *p*=0.108
AUCg	*r*=0.19 *p*=0.339	*r*=0.01 *p*=0.944	*r*=0.20 *p*=0.295	*r*=0.30 *p*=0.128	***r*=0.88** ***p*<0.001**	***r*=0.38** ***p*=0.075**	
				Normotensive group			

Cort.: Cortisol; CAR: Cortisol awakening response; AUCg: Area under the curve with respect to the ground.

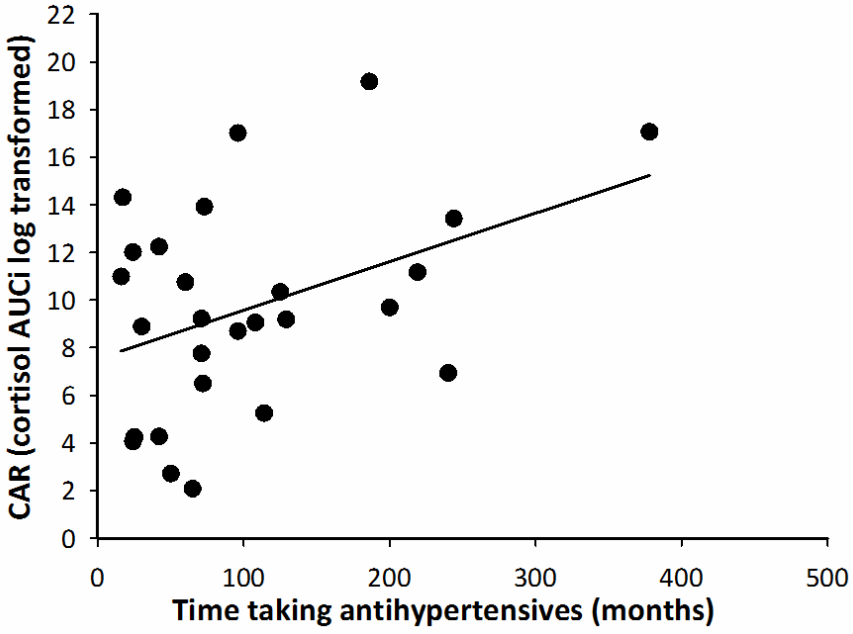

Figure 2. Scatter plot for the relationship between time taking antihypertensive medication and CAR (*r*=0.40; *p*=0.036). This relationship is also significant if age and body mass index are included as covariates (*r*=0.40, *p*=0.047).

Table 3. *Step 2* and *Step 3* of the regression analyses with CAR as a predictor and cognitive performance as dependent variable.

	CAR			CAR*Group
	Adj. R^2	β	*p*	*p*
W-C naming	0.21	0.47	**0.001**	0.106
Stroop Interference	-0.02	-0.02	0.905	0.248
TMT-A	0.18	0.06	0.624	0.991
TMT-B	0.43	-0.25	**0.026**	0.533
Digit Span Forward	0.38	0.14	0.231	0.791
Digit Span Backward	0.24	0.05	0.730	0.305
RAVLT Learning	0.12	0.24	**0.088**	0.962
RAVLT Immediate	0.00	0.12	0.427	0.847
RAVLT Delayed	020	0.07	0.588	0.616

Adj: Adjusted; W-C naming: Word and Color task of the Stroop test; TMT: Trail Making Test; RAVLT: Rey Auditory Verbal Learning Test. *Step 1 (covariates)* = In the first step of the regression analyses we included as covariates: age, BMI, SES, mean cortisol levels during the session, change in cortisol levels during the session, Sex (0=Women; 1=Men), Group (0=Hypertensive; 1=Normotensive), time of waking and mean sleep time, TMT-A scores (for analyses with TMT-B) and Digit Span Forward (for analyses with Digit Span backward).

4. Discussion.

In the present study, hypertensive and normotensive older people did not show differences in CAR, (i.e., the dynamic of the cortisol increase after awakening), but lower cortisol at awakening and AUCg (a measure of the overall cortisol exposure) were observed in participants with systemic hypertension. In normotensive and hypertensive participants, higher CAR was related to better performance on executive functioning (TMT-B) and processing speed (Color reading and Word naming on the Stroop test). Additionally, the hypertensive participants who had being taking antihypertensive medication for a longer time showed higher CAR. All these results were also observed when we excluded from the analyses the participants with a possible delay in performing the first salivary sample of the CAR.

Our results for AUCg and cortisol awakening agree with previous studies showing a relationship between lower overall morning cortisol exposure and higher BP (Kuehl et al., 2015) and cardiovascular risk factors, including high BP (DeSantis et al., 2011; Rosmond and Björntorp, 2000). In contrast to these previous studies, in the present study we controlled for self-reported time of awakening, showing that hypertensive partici-

pants woke up earlier in the morning and slept less time than normotensive participants. These results agree with previous research investigating sleep characteristics in hypertensive individuals (Gottliev et al., 2006; Vgontzas et al., 2009). Thus, our results suggest that the differences in AUCg and cortisol awakening observed in our study and previous research could be explained by the fact that hypertensive participants woke up earlier in the morning, when cortisol levels were lower (Elder et al., 2014). Along these lines, our study also suggests that an HPA-axis dysregulation during the night may contribute to these sleep differences because higher cortisol levels the preceding night were related to less sleep time and an earlier waking time in hypertensive participants, but not in normotensive participants. This idea agrees with previous studies showing that a dysregulated HPA-axis functioning during the night is related to sleep problems (Elder et al., 2014; Van Cauter et al., 2000). Further research is needed to investigate whether hypertensive and normotensive people differ in their night-time cortisol rhythms, and if this difference is related to waking time and cortisol levels on awakening.

With regard to the dynamic of the cortisol increase after awakening (i.e., the CAR), we observed no statistical differences between hypertensive and normotensive participants. This finding coincides with Strahler et al. (2010b), who showed no effects of hypertension on the CAR of older people. However, it does not agree with Wirtz et al. (2007), who reported an attenuated CAR in middle-aged people with systemic hypertension. In our opinion, one important difference in the studies could explain these contradictory results and deserve attention in further research. It is worth noting that, in our study and Starhler's, but not in Wirtz et al. (2007), all the hypertensive participants were undergoing treatment with antihypertensive medication. Therefore, it is possible that no differences were observed in morning cortisol increases because antihypertensive medication might contribute to a regularization of the CAR. This idea is supported by the positive relationship observed between the time taking antihypertensives and the magnitude of the CAR in our study. This is an important result because it suggests a potential clinical application for antihypertensive medication. More research is needed in order to discover how antihypertensives might affect CAR. If an effect of antihypertensives on CAR is confirmed in future studies, a clinical use of anti-

hypertensives might be considered in pathologies that also show an attenuated CAR, such as type 2 diabetes, burnout or posttraumatic stress disorder (Chida and Steptoe, 2009; Fries et al., 2009; Keeshin et al., 2014; Marchand et al., 2014).

In the present study, we observed that a higher CAR was related to better performance on an attention-switching task (TMT-B) and on processing speed (Word reading and Color naming on the Stroop test). The diagnosis of hypertension does not moderate these relationships. Our results are consistent with previous research showing a positive association between CAR and frontal cortex-related cognitive tasks in healthy older people and young children (Almela et al., 2012; Bäumer et al., 2014b; Evans et al., 2012). Indeed, Evans et al. (2012) observed the same relationship between CAR and the TMT-B in a sample of healthy older people. It has been suggested that the CAR may be critical for the process of wakefulness, and that the CAR's dynamic closely parallels the reactivation of the prefrontal cortex (Clow et al., 2010a). Thus, a higher CAR would be related to a healthier frontal cortex and, consequently, to better cognitive performance. Our study extends these findings to hypertensive older people.

It has been observed that systemic hypertension affects frontal cortex-related abilities, but that long-term treatments with antihypertensives could decrease the risk of cognitive decline (Tzourio et al., 2014). Our study suggests that this protective effect of antihypertensives may be due, at least in part, to their effect on CAR. Hypertensive participants (undergoing treatment for systemic hypertension) did not differ from the normotensive participants on cognitive performance in the present study. Additionally, as mentioned above, higher CAR was related to a longer time taking antihypertensive medication, and to better executive function and processing speed. Thus, it is possible that antihypertensive treatment increases the CAR in older people with systemic hypertension, in the long run improving the executive function and processing speed performance that would be impaired before treatment. To confirm this idea, it is important to investigate in a longitudinal study whether changes in CAR and cognitive performance are observed after several years of treatment.

Importantly, some studies observed a positive relationship between CAR and a frontal cortex-related memory task (Almela et al., 2012; Bäumer et al., 2014b), but here we

only observed a marginally significant association with learning a word-list (p=0.088). It is possible that the type of memory tasks used in this study were not sensitive enough to observe effects on memory, as observed previously. Further studies are needed to investigate the relationship between CAR and several memory tasks (e.g., visual and verbal memory, implicit and explicit tasks).

A limitation of this study is that we did not use any electronic devices to objectively assess time of awakening. To control for this possible confounder, we identified the participants with a possible delay in performing the first salivary sample, which can affect the reliability of the CAR measurements. Most of the statistical conclusions of the study do not change if we eliminate these participants from the analyses. Thorn et al. (2006) indicates that this methodology should be used with caution in people with cardiovascular disease because it might exclude adherent participants with aberrant CAR. Importantly, it should be noted that all the participants in this study showed a positive CAR on at least one day, and that there was no difference in the number of hypertensive and normotensive participants who showed a positive CAR on only one day of sampling. This suggests that a negative CAR was not a characteristic of a specific number of participants, and that a delay in the first salivary sample is a possible explanation. Finally, given that this study has a cross-sectional design, no causality can be endorsed.

In conclusion, this study indicates that an earlier time of awakening and shorter sleep time in hypertensive older people might underlie the lower cortisol levels on awakening and lower overall morning cortisol secretion observed here and in previous studies. Moreover, we observed that hypertensive (receiving antihypertensive treatment) and normotensive participants did not differ in the magnitude of the CAR. Our results suggest a potential clinical application of antihypertensives in cases of attenuated CAR. Finally, our study replicates previous research showing a positive relationship between CAR and frontal cortex-related cognitive functioning, suggesting that the possible protective effect of antihypertensives on cognition could be mediated by their effects on CAR.

Chapter 7

Discussion of the main findings

The empirical studies of this doctoral dissertation investigated the relationship between acute and daily cortisol levels and cognitive performance in older people. We studied the contribution of different components of HPA-axis functioning to cognitive performance: (i) The first and second studies investigated the acute effects of stress-induced cortisol response on memory performance; (ii) the third and fifth studies investigated the relationship between the circadian activity of the HPA-axis (CAR and diurnal cortisol) and long-term endogenous cortisol exposure and cognitive performance; and (iii) the fourth study investigated the relationship between CAR and walking speed, a measure of physical performance that has been consistently related to cognitive performance and health condition in older people. The main results have been discussed in the chapters that contain each study. In this chapter, we will present a short general discussion of the main results, and we will elaborate on the clinical implications, general limitations and future directions stemming from the findings of this dissertation.

1. Summary of the main findings and general discussion.

1.1. Stress-induced HPA-axis response and memory performance.

Some authors suggest that older people may be more vulnerable to the effect of stress on cognitive performance than young people (e.g., Lupien et al., 2009; Sindi et al., 2014; Allen et al., 2014). However, while this may be true for the effect of chronic stress on memory (or even cognition in general), to date there is not consistent evidence to support the idea that memory in older people is more affected by acute stress-induced cortisol increases than in young adults. Only a few studies have investigated this issue in the older population (Lupien et al., 1997; Wolf et al., 2001; Porter et al., 2002; Yehuda et al., 2007; Almela et al., 2011a; Hidalgo et al., 2014), compared to the large number of studies performed in young adults (e.g., de Quervain et al., 2000; Buchanan and Lovallo, 2001; Cahill et al., 2003; Roozendaal et al., 2004; Elzinga and Roelofs, 2005; Kuhlmann, 2005a, 2005b; Oei et al., 2006; Smeets, 2008; Buchanan and Tranel, 2008; Schoofs et al., 2008; Duncko et al., 2009; Luethi et al., 2009; Schoofs et al., 2009; Smeets, 2011; Terfehr et al., 2011; Schoofs et al., 2013; Stauble et al., 2013). Among the studies performed in older people, only two studies have observed that

cortisol increases after stress affect memory performance, an effect that seems to be specific to retroactive interference (Lupien et al., 1997; Almela et al., 2011a). Moreover, studies investigating the age-related changes in the HPA-axis activity have indicated that with age there is a reduction in the density and sensibility of glucocorticoid receptors in the aging brain, suggesting that older people might be less vulnerable to the acute effects of cortisol increases. From this starting point, the effect of stress on long-term memory retrieval and working memory in older people was studied.

In the **first study** we investigated the effect of a stress task on long-term memory retrieval performance in a group of healthy older people. The stress task did not affect memory performance. Additionally, the overall cortisol secretion and the cortisol response to stress were not related to memory. The **second study** presented the results of two experiments performed to explore the effect of an acute stress task on working memory. This study showed that older women performed better on memory span after the stress task, and that high circulating cortisol levels after stress were related to memory span performance only in older women, but not in men. The executive component of working memory was not affected by stress, and it was not related to cortisol response or acute circulating cortisol levels after stress in older men and women.

Together, the results of these two studies support the idea that older people may be less sensitive to the acute effects of the cortisol response to stress on memory performance than expected. These are important findings because they indicate that in healthy older people the response to a threatening and stressful situation may be less adaptive. As indicated previously, it has been proposed that the specific effects of the cortisol response to stress on memory are part of a complex mechanism aimed to improve the survival of individuals. If cortisol is not able to affect working memory and impair long-term memory, and at the same time it seems to enhance retroactive interference (Almela et al., 2011a), it is possible that older people will have worse memories of stressful or dangerous situations. This condition would make them more vulnerable to the environment, as they would not benefit from learning necessary information to avoid potential threats.

These findings from the two studies presented in this thesis have specific characteristics, such as the type of memory tasks used (digit span, letter-number sequencing, word-list, pictures and histories) the stress task (TSST), and the sample (healthy older people), among others. Thus, although the results are consistent with previous studies (Wolf et al., 2001; Porter et al., 2002; Yehuda et al., 2007; Almela et al., 2011a; Hidalgo et al., 2014), it is important to replicate these results and investigate more in depth in what conditions stress may or may not affect memory performance in older people. Along these lines, in our opinion, one of the most important contributions of these two studies is that they point out that the effect of acute stress on memory in older people is understudied, and that it deserves much more attention because the effect in older people probably differs from the one observed in young adults.

1.2. Basal HPA-axis activity and cognitive performance.

A large number of studies (using salivary, blood or urine samples) have observed that a worse cognitive performance may be observed in older people who show a dysregulation of the diurnal HPA-axis activity (represented as a flatter diurnal cortisol pattern or higher diurnal cortisol outputs) (e.g., Hodgson et al., 2004; Karlamangla et al., 2005; MacLullich et al., 2005; Li et al., 2006; Kuningas et al., 2007; Lee et al., 2007, 2008; Beluche et al., 2010; Comijs et al., 2010; Evans et al., 2011; Franz et al., 2011; Gerritsen et al., 2011; Johansson et al., 2011). However, little is known about the relationship between changes in long-term endogenous cortisol exposure and cognitive performance in healthy older people. With this in mind, in the **third study** we investigated whether the endogenous cortisol exposure during the previous three months (measured in hair samples) was related to cognitive performance in healthy older people. Higher cortisol levels during the previous three months were related to better working memory, learning, and short and long-term verbal memory. In line with previous studies, higher daily salivary cortisol output was related to worse attention and short-term verbal memory. Importantly, we observed that those individuals with lower long-term cortisol exposure would be more vulnerable to the negative effects of HPA-axis dysregulation on cognition.

The results of this study are important because they show that lower long-term endogenous cortisol exposure might be as detrimental as a dysregulation of the diurnal HPA-axis. Additionally, an important finding of this study is that lower exposure to cortisol levels in the previous months in healthy people may facilitate the negative effects of a dysregulation of the HPA-axis functioning. In healthy older people, higher levels of cortisol (in a normal or healthy range) might be a consequence of a more activating and stimulating style of life. Thus, it is possible that the positive effects of an "active" life style on cognition in older people is due, at least in part, to the fact that activating and stimulating activities every day keep long-term cortisol levels in a healthy high range.

Research investigating the relationship between CAR and cognitive performance is scarce, and more studies are needed to understand what factors may play a role in this association. In the **fourth study** we investigate the relationship between CAR and walking speed in older people. Walking speed is used as a measure of physical activity, and previous studies have shown that slower walking speed in older people is closely associated with worse performance in frontal cortex functioning (Herman et al., 2010; Cooper et al., 2011). In line with previous research, we found that lower CAR was related to slower walking speed in older people. In the **fifth study** we investigate whether the CAR may be dysregulated in systemic hypertension, and whether it may be related to cognitive performance. We focused on frontal cortex-related cognitive tasks due to the previous relationship observed between these kinds of tasks and both hypertension and CAR. Results showed that hypertensive participants woke up earlier and slept fewer hours than normotensive participants, leading to lower overall morning cortisol secretion. Results suggest that an alteration in the nocturnal HPA-axis activity might underlie the sleep characteristics of hypertensive older people. This would result in a lower morning cortisol exposure that might be detrimental in the long run. No differences in CAR were observed, but interestingly, those participants undergoing treatment for hypertension for a longer time showed a higher CAR. Moreover, a higher CAR was related to better executive function and processing speed in both groups. Together, this study makes an important contribution to the literature by adding more evidence about the association between CAR and frontal cortex-related functioning in

older people, and at the same time, it suggests that antihypertensive medication may affect cognitive performance through its effects on the regularization of the CAR.

2. Clinical implications.

On the basis of the results from the first and second studies, an important clinical implication related to the treatment of anxiety disorders can be drawn. In the last decade, it has been proposed that the acute effects of stress-induced cortisol increases on memory (impairment of the process of retrieval and enhancement of consolidation) observed in young adults may have an important clinical application in the treatment of anxiety disorders (for detailed reviews see: de Quervain and Margraf, 2008; Bentz et al., 2010). The most effective psychotherapy for anxiety disorders, such as specific phobia, social phobia and posttraumatic stress disorder, is exposure psychotherapy (Chambless and Ollendick, 2001). Learning and memory play an important role in this kind of psychological treatment because the process of extinction memory is a core element in this therapy. In this context, de Quervain et al. (2011) demonstrated in patients with specific phobia that, compared to a placebo, oral cortisol (20mg) administration one hour before three sessions of an extinction-based psychotherapy resulted in a greater reduction in phobic symptoms one month later. The authors suggest that cortisol administration would facilitate the extinction processes in two ways: First, due to the well-known cortisol-induced impairment in memory retrieval, an aversive cue is no longer followed by a complete retrieval of the fear memory and related clinical symptoms (e.g., anxiety). Instead, it becomes related to a new less aversive experience, which is stored as extinction memory. Second, because cortisol enhances memory consolidation of new information, in this case cortisol enhances the storage of the extinction memory (i.e., the corrective experience) (de Quervain et al., 2011). This cortisol effect in psychotherapy has been replicated in a study using in vivo exposure-based group therapy for spider phobia (Soravia et al., 2014), and similar results for cortisol in social phobias have been observed (Soravia et al., 2006). Interestingly, not only an administration of exogenous cortisol enhances exposure-based therapy. Recent studies have observed that diurnal variations in endogenous cortisol levels may also modulate the success of exposure therapy for spider phobia (Meuret et al., 2015;

Lass-Hennemann and Michael, 2014). These studies have shown that a greater reduction in phobic symptoms may be observed in patients treated in the morning (when endogenous cortisol levels are high) than in patients treated in the evening (when endogenous cortisol levels are low).

Taken together, these studies performed in young people indicate that the acute effects of cortisol on memory may be a key component in the effectiveness of exposure therapy for anxiety disorders, and that exogenous administration of this hormone may be used to enhance the efficacy of the treatment. However, based on the results of the first and second study, showing a lack of effects of acute cortisol increases on memory in older people, it is important to ask the following questions: Is it possible that the efficacy of exposure therapy for anxiety disorders is reduced in older people due to a low sensitivity to acute cortisol effects on memory?, and, is it possible that older people may benefit less from using this new cortisol-based psychopharmacotherapy approach?.

With regard to the first question, a recent meta-analysis and meta-regression of randomized controlled trials showed that the effectiveness of cognitive behavioral based therapies for anxiety disorders is reduced in older people, compared to working-age adults (including exposure therapy as a treatment for phobias) (Gould et al., 2012). Thus, it seems that the effectiveness of this therapy in older people is reduced in comparison with young adults. Nevertheless, this meta-analysis was performed in anxiety disorders in general and, to the best of our knowledge, there are no studies focused specifically on exposure-based therapy in phobias or posttraumatic stress disorders in older people. The literature on the treatment of anxiety disorders in general, and phobias in particular, among older people is less developed than the comparable literature for young adults. Thus, we think the current evidence justifies more research testing the effectiveness of this treatment in older people. With regard to the second question, due to the lack of studies in older people, it is not possible to determine the efficacy of cortisol administration on exposure-based psychotherapy in older people; however, based on the findings of this dissertation, it is likely that this kind of treat-

ment may be less useful, and that different approaches might be needed in the older population.

The results of the third study also have interesting clinical implications. Saleem et al. (2013) suggested that higher cortisol levels in hair in adults at the beginning of coronary artery disease may be used as a predictor of a better recovery of the memory impairment. Along these lines, this dissertation also suggests that lower cortisol levels in the previous three months may be used as a biomarker to detect worse cognitive performance in older people. The results of this study do not allow conclusions about causality, but if future studies confirm that a decline in hair cortisol levels is followed by a decrease in cognitive performance, cortisol levels in hair samples might be used as a biomarker to detect those individuals with a higher vulnerability to showing HPA-axis dysregulation-related cognitive impairment.

The findings of the fifth study offer a potential clinical use of antihypertensive medication in diseases that show a dysregulation of the CAR. The results of this study confirmed a previous study showing no differences in CAR between normotensive and hypertensive older people who were undergoing treatment with antihypertensive medication (Strahler et al., 2010b). Additionally, it was observed that the hypertensive individuals who had been taking antihypertensive medication for a longer time showed higher CAR. If an enhancement effect of antihypertensives on the CAR is confirmed in further studies, this kind of medication might be used to contribute to the regulation of an attenuated CAR, observed in some diseases and unhealthy conditions such as type II diabetes, posttraumatic stress disorder, chronic fatigue, and burnout, among others (Chida and Steptoe, 2009; Fries et al., 2009; Keeshin et al., 2014; Marchand et al., 2014). Therefore, the findings of the fifth study suggest a potential clinical use of antihypertensives that might contribute to a regularization of the CAR in these cases. More research is clearly needed to better understand what the role of the CAR is in these conditions and how antihypertensive medication might be used.

Finally, these studies suggest that a dysregulation of the diurnal HPA-axis may contribute to worse cognitive performance in older people. Interestingly, changes in the stress response may also be related to worse cognitive performance. This idea is

supported by a recent study that was not included in this dissertation, but that is closely related to this research. In this study we showed that an attenuated cortisol response to stress is associated with worse cognitive performance in basal conditions in older people (Almela et al., 2014). Thus, all these findings show that a dysregulation of the HPA-axis in both basal and stressful conditions could be considered as a biomarker of cognitive function in older people.

3. General limitations.

In each study included in this dissertation, its specific limitations have been discussed. Here we will present some general limitations that need to be considered in order to properly interpret and generalize the findings of this dissertation.

Based on the lack of previous research, the first two studies were designed to investigate the acute effects of stress on long-term memory and working memory in older people. The results were agreed with previous research in older people, and at the same time, they contrasted with most of the vast research performed in young people. In spite of this evidence, a direct comparison of the effect of stress on memory in a young and older sample would make it possible to draw more solid conclusions about the results. In this regard, we are carrying out a comparison of the results observed in the older participants included in the first study with a new young sample performing the same picture memory task. The first results of this comparison support the idea of a reduced stress-induced cortisol effect on memory in older people. Finally, in order to reduce the possible effects of confounder variables, the sample used in this thesis is characterized by having a generally good psychological and physical health condition (except for the diagnosis of hypertension). This allows us to control for unknown interactions among HPA-axis activity, cognition and medication or disease. However, it also implies the existence of a possible bias in the sample, reducing the generalization of the results observed to older people with some age-related diseases.

4. Future directions.

The findings raise new exciting and important research questions. Most of them have been discussed in the different sections included in this dissertation, but some

other questions also deserve attention in future studies. It is well known that the sleep process is crucial for learning and memory (Walker, 2009). Additionally, it is also known that sleep is affected in older people (Cirelli, 2012). Thus, it would be very important to study whether differences in sleep characteristics may moderate the effect of stress on memory in young and older people. Another interesting question comes from a complementary result observed in the second study, which showed that older people with a higher subjective socioeconomic status showed a lower stress-induced cortisol response. In this regard, it may be interesting to investigate whether social and psychological variables that moderate the cortisol response may also affect the relationship between acute cortisol increases and memory in older people.

Results of the third study showed that older individuals with lower exposure to endogenous cortisol levels in the previous months might be more vulnerable to the effect of a dysregulation of the diurnal HPA-axis functioning. Along these lines, it would be interesting to investigate whether similar results can be observed with the CAR and its relationship with cognitive performance. Finally, and related to the latter idea, it is well known that individual differences in the cognitive processes or neural networks underlying task performance allow some people to cope better than others with brain damage, which is known as cognitive reserve. Thus, it may be important to investigate whether cognitive reserve can prevent some of the possible effects of HPA-axis dysregulation on cognitive performance.

Chapter 8

Conclusions

The following main conclusions can be drawn from the studies included in this doctoral dissertation:

1. The present dissertation shows that stress-induced cortisol increases do not affect long-term memory retrieval and working memory in older people.

2. It highlights the need for more studies focused on investigating the effect of acute stress on memory performance in older people and the specific processes involved.

3. It shows that older people with lower long-term endogenous cortisol exposure have worse cognitive performance.

4. It suggests that older people with lower endogenous cortisol exposure in the previous months may be more vulnerable to the detrimental effects of a dysregulation of the diurnal HPA-axis activity on cognitive performance.

5. It demonstrates that lower CAR is related to slower walking speed, a measure of physical performances that is closely related to cognitive performance.

6. It suggests that the dysregulation of the morning cortisol levels observed in previous studies in older people with systemic hypertension may be due to sleep characteristics.

7. It indicates that the CAR does not differ between normotensive and hypertensive older people who are undergoing treatment with antihypertensive medication.

8. It confirms previous studies showing that a higher CAR is related to better frontal cortex-related functioning in older people, and it extends this finding to older people with hypertension.

9. It provides a potential clinical application of antihypertensives in diseases that show an attenuated CAR.

As a general conclusion, the present doctoral dissertation adds relevant evidence to the literature on the relationship between the activity of the HPA-axis and cognitive performance in older people. As people age, we can expect a certain dysregulation of the HPA-axis activity. This age-related dysregulation can contribute to inter-individual differences in cognitive performance, but, at the same time, it can reduce the acute effects that cortisol can have on memory processes in stressful conditions.

Chapter 9

Resumen general en español

(General summary in Spanish)

1. Introducción

1.1. Eje hipótalamo-hipófiso-adrenal y glucocorticoides.

Uno de los mayores logros conseguidos por los países desarrollados es el aumento de la esperanza de vida de sus habitantes. Este incremento es tan significativo que niños nacidos después del 2011 tienen una posibilidad entre tres de alcanzar los 100 años y, específicamente en Europa, un cuarto de las personas serán mayores de 60 años en 2020 (European Commission, 2014). Este es un importante cambio en la sociedad que está ocurriendo por primera vez en la historia de la humanidad y está dando lugar importantes nuevos retos que necesitan ser abordados; especialmente aquellos relacionados con enfermedades asociadas al envejecimiento. Es este sentido, uno de los principales objetivos de los nuevos programas de investigación, como por ejemplo Horizonte 2020 en Europa, es el incremento de las posibilidades de mantener un buen estado de salud e independencia en las personas mayores. Para conseguir este objetivo, es crítico conocer qué procesos pueden provocar, o al menos contribuir, a un incremento en las condiciones de vulnerabilidad en el envejecimiento, ya que esto permitirá desarrollar nuevas estrategias de prevención e intervención en población mayor.

Conservar un buen funcionamiento de las capacidades cognitivas resulta especialmente importante para el mantenimiento de una vida independiente. Así, resulta esencial identificar aquellos mecanismos que puedan afectar el rendimiento cognitivo en personas mayores y su adaptación al medio. En este sentido, cambios en la actividad del eje hipotálamo-hipófiso-adrenal (HHA) han sido relacionados con cambios en el rendimiento cognitivo tanto en jóvenes como en adultos, por lo que se convierte en una diana de investigación que pueda arrojar más luz sobre los cambios producidos en el envejecimiento. Siguiendo esta línea, esta tesis presenta los resultados de un trabajo de investigación centrado en estudiar la relación entre el rendimiento cognitivo y la actividad del eje HHA en personas mayores.

El eje HHA es un complejo sistema neuroendocrino que tiene un rol central en el control de la homeostasis en situaciones basales y en situaciones de estrés. Su actividad sigue un patrón circadiano, pero puede ser activado de forma aguda en condi-

ciones de estrés psicológico y/o físico. El eje HHA se compone de tres estructuras localizadas en el hipotálamo, la hipófisis y las glándulas adrenales. Ante la presencia de un estresor físico o psicológico, el núcleo paraventricular del hipotálamo secreta la hormona liberadora de corticotropina y vasopresina desde las terminaciones nerviosas neurosecretoras de la eminencia media al sistema porta hipofisario. Una vez en el lóbulo anterior de la hipófisis, la hormona liberadora de corticotropina y la vasopresina inducen la liberación de hormona adrenocorticotropa en el torrente sanguíneo. Ésta estimula el córtex de la glándula adrenal donde, finalmente, los glucocorticoides son liberados al torrente sanguíneo (Jacobson et al., 2005). El cortisol es el principal glucocorticoide producido por el eje HHA en humanos y esta tesis se centra especialmente en sus efectos.

En situaciones de estrés agudo, los niveles de cortisol alcanzarán sus niveles máximos entre 15 y 20 minutos después de la aparición del estresor, volviendo a sus niveles basales hasta una hora después (Sapolsky et al., 2000). En situaciones no estresantes, en cambio, la secreción de cortisol sigue un ritmo circadiano que puede ser separado en tres componentes discretos: (i) en primer lugar, inmediatamente después de despertar se produce un incremento agudo de los niveles de cortisol que alcanza sus pico entre 30 y 45 minutos después. Este incremento es conocido como la respuesta cortisol (CAR, por su nombre en inglés: *Cortisol Awakening Response*). (ii) Posteriormente, se produce un descenso gradual de los niveles de esta hormona hasta alcanzar sus niveles más bajos a mitad de la noche. (iii) Finalmente, en el último tramo de la noche, mientras el individuo está durmiendo, comienza un incremento gradual de los niveles de cortisol hasta el despertar (Elder et al., 2014). Cada componente del eje HHA y su posible desregularización pueden ser estudiados de forma independiente.

Un característica importante de esta hormona es que tiene propiedades liposolubles, por lo que puede cruzar la barrera hematoencefálica, llegar al sistema nervioso central y afectar estructuras relacionadas con la actividad cognitiva y las emociones (de Kloet et al., 2005). El cortisol produce estos efectos gracias a un importante número de dos tipos de receptores, receptores de glucocorticoides y receptores de mineralocorticoides, que se localizan en muchas de las células del cuerpo humano. En el sistema

nerviosos central, estos receptores se encuentran sobre todo en el hipotálamo, la hipófisis, el córtex frontal, el hipocampo y amígdala. Estas estructuras cerebrales, además, se encargan de regular los niveles de cortisol enviando señales para que se disminuya su producción cuando detectan niveles elevados en el torrente sanguíneo (Sullivan y Gratton, 2002; Jankord y Herman, 2008). Especialmente relevante para el rendimiento de importantes funciones cognitivas, tales como memoria, atención y funciones ejecutivas son el córtex frontal, el hipocampo y amígdala. Relacionado con esto, cambios en los niveles de cortisol pueden afectar el rendimiento de estas estructuras gracias a los receptores de cortisol localizados en ellas. (Sapolsku et al., 2000; Lupien et al., 2005).

1.2. Cambios en la actividad del eje HHA en personas mayores.

La evidencia científica sugiere que durante el proceso de envejecimiento se producen cambios importantes en la actividad del eje HHA. Se ha propuesto que estos cambios se deben principalmente a la excesiva exposición al cortisol que se produce a lo largo de la vida en la personas mayores. Este es el punto de partida de la hipótesis de la cascada de glucocorticoides propuesta por Robert Sapolsky (Sapolsky, 1986). Este autor propuso esta hipótesis basándose en la idea de que una excesiva exposición al cortisol produce una reducción de los receptores de cortisol en el hipocampo. Debido al papel importante del hipocampo en la regulación de los niveles de cortisol, esto provocaría una desregularización de ese feedback negativo, dando como resultado final un aumento de los niveles de cortisol. Finalmente, debido a los efectos dañinos del cortisol, éste provocaría daño neuronal. Efectos similares podrían ser observados en el córtex prefrontal (Lowy et al., 1995). Años después, esta teoría se renombro como la hipótesis de la neurotoxicidad (Gilbertson et al., 2002), ya que se propuso que una exposición prolongada al cortisol incrementaría las posibilidades de las neuronas a ser dañadas por otras amenazas tóxicas. El daño de estas estructuras provocaría, además, una reducción en el número de receptores de cortisol, lo que incrementaría, a su vez, la desregularización del eje HHA. Cabe destacar que no todos las personas mostrarán el mismo grado de desregularización del eje HHA y que importantes diferencias entre individuos pueden ser observadas (Lupien et al., 1996; Seeman et al., 1997). Esta va-

riabilidad estaría explicada por diferentes factores de vulnerabilidad, tales como factores genéticos, personalidad o estilos de vida, que predispondrán a algunos individuos a mostrar una mayor desregularización del eje HHA que otros, lo que se conoce como hipótesis de la vulnerabilidad (Lupien et al., 2009).

En línea con estás hipótesis, diversos estudios han mostrado que con el envejecimiento se produce una disminución en la densidad y sensibilidad de los receptores de cortisol, especialmente en el córtex prefrontal y el hipocampo (Reul et al., 1991; Newcomer et al., 1995; Bhatnagar et al., 1997; Heuser et al., 2000; Heffelfinger y Newcomer, 2001; Nichols et al., 2001; Gupta y Morley, 2004; Giordano et al., 2005; Mizoguchi et al., 2009; Wang et al., 2013). Estos cambios asociados a la edad se verán reflejados en la actividad del eje HHA tanto en situaciones de estrés como en la actividad diurna.

Diversos estudios han mostrado que con la edad se produce una desregularización de la respuesta de cortisol al estrés que se vería reflejado en un mayor incremento en los niveles de cortisol y una recuperación de niveles basales más tardía en comparación con personas jóvenes (Seeman et al., 2001; Kudielka et al., 2004; Traustadóttir et al., 2005; Strahler et al., 2010b; Almela et al., 2011b; pero ver Nicolson et al., 1997 y Rohleder et al., 2002). Acorde con estos resultados, se ha propuesto que el envejecimiento se caracteriza por una pérdida gradual de la habilidad del sistema nervioso central para mantener la homeostasis y adaptarse a situaciones cambiantes, tales como las situaciones estresantes (Bloss et al., 2010; Pardon et al., 2007).

Con respecto a la actividad basal del eje HHA, estudios que han utilizado muestras de saliva o sangre para medir niveles de cortisol han mostrado que con el envejecimiento se produce un aplanamiento de la actividad diurna, mostrando menores niveles en la mañana y mayores en la noche en comparación con personas jóvenes (Dmitriva et al., 2013; Veldhuis et al., 2013; Van Cauter et al., 1996; Ferrari et al., 2001; Kumari et al., 2010; Milcu et al., 1978). Este cambio provocaría un aumento en los niveles de cortisol total en estudios en los que se mide el cortisol con muestras de saliva y sangre. Sin embargo, este aumento mostraría una gran variabilidad entre individuos, y mientras algunas personas mostrarían altos incrementos en sus niveles de cortisol, en otras

personas este incremento sólo serán moderados (Lupien et al., 1996; Seeman et al., 1997).

Observando los cambios que se producen concretamente en el CAR, los resultados obtenidos en estudios previos son contradictorios (Pruessner et al., 1997; Wust et al., 2000; Kudielka et al., 2003; Almeida et al., 2009; Kumari et al., 2010) y hasta el momento no se puede concluir que existan cambios asociados a la edad en este componente del HHA. Sin embargo, sí existe evidencia que parece indicar que una desregularización del eje HHA podría estar asociada a enfermedades que suelen ocurrir en el envejecimiento (Rhebergen et al., 2015; Varadhan et al., 2008; Bruehl et al., 2009). Esta tesis se centrará en la posible relación entre el CAR y la capacidad física, y el CAR en personas mayores con hipertensión.

El tiempo necesitado para caminar unos pocos metros es comúnmente utilizado en clínica como una medida de capacidades físicas en personas mayores (Guralnki et al., 1994). Un bajo rendimiento en esta prueba en personas mayores se ha relacionado con diversos problemas de salud asociados a la edad, tales como demencia, factores de riesgo cardiovasculares y probabilidad de futuras fracturas (Cooper et al., 2011). De hecho, la velocidad al caminar se considera como un buen indicador del estado de salud de un individuo mayor (Fritz y Lusardi, 2009). La evidencia científica ha demostrado que una reducida variabilidad en los niveles de cortisol (bajos niveles por la mañana y altos al final de la tarde) se relaciona con un caminar más lento (Gardner et al., 2011, 2013; Kumari et al., 2010) y se ha propuesto que puede estar debido a que una desregularización del eje HHA provocaría pérdida de masa muscular y fuerza. Sin embargo, la relación entre el CAR y la velocidad al caminar ha sido menos estudiada. Estudios previos han mostrado tanto relaciones positivas como negativas, e incluso ausencia de relación entre CAR y velocidad al caminar (Kumari et al., 2010; Gardner et al., 2011, 2013; Johar et al., 2014). En conjunto, los resultados parecen indicar que puede existir una relación entre estas dos variables, pero se necesitan más estudios para poder entender la dirección de esta relación.

La hipertensión es el factor de riesgo para enfermedades cardiovasculares más importante y el problema de salud más común entre personas mayores (Virdis et al.,

2011). Diversos estudios sugieren que existe una alteración de la secreción de cortisol inmediatamente después de despertar. Wirtz y col. (2007) mostraron que personas con hipertensión sistémica presentaban un CAR atenuado. De forma similar, una secreción total de cortisol menor después de despertar se ha visto relacionada con alta presión arterial (Kuehl et al., 2005), así como con síndrome metabólico (DeSantis et al., 2011) y factores de riesgo cardiovasculares (Rosmond y Björntorp, 2000), incluida alta presión arterial. Cabe destacar que esto tres últimos estudios no midieron específicamente el CAR, sino un índice que representa producción de cortisol por la mañana, lo que representa tanto el CAR como diferencias en los niveles de cortisol justo en el momento de despertar.

1.3. Eje HHA y rendimiento cognitivo.

Como se ha comentado antes, el cortisol tiene un rápido acceso a importante estructuras cerebrales como son el hipocampo, córtex prefrontal y amígdala, debido a su alto número de receptores. Esto conlleva que cambios agudos y crónicos en la actividad del eje HHA puedan afectar el rendimiento en tareas cognitivas dependientes de estas estructuras. En relación con esto, resulta importante investigar si los cambios en el eje HHA que se producen durante el envejecimiento pueden cambiar los efectos que el eje HHA tiene sobre el rendimiento cognitivo.

Con respecto a los efectos de la actividad aguda del eje HHA en situaciones de estrés en jóvenes, son muchos los estudios que han mostrado que incrementos en los niveles de cortisol benefician la consolidación de recuerdo, mientras que parecen perjudicar el recuerdo a largo plazo y la memoria de trabajo (por ejemplo: Buchanan y Lovallo, 2001; de Quervain et al., 2000; Cahill et al., 2003; Roozendaal et al., 2004; Elzinga y Roelofs, 2005; Kuhlmann et al., 2005a, 2005b; Smeets et al., 2008, 2011; Schoofs et al., 2013). Roozendaal (2002) propuso que estos efectos de cortisol en situaciones de estrés se explicarían desde un punto de vista adaptativo en el que el cortisol facilitaría el aprendizaje de estímulos importantes que ocurren durante situaciones estresantes y peligrosas, pero a su vez perjudicaría el recuerdo de material antiguo que pudiese interferir en ese aprendizaje, lo que se conoce como interferencia retro-

activa. Esta información sería de gran utilidad en futuras situaciones para la supervivencia del individuo.

A diferencia de la amplia evidencia en personas jóvenes, muy pocos estudios se han realizado en personas mayores y los resultados parecen indicar que el efecto agudo del cortisol podría estar atenuado en esta población. Estudios previos sugieren que aumentos agudos de forma farmacológica de los niveles de cortisol no afectarían la memoria de trabajo, y que la exposición a estrés afectaría específicamente el rendimiento en interferencia retroactiva (Bohnen et al., 1990; Lupien et al., 1997; Domes et al., 2002; Wolf et al., 2001; Porter et al., 2002; Yehuda et al., 2007; Almela et al., 2011a; Hidalgo et al., 2014). Sin embargo, ningún estudio ha investigado los efectos del estrés en tareas de recuerdo a largo plazo o en tareas de memoria de trabajo en personas mayores.

En referencia a los efectos de la actividad diurna del eje HHA en el rendimiento cognitivo, muchos estudios han mostrado que una desregularización del eje HHA (expresado en altos niveles de cortisol diurno) se relacionaría con un peor rendimiento cognitivo en personas mayores (Hodgson et al., 2004; Karlamangla et al., 2005; MacLullich et al., 2005; Li et al., 2006; Kuningas et al., 2007; Lee et al., 2007, 2008; Beluche et al., 2010; Comijs et al., 2010; Evans et al., 2011; Franz et al., 2011; Gerritsen et al., 2011; Johansson et al., 2011). Cabe destacar que estos estudios se han realizado utilizando muestras de saliva o sangre para medir la actividad del eje HHA, por lo que estos han medido la actividad circadiana de esta hormona. Sin embrago, hasta el momento no se ha estudiado si cambios en la exposición durante largos periodos de tiempo en niveles endógenos de cortisol pueden estar relacionados con cambios en el rendimiento cognitivo en personas mayores sanas.

Por último, varios estudios han mostrado que un CAR atenuado se relaciona con un mejor rendimiento en tareas dependientes de córtex frontal (Almela et al., 2012; Evans et al., 2012; Bäumer et al., 2014a, 2014b). La velocidad al caminar, como medida de capacidad física, ha sido consistentemente relacionada con el rendimiento cognitivo en pruebas dependientes de córtex frontal y con factores de riesgo cardiovasculares en personas mayores. Siguiendo estos hallazgos, resulta de gran interés determinar si el

CAR se encuentra relacionado con la velocidad al caminar, debido a que ambos parecen estar relacionados con similares cambios asociados a la edad. Por último, resulta importante investigar si cambios en la secreción de cortisol inmediatamente después de despertar debido a problemas de salud, como por ejemplo el caso de personas con hipertensión, puede relacionarse con el rendimiento cognitivo en estás personas.

2. Objetivo

El objetivo general de esta tesis doctoral es investigar si la actividad del eje HHA en situaciones de estrés y en situaciones basales puede afectar o se encuentra relacionado con el rendimiento cognitivo en personas mayores.

2.1. Objetivos específicos e hipótesis de cada estudio

Estudio 1: Investigar los efectos de estrés agudo en el rendimiento en tareas de recuerdo a largo plazo en personas mayores. Considerando los resultados en personas jóvenes, se espera que incrementos agudos de cortisol perjudiquen el rendimiento en estas tareas.

Estudio 2: Investigar los efectos de estrés agudo en el rendimiento en tareas de memoria de trabajo en personas mayores. Dos predicciones posibles pueden hacerse en este estudio: Considerando los resultados en estudios previos con administraciones exógenas de cortisol, no se espera encontrar un efecto del estrés en estas tareas, al menos en hombres. Sin embargo, considerando el efecto previo del estrés observado en interferencia retroactiva, se espera un efecto perjudicial del estrés en el rendimiento en memoria de trabajo, al menos en mujeres.

Estudio 3: Investigar si diferencias individuales en la exposición a largo plazo de niveles de cortisol y de la actividad diurna del eje HHA se relaciona con diferencias individuales en el rendimiento cognitivo en personas mayores. En base a estudios previos, se espera que mayores niveles de cortisol los meses previos y una mayor desregularización del eje HHA se relacionen con un peor rendimiento cognitivo.

Estudio 4: Investigar la relación entre el CAR y la velocidad al caminar en personas mayores. Teniendo en cuenta los resultados en estudios previos, se espera que un CAR atenuado se relacione con una caminar más lento.

Estudio 5: Investigar las diferencia en CAR y cortisol inmediatamente después de despertar en personas mayores normotensas e hipertensas. Además, se estudiará si el CAR en estas personas se relaciona con su rendimiento en tareas cognitivas especialmente dependientes de córtex frontal. En base a estudios previos, se espera observar un CAR atenuado, menor cortisol matutino y peor rendimiento cognitivo en personas con hipertensión. Además, se espera una relación positiva entre CAR y rendimiento cognitivo.

3. Metodología

3.1. Descripción general de los participantes

La muestra de los cinco estudios que se presentan en esta tesis proviene de un programa de estudios de la Universidad de Valencia para personas mayores de 55 años (NAU GRAN). Los criterios de exclusión generales fueron: presencia de problemas importantes de visión o audición, fumar más de diez cigarrillos al día, abuso de alcohol u otras drogas, presencia de enfermedades neurológicas, psiquiátricas o endocrinas, uso de medicación relacionada con el rendimiento cognitivo o el control emocional, o que pueda afectar los niveles hormonales, tales como glucocorticoides, medicación para diabetes, antidepresivos, anticoagulantes, benzodiacepinas o antipsicóticos. Además se excluyeron aquellas personas que estuvieron bajo los efectos de anestesia general o que hubiesen vivido un evento vital estresante en algún momento desde un año antes de la participación en los estudios. Todas las mujeres eran postmenopáusicas y habían tenido su último periodo menstrual al menos un año antes.

3.2. Procedimiento

Estudio 1: Este estudio fue diseñado para investigar el efecto del estrés agudo en el recuerdo a largo plazo. Para esto, 76 participantes (38 hombres y 38 mujeres) de entre 56 y 76 años realizaron una tarea de aprendizaje por la mañana. Aprendieron

una serie de 30 imágenes (10 emocionalmente positivas, 10 negativas y 10 neutras), una lista de 15 palabras (el test de aprendizaje verbal de Rey) y dos historias (subtest de historia del test Rivermead). Al día siguiente por la tarde, la mitad de esas personas participaron en una tarea estandarizada de laboratorio de estrés psicosocial (Trier Social Stress Test; TSST) y la otra mitad en una tarea control no estresante. Posteriormente se les pidió que recordaran el material que habían aprendido el día anterior. Se recogieron muestras de saliva a lo largo de las dos sesiones para poder medir sus niveles de cortisol y alfa-amilasa (una enzima que es considerada un indicador indirecto de la actividad simpática).

Estudio 2: En este estudio se diseñaron dos experimentos para investigar el efecto del estrés agudo en memoria de trabajo. En el primer experimento 63 personas (30 hombres y 33 mujeres) de entre 55 y 77 años realizaron la tarea de Digit Span Directo y Digit Span Inverso inmediatamente antes de participar en el TSST y 10 minutos después de éste. En el segundo experimento, 37 participantes (19 hombres y 18 mujeres) de entre 56 y 76 años participaron en el TSST y 10 minutos después realizaron la tarea de secuenciación de Letras y Números. Un segundo grupo de 39 participantes (19 hombres y 20 mujeres) de entre 56 y 76 años participaron en una tarea control y 10 minutos después realizaron la tarea de secuenciación de Letras y Números. Se recogieron muestras de saliva a lo largo de los dos experimentos para poder medir sus niveles de cortisol (experimento 1 y 2) y alfa-amilasa (experimento 2).

Estudio 3: En este estudio 57 personas (14 hombres y 43 mujeres) de entre 56 y 77 años participaron en una evaluación neuropsicológica en las que se les evaluó su rendimiento en tareas de atención y función ejecutiva (Trail Making Test A y B), memoria de trabajo (Digit Span Directo e Inverso), aprendizaje, recuerdo a corto plazo y recuerdo demorado (el test de aprendizaje verbal de Rey y subtest de historia del test Rivermead). Además, se les extrajo una muestra de cabello de al menos tres centímetro de largo del vertex posterior de la cabeza. Esta muestra se utilizó para medir los niveles de cortisol endógeno a los que estuvo expuesta la persona durante los tres meses previos al estudio. Por otro lado, estos participantes recogieron 3 muestras de saliva (inmediatamente después de despertar, 30minutos después de despertar y a las

23hs) dos días consecutivos entre semana. Estas muestras se utilizaron para medir la actividad diurna del eje HHA.

Estudio 4: En este estudio 86 personas (41 hombres y 45 mujeres) de entre 56 y 72 años realizaron una prueba de velocidad de caminar. Los participantes caminaron a lo largo de 10 metros a una velocidad normal, se dieron la vuelta y caminaron nuevamente esos 10 metros lo más rápido posible sin llegar a correr. Se midió el tiempo para caminar los seis metros centrales en cada dirección. Además, todos los participantes realizaron muestras de saliva inmediatamente después de despertar, 30 minutos y 45 minutos después de despertar durante dos días seguidos para medir el CAR.

Estudio 5: En este estudio 29 personas normotensas (14 hombres y 15 mujeres) y 27 personas con hipertensión sistémica (14 hombres y 13 mujeres) de entre 56 y 78 años participaron en una evaluación neuropsicológica focalizada en evaluar especialmente su rendimiento en tareas dependientes de córtex frontal, tales como tareas de atención, velocidad de procesamiento (Trail Making Test A y Palabras y Colores del test de Stroop), función ejecutiva (Trail Making Test B y tarea de interferencia de Stroop), memoria de trabajo (Digit Span Directo e Inverso), aprendizaje, recuerdo a corto plazo y recuerdo demorado (el test de aprendizaje verbal de Rey). Además, durante dos días recogieron muestras de saliva inmediatamente después de despertar, 30 minutos y 45 minutos después de despertar, y los días previo, inmediatamente antes de dormir.

4. Resultados y principales conclusiones

Los resultados del **primer estudio** mostraron que el estrés no afectó el rendimiento en recuerdo a largo plazo de imágenes, palabras o historias en hombres y mujeres mayores. Además, la secreción total de cortisol y, específicamente, la respuesta de cortisol a estrés no se relacionaron con el rendimiento en memoria de los participantes. Los resultados de los dos experimentos del **segundo estudio** mostraron que las mujeres mayores tuvieron un mejor rendimiento en la tarea de Digit Span Directo (una tarea que evaluá el componente de span de memoria de la memoria de trabajo) después de participar en una tarea de estrés. Sin embargo, el rendimiento en Digit Span Inverso y secuenciación de Letras y Números (dos tareas que evalúan el componente

ejecutivo de la memoria de trabajo), no se vio afectado por el estrés. Los resultados mostraron que altos niveles de cortisol (después de la tarea estrés) en el momento de la tarea de memoria de trabajo, se relacionaban solamente con el rendimiento en el componente de span de memoria de la memoria de trabajo en mujeres. La respuesta de cortisol a la tarea de estrés no se relacionó ni con el componente de span de memoria, ni con el componente ejecutivo de la memoria de trabajo en ambos sexos.

En conjunto, los resultados de estos dos primeros estudios apoyan la idea de que las personas mayores podrían ser menos sensibles a los efectos agudos de la respuesta de cortisol en el rendimiento en memoria. Una posible explicación para esta sensibilidad reducida sería la disminución en densidad y sensibilidad de receptores de cortisol que se ha observado especialmente en el córtex prefrontal y el hipocampo y que estaría asociado al proceso de envejecimiento, (Reul et al., 1991; Newcomer et al., 1995; Bhatnagar et al., 1997; Heuser et al., 2000; Heffelfinger y Newcomer, 2001; Nichols et al., 2001; Gupta y Morley, 2004; Giordano et al., 2005; Mizoguchi et al., 2009; Wang et al., 2013). Este es un importante hallazgo ya que pone de manifiesto que, en personas mayores, la respuesta ante situaciones amenazantes y estresantes podría ser menos adaptativa que en jóvenes. Como se indicó previamente, se ha sugerido que el efecto específico que tiene el estrés y el cortisol en el rendimiento en memoria es parte de un complejo mecanismo que persigue aumentar las posibilidades de supervivencia del individuo. En personas jóvenes, el estrés mejoraría la consolidación de información aprendida, mientras que perjudicaría el recuerdo (por ejemplo: Buchanan y Lovallo, 2001; de Quervain et al., 2000; Cahill et al., 2003; Roozendaal et al., 2004; Elzinga y Roelofs, 2005; Kuhlmann et al., 2005b; Smeets et al., 2008, 2011; Schoofs et al., 2013), facilitando de este modo el aprendizaje de nueva información relevante para evitar y prevenir futuras situaciones peligrosas (Roozendaal, 2002). Si el cortisol no es capaz de afectar el rendimiento en personas mayores, y a su vez, parece que podría aumentar el efecto de interferencia retroactiva (Almela et al., 2011a), es posible que estas personas tengan, en un futuro, peor recuerdo de situaciones estresantes y amenazantes. Esta condición aumentaría la vulnerabilidad de las personas mayores ya que éstas no se beneficiarían del aprendizaje de información crítica para evitar futuros peligros.

Cabe destacar que estos resultados tienen, además, una posible implicación clínicas. Se ha sugerido que el efecto del cortisol en memoria podría ser un factor clave para la eficacia de las terapias de exposición en el tratamiento de fobias específicas ya que altos niveles de cortisol facilitarían el aprendizaje de extinción necesario en este tipo de terapias. Si los resultados de esta tesis se confirman en futuros estudios, esto podría indicar que la eficacia de las terapias de exposición se vería reducida en personas mayores.

Los resultados del **tercer estudio** muestran que mayores niveles de cortisol en muestras de cabello (que indica en este caso una exposición a mayores niveles de cortisol endógenos los tres meses previos al estudio) se relacionan con un mejor rendimiento en tareas de memoria de trabajo, aprendizaje, recuerdo a corto plazo y recuerdo demorado. Además, mayores niveles de cortisol en muestras de saliva (que indica en este caso una desregularización de la actividad diurna del eje HHA) se relacionaron con un peor rendimiento en tareas de atención, span de memoria y recuerdo a corto plazo. Finalmente, aquellos individuos con exposición a bajos niveles de cortisol los meses previos, mostraron un peor rendimiento en memoria de trabajo y memoria a corto plazo cuando presentaban una mayor desregularización del eje HHA.

Los resultados de este estudio van en línea con la literatura previa que ha observado que una desregularización de la actividad diurna del eje HHA se relaciona con peor rendimiento cognitivo (por ejemplo: Hodgson et al., 2004; Karlamangla et al., 2005; MacLullich et al., 2005; Li et al., 2006; Kuningas et al., 2007; Lee et al., 2007, 2008; Beluche et al., 2010; Comijs et al., 2010; Evans et al., 2011; Franz et al., 2011; Gerritsen et al., 2011; Johansson et al., 2011). Pero además, estos resultados son importantes ya que muestran que bajos niveles de cortisol los meses previos podría ser igual de perjudicial que una desregularización de la actividad diurna del eje HHA. Además, este estudio sugiere que una exposición a bajos niveles de cortisol podría aumentar la vulnerabilidad de estas personas mayores a que una desregularización del eje HHA perjudique su rendimiento cognitivo.

Los resultados del **cuarto estudio** mostraron que un CAR atenuado se relaciona con un caminar más lento en personas mayores. Esto pone de manifiesto que una des-

regularización del CAR se vería relacionado con unas peores capacidades físicas que, a su vez, se encuentran estrechamente relacionados con un peor rendimiento cognitivo en tareas dependientes de córtex frontal y con factores de riesgos cardiovasculares.

Finalmente, el **quinto estudio** muestra que los participantes con hipertensión se despertaron antes y durmieron menos horas que los participantes normotensos, lo que dio lugar a que presentaran menores niveles de cortisol al despertar. Este estudio no mostro diferencias en la magnitud del CAR entre los dos grupos, pero un resultado muy importante es que el tiempo que aquellas personas que más tiempo levaban en tratamiento con antihipertensivos presentaron un mayor CAR. Además, se observó que un mayor CAR se relacionó con mejor rendimiento en tareas de función ejecutiva y de velocidad de procesamiento. En conjunto, este estudio hace una importante contribución a la literatura añadiendo más evidencia a la relación positiva entre rendimiento cognitivo en tareas dependientes de córtex frontal y el CAR en personas mayores (Almela et al., 2012; Evans et al., 2012). Además, sugiere un potencial uso clínico de los antihipertensivos en aquellas enfermedades en las que también se han observado atenuaciones de la magnitud del CAR, como pueden ser diabetes tipo II, burnout o estrés postraumático (Chida y Steptoe, 2009; Fries et al., 2009; Keeshin et al., 2014; Marchand et al., 2014). Estos resultados sugieren que el efecto protector de los antihipertensivos en el funcionamiento cognitivo podría estar mediado por su efecto en el CAR.

En resumen, las siguientes conclusiones generales se pueden extraer de los estudios que comprenden esta tesis doctoral:

1. Esta tesis muestra que incremento agudos de cortisol debido a estrés no afecta el rendimiento en recuerdo a largo plazo y en memoria de trabajo en personas mayores.

2. Pone de manifiesto la necesidad de realizar más estudios centrados en los efectos del estrés agudo en el rendimiento en memoria en personas mayores y en los procesos específicos involucrados.

3. Muestra que bajos niveles de cortisol endógeno durante largos periodos de tiempo están relacionados con peor rendimiento cognitivo en personas mayores.

4. Sugiere que aquellas personas mayores exposición a bajos niveles de cortisol endógeno los meses previos pueden ser más vulnerables a los efectos negativos en el rendimiento cognitivo de una desregularización de la actividad diurna del eje HHA.

5. Demuestra que un CAR atenuado se relacionaría con un caminar más lento, una medida de capacidades física que se encuentra estrechamente relacionado con el rendimiento cognitivo en personas mayores.

6. Sugiere que la posible desregularización de los niveles matutinos de cortisol observados en estudios previos en personas mayores con hipertensión pueden deberse a las características de sus patrones de sueño.

7. Indica que la magnitud del CAR no difiere entre personas mayores normotensas e hipertensas que se encuentran bajo tratamiento con antihipertensivos.

8. Confirme resultados de estudios previos que muestran en personas mayores una relación entre un mayor CAR y un mejor rendimiento en tareas cognitivas relacionadas con córtex frontal, y extiende estos resultados a personas con hipertensión.

9. Propone un potencial uso clínico de los antihipertensivos en enfermedades que muestran un CAR atenuado.

Como conclusión general, esta tesis doctoral añade evidencias importantes a la literatura centrada en investigar la relación entre la actividad del eje HHA y el rendimiento cognitivo en personas mayores. Conforme envejecemos, podemos esperar una cierta desregularización en la actividad del eje HHA. Este cambio asociado a la edad contribuye a las diferencias interindividuales en el rendimiento cognitivo observado en personas mayores, pero al mismo tiempo, estos cambios pueden reducir el efecto que el cortisol tiene sobre los procesos de memoria en situaciones estresantes.

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