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Department of Psychobiology

Stress effects on cognitive function in healthy adults

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Presented by:
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Promotor:
Dra. Alicia Salvador Fernández-Montejo

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THESIS OUTLINE

In our lives, we are constantly exposed to different sources of stress, specifically psychological or social. Our reaction to stress is an adaptive response due to its role in facilitating survival. However, stress can also have immediate and delayed damaging consequences for health, and it is considered one of the most significant health problems of the 21st century, according to the World Health Organization (2001).

The impact of stress extends to most of the physiological systems (i.e. cardiovascular, digestive, immune, neuroendocrine or nervous), resulting in numerous diseases. Cognitive problems stand out among the stress effects related to the nervous system. Given the large impact that these problems can have on society in general, and on individuals in particular, the need to understand more about this link is clear. This is one of the reasons for the growing interest in investigating the main mechanisms underlying the stress impact on different cognitive processes, such as memory, attention or executive functions. Several factors related to the characteristics of the stressor, the individual and the cognitive process assessed seem to play an important role in determining the direction of these stress effects. Thus, this thesis focuses on the way stress affects cognition, specifically memory performance, in healthy adults, analyzing the role of some of these factors.

The first section of the first chapter discusses the evolution of the stress concept and explains what the stress response is. In the second part, the link between stress and memory is explained, detailing which brain structures are related to the control of the stress response and the cognitive processes. Then, a brief summary of the studies about the effect of acute stress on memory performance is presented. Moreover, the Hypothalamus-Pituitary-Adrenal axis (HPA-axis) in basal conditions (non-stress) is addressed, again summarizing the studies that have investigated the relationship between HPA-axis functioning and cognitive performance. Finally, the chapter ends with the main goals and

hypothesis of this thesis and a general description of the material and methods used in the empirical chapters.

In the second chapter, the first study is presented. In this study, we examined the effects of stress-prior learning on two types of memory (i.e. non-declarative and declarative memory) in young adults. Here, the material to be remembered is neutral, and the role of sex is considered. Next, in the third chapter of the thesis, following a similar design, the second study carries out a direct comparison of older and young adults.

The fourth chapter describes the third study, which investigates the stress effects on memory retrieval. Now, the stressor is applied before the retrieval tasks, and the material to be remembered is neutral and emotional. Again, older and young adults of both sexes are compared.

In the last study, the fifth chapter analyzes the relationships between the cortisol awakening response (CAR) and the diurnal cortisol slope (DCS), two different components of the diurnal cortisol cycle, and different memory tasks.

The sixth chapter contains a general discussion and the main findings of the aforementioned studies; the strengths and limitations of this thesis and the direction of the next steps in the research on this topic are discussed here. Finally, the seventh chapter presents the main conclusions of the studies included in this thesis.

ABBREVIATIONS

ACTH = Adrenocorticotropic Hormone

ANS = Autonomic Nervous System

AVP = Arginine Vasopressin

BLA = Basolateral Amygdala

BMI = Body Mass Index

CAR = Cortisol Awakening Response

CAs = Catecholamines

CRH = Corticotropin Releasing Hormone

DCS = Diurnal Cortisol Slope

ENS = Enteric Nervous System

GCs = Glucocorticoids

GRs = Glucocorticoid Receptors

HPA-axis = Hypothalamic-Pituitary-Adrenal axis

MRs = Mineralcorticoid Receptors

PFC = Prefrontal Cortex

PNS = Parasympathetic Nervous System

PVN = Paraventricular Nucleus

sAA = Salivary Alpha-Amylase

SES = Socioeconomic Status

SCN = Suprachiasmatic Nucleus

SNS = Sympathetic Nervous System

TSST = Trier Social Stress Test

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CHAPTER I

GENERAL INTRODUCTION

1.1. WHAT IS STRESS?

1.1.1. Evolution of the stress concept

The term "stress" was employed for the first time in the field of Physics as the force exerted on an object that was "distorted" (strain). But the most important use comes from Physiology. Thus, Walter Cannon introduced the homeostasis concept to refer to physiological coordinated processes that act to keep most organisms' states constant (an organism's internal regulatory balance). He highlighted the importance of Autonomic Nervous System (ANS) activation through its preparatory function to cope with the situation, emitting "fight or flight" behaviors (Cannon, 1932).

Years later, the physiologist Hans Selye defined stress as a non-specific response to a stimulus that disturbs the homeostasis, and he extended Cannon's theory, arguing that in the stress response, both ANS and Hypothalamic-Pituitary-Adrenal axis (HPA-axis) activation occur. Selye described a general adaptation phenomenon in rats exposed to a wide variety of noxious stimuli; rats went into an adaptation phase (maladaptation) leading to death from a non-specific reaction (Selye, 1936). The entire response process was called the "General Adaptation Syndrome" (Selye, 1956), with three stages: (i) the Alarm Reaction: this is the initial response to the stressor, characterized by ANS activation, and the physiological changes are designed to obtain the maximum energy resources to deal with the stressor by fighting or fleeing. This stage cannot be maintained continuously in time; therefore, if the stressor continues and the organism is not dead, the next stage will take place. (ii) The Resistance Stage: this is characterized by HPA-axis activation, where the main goal is to ensure an efficient distribution of energy. The energy mobilization and inhibition of sexual and reproductive activities (both activities without an immediate purpose for survival) are the main physiological changes produced in this stage. (iii) The Exhaustion Stage: this is the final stage of the

syndrome, and at this point, the organism has lost the ability to adapt. Consequently, a number of diseases (diseases of adaptation) will occur, such as hypertension, gastrointestinal ulcers, nervous disorders, among others.

However, later there was a change of focus in explaining stress. To date, studies have focused on the physical aspects of stress, but based on the idea that there are individual differences in the way the same situation produces different responses in different individuals, the psychological aspects of stress began to be considered. First, Mason (1968) described three main psychological characteristics that would cause a situation to be interpreted as stressful by the individual: novelty, unpredictability and lack of control. After that, Lazarus and Folkman (1984) proposed an integrative approach. In their stress model, these authors included two new concepts that may help to explain this variability: the appraisal (cognitive assessment made by the subject about the stressful situation) and coping (strategy adopted by the individual to deal with the stressful situation based on the appraisal). Then, they considered: first, the interaction between the stressors and the stress reaction of the subject; second, the appraisal about the situation and their own abilities to solve it or not; and, finally, the strategies adopted by the individual and the efficiency in obtaining the adaptation to the situation.

Years later, based on the allostasis concept proposed by Sterling and Eyer (1988), which describes the organism's ability to maintain the homeostasis or stability through changes, McEwen and Stellar (1993) introduced the allostatic load concept as the key to understanding the stress/disease binomial. Thus, the body has systems (i.e. the ANS, the HPA-axis, the metabolic and immune systems) that promote this allostasis in stressful situations. However, at the same time, these systems can also cause problems for the body if they are overactive or underactive. Therefore, the allostatic load is the price the organism pays for striving to maintain homeostasis.

1.1.2. Stress response

The stress response is the set of physiological and psychological changes that occur in the individual when exposed to a stressful situation/stimulus or one considered stressful. These changes are produced in order to enable the individual to cope with the stressor and/or suffer the least possible damage. The integration of the information about the stimulus or stressful situation converges in the hypothalamus, more specifically, in the Paraventricular Nucleus (PVN), which activates the ANS and HPA-axis, the most important systems involved in the stress response. It is important to note that, in addition to the physiological changes described below, psychological changes also occur in a stressful situation (Lupien and Schramek, 2006). Although there are differences among individuals in how they perceive the stressor, it is usually a negative experience that increases anxiety and negative mood while reducing positive mood.

1.1.2.1. Autonomic Nervous System (ANS)

First, the ANS is activated by the stressor promoting the "fight or flight" response. This system is composed of three branches: (i) Sympathetic Nervous System (SNS), (ii) Parasympathetic Nervous System (PNS), and (iii) Enteric Nervous System (ENS). In a stressful situation, the SNS is activated and, consequently, large amounts of catecholamines (CAs, noradrenaline and adrenaline) are released. Specifically, noradrenaline is secreted at sympathetic nerve endings in tissues and glands throughout the body, while sympathetic preganglionic neurons activate the adrenal medulla, which releases adrenaline and, to a lesser extent, noradrenaline (Granger et al., 2007). The binding of CAs to the adrenergic receptors (i.e. α1, α2, β1 and β2) leads to physiological changes, such as enhanced cardiovascular tone, respiratory rate, blood flow, elevated glucose in blood, dilation of pupils and

diminishing vegetative functions (Chrousos, 2009; Chrousos and Gold, 1992). These changes are designed to ensure the individual's adaptation to the situation, as they provide greater blood flow and energy to the organs needed to overcome the stressful situation, to the detriment of other organs that are less relevant or not at all relevant in confronting stress.

Recently, a growing body of research considers salivary alpha-amylase (sAA) as a target biomarker in the ANS reactivity to stress (for a review of this topic see: Granger et al., 2007; Nater and Rohleder, 2009; Rohleder and Nater, 2009). sAA is an oral enzyme secreted by the salivary glands (mainly parotid glands) as a result of the action of noradrenaline released from sympathetic nerve endings on β-adrenergic receptors located in the salivary glands. Its secretion takes place completely in the oral cavity, and the sAA levels in saliva do not represent a portion of sAA levels circulating because it is not actively transported or passively diffused into saliva from the bloodstream, in contrast to other analytes present in saliva. Some of its functions are: the digestion of carbohydrates (Baum, 1993) and maintaining oral health, preventing and eliminating bacteria from the mouth (Scannapieco et al., 1993).

The sAA has been proposed as a potential substitute for catecholamines in psychoneuroendocrinological studies because, on the one hand, it is easily accessible and obtained from human saliva in a non-invasive way and, on the other, it reflects the SNS activity.

1.1.2.2. Hypothalamic-Pituitary-Adrenal axis (HPA-axis)

Minutes after the onset of the stressor, the next stress system activated is the HPA-axis. Its functioning is established through communication among three structures, the hypothalamus, the pituitary gland and the adrenal gland. Thus, when

the individual is facing a physical or psychological stressor, the HPA-axis is activated, resulting in large amounts of corticotropin releasing hormone (CRH) and arginine-vasopressin (AVP) from the PVN of the hypothalamus. These two hormones cause the release of adrenocorticotropic hormone (ACTH) in the bloodstream by the pituitary gland action. Then, the ACTH stimulates the adrenal glands, and as a result, glucocorticoids (GCs) are released into the bloodstream (Ulrich-Lai and Herman, 2009). In humans, the most important glucocorticoid is cortisol.

HPA-axis functioning under basal situations (non-stress) follows a circadian pattern, where the cortisol levels achieve a peak 30 minutes after awakening, in order to prepare the organism to cope with the daily demands, and they decrease throughout the day with lower levels at night. Moreover, the HPA-axis activity is controlled by a negative feedback system because the continued exposure to high levels of GCs can cause serious health problems for the individual. There are two ways to regulate this negative feedback system: hormonal and neural inhibition. The former occurs when large amounts of GCs are in the bloodstream and the release of CRH and AVP is inhibited by the PVN (Whitnall, 1993). The latter takes place through the inhibitory action of the hippocampus and prefrontal cortex on the PVN (Patel et al., 2000). Further explanation of the latter will be provided in point 2 of this introduction.

Two important GC actions in the organism are worth noting: (i) modulating actions and (ii) preparative actions (for a review on this topic see: Sapolsky et al., 2000). The modulating actions are those that affect the organism's response to the stressor. Among this type of actions, the authors distinguish permissive, suppressive and stimulating actions. Permissive actions are carried out by the basal GCs present before the stress, and they strengthen the defense mechanisms through which the organism responds to the stressor. By contrast, suppressive actions are exerted by the stress-induced GC peak; therefore, they are delayed actions and take place

about one hour from the onset of the stressor. Their final aim is to stop the stress-activated defense reactions, returning the organism to homeostasis, in order to protect it from the damage caused by an overshoot. Likewise, the stimulating actions are exerted by the stress-induced GC peak after about one hour from the onset of the stressor, but the difference between this type of actions and the previous actions is that the stimulating actions reinforce the first wave of hormonal response to the stressor, like the permissive actions. Finally, the preparative actions are those that do not affect the immediate response to a stressor, but instead modulate the organism's response to a subsequent stressor through mediating or suppressive actions.

The GCs' actions can be grouped in two categories characterized by the time the GCs need to exert their effects: (i) non-genomic and (ii) genomic effects (for a review on this topic see: Stahn and Buttgereit, 2008). The non-genomic effects or non-classical mechanism is the faster route because the GCs exert their effects in a short period of time, from seconds to minutes, once there is an increase in GC levels. These fast effects include rapid negative feedback-inhibition of the HPA-axis. It is not clear whether these non-genomic effects occur through the membrane actions of the nuclear receptors (GRs and MRs) or through other unidentified membrane receptors (Ulrich-Lai and Herman, 2009). The genomic effects or classical mechanism is the slower route because the GCs exert their effects over a long period of time, from minutes to hours. These effects are determined by intracellular receptors found in the cytosol and bound to stabilizing proteins in their inactive state. The GCs penetrate the cellular membrane, thanks to their lipophilic nature, and they bind to intracellular receptors, forming a "hormone-receptor" complex. This union translocates to the inside of the nucleus, where it binds to specific DNA sites and regulates the gene transcription of different proteins (Herman and Spencer, 1998; Ulrich-Lai and Herman, 2009).

There are two types of receptors for GCs, the mineralcorticoid receptors (MRs), or Type I, and the glucocorticoid receptors (GRs), or Type II. They show two main differences: affinity, and their distribution in the brain. Regarding their affinity, the MRs have from 6 to 10 times more affinity to GCs than GRs (Reul and de Kloet, 1985). Under basal situations, the MRs are occupied, so that this type of receptor is responsible for the effects of low cortisol levels. However, when cortisol levels are higher due to a stressful situation or circadian peak of cortisol (30 minutes after awakening), the MRs are saturated, and the rest of the cortisol has to bind to GRs. This type of receptor is involved in the stress-induced glucocorticoid effects and in the feedback inhibition of the HPA-axis (de Kloet et al., 2005). In addition, their distribution in the brain is different. While MRs are located especially in the hippocampus, the GRs are found mainly in the prefrontal cortex (Lupien et al., 2007).

1.2. THE LINK BETWEEN STRESS AND MEMORY

There is a close interaction between stress and memory, probably due to its adaptive function throughout evolution. This fact could explain the high degree of overlap between the neurobiological systems that regulate both the stress response and the memory function. Thus, the brain areas related to the control of the stress response (i.e. hippocampus, prefrontal cortex and amygdala) are also involved in the memory function (Lupien and Lepage, 2001; Lupien et al., 2007; Roozendaal et al., 2009). Therefore, it is not unusual to expect stress-related changes in these brain areas to be reflected in changes in memory performance.

2.1. How stress affects memory

As mentioned above, in response to stress, the SNS and HPA-axis are activated, and consequently, large amounts of CAs and GCs are secreted. These stress biomarkers exert their effects in two different ways. While GCs exert their action by crossing the blood-brain barrier and binding to MRs and GRs located in the hippocampus, prefrontal cortex and amygdala, CAs activate the β-adrenergic receptors on vagal afferents projecting to the nucleus of the solitary tract in the brainstem (McGaugh, 2000), and these noradrenergic projections influence the neuronal activity of the amygdala (Packard et al., 1995).

1.2.1.1. Hippocampus

The hippocampus, located deep within the medial temporal lobe, is considered a main structure of the limbic system. This area presents the largest number of receptors for GCs, and its functions control the circadian rhythm of GCs (Fischette et al., 1980) and inhibit the HPA-axis activity in response to stress (Fendler et al., 1961). This inhibitory action occurs because the glutamatergic hippocampal projections activate the GABAergic neurons of the bed nucleus of the stria terminalis, the medial preoptic area, the dorsomedial hypothalamus, and other hypothalamic nuclei, which in turn, exert an inhibitory action on the PVN of the hypothalamus (Cullinan et al., 1993; Herman et al., 2003).

As mentioned above, this structure is also involved in memory processes. This was supported by the well-known case of the patient H.M. This person suffered from epilepsy, and in order to avoid it, his medial temporal lobe (mainly the hippocampus, but also the amygdala and other temporal cortical structures) was removed. Consequently, H.M. developed anterograde amnesia, the inability to store new knowledge in long-term memory, although his short-term and long-term

memories from before the surgery and his ability to acquire new motor skills were intact. This finding illustrated the relevance of the hippocampus in declarative memory (Scoville and Milner, 1957).

In animals, spatial memory has been studied as an analogous form of declarative memory, using the water maze designed by Morris. On this task, the animals have to learn, aided by contextual cues, where the platform is. Good performance on this task requires the integrity and functional activation of the hippocampus (Morris et al., 1982). It seems that the hippocampus underlies the processes of spatial learning and memory because it has "place cells" (i.e. type of cells that fire in response to the animal's specific location in the environment) and virtually creates a "cognitive map" of the environment (O'Keefe and Nadel, 1978). Interestingly, in humans the hippocampus is activated during spatial memory tasks in taxi drivers (Maguire et al., 1998).

1.2.1.2. Prefrontal cortex (PFC)

The PFC is located in the most anterior part of the frontal lobes. The PFC is involved in integrating information from the stressful stimuli. Like the hippocampus, it has numerous GC receptors and exerts an inhibitory action on the HPA-axis through PVN inhibition resulting from the GABAergic neuron activation in the preoptic area of the hypothalamus, the nucleus of the solitary tract, and the bed nucleus of the stria terminalis, among others. Given that the PFC connects to the hippocampus and the amygdala, it is considered one of the most important areas in the coordination and control of HPA-axis functioning in response to stress (Ulrich-Lai and Herman, 2009).

Moreover, the PFC is also involved in several cognitive processes, such as affect, emotion, social behavior, language and intelligence. Some of its functions

include its role in executive function, the ability to temporally organize purposeful behavior, language and reasoning, and it includes attention, working memory, planning, temporal integration, decision-making, monitoring and inhibitory control (Fuster, 2008). Therefore, the PFC is crucial for working memory (Galloway et al., 2008).

1.2.1.3. Amygdala

The amygdala is a bilateral structure located deep inside the medial temporal lobe; it forms part of the limbic system, like the hippocampus, and contains both GCs and adrenergic receptors. It has an excitatory effect on the HPA-axis, which is mediated by its GABAergic projections, which inhibit, in turn, the neurons of the bed nucleus of the stria terminalis, the preoptic area and other hypothalamic nuclei. Moreover, it coordinates behavioral, autonomic and endocrine response to a stressful situation (Sandi et al., 2001).

This brain structure is involved in processing emotions (mainly fear and anger) and also in emotional memory. In our context, the main role of the amygdala is the strengthening of declarative memory that occurs when the material to be recalled is emotional (Cahill and McGaugh, 1998). Two types of neurobiological mechanisms would be involved in this strengthening: neural and hormonal. Thus, at the neural level it would occur through direct neural projections from the amygdala to other brain structures such as the hippocampus and the prefrontal cortex. And at the hormonal level, it would occur through the connection between the amygdala and the hypothalamus, which can modulate the HPA-axis activity and the ANS activity. At this point, it has been indicated that noradrenergic activation of the basolateral amygdala is necessary to observe the effects of stress hormones on

consolidation and retrieval processes (Roozendaal et al., 2009). In addition, it is a necessary structure for fear conditioning (Sandi et al., 2001).

1.2.2. Stress effects on memory[1]

It is well known that memory function is affected by stress in both animals and humans. However, to define these stress effects, it is necessary to deal with multiple factors related to both the stressor and memory functions because they can moderate this relationship. Some of the most important factors related to the stressor are its intensity, nature (exogenous vs. endogenous) and duration (acute vs. chronic) (Sandi, 2013). To investigate the acute stress effects on memory function, in recent years several tasks have been proposed in order to obtain a more realistic stress response and, consequently, greater ecological validity. Thus, the use of stress paradigms in the laboratory has meant an advantage over the pharmacological challenge studies because they trigger the SNS and HPA-axis activation. Among them, the Trier Social Stress Test (TSST; Kirschbaum et al., 1993), a combination of a public-speaking task and an arithmetic task, has been widely used. This task is able to induce a consistent stress response at different levels, endocrine, cardiovascular, immune and subjective (Kudielka et al., 2007), probably because it simulates an uncontrollable social evaluative situation (Dickerson and Kemeny, 2004).

With regard to memory function, it is important to note that the type and phase of memory should also be considered in the memory consequences of stress. According to the level of consciousness, memory has been classified as explicit or declarative memory, which requires conscious recollection of previous experiences (Milner et al., 1998), or implicit or non-declarative memory, which represents the

[1] This section is part of a review article that is currently being prepared: Hidalgo, V., Pulopulos, M.M., and Salvador, A. Acute stress and memory: the role of age and sex.

effect of unconscious prior experience on subsequent behavior (Graf et al., 1984). These two different memory systems operate in parallel, supporting behavior (Squire, 2009). In addition, memory is a dynamic process because the information is encoded, consolidated and retrieved. After encoding new information from outside, this information is maintained for a short time. Finally, this information that has been stored returns to the consciousness. Thus, the memory process consists of three phases: encoding, consolidation and retrieval. Stress can affect all these phases depending on when individuals are stressed: before encoding/learning, consolidation or retrieval. Whereas most of the work addressed to investigating the impact of stress on memory performance has focused on these phases, there is recent evidence that stress can also affect reconsolidation/or extinction processes if the stressor is applied after retrieval (for a review see: Schwabe et al., 2012).

However, we must not forget the importance of individual differences in this stress-memory relationship. Factors like the age or the sex of the individuals can also moderate the stress effects on memory function.

In the following sections, a brief summary of the main findings reported in the literature about the effects of acute stress on memory performance, considering these moderating factors, will be explained. We will focus mainly on the variables addressed in this thesis. In addition, a systematic review of the effects of acute stress on two types of memory (i.e. non-declarative and declarative memory) and on two phases of memory (i.e. learning and retrieval) is presented.

1.2.2.1. Acute stress effects on non-declarative memory

In human studies, one of the most widely-used tasks to assess non-declarative memory performance has been a word-stem completion task. This task consists of word stems as cues to recover recently presented words and assess the

priming effect. Priming refers to an improvement in the ability to identify or process a stimulus as the result of a recent prior experience with the same stimulus or a related one (Tulving and Schacter, 1990). It seems to be an advantageous effect because it improves the speed and accuracy with which organisms interacts with a familiar environment (Squire, 2009). Regarding the brain areas related to priming, previous neuroimaging studies have shown that it is related to reduced activity in the neocortical regions involved in the task (Wiggs and Martin, 1998; Schacter et al., 2007).

Studies investigating the stress effects on priming are scarce and report mixed results. Thus, no effects of psychosocial stress were found in older men and women (Lupien et al., 1997) or in women from middle to older ages (Domes et al., 2002). However, enhancing effects have also been shown in men and women from 18 to 65 years old employing a physical stress (i.e. running a marathon) (Eich and Metcalfe, 2009). Unfortunately, they did not measure the cortisol and/or sAA response, and so it is impossible to know whether this enhancement was due to HPA-axis and/or SNS activation. The only study that checked the impact of cortisol administration on the priming effect failed to find effects of high cortisol concentrations in young men (Kirschbaum et al., 1996). Therefore, the purpose of the first study in this thesis will be to address whether the SNS and HPA-axis reactivity to stress affect the priming of non-declarative memory.

1.2.2.2. Acute stress effects on declarative memory

1.2.2.2.1. Acute stress prior-learning

In contrast to non-declarative memory, the relationship between stress and declarative memory has been investigated more thoroughly, mainly in young people.

When young people were exposed to an acute stressor, prior learning of neutral material worsened (Jelicic et al., 2004; Kirschbaum et al., 1996; Payne et al., 2006; 2007; Smeets et al., 2006), was enhanced (Nater et al., 2007; Schwabe et al., 2008) and even showed no effect (Elzinga et al., 2005; Wolf et al., 2001b) on short-term memory performance (for more details see Table I.1). However, in only a few studies, the impact of the HPA-axis reactivity to stress was related to memory performance. Thus, a stress-induced cortisol increase was negatively related to declarative memory performance (Kirschbaum et al., 1996; Wolf et al., 2001b). By contrast, those who had higher cortisol responses to stress performed better on the declarative memory task than those who had lower cortisol responses (Nater et al., 2007). On the other hand, three studies investigated the SNS response to stress (Elzinga et al., 2005; Payne et al., 2007; Schwabe et al., 2008), but only Schwabe et al. (2008) showed that autonomic arousal facilitated memory recall of neutral words.

Despite evidence indicating the moderating role of sex in the relationship between acute stress and declarative memory performance (Andreano et al., 2008; McEwen, 2002; Shors, 2006), many of the previous studies did not address this issue. For example, Nater et al. (2007) only studied men, while Elzinga et al. (2005) included only women. Others studied mixed-sex samples, but they did not control the menstrual cycle phase of the women (Kirschbaum et al., 1996; Jelicic et al., 2004; Payne et al., 2006; 2007; Smeets et al., 2006), although differences in the cortisol response have been reported depending on sex hormone levels (Kirschbaum et al., 1999). Only two previous studies considered the sex hormone levels, reporting mixed results. While no sex differences were found between men and women oral contraceptive users (Schwabe et al., 2008), a negative correlation was found between cortisol response and memory performance only in men and not in women in the luteal phase of their menstrual cycle (Wolf et al., 2001b).

To summarize, the effects of prior acute stress on memory performance are unclear. Moreover, the impact of the HPA-axis and SNS reactivity to stress on memory performance, as well as the role of sex and the sex hormone levels in this relationship, has not been studied in depth. Therefore, these issues are going to be addressed in the first study of this thesis.

Little attention has been paid to pre-learning effects on memory in older people. To our knowledge, only a few studies have focused on this topic. Two previous studies failed to find stress effects in women from 41 to 69 years of age (Bohnen et al., 1990) and from 32 to 68 years of age (Domes et al., 2002). Based on these studies, it is impossible to distinguish sex differences. Only one study investigated the role of sex in the stress effects on memory performance in older people. Thus, only among women, the stress-induced response had an acute differential impact on memory performance (Almela et al., 2011a). Previously, Wolf et al. (2001a) published an article with a direct comparison of older and young people. In their study, a hydrocortisone (a cortisol agonist) injection administered prior to learning did not influence the recall of a list of neutral words in young (19 to 30 years old) and older (59 to 76 years old) men. However, there are important differences between the GC increases induced by pharmacological administration and those produced by exposure to stress (for more details see Table I.1). As mentioned above, in addition to the cortisol increase that occurs with drug administration, stress provokes other physiological (i.e. SNS activation) changes (Lupien and Schramek, 2006). Hence, the use of stress paradigms in the laboratory allows a more complete study of stress effects on memory performance. Therefore, the second study in this thesis aimed to compare the effects of acute stress on memory performance in older and young people, and find out whether there were sex differences in this comparison.

Table I.1. Acute cortisol administration or stress prior-learning effects on declarative memory performance.

Authors	Participants	Stressor	Memory Task	Main Results
Bohnen et al. (1990)	12 ♀ (41-49 years) 12 ♀ (61-69 years)	4-hour mental task	Neutral word list	No stress effects on memory performance No age differences
Kirschbaum et al. (1996)	8 ♂ + 5 ♀ (Students, age not specified)	TSST	Neutral word list	Negative correlation between stress-induced cortisol levels and memory performance No sex differences
Wolf et al. (2001a)	9 ♂ (19-30 years) 11 ♂ (59-76 years)	0.5 mg/kg cortisol injection	Neutral word list Paragraph recall test	Cortisol did not influence memory recall in both age groups
Wolf et al. (2001b)	33 ♂ + 25 ♀ (L) SG (mean 24.9 years) CG (mean 23.6 years)	TSST	Neutral word list	No stress effects on memory performance Negative correlation between stress-induced cortisol levels and memory performance This correlation was solely caused by the strong association in men
Domes et al. (2002)	32 ♀ (32-68 years)	TSST	Neutral word list	No stress effects on memory performance Regardless of experimental condition, high responders showed increased memory performance
Jelicic et al. (2004)	9 ♂ + 31 ♀ (mean 20.1 years)	TSST	Neutral and emotional word list	Stress impaired recall of neutral words, whereas it enhanced recall of emotional words. These stress effects on memory performance were not mediated by cortisol
Elzinga et al. (2005)	16 ♀ (mean 21.4 years)	Cognitive challenge	Paragraphs	No stress effects on memory performance

Table I.1 (continued). Acute cortisol administration or stress prior-learning effects on declarative memory performance.

Authors	Participants	Stressor	Memory Task	Main Results
Payne et al. (2006)	53 ♂ + 64 ♀ (Psychology students, years not specified)	TSST	Neutral and emotional narrated slides	Stress disrupted memory of non-emotional material, whereas it preserved or even enhanced memory of emotional material Higher cortisol levels were associated with poorer memory performance in men, but not in women
Payne et al. (2007)	32 ♂ + 44 ♀ (Undergraduate students, age not specified)	TSST	Neutral and emotional narrated slides	Stress disrupted long-term memory of neutral material, but facilitated long-term memory of emotional material No sex differences
Smeets et al. (2006)	30 ♂ + 30 ♀ (mean 19.65 years)	TSST	Neutral and emotional word list	Stress only impaired recall of neutral words No sex differences
Nater et al. (2007)	20 ♂ (mean 23.75 years)	TSST	Neutral word list	No general stress effect on memory performance High responders: better immediate free recall after stress
Schwabe et al. (2008)	48 ♂ + 48 ♀ (OC) (mean 23.3 years)	CPT	Neutral and emotional word list	Stress enhanced the recall of neutral words independently of cortisol response Cortisol responders: better short-term free recall of negative words
Almela et al. (2011a)	16 ♂ + 16 ♀ (PM) (54-72 years)	TSST	Neutral word list	Only in ♀: enhanced attention (trial 1) and impaired working memory (trial 6)

Table I.1 (continued). Acute cortisol administration or stress prior-learning effects on declarative memory performance.

Authors	Participants	Stressor	Memory Task	Main Results
Espin et al. (2013)	32 ♂ + 87 ♀ (30 F, 34 L, 23 OC) (18-25 years)	TSST	Neutral word list	In CC, all groups of women recalled more words than men, but these differences disappeared in the group exposed to TSST, given that men's performance improved to the level of women's.

The studies are listed in chronological order. TSST: Trier Social Stress Test. SG: Stress Group. CG: Control Group. CC: Control Condition. CPT: Cold Pressor Test. F: Follicular. L: Luteal. OC: Oral Contraceptive. PM: Postmenopausal

2.2.2.2. Acute stress prior-retrieval

Pharmacologically-induced or stress-induced increases in cortisol levels usually enhance memory consolidation (Buchanan and Lovallo, 2001; Cahill et al., 2003; Smeets et al., 2008). However, when this cortisol increase takes place before retrieval due to pharmacological administration (de Quervain et al. 2000; 2003; Kuhlmann and Wolf, 2005; Kuhlmann et al., 2005a) or stress exposure (Buchanan et al., 2006; Buchanan and Tranel, 2008; Domes et al., 2004; Kuhlmann et al., 2005b; Oei et al., 2006; Smeets, 2011; Smeets et al., 2008), it usually impairs memory retrieval (but see: Schoofs and Wolf, 2009; Beckner et al., 2006; Wolf et al., 2002 for non-effects) (for more details see Table I.2). According to Roozendaal (2002), these different effects (i.e. enhancement of consolidation and impairment of retrieval) occur because cortisol has a blocking effect on retrieval processes, in favor of consolidation processes, facilitating the consolidation of new important information that can be necessary in future situations.

The cortisol response to stress was especially involved in these impairing effects of stress. Thus, a negative correlation between memory retrieval and the cortisol response to stress was reported (Domes et al., 2004; Oei et al., 2006; Smeets, 2011; Smeets et al., 2008). On the other hand, Buchanan and Tranel (2008) and Buchanan et al. (2006) found negative stress effects among those participants who showed both autonomic and cortisol responses. Interestingly, apart from these two studies, only two other studies investigated the SNS response to stress (Oei et al., 2006; Smeets et al., 2008), but only Smeets et al. (2008) found a negative relationship between sAA response and memory retrieval.

It is important to note that these studies have been carried out with young people, and so little is known about the role of age in these effects of stress on memory retrieval. To date, only two studies have investigated this matter in older people. Thus, Wolf et al., (2001a) observed that a higher cortisol increase after an

injection of hydrocortisone impairs memory retrieval for words in both young and older men. By contrast, Pulopulos et al. (2013) reported no effects of stress-induced cortisol and sAA increases on memory retrieval of pictures, words and stories in a mixed-sex sample. Hence, no study has compared the impact of the HPA-axis and SNS activation on memory retrieval in young and older people of both sexes. The third study in this thesis has been proposed to answer this question.

Finally, previous studies have observed greater cortisol effects for emotionally arousing material than for neutral material (Domes et al., 2004; Kuhlmann et al., 2005b; Buchanan et al., 2006; Smeets et al., 2008) (for more details see Table I.2). This can be explained by a greater noradrenergic activation of the amygdala provoked by emotional material than neutral material. As has been reported, noradrenergic activation of the basolateral amygdala (BLA) and interactions between the amygdala and hippocampus are crucial to observe cortisol effects on memory performance related to hippocampus functioning (Roozendaal et al., 2009). However, whether there are age differences in the stress effects on memory retrieval of emotional and neutral material is an issue that remains unanswered. Therefore, in the third study of this thesis, positive, negative and neutral materials have been included in order to address this question.

Table I.2. Acute cortisol administration or stress prior-retrieval effects on declarative memory performance.

Authors	Participants	Administration/Stressor	Memory Task	Main Results
de Quervain et al. (2000)	18 ♀ + 18 ♀ (mean 28.8 years)	25 mg cortisone	Neutral word list	Cortisone impaired free recall of verbal material, but left recognition unaffected Sex differences were not studied
Wolf et al. (2001a)	9 ♂ (19-30 years) 11 ♂ (59-76 years)	0.5 mg/kg cortisol injection	Neutral word list	Cortisol reduced memory recall in both age groups Sex differences were not studied
Wolf et al. (2002)	22♂ + 18 ♀ (L)	TSST	Neutral word list	Stress did not affect long-term (4 weeks) memory performance Sex differences were not studied
de Quervain et al. (2003)	40 ♂ (mean 22.7 years)	25 mg cortisone	Two neutral word-pair lists	Cortisone impaired cued recall of word pairs, but did not affect recognition
Domes et al. (2004)	60 ♂ (mean 25.3 years)	TSST	Neutral and emotional word list	Stress did not affect free recall For recognition, no main effect of stress, but recognition for positive words was impaired when stress was before retrieval Positive correlation between cortisol response and errors of commission
Kuhlmann et al. (2005a)	16 ♀ (No OC) (mean 26.56 years)	30 mg hydrocortisone	Neutral and negative word list	Overall cortisol impaired memory retrieval Cortisol significantly impaired retrieval of negative words, no effects on neutral words
Kuhlmann and Wolf (2005)	47 ♀ (20 OC, 14 L, 13 M) (mean 24.81 years)	30 mg hydrocortisone	Neutral and negative word list	Overall cortisol impaired memory retrieval, especially for emotional words Only the free cycling women were significantly affected

Table I.2 (continued). Acute cortisol administration or stress prior-retrieval effects on declarative memory performance.

Authors	Participants	Administration/Stressor	Memory Task	Main Results
Kuhlmann et al. (2005b)	19 ♀ (mean 24.58 years)	TSST	Neutral and emotional word list	Stress impaired free recall of memory retrieval Emotional words were affected, but not neutral words
Buchanan et al. (2006)	16 ♂ + 16 ♀ (mean 18.9 years)	CPT	Neutral and negative word list	Cortisol responders recalled fewer words than non-responders and controls Effect most pronounced for moderately arousing words No sex differences
Oei et al. (2006)	20 ♂ (mean 21.86 years)	TSST	Neutral and emotional paragraphs	Stress disrupted long-term memory of neutral material, but facilitated long-term memory of emotional material No sex differences
Beckner et al. (2006)	157 ♂ + ♀ (64%) (mean 18.77 years)	Anticipation and preparation a public speech	Film recognition and paragraphs	Stress did not affect memory retrieval No sex differences
Buchanan and Tranel (2008)	20 ♂+ 20 ♀ (mean 20 years)	TSST modified	Neutral and negative pictures	Cortisol responders showed reduced memory retrieval Men and women in SC with no cortisol response: increased retrieval for negative pictures versus CC

Table I.2 (continued). Acute cortisol administration or stress prior-retrieval effects on declarative memory performance.

Authors	Participants	Administration/Stressor	Memory Task	Main Results
Smeets et al. (2008)	6 ♂ + 84 ♀ (mean 20.6 years)	CPT	Neutral and emotional word lists	Stress impaired memory retrieval, predominantly for emotional words This effect was strongly related to stress-induced cortisol and sympathetic activity
Schoofs and Wolf (2009)	36 ♀ (L) (mean 24.47 years)	TSST	Neutral and emotional word list	Stress did not affect memory retrieval No differences between cortisol responders and non-responders No correlation between stress-induced cortisol increase and memory
Smeets, 2011	34 ♂ + 42 ♀ (31 L, 11 F) (mean 19.9 years)	SECPT	Neutral and negative word list	Stress impaired memory retrieval This effect was larger for negative than for neutral words and was associated with cortisol response
Pulopulos et al. (2013)	38 ♂ + 38♀ (PM) (56-76 years)	TSST	Neutral and emotional pictures Neutral word list Paragraphs	Stress did not affect memory retrieval on any memory task

The studies are listed in chronological order. TSST: Trier Social Stress Test. CPT: Cold Pressor Test. SC: Stress Condition. CC: Control Condition. L: Luteal. M: Mensis. OC: Oral Contraceptive. F: Follicular. PM: Postmenopausal

1.2.3. Diurnal cortisol cycle and memory

So far, our focus has been on the HPA-axis activity in stress situations and how this reactivity affects memory function. However, the diurnal HPA-axis functioning (under normal or non-stress situation) reflects the general neuroendocrine health status and, consequently, is related to physical, emotional and cognitive health (Adam and Kumari, 2009). Hence, cortisol is not only the end product of the HPA-axis activation in response to stress, but also an indicator of HPA-axis health (Hellhammer et al., 2007; Miller et al., 2007). In support of this, exposure to chronic stress has been associated with HPA-axis dysregulation (Miller et al., 2007). Therefore, HPA-axis functioning can also reflect the exposure to stress across the lifetime.

The diurnal HPA-axis activity follows a circadian rhythm. Thus, in the morning, an acute and rapid increase in cortisol levels occurs, reaching a peak between approximately 30 and 45 minutes after awakening (Pruessner et al., 1997; Wüst et al., 2000). Afterwards, the cortisol levels gradually decrease to lower levels at the end of the day. Therefore, two components are distinguished in the diurnal cortisol cycle: (i) the cortisol awakening response (CAR), and (ii) the diurnal cortisol slope (DCS).

A healthy HPA-axis function requires strong CAR and DCS slopes, while flattened CAR and DCS slopes reflect an unhealthy HPA-axis function. However, in spite of the importance of these two different HPA-axis components, most studies investigating HPA-axis functioning and cognitive function did not make an effort to collect the entire cortisol profile using urinary, blood or salivary samples (Karlamangla et al., 2005; MacLullich et al., 2005; Li et al., 2006; Kuningas et al., 2007; Lee et al., 2007; Lee et al., 2008; Comijs et al., 2010; Seeman et al., 1997; Souza-Talarico et al., 2010; Schrijvers et al., 2011; Potvin et al., 2012; Pulopulos et al., 2014) (for more details see Table II.3).

The following sections briefly explain these two components of diurnal HPA-axis functioning and their relationship with the memory performance of older adults.

1.2.3.1. The cortisol awakening response and memory

As its name suggests, the cortisol awakening response (CAR) is a response to morning awakening characterized by a sharp cortisol increase from about 50 to over 100% (Clow et al., 2004) 30-45 minutes post-awakening (for a review about this topic see: Clow et al., 2010a; 2010b; Fries et al., 2009; Kudielka and Wüst, 2010). The CAR was first established by Pruessner et al. (1997) as a good index of adrenocortical activity. It is usually collected by saliva samples, providing ecological validity because the samples can be taken by the participants in their own homes. However, at the same time, problems with adherence to the protocol can appear (Thorn et al., 2006). Normally, participants are instructed to provide saliva at awakening, and 15, 30, 45 and, sometimes, 60 minutes post-awakening on more than one day, given the difficulty of capturing this dynamic index of cortisol secretion. The CAR is a discrete component superimposed on the circadian rhythm and distinct from the rest of the cortisol secreted during the day.

As mentioned in the previous section, the HPA-axis is mainly regulated by the hippocampus, the PFC and the amygdala. Specifically, the hippocampus may exert its control on it differently. Thus, in addition to the inhibitory role that it usually plays in the control of HPA-axis activity during the rest of the day, the hippocampus would also have a permissive role in the regulation of the CAR (Fries et al., 2009). Accordingly, two studies reported that when the hippocampus is damaged, the CAR is absent (Buchanan et al., 2004; Wolf et al., 2005). Moreover, larger hippocampal volume was related to a greater CAR (Pruessner et al., 2007). In sum, these results

support the notion that the hippocampus plays a central role in the regulation of the CAR. However, although the PFC and the amygdala also regulate the HPA-axis activity, their specific role in the control of the CAR is still not known (Fries et al., 2009). In addition, other mechanisms are involved in the regulation of the CAR. The suprachiasmatic nucleus (SCN), the light-sensitive endogenous biological clock, exerts its function through two pathways: (i) via input to the PVN and the HPA-axis cascade (CRH and ACTH) and (ii) via its direct sympathetic innervation to the adrenal gland by the splanchnic nerve (for a review see: Fries et al., 2009).

Despite the numerous studies investigating the CAR, the exact function of this aspect of HPA-axis activity remains unclear. However, it has been proposed that it may be involved in the transition from sleep to awakening (full alertness) and in the body's synchronization to the sleep-wake and light-dark cycle (Clow et al., 2010a). Moreover, it may play a part in the immune system balance and voluntary motor function following nighttime sleep (Clow et al., 2010b), as well as in the preparation of the organism to meet the physical and mental demands of the coming day (Fries et al., 2009).

Among the factors that can influence the CAR, age and sex have been two of the most studied (Fries et al., 2009). Regarding age, there is no consensus, as the few existing studies reported mixed results. While two studies failed to find age effects on the CAR (Pruessner et al., 1997; Wüst et al., 2000), another showed a negative relationship between age and CAR (Kudielka and Kirschbaum, 2003). On the other hand, although sex differences could be expected in the CAR due to sexual dimorphisms in brain structures modulating the HPA-axis, sex seems to have a low impact on the CAR (Fries et al., 2009).

To our knowledge, few studies have investigated the possible relationship between the CAR and memory performance in healthy older people, and the results are far from conclusive. Thus, Evans et al. (2011) found a positive relationship

between the CAR and memory tests related to prefrontal cortex functioning (i.e. working memory and verbal fluency), but after controlling for age, this relationship disappeared. Franz et al. (2011), after controlling for several confounding factors, failed to find any relationship between the CAR and several memory domains (i.e. verbal, visual spatial, short-term and working memory). Finally, Almela et al. (2012) showed that the CAR was associated with poorer declarative memory (in men and women) and better working memory (only in men) (for more details see Table II.3).

1.2.3.2. The diurnal cortisol slope and memory

In contrast to the CAR, the cortisol secreted during the rest of day has been understudied. One diurnal cortisol measure frequently used is the diurnal cortisol slope (DCS). This index represents the degree of change (typically decline) in the daily cortisol levels from morning to late evening (Adam and Kumari, 2009). A steeper decline has been associated with better psychosocial and physical health. In support of this, a flattened DCS has been related to chronic and acute psychosocial stress (Adam et al., 2006), sub-clinical disease (Matthews et al., 2006) and increased mortality from breast cancer (Sephton et al., 2000).

However, there is no consensus about different aspects of its collection: the number of samples, the exact time or the method used to calculate the typical slope. For this reason, it is not surprising that there are large methodological differences among the studies investigating the relationship between this component of the diurnal HPA-axis activity and cognition function.

To our knowledge, only a few studies have investigated the relationship between the DCS and memory performance in older people, reporting mixed results. Thus, flatter DCS has been related to poorer declarative memory (Abercrombie et al., 2004; Evans et al., 2011; Gerritsen et al., 2011); accordingly,

higher total cortisol output across the day (AUCg; Franz et al., 2011) was related to poorer cognitive performance. However, a steeper DCS was also associated with poorer declarative memory (O'Hara et al., 2007). Moreover, no associations between the DCS and memory performance were reported (Beluche et al., 2010; Fiocco et al., 2006; Sing-Manoux et al., 2014) (for more details see Table II.3).

Taken together, in light of the mixed and scarce results, the need to investigate the link between the CAR and the DCS and memory performance in older people seems clear. Therefore, the fourth study of this thesis has been proposed to investigate this issue.

Table I.3. Summary of the main studies investigating the relationship between the diurnal cortisol cycle and cognitive function.

Authors	Participants	Cortisol samples	Cortisol indices	Cognitive domains
Seeman et al. (1997)	1313 ♂/♀ (70-79 years)	Urine: 12-h overnight	--	Language, abstraction, spatial ability and memory (delayed recall of a story)
Abercrombie et al. (2004)	31 ♀ (mean 56 years)	Saliva (3 days): awakening, 12:00, 17:00, 21:00	Mean, DCS	Memory (RAVLT)
MacLullich et al. (2005)	97 ♂ (65-70 years)	Plasma: 9:00, 14:30, post-dex (9:00)	--	Non-verbal reasoning, verbal memory (logical memory + RAVLT), visual memory (visual reproduction + BVRT-A)
Li et al. (2006)	79 ♂/♀ (mean 77.9 years)	Saliva: 8:00, 15:00, 23:00	--	Global cognitive function, verbal memory (PI + paragraph recall, visual memory (delayed object recall), attention and language
Fiocco et al. (2006)	42 ♂/♀ (mean 68.36 years)	Saliva (2 days): awakening, 14:00, 16:00, bed	DCS	Short-term memory (DS), verbal fluency declarative memory (cued recall test)
Kuningas et al. (2007)	563 ♂/♀ (85-90 years)	Plasma: before 11:00	--	Global cognitive function, attention, processing speed, immediate and delayed recall memory (WLTI, WLTD)
O'Hara et al. (2007)	51 ♂ + 103 ♀ (mean 71.13 years)	Saliva (2 days): awakening, +30min, 12:00, 17:00, 21:00	Awakening DCS	Verbal memory (RAVLT), information processing speed, attention, verbal naming and visuospatial ability

Table I.3 (continued). Summary of the main studies investigating the relationship between the diurnal cortisol cycle and cognitive function.

Authors	Participants	Cortisol samples	Cortisol indices	Cognitive domains
Lee et al. (2007; 2008)	967 ♂/♀ (50-70 years)	Saliva: hours undetermined (before, during, after cognitive test and final session)	First sample, mean, AUCi, AUCg, Variance and slope (s. 1-2; 3-4)	Language, processing speed, eye-hand coordination, executive functioning, verbal memory and learning (RAVLT), visual memory (Rey Complex Figure delayed recall, symbol digit) and visuoconstruction
Comijs et al. (2010)	1154 ♂/♀ (65-88 years)	Blood: before 10:00 h	--	Global cognitive performance, episodic memory (RAVLT) and information processing speed
Souza-Talarico et al. (2010)	40 ♂/♀ (mean 72.2 years)	Saliva: within two hours of awakening	--	Visual declarative memory (sub-item of BCSB), category fluency and clock drawing
Beluche et al. (2010)	111 ♂ + 86♀ (65-90 years)	Saliva: 8:40, 15:40, 21:40	DCS	Verbal and non-verbal recall, visual memory (BVRT) and executive function
Gerritsen et al. (2011)	911 ♂/♀ (mean 74.5 years)	Saliva: within 30 min after awakening and bed	Tertiles of delta	Global cognitive function, verbal memory (Word learning test) and processing speed
Stawski et al. (2011)	1500 ♂/♀ (mean 57 years)	Saliva (4 days): awakening, +30min, before lunch, bed	Morning rise DCS	Episodic verbal memory (RAVLT), working memory (DSB), executive function, reasoning and speed of processing
Evans et al. (2011)	16 ♂ + 34♀ (mean 74 years)	Saliva (2 days): awakening, +15 min, +30 min, +45 min, +3h, +6h, +9h, +12h	Mean (Aw-45min; 3-12h), overall mean, diurnal fall, CAR (+30min-Aw)	Declarative memory (HVLT) and executive function

Table I.3 (continued). Summary of the main studies investigating the relationship between the diurnal cortisol cycle and cognitive function.

Authors	Participants	Cortisol samples	Cortisol indices	Cognitive domains
Franz et al. (2011)	778 ♂ (51-60 years)	Saliva (3 days): awakening, +30 min, 10:00, 15:00, bed	AUCg, CAR (+30min-Aw)	Verbal ability, visual-spatial ability, verbal memory (CVLT-2 + Logical Memory), visual spatial memory, short-term memory (DSF + SSF), working memory (DSB, SSB, LNS), executive function, verbal fluency, abstract reasoning and processing speed
Almela et al. (2012)	44 ♂ + 44 ♀ (55-77 years)	Saliva (2 days): awakening, +30 min, +45 min, +60 min	CAR (AUCi), AUCg	Declarative memory (Logical memory and RAVLT) and working memory (Spatial span and spatial working memory)
Evans et al. (2012)	16 ♂ + 34 ♀ (mean 74 years)	Saliva (2 days): awakening, +15 min, +30 min, +45 min	CAR (mean increase[1])	Declarative memory (HVLT) and executive function
Pulopulos et al. (2014)	57 ♂/♀ (mean 64.75years)	Saliva (2 days): awakening, +30 min, 23:00	Mean 3 samples AUCg	Learning and verbal memory (RAVLT, Rivermead), psychomotor speed, attention, executive function, working memory (DS)
Singh-Manoux et al. (2014)	3229 ♂/♀ (mean 61 years)	Saliva: awakening, +30min, +2.5h, +8h, +12h, bed	Aw, CAR (+30min-aw), DCS, bed, AUCg	Short-term memory (free recall test), verbal fluency, inductive reasoning

The studies are listed in chronological order. [1]: Mean Increase: sample 2+ s3+s4)/3 – s1. RAVLT: Rey Auditory Verbal Learning Test. BVRT-A: Benton Visual Retention Test A. PI: Proactive Interference. WLTI: Word Learning Test Immediate Recall. WLTD: Word Learning Test Delayed Recall. BCSB: Brief Cognitive Screening Battery. DS: Digit Span. HVLT: Hopkins Verbal Learning Test. CVLT: California Verbal Learning Test. DSF: Digit Span Forward. SSF: Spatial Span Forward. DSB: Digit Spatial Backward. SSB: Spatial Span Backward. LNS: Letter Number Sequencing. AUCi: Area under the curve with respect to increase. AUCg: Area under the curve with respect to ground. CAR: Cortisol Awakening Response. DCS: Diurnal Cortisol Slope. s: sample.

1.3. OBJECTIVES AND HYPOTHESIS

We performed four studies in order to shed light on these mixed results and provide evidence to fill the gaps in the literature on this issue. The objectives and hypotheses of this thesis are presented below:

General objective 1. Determine the impact of the acute stress-prior learning on the memory performance of neutral material in healthy adults.

- *Specific objective 1.1:* Study the stress effects on non-declarative and declarative memory performance in young adults.

- *Specific objective 1.*2: Compare the stress effects on declarative memory performance in older and young adults.

- *Specific objective 1.*3: Investigate the role of sex in the specific objectives mentioned above.

Due to the few studies carried out on non-declarative memory and the mixed results in the literature about the stress effects on declarative memory, we have no specific hypotheses about the effects of stress-prior learning on these two types of memory in young adults. However, age differences are expected in the stress effects on declarative memory. Moreover, based on previous findings of our group (Almela et al., 2011a), we expect greater negative stress effects in older women.

We will try to respond to the specific objective 1.1 in study 1, where we test the hypothesis that both stress biomarkers' responses to TSST are related to non-declarative and declarative memory performance. Moreover, in this study, the specific objective 1.3 is addressed too, as we studied men and women. Study 2 aims to respond to specific research objectives 1.2 and 1.3 because in this study we have

directly compared the stress effects on memory performance in two different age groups with a similar number of participants of both sexes.

General objective 2. Determine the impact of the acute stress-prior retrieval on the memory performance of positive, negative and neutral material in healthy adults.

- *Specific Objective 2.1:* Compare the stress effects on memory retrieval between older and young people.

- *Specific Objective 2.*2: Investigate the role of sex in the specific objective above.

The fact that no previous studies have directly investigated the effects of stress on memory retrieval comparing young and older people makes it very difficult to propose a hypothesis about this general objective. However, taking into account the findings of prior studies with similar designs that investigate these age groups separately, we expect a negative effect of the stressor only in the young group and not in the older group. Moreover, this effect will be stronger for emotionally arousing material than for neutral material (Wolf et al., 2004). Finally, sex differences will appear, given that the magnitude of the stress response depends on the levels of sexual hormones.

The aims of study 3 focus on answering these objectives.

General objective 3. Examine the relationship between the diurnal HPA-axis activity (non-stress) and memory performance in healthy older adults.

- *Specific objective 3.1:* Study the association between the CAR and memory performance in healthy older adults.

- *Specific objective 3.2*: Explore the relationship between the DCS and memory performance in healthy older adults.

- *Specific objective 3.3*: Investigate the role of sex in these general and specific objectives.

We expect that the diurnal HPA-axis functioning will be related to memory performance in older people, but this relationship will be different depending on the component of the diurnal cortisol cycle studied. Moreover, although there have been no reported sex-related differences in the diurnal cortisol profiles, it is possible to find sex differences in the relationship between the diurnal cortisol cycle and memory performance, given the crucial role that this factor plays in the relationship between HPA-axis activity and cognitive performance in older people (Almela et al., 2011a; Seeman et al, 1997).

These objectives will be addressed in study 4.

1.4. GENERAL MATERIAL AND METHODS

To provide a global view of the methodology used in the following four studies, the section below presents a brief summary of the subjects who participated in the studies, the procedure used, and the variables studied.

1.4.1. Participants

In order to ensure the homogeneity of the sample and the comparison of older and young people, the sample used in the current thesis was composed of healthy adults who were cognitively active. They were students enrolled in a study program at the University of Valencia for people over 50 year of age (NAU GRAN) (older group) or college students from different areas (young group). Depending on the aim of each study, we used only a young group (study 1) or an older group (study 4) or both age groups (studies 2 and 3). The age range was similar within each age group across the studies, although they differed slightly. In the older group, the general age range was from 54 to 76 years old (study 2: 54-72, study 3: 56-76, and study 4: 57-76), while in the young group, the general age range was from 18 to 35 years old (studies 1 and 2: 18-35, and study 3: 18-27).

Moreover, in all studies the sex factor was taken into consideration. Thus, the samples were composed of a similar number of men and women in each age group, except in study 1, whose aim was to investigate, in addition to sex, the use of contraceptives.

1.4.2. Procedure

The procedure carried out in this thesis was different depending on the aim of each study. There are three different procedures.

Studies 1 and 2 used a within-subject design with two randomized and counterbalanced conditions (stress or control) in two separate (less than 10 days) sessions. The sessions consisted of the same phases with equal durations, they started at the same hour, and the saliva samples were taken at the same time point. The two conditions differed only in the task (stress or control task). While in the

stress condition, participants were asked to perform a stressful task (TSST, this task will be explained in more detail in the following section), in the control condition, they had to perform a control task (a task with a similar mental workload and global physical activity, but without the evaluative threat and uncontrollability). Moreover, in both conditions, after the stress or control task, participants performed the memory tasks under study (study 1: non-declarative and declarative memory tasks and study 2: declarative memory task). Therefore, the stress was prior-learning.

The procedure in study 3 consisted of two consecutive separate sessions. In the first session (acquisition session), participants were presented the material to be remembered (pictures). This session was equal for all participants. In the second session (retrieval session), participants were randomly assigned to the stress or the control condition. The tasks in these two conditions were similar to the stress and control tasks described above. It is worth noting that an important difference between this design and the design employed for studies 1 and 2 is that here the stress was prior-retrieval.

Finally, the procedure used in study 4 was quite different because our aim was to delve into the relationship between diurnal HPA-axis functioning and memory performance in older people. Thus, participants provided 7 saliva samples on two consecutive weekdays to measure the diurnal cortisol cycle, and from this, the CAR and DCS components. In addition, they underwent a neuropsychological assessment with different memory tests to assess cognitive performance.

1.4.3. Variables

1.4.3.1. Stress Task

To induce stress, we used an acute psychosocial stressor in the laboratory, the Trier Social Stress Test (TSST; Kirschbaum et al., 1993). It is a widely-used tool by researchers in the field of psychoneuroendocrinology, as it is indeed able to provoke a stress response similar to those that occur in a real situation. After an introduction phase, where participants are given the instructions to the TSST, and a preparation phase, with time to prepare a free speech, they performed two tasks: (i) free speech: participants have to perform a free speech in order to convince a committee that they are the best candidate for a position previously characterized as interesting for them, and (ii) an arithmetic task: participants have to perform a mental arithmetic task (subtraction). Both tasks have a duration of 5 minutes, and they are performed in front of a committee of supposed experts. According to Dickerson and Kemeny (2004), the effectiveness of the TSST is explained by its characteristics of uncontrollability and social-evaluative threat.

1.4.3.2. Demographic, anthropometric and psychological variables

To ensure the homogeneity of the samples and comparisons among them, avoiding possible confounding factors that could interfere with both the stress response and memory performance, in all the studies we measured the same demographic and anthropometric variables, as follows:

- *Body Mass Index (BMI)*. Once participants arrived at the laboratory, we measured their height and weight in order to then calculate the BMI (Kg/m2). This index has been associated with HPA-axis functioning (Dettenborn et al., 2012) and cognitive performance (Cournot et al., 2006).

- *Subjective Socioeconomic Status (SES)*. SES was assessed using the MacArthur Scale of Subjective Social Status (Adler et al., 2000). Participants had to rate their subjective socioeconomic status in comparison to other Spanish people. The scale ranging from 1 (people who have low socioeconomic status because they have less money, education and a less respected job or even no job) to 10 points (people who have a high socioeconomic status because they have the most money, education and respected job). This variable has been related to HPA-axis activity (Cohen et al., 2006).

- *Educational level*. The educational level was determined by asking participants what educational level they had completed. Participants had to choose one option among some possibilities: 0= no studies, 1= primary school, 2= secondary education, 3=university and higher education, and 4= postgraduate studies (master, PhD).

In the third study, a psychological variable was included to assess the stress response at the psychological level.

- *The situational appraisal*. We measured how participants perceived the stress (TSST) or the control tasks by using 5 questions about particular aspects of the tasks. Specifically, the questions were related to the degree of stress, difficulty, frustration, effort and motivation that the task provoked in the participants. These questions were previously elaborated in our group (Gonzalez-Bono, 2002) from the existing evidence about this topic (Baggett et al., 1996).

1.4.3.3. Enzymatic and hormonal variables

To study the SNS and HPA-axis functioning, we analyzed the sAA and salivary cortisol levels. However, depending on the aim we wanted to address, they were analyzed in different situations: in response to stress and/or in a basal situation. It is important to note that the biomarkers were obtained from saliva because is a non-invasive and readily accessible tool. Moreover, measuring cortisol levels in saliva allows us to determine only the free hormone fraction, which is biologically active (Foley and Kirschbaum, 2010).

- *Salivary alpha-amylase (sAA)*. We measured the SNS activity through the sAA levels. To do this, participants provided several saliva samples at different time points, according to each study procedure, using salivettes (Sarstedt, Nümbrecht, Germany). For 1 minute, participants had to keep the cotton swab in their mouths, moving it in a circular pattern to collect saliva from all the salivary glands (Rohleder and Nater, 2009). After the samples were frozen, sAA levels were obtained by an enzyme kinetic method. Specifically, sAA was studied in response to stress (TSST) or the control condition in studies 1, 2 and 3.

- *Salivary cortisol*. The HPA-axis activity was measured through the salivary cortisol levels. According to the aim of each study, the saliva samples were provided by participants using salivettes and/or by depositing 5 ml of saliva in plastic vials for no more than 5 minutes. The concentrations of cortisol were analyzed by a competitive solid-phase radioimmunoassay. This biomarker was studied in different situations: in response to the stressor (TSST) in studies 1, 2 and 3 and in the basal situation in study 4, given that in this latter study we collected saliva samples at different time points on two consecutive weekdays to obtain the diurnal pattern of cortisol, including the

CAR. Moreover, in the same study, we obtained the pre- and post-neuropsychological assessment salivary cortisol levels.

1.4.3.4. Cognitive variables

The cognitive process investigated in the present thesis was the memory performance, and more specifically, the declarative memory performance. However, other types of memory have also been considered.

- *Non-declarative memory*. This type of memory includes classical conditioning, non-associative learning, motor, perceptual, and cognitive skill acquisition and priming effects (Daum and Ackerman, 1997). In this thesis, we studied this type of memory through the latter (study 1). Priming refers to a change in the speed, bias, or accuracy of the processing of a stimulus after prior experience with the same or a related stimulus (Henson, 2003). To assess it, we used a word-stem completion task.

- *Declarative memory*. In this thesis, this memory has been addressed in all four studies. To assess it, we used a word list (studies 1 and 2), pictures (study 3), and paragraphs, a word pairs list, and pictures (study 4).

- *Working Memory*. This type of memory allows us to store and manipulate a limited amount of information in the short-term. In our research, it has been tackled in study 4 using three different types of tasks: a digit task, a letters and digits task, and a spatial task.

1.4.3.5. Moderating variables

As I summarized above, the direction of the stress effects on memory depends on several factors, some related to characteristics of the individual (i.e. sex and gender) and others associated with aspects of the memory task (i.e. arousal and valence of the material to be remembered). Therefore, we considered these factors in our research.

- *Sex*. This factor has been considered in all four studies. Thus, we systematically compared men and women. Given that the moderator role of sex could be explained by the different sex hormone levels, in study 1 the menstrual cycle phase and the intake of contraceptives were taken into account. Moreover, all the older women were post-menopausal, and none of them were receiving estrogen replacement therapy (studies 2, 3 and 4).

- *Age*. With regard to this factor, we compared men and women, as well as the stress effects on memory between young and older people (studies 2 and 3). Only young and only older people were included in the samples of studies 1 and 4, respectively. The age range in the young people was from 18 to 35 years old, and in older people from 54 to 76 years old.

- *Type of material to be remembered*. We addressed this factor by investigating only neutral material in studies 1, 2 and 4. In study 3, participants had to learn neutral and emotional material (i.e. positive and negative).

CHAPTER II

STUDY 1

STUDY 1

The effects of stress prior-learning on memory performance in young people[1]

[1] The main results of this study have been published in: Hidalgo, V., Villada, C., Almela, M., Espin, L., Gomez-Amor, J., and Salvador, A. (2012). Enhancing effects of acute psychosocial stress on priming of non-declarative memory in healthy young adults. *Stress: The International Journal on the Biology of Stress.* 15(3): 329-338.

2.1. INTRODUCTION

Exposure to stress can have impairing or enhancing effects on memory, attention and executive functions (for reviews see: Shors, 2006; Lupien et al., 2007; Schwabe et al., 2010). The influence of stress on these cognitive processes has been related to the stress-induced activation of both the hypothalamus-pituitary-adrenal axis (HPA-axis) and the sympathetic nervous system (SNS). In fact, it has been demonstrated that the release of cortisol, the end-product of HPA-axis activity, and several SNS biomarkers (e.g.: cathecolamines) can influence cognitive processes (for review see: Roozendaal, 2002). Among the SNS biomarkers, salivary alpha-amylase (sAA), an oral cavity enzyme, has increasingly been used as an indicator of SNS activation because is easier to measure than cathecolamines (for reviews see: Nater and Rohleder, 2009; Rohleder and Nater, 2009). The current study investigated whether HPA-axis and SNS activation in response to acute psychosocial stress affects different memory systems (implicit and explicit systems).

It is well known that the impact of stress on implicit memory has been understudied. Implicit memory represents the effect of unconscious prior experience on subsequent behavior (Graf et al., 1984). This type of memory includes priming effects, classical conditioning and non-associative learning, as well as motor, perceptual and cognitive skill acquisition (Daum and Ackerman, 1997). According to Henson, priming refers to a change in the speed, bias or accuracy of the processing of a stimulus, following prior experience with the same, or a related, stimulus (Henson, 2003). Only a few studies have investigated the impact of acute stress on priming, and results from these studies are inconclusive. No effects of acute stress on priming have been reported among people from middle- to older ages (Lupien et al 1997; Domes et al., 2002), but more recently Eich and Metcalfe (2009) found in a younger sample that a physical stressor (running a marathon) was associated with an enhancement of priming effects. However, Eich and Metcalfe did not include physiological measures in their study; therefore, we cannot know if this enhancing effect was

related to HPA and/or SNS activation. To our knowledge, only one study, in young men, has directly investigated the impact of cortisol administration on priming, finding that high cortisol concentrations did not have any effect on this kind of implicit memory (Kirschbaum et al., 1996). The current study further investigated whether the stress-induced change in the activity of the HPA-axis (i.e. cortisol) and SNS (i.e. sAA) affects implicit memory measured by priming.

Although the effects of stress on non-declarative memory have not been studied in detail, the relationship between stress and declarative memory has been investigated more thoroughly. It has been shown that cortisol exerts a modulatory effect on declarative memory performance through its action on brain areas that are also important for memory functioning. These brain areas are mainly the hippocampus and the prefrontal cortex, which have a large number of receptors for cortisol (de Kloet et al., 1999; Roozendaal, 2000; Lupien et al., 2009). Cortisol can have either enhancing or impairing effects on declarative memory performance, depending on several factors such as the memory phase under investigation (i.e. acquisition, consolidation or retrieval), or the emotional valence of the material to be remembered (i.e. emotional or neutral). Cortisol has been shown to enhance memory consolidation but impair memory retrieval (Roozendaal, 2002); moreover, due to the moderating role of the amygdale, the impact of cortisol on memory performance is stronger for emotionally arousing material than for neutral material (McEwen, 2002; Roozendaal, 2002; Lupien et al., 2005, 2007; Sandi and Pinelo-Nava, 2007).

Our study investigated the impact of psychosocial stress on priming and declarative memory performance when stress was applied prior to learning, using neutral content. Previous studies with a similar design have found mixed results. Some studies show an impaired short-term declarative memory recall after exposure to stress (from 20 to 60 min after learning) compared to a control group (Jelicic et al., 2004; Payne et al., 2006, 2007; Smeets et al., 2006), while others found no effect (Wolf et al., 2001b; Elzinga et al., 2005), or even an

enhancing effect of stress on declarative memory performance (Schwabe et al., 2008). The majority of the studies showing that stress induction affected declarative memory performance failed to find that the release of cortisol during stress was proportionally related to declarative memory performance, either because these studies did not investigate this or because the results were non-significant (Jelicic et al., 2004; Payne et al., 2006, 2007; Smeets et al., 2006; Schwabe et al., 2008). In fact, only two studies have shown that the stress-induced cortisol increase was indeed negatively related to declarative memory performance when stress was applied prior to learning (Kirschbaum et al., 1996; Wolf et al., 2001b). In contrast, Nater et al. (2007) found the opposite result that the high cortisol responders to stress performed better on the declarative memory task than the low cortisol responders.

Only a few studies have investigated whether the stress-induced sAA release had an effect on declarative memory performance. These studies found enhancing effects of sAA release on memory performance (Segal and Cahill, 2009; Smeets et al., 2009) and no effects (Preuβ and Wolf, 2009).

The current study investigated, among young people, the hypothesis that cortisol and sAA responses to acute psychosocial stress would be associated with priming and declarative memory performance. It has been suggested that the relationship between acute stress and memory processes could be moderated by sex (Andreano et al., 2008). However, previous studies either only included one sex (Nater et al., 2007) or they included both sexes but without registering the menstrual cycle phase of the women, which should be taken into account when studying the impact of cortisol reactivity on acute stress (Kirschbaum et al., 1996; Jelicic et al., 2004; Elzinga et al., 2005; Payne et al., 2006, 2007; Smeets et al., 2006). Therefore, in this study we included women in their early follicular phase and women using hormonal contraception, both groups usually showing responses to stress that differ more than those of women in their luteal phase of the menstrual cycle when compared to men's responses. In a crossover design,

participants were exposed to both psychosocial stress (TSST) and a control condition. Based on previous studies in young people, we expect a higher cortisol response to stress in men than in women (Kirschbaum et al., 1999) and no sex difference in the sAA response to stress (Rohleder and Nater, 2009). Due to the mixed results of acute stress on priming and declarative memory, we explored whether acute stress affected these memory processes, taking in account the sex and hormonal state of the participants. Finally, we investigated whether the cortisol and sAA reactivity to stress had an effect on priming and declarative memory performance, and whether this effect was different for men and women.

2.2. METHOD

2.2.1. Participants

The final sample was composed of 52 subjects: 18 men, 17 women in the early follicular phase (2-5 days), and 17 women using oral contraceptives (monocyclic formulas) for at least 6 months. The age of participants was between 18 and 35 years (Total sample: *M* = 21.56; *SEM* = 0.55).

The subjective socioeconomic status (Subjective SES Scale, Adler et al., 2000) was medium-high, and there were no significant differences between groups (Total sample: *M* = 6.33; *SEM* = 0.13). The groups did not differ with respect to age or body mass index (BMI). Most of them (94%) were college students from different areas. One hundred fifty-nine volunteers were interviewed and completed a standardized questionnaire to check whether they met the study prerequisites. The criteria for exclusion were: smoking more than five cigarettes a day, alcohol or other drug abuse, visual or hearing problems, presence of a cardiovascular, endocrine, neurological or psychiatric disease, having been under general anesthesia once or more than once in the past year, the presence of a stressful life event during the last year, or using any medication

directly related to cardiac, emotional or cognitive function, or one that was able to influence hormonal levels, such as glucocorticoids or β-blockers. One hundred and seven volunteers were dropped from the sample for two reasons: thirty-four of them did not meet the exclusion criteria mentioned above; and the rest, seventy-three, because their schedules were incompatible with the experiment's features (two days, four hours, and only in the afternoon).

Participants meeting the criteria were contacted by telephone and asked to attend two sessions that took place in a laboratory at the Faculty of Psychology. No economic payment was made for participation, although they received a pen drive (approximately 15 €). Before each session, participants were asked to maintain their general habits, sleep as long as usual, refrain from heavy activity the day before the session, and not consume alcohol since the night before the session. Additionally, they were instructed to only drink water and not eat, smoke or take any stimulants, such as coffee, cola, caffeine, tea or chocolate, two hours prior to the session. The study was conducted in accordance with the Declaration of Helsinki, and the protocol and conduct were approved by the University of Valencia Ethics Research Committee. All the participants received verbal and written information about the study and signed an informed consent form.

2.2.2. Procedure

The procedure was similar to the one employed previously in a sample with old people (Almela et al., 2011a). It was a within-subject design with two completely randomized and counterbalanced conditions in two separate sessions: a stress condition and a control condition, with less than two weeks between sessions, except for the women in the follicular phase with four days. The test-retest interval was different in this group in order to ensure the same phase of the menstrual cycle in both conditions. The sessions consisted of several phases of equal duration for both conditions. Sessions took 1 hour and 50

minutes to complete, and they were always held between 16.00 and 20.00 hours. Each participant started his or her two sessions at the same hour (see Figure II.1). Upon arrival at the laboratory, the weight and height of the participants were measured, and the experimenter checked to see whether they had followed the instructions given previously.

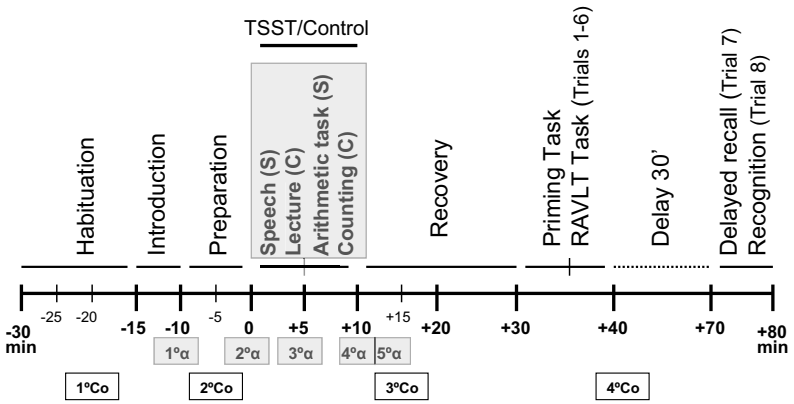

Figure II.1. Timeline of the TSST (S) and control (C) conditions. Salivary cortisol samples = 1ºCo, 2ºCo, 3ºCo, 4ºCo. Salivary alpha-amylase samples = 1ºα, 2ºα, 3ºα, 4ºα, 5ºα. RAVLT = Rey auditory verbal learning test.

Stress Condition. To produce stress, we subjected the participants to the Trier Social Stress Test (TSST, Kirschbaum et al., 1993). The stress task consisted of 5 min of free speech (job interview) and a 5 min arithmetic task, and it was performed in front of a committee composed of a man and a woman. The participants remained standing at a distance of 1.5 meters from the committee. Additionally, a video camera and a microphone were clearly visible. Both the speech and arithmetic tasks were filmed.

The protocol started with a habituation phase of 15 min to allow the participants to adapt to the laboratory setting. During this phase, the participants remained seated. Five minutes after the start of this phase, subjects provided the first cortisol saliva sample (-20 minutes pre-stress). After the

habituation phase, the introduction phase started (duration 5 min). In this phase the participants were informed about the procedure for the stress task. They received the instructions in front of the committee in the same room where the task took place. After this, subjects provided the first sAA sample (-10 minutes pre-stress). Next, the participants had 10 minutes to prepare for the task at hand. At that moment, they provided the second cortisol saliva sample (-5 minutes pre-stress), and the second sAA sample was provided when this phase ended (0 minute).

Following the preparation phase, the stress task was carried out. During the stress task, participants provided the third (after speech, +5 minutes) and fourth sAA sample (after the arithmetic task, +10 minutes). Then, subjects had 20 minutes to recover after the stress task, and they provided the fifth sAA sample (+14 minutes post-stress) and the third cortisol saliva sample (+15 minutes post-stress) during this recovery period. Each participant then performed two memory tests. Participants first did a priming task, more concretely, a word-stem completion task, to assess non-declarative memory, and then they performed a standardized memory test consisting of 8 trials (Rey Auditory Verbal Learning Test, RAVLT), in order to measure declarative memory. The participants completed the first six trials between 30 to 40 minutes after the TSST. After trial 6, they waited 30 minutes (delay period) before they continued with the memory test. During the delay period, the participants provided the fourth saliva sample (+40 minutes post-stress). After the delay period, they finished the memory test with trials 7 and 8 and, finally, were debriefed.

Control Condition. The control condition was similar to the experimental condition, except that the stressful task was replaced by a control task. This task was designed to be similar to the stress task in mental workload and global physical activity, but without the main components capable of provoking stress, such as evaluative threat and uncontrollability (Dickerson and Kemeny, 2004). The control task was composed of 5 minutes of reading aloud and 5 minutes of

counting. In the preparation phase, the participants did not prepare for their task, but instead they read a book with neutral content. The timing of the saliva samples and the phase durations were the same for the two conditions.

2.2.3. Memory

Priming. We used a word-stem completion task to assess priming. Two parallel word lists were used to avoid a learning effect (List A and List B). The order of the two versions was randomized and counterbalanced. First, the experimenter presented a list of 26 neutral words. The participants had to read each word aloud and rate its degree of familiarity on a Likert scale ranging from 1 (unfamiliar) to 7 (extremely familiar). After this step, subjects performed a distracting task that lasted two minutes. The distracting task consisted of writing words beginning with the letters "b" and "l" (List A) or "d" and "p" (List B). Finally, the word-stem completion task was performed. The participants had to complete a list containing 78 stems of words (first three letters). Among these words were the 26 words read previously. No restriction was imposed as to the category of word that could be given as a completion. The participants were instructed to complete the list of stems as fast as possible and with the first word that came to mind. This instruction, which provokes the priming effect through the implicit recall of the words presented previously, differs from the "word-stem cued-recall", which explicitly instructs participants to complete the stems using words that have been presented previously (Henson, 2003).

We obtained three scores: (i) number of frequent words, (ii) number of non-frequent words and (iii) number of total words (sum of frequent and non-frequent words) recalled from the target list. To control the effect of chance, another group of 31 young subjects did the word-stem completion task, but without the target lists being presented previously. This group was called the 'priming baseline group'. The number of words from the two lists that could be

correctly completed by chance was subtracted from the scores of the experimental subjects (Lupien et al. 1994, 1997).

Declarative memory. To measure declarative memory, the Spanish version of Rey´s Auditory –Verbal Learning Test (RAVLT) was used (Miranda and Valencia, 1997). This test has several versions, and for each participant a different version of the RAVLT was used in the second session to avoid learning effects. The order of the two versions was randomized and counterbalanced. The RAVLT is composed of different trials. In the first five trials the experimenter read aloud a target list of 15 neutral words, and each participant had to repeat as many words as possible in each of the five trials. The performance on these first five trials reflects the rate of learning (Trials 1 to 5: *Learning curve*). After trial 5, the experimenter read aloud an interference list of 15 words and tested the retention of these new words. Following this step, participants were requested to recall the words from the target list (Trial 6: *Recall after interference*); after a delay of 30 minutes they had to recall them a second time (Trial 7: *Delayed recall*). In trial 8 (*Recognition*), participants had to recognize the memorized words from a list presented verbally containing 15 new and 15 previously learned words. Trial 8 was divided into two different scores: *Hits*, the number of words correctly recognized as being on the target list; and *False alarms*, the number of words incorrectly recognized as being on the target list.

2.2.4. Biochemical Analyses

Cortisol. Participants provided four saliva samples by depositing 5 ml of saliva in plastic vials. They took approximately 5 minutes to fill the vial. The samples were frozen at -80ºC until the analyses were performed. The samples were analyzed by a competitive solid phase radioimmunoassay (tube coated), using the commercial kit Coat-A-Count C (DPC, Siemens Medical Solutions Diagnostics). Assay sensitivity was 0.5 ng/ml. For each subject, all the samples

were analyzed in the same trial. The within- and inter- assay variation coefficients were all below 8%.

Alpha-amylase (sAA). Saliva was collected using salivettes (Sarstedt, Nümbrecht, Germany). Participants were instructed to introduce the cotton swab into their mouths for exactly 1 min, not chew the cotton, and move the swab around in a circular pattern to collect saliva from all the salivary glands (Rohleder and Nater, 2009). The samples were frozen at -20º C after the completion of the session until the analyses took place. The samples were shipped to Dresden and analyzed at the Kirschbaum lab, Technical University of Dresden. Concentration of alpha-amylase in saliva was measured by an enzyme kinetic method according to the protocol specified in Rohleder et al. (2006). The lowest detectable concentration in our assay was 1.56 U/ml. Inter- and intra-assay variation was below 10%. Analyses of sAA failed to detect the sAA concentrations in the samples of one man and one OC user.

2.2.5. Statistical Analyses

Data were checked for normal distribution and homogeneity of variance using Kolmogorov-Smirnov and Levene's tests before statistical procedures were applied. Since neither the cortisol nor the sAA data had a normal distribution, they were square root transformed.

One-way ANOVAs were used to investigate group demographic and anthropometric differences. Cortisol and sAA responses were assessed using ANOVAs for repeated measures with a between-subject factor (Group: men, M; women in follicular phase, F; and women oral contraceptive users, OC) and two within-subject factors, Condition (stress vs. control) and Time (Cortisol: -20, -5, +15, +40; sAA -10, 0, +5, +10, +14).

Student's *t*-tests were used to investigate the priming effect between groups, the experimental groups and the priming baseline group. We used an ANOVA for repeated measures to analyze non-declarative memory, employing Condition as a within-subject factor and Group (M, F and OC) as a between-subject factor.

The declarative memory test used (RAVLT) provides one score for each trial performed, consisting of the number of correct words recalled in each trial. In trials 1 to 7, the words from the same target list have to be recalled; for this reason, we performed an ANOVA for repeated measures. We used Condition (stress vs. control) and Trials (trials 1 to 7) as within-subject factors and Group as a between-subject factor. To analyze the effects on recognition (trial 8), we used d-prime (d´), which is the difference between the standardized proportion of correct hits and the standardized proportion of false alarms.

Due to the great variability among subjects in their cortisol reactivity to psychosocial stress, we divided the sample into responders and non-responders, according to Schommer et al. (2003). Responders were those individuals who had an increase of at least 2.5 nmol/L in cortisol levels from the baseline levels (-20 min) to the third cortisol sample (+15 min), the sample immediately after the stress test. In addition, stress-induced sAA reactivity was calculated by subtracting sAA levels in the sample immediately after the TSST (+10) and baseline levels (-10). Pearson's correlations were calculated in order to assess whether cortisol reactivity and sAA reactivity to the stress task were related to priming and explicit memory performance.

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. All *p*-values reported are two-tailed, and the level of significance was marked at <0.05. When not otherwise specified, results shown are means ± standard error of means (SEM). We used SPSS 15.0 to perform the statistical analyses. For an easy interpretation

of the figures, the values in the figures represent raw values and not square root transformed values.

2.3. RESULTS

2.3.1. Stress Response

Cortisol. The repeated measures ANOVA with cortisol levels as the dependent variable showed main effects for Condition (F (1, 45) = 14.362, p < 0.001), Time (F (1.64, 74.1) = 10.052, p < 0.001), and their interaction: Condition×Time, F (1.54, 69.49) = 50.132, p < 0.001. There were no baseline differences between conditions (p > 0.2). In the stress condition, cortisol levels increased immediately after the stress task (p < 0.001), and they decreased, recovering baseline levels, in the last saliva sample (p > 0.7).In the control condition, cortisol levels decreased over time according to the normal cortisol circadian rhythm (for all $p \leq 0.001$).

The Group (M, F and OC) factor was significant, (F (2, 45) = 4.608, p = 0.015) as was the interaction between Condition, Time and Group *(F* (3.09, 69.49) = 3.699, p = 0.015). Baseline cortisol did not differ between groups (all p > 0.1).However, five minutes before the TSST, men had higher cortisol levels than the F group (p = 0.028), but not the OC users (p = 0.2). After the exposure to the stressor, men had higher cortisol levels than both groups of women in the +15 sample (for all $p \leq 0.006$), and in the +40 sample (for all $p \leq 0.024$) (see Figure II.2A). Both groups of women had a lower cortisol response to stress than men, but their cortisol levels increased in response to stress, as they were higher in the stress condition than in the control condition in samples +15 (for both $p \leq$ 0.045) and +40 (for both $p \leq 0.018$). In the two groups of women, the cortisol response to stress was not different (p > 0.9). In the control condition, there were no differences between groups for any cortisol sample (p > 0.3) (see Figure II.2B).

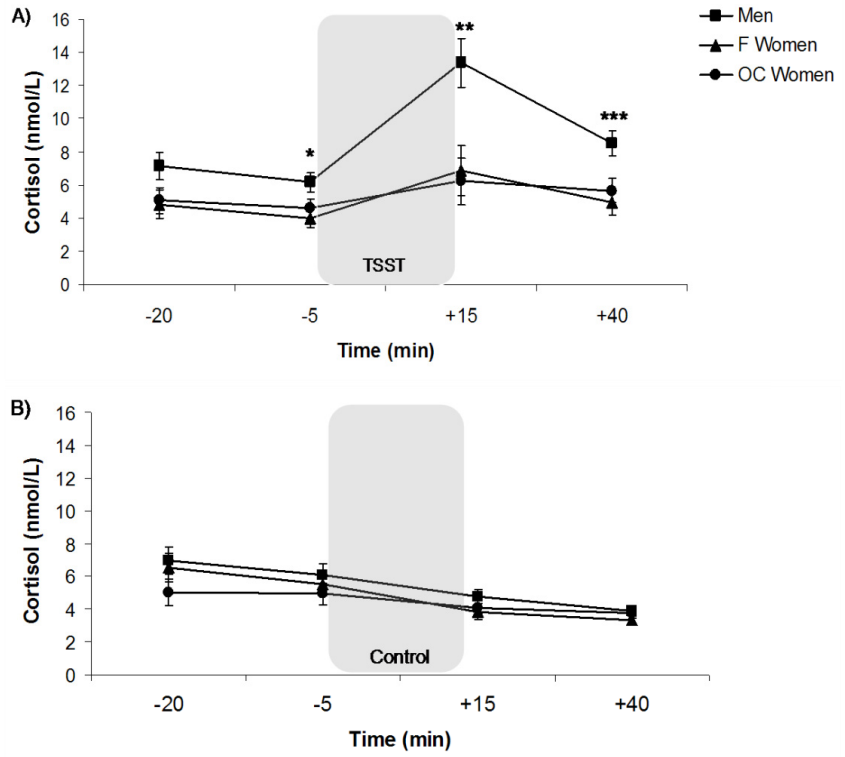

Figure II.2. Salivary cortisol concentrations in the stress, TSST (A) and control (B) conditions for men (*N* = 16), follicular women, F (*N* = 15), and oral contraceptive women, OC (*N* = 17). In the stress condition (A), the repeated measures ANOVA showed that men had higher cortisol levels than F women in the -5 sample (**p* = 0.028) and that men had higher cortisol levels than F and OC women in the +15 sample (**for both $p \leq 0.006$) and in the +40 sample (***for both $p \leq 0.024$). In the control condition (B) there were no significant group differences in cortisol levels (for all $p > 0.3$). Depicted values are means and error bars represent the standard error mean.

Salivary Alpha-amylase (sAA). The repeated measures ANOVA with sAA levels as the dependent variable showed main effects for Condition (F (1, 45) = 27.764, $p < 0.001$), Time (F (4, 18) = 25.795, $p < 0.001$), and their interaction: Condition×Time (F (3.19, 143.42) = 6.833, $p < 0.001$). The Group factor and its interactions with the other factors were not significant, (for all $p > 0.3$).

Baseline sAA levels were similar between conditions (p > 0.5). In the stress condition, one minute before the TSST there was an anticipatory increase in sAA levels (p = 0.006). The sAA levels continued increasing, reaching their peak at the end of the speech (p = 0.002), and remaining constant at the end of the arithmetic task (p > 0.99). Participants had recovered baseline in the last saliva sample (p > 0.1). In the control condition, the response profile was similar to that of the stress condition, except that there was no anticipatory response (p > 0.5). However, all the sAA levels, except baseline, were lower in the control condition than in the stress condition (for all p < 0.001) (see Figure II.3).

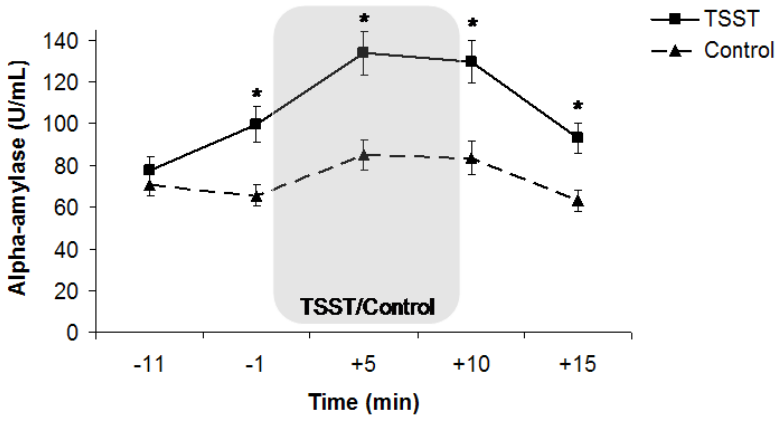

Figure II.3. Salivary alpha-amylase (sAA) concentrations in the stress (TSST) and control conditions for total sample (N = 48). The repeated measures ANOVA showed significant differences in sAA levels between conditions. Participants had higher sAA levels in the stress condition than in the control condition in the -1, +5, +10, and +15 saliva samples (*for all p < 0.001). There was no difference in baseline sAA levels between conditions (p > 0.5). Depicted values are means and error bars represent the standard error mean.

2.3.2. Memory

Priming. Participants correctly completed 6 (±0.41) words from List A, and 6.12 (±0.28) words from List B. The priming baseline group completed 2.94

(±0.36) words from List A and 3.86 (±0.60) words from List B. Therefore, there was a significant priming effect for the participants compared with the priming baseline group (List A: t (55.70) = 5.602, p < 0.001; List B: t (64) = 3.672, p < 0.001).

The repeated measures ANOVA with priming as a dependent variable revealed the main effect of Condition, F (1, 45) = 5.732, p = 0.021. The Group factor and its interaction with Condition were non-significant (for all p > 0.7) (see Figure II.4). The participants recalled more words from the target list in the stress condition than in the control condition. The frequency of the words did not affect their priming (for all p > 0.1).

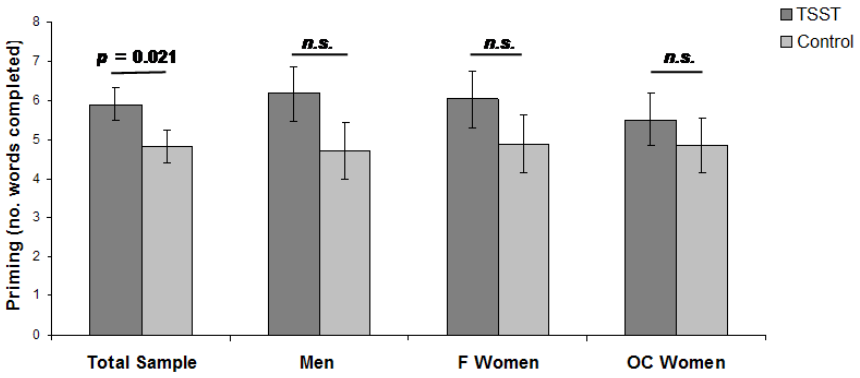

Figure II.4. The effect of condition (stress, TSST and control) on priming represented for the total sample (N = 48), for men (N = 16), follicular women, F (N = 15), and oral contraceptive women, OC (N = 17). The repeated measures ANOVA revealed significant differences in priming between conditions only for the total sample (p = 0.021). Depicted values are means and error bars represent the standard error mean.

Declarative Memory. The repeated measures ANOVA with declarative memory as the dependent variable only revealed a main effect of Trial, (F (3.747, 161.113) = 196.223, p < 0.001), but not Condition, Group, or the interactions between these factors (for all p > 0.4). Across both the stress and control

conditions, there was a positive learning curve from trial 1 to trial 4 (for all $p < 0.001$). No more words were learned from trial 4 to trial 5 ($p > 0.2$). Participants recalled fewer words in the trial immediately after the interference list (trial 6) than in the trial before it (trial 5) ($p < 0.001$). Finally, they recalled a similar number of words after the 30-minute delay (trial 7) and before this delay (trial 6) ($p > 0.9$). The repeated measures ANOVA with recognition as dependent variable did not show any main effect for Condition or Group, nor was there an interaction between these factors (for all $p > 0.4$) (see Figure II.5).

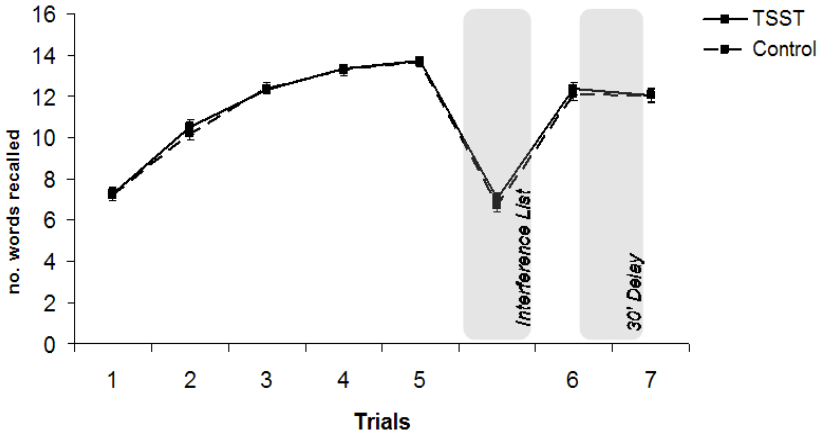

Figure II.5. Number of words recalled in the stress (TSST) and control condition represented for the total sample ($N = 46$) in each trial of the RAVLT (Rey Auditory Verbal Learning Test). Depicted values are means and error bars represent the standard error mean.

2.3.3. Stress Reactivity and Memory

Priming. Cortisol reactivity to the stress induction was not correlated with the number of words correctly completed in the priming test ($p > 0.6$). There were no differences between the cortisol responders and non-responders to the stress induction in priming ($p > 0.2$). However, sAA reactivity was positively

correlated with priming test performance (r = 0.339, p = 0.018). Thus, those who increased their sAA levels more in response to the stress induction completed more words from the target list on the priming test (see Figure II.6).

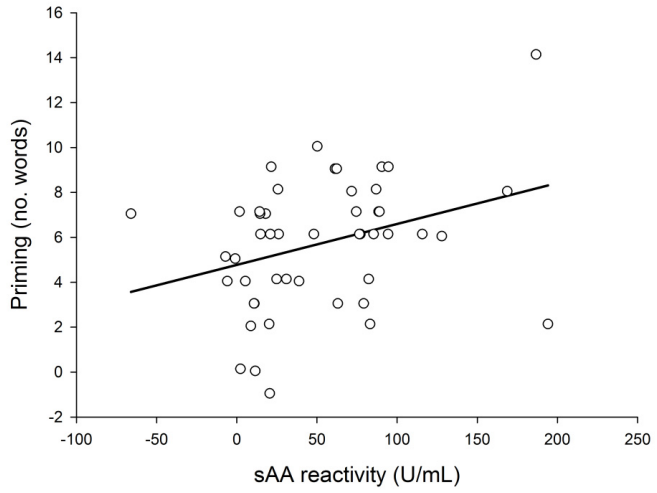

Figure II.6. The relationship between salivary alpha-amylase (sAA) reactivity and priming in the stress condition for the total sample (N = 48; r = 0.339, p = 0.018).

Declarative Memory. Cortisol reactivity did not correlate with declarative memory performance (for all p > 0.3). Declarative memory performance did not differ between cortisol responders and non-responders to the stress induction (for all p > 0.1). The stress-induced sAA increase did not correlate with declarative memory performance (for all p > 0.5).

2.4. DISCUSSION

The aim of our study was to analyze the effects of acute psychosocial stress on non-declarative memory, measured by priming, and on declarative memory performance in young men and women. The main results of our study

were that acute stress was associated with an enhancement of priming effects, and that this improvement in performance was positively related to the stress-induced sAA increase.

To provoke stress we employed the TSST, which is a standardized psychosocial stressor that has been shown to produce a consistent stress response (Dickerson and Kemeny, 2004). The TSST was indeed able to induce stress, since it stimulates an increase in the participant's cortisol levels and a higher sAA release compared to the control condition. Sex had a modulator effect on the stress-induced cortisol response, as has been shown in other studies (Kirschbaum et al., 1992, 1999; Preuβ and Wolf, 2009; Childs et al., 2010). Women in the follicular phase of their menstrual cycle or using oral contraceptives did not differ in their cortisol levels in any sample of the stress condition or the control condition. However, men increased their cortisol levels more in response to the TSST than both groups of women. Moreover, although with a blunted response, women did respond to the stress induction with an increase in their cortisol levels, because they had higher cortisol levels in the salivary samples taken after the TSST than in the salivary samples taken after the control task. Conversely, there were no sex differences in the stress-induced sAA release, which is consistent with previous findings (Rohleder and Nater, 2009; Almela et al., 2011b).

The stress response was associated with an enhancement of the number of words correctly completed on the priming test. Additionally, this improvement effect was not different between men and women, suggesting that neither sex nor oral contraceptive intake had an influence on this effect. Previously, Eich and Metcalfe (2009) found a similar result when measuring priming also with a word-stem completion task after exposure to a physical stressor (running a marathon). Others have reported enhancing effects of acute stress when measuring implicit memory through other kinds of strategies. For example, Luethi et al. (2009) found that the exposure to the TSST improved classical conditioning only for

negative stimuli, and others have found that acute psychosocial stress enhances fear conditioning (Jackson et al., 2006; Zorawski et al., 2006). Furthermore, Schwabe et al. (2007) reported that the exposure to the TSST increased the classical-conditioning learning strategy for neutral material over spatial learning strategies, which require more conscious processing.

Taken together, these findings support the hypothesis that acute stress induces a shift between memory systems. Thus, learning strategies that require less conscious processing, and therefore are faster and less demanding, are favored under acute stress over strategies that require awareness and more complex processes (Schwabe et al., 2007). Additionally, in our study, the enhancing effect of stress on priming was higher among those who responded to stress with a larger sAA increase. Nevertheless, the cortisol response to stress apparently was not related to the outcome of the priming test. To our knowledge, this positive relationship between sAA reactivity to stress and an enhancement of priming has not been reported previously. This finding suggests that SNS activation is crucial for the enhancing effect of stress on implicit memory. In fact, using a 3D spatial task, Schwabe and colleagues found that the participants used more implicit learning strategies to solve the task after being exposed to the TSST, which induces the activation of both the HPA-axis and SNS (Schwabe et al., 2007), but they employed more explicit learning strategies to solve the task after the infusion of glucocorticoids (Schwabe et al., 2009). Similarly, Kirschbaum et al. (1996) found that the administration of glucocorticoids did not have any effect on a short-term priming test. In our opinion, an involvement of HPA-axis activity on the modulation of implicit memory by stress cannot be completely discarded because animal research has shown that cortiscosterone enhances long-term memory consolidation of implicit memory through its action on the dorsal striatum (Quirarte et al., 2009). In our study, we only measured short-term implicit memory; therefore, we could not know about long-lasting effects of HPA-axis activation on implicit memory. Further research is needed to disentangle these relationships.

In the current study, we did not find any effect of acute stress on declarative memory. In fact, throughout the five trials of the learning curve, the participants learned, recalled (immediate and delayed recall) and recognized a similar number of words in both the stress and control conditions. This result contrasts with the results of other studies that found an impairing effect of acute stress on declarative memory for neutral material when stress was applied prior to learning. In our opinion, a main reason for this divergent result could be related to the magnitude of the stress-induced cortisol reactivity, since cortisol reactivity to stress has been identified as a main factor involved in short-term declarative memory impairment (Kirschbaum et al., 1996; Wolf et al., 2001b). The other studies were performed only in men (Nater et al., 2007), in men and women without controlling their menstrual cycle or oral contraceptives intake (Kirschbaum et al., 1996; Jelicic et al., 2004; Payne et al., 2006, 2007; Smeets et al., 2006), or in men and women in their luteal phase (Wolf et al., 2001b). It has been shown that men react to stress with larger cortisol increases than women in their follicular phase or women taking oral contraceptives. Moreover, women in their luteal phase have a cortisol reactivity to stress that is comparable to men's (Kirschbaum et al., 1999). Therefore, it is likely that the null effects found in our study were due to the fact that the magnitude of the cortisol response in the majority of the sample (i.e. more women in their follicular phase or taking contraceptives than men) was low. Even when we divided the sample into responders and non-responders, we failed to find effects of cortisol response to stress on declarative memory. This could be explained by the fact that due to our design, learning and retrieval processes occurred under the same stressful conditions, leading to a compensatory process. With Roozendaal's model in mind, which indicates that cortisol enhances the consolidation memory and impairs the retrieval memory (Roozendaal, 2002), this compensatory process would be explained because the enhancing effects of stress on consolidation might be canceled by impairing effects on retrieval.

Some limitations have to be considered in order to interpret our results. We aimed to test one group of women in their early follicular phase and this meant that this group of women had a different test-retest interval compared to the other two groups, which could have affected our results. Additionally, related to the null effects found of stress on declarative memory, it would be advisable in future studies to also include a group of women in the luteal phase, in order to ensure the comparability of the cortisol response between men and women. Finally, similarly to other studies (Kirschbaum et al., 1996; Lupien et al., 1997), the order of the priming test and the declarative test were not counterbalanced, which made it impossible to know whether the effect found is specific to the priming task or whether it is specific to the point in time when the priming task took place.

In conclusion, we have confirmed that acute stress may not only affect declarative memory but also implicit memory, and that this enhancing effect is related mainly to the activity of the sympathetic nervous system.

CHAPTER III

STUDY 2

STUDY 2

The impact of stress prior learning on memory performance: the role of age and sex[3]

[3] The main results of this study have been published in: Hidalgo, V., Almela, M., Villada, C., and Salvador, A. (2014). Acute stress impairs recall after interference in older people, but not in young people. *Hormones and Behavior*. 65(3): 264-252.

3.1. INTRODUCTION

Stress has been suggested as a main factor related to negative changes observed during the aging process. However, little is known about the role of age in acute stress effects on memory performance. Given that there are data suggesting age differences in the reactivity to stress, the need to obtain evidence to fill this gap in the literature seems clear.

Stress, particularly, provokes the activation of two systems: (i) the sympathetic nervous system (SNS) and (ii) the hypothalamus-pituitary-adrenal axis (HPA-axis). The fast SNS response includes the release of the catecholamines (adrenaline and noradrenaline), which are responsible for different physiological changes preparing the organism for a "fight-or-flight" response. Minutes after the onset of the stressor, HPA-axis activation occurs and, consequently, large amounts of glucocorticoids are secreted in the adrenal cortex. There are numerous glucocorticoid receptors in the brain areas involved in the memory process, such as the hippocampus, the frontal lobe and the amygdala (Roozendaal, 2000; Lupien and Lepage, 2001; Lupien et al., 2009;), which also play an important role in the regulation of the HPA-axis (Lupien and Lepage, 2001; Herman et al., 2005). Thus, cortisol, the main glucocorticoid hormone in humans, would have important effects on memory, although the direction of these effects remains unclear. They can differ depending on several factors, some related to the task (such as the type of memory or the nature of the material, neutral or emotional) and others associated with characteristics of the individual (including age and sex). In addition, it has been well established that SNS activation can also affect memory performance through the influence of cathecolamines on the limbic brain structures. According to Roozendaal et al. (2009), the noradrenergic activation of the amygdala and the interactions between the amygdala and hippocampus are crucial to finding cortisol effects on hippocampus-dependent memory performance.

The majority of studies about the relationship between the exposure to an acute stressor and memory have been performed on declarative memory in young people, reporting mixed results. When subjects have to learn neutral material after stress induction, worsening effects (Kirschbaum et al., 1996; Jelicic et al., 2004; Payne et al., 2006, 2007; Smeets et al., 2006), enhancing effects (Schwabe et al., 2008; Espin et al., 2013), and even a lack of effects (Wolf et al., 2001b; Hidalgo et al., 2012) have been described in mixed-sex samples. When studying only one sex, enhancing effects were found in young men (Nater et al., 2007), but non-effects were detected in women when they were grouped without taking age into account (32-68 years) (Domes et al., 2002). Bohnen et al. (1990) compared two groups of women (41-49 vs. 61-69 years) exposed to a 4-hour mental task, finding no significant differences.

To our knowledge, only Wolf et al. (2001a) have investigated the pre-learning cortisol effects on short-term memory considering the role of age by directly comparing young and older people, specifically men. These authors reported that cortisol did not influence the recall of a list of neutral words learned after they injected a cortisol agonist (hydrocortisone). However, there are important differences between the glucocorticoid increases induced by pharmacological administration and those produced by exposure to stress. As mentioned above, in addition to the cortisol increase that occurs with drug administration, stress provokes other physiological (i.e. SNS activation) changes (Lupien and Schramek, 2006). Hence, the use of stress paradigms in the laboratory allows a more complete study of stress effects on memory performance. In recent years, SNS activation has been measured by means of the salivary alpha-amylase (sAA), an oral enzyme secreted by the salivary glands (mainly parotid glands) due to parasympathetic and sympathetic nerve stimulation innervating the salivary glands. sAA is involved in converting starch into glucose and maltose in the oral cavity (Baum, 1993), eliminating bacteria from the mouth, and preventing bacterial attachment to oral surfaces (Scannapieco et al., 1993). A growing body of literature considers sAA to be a

sensitive biomarker for stress-related changes in the body reflecting sympathetic nervous system activation (Granger et al., 2007; Nater and Rohleder, 2009; Rohleder and Nater, 2009). Moreover, as it is readily accessible and easily obtained, sAA is a good surrogate for catecholamines in psychoneuroendocrinological research.

Reactivity to stress changes throughout the lifespan; while the role of age in the cortisol response has been investigated more extensively, with most studies reporting that older people have a higher cortisol response than young people (For a review see: Kudielka et al., 2009), for the sAA response, results are fewer and mixed (Strahler et al., 2010; Almela et al., 2011b). Thus, the HPA-axis and the SNS activity could influence memory performance differently as a function of age. Furthermore, since both the HPA-axis and the SNS work in alliance to generate the stress response, in addition to the action of each system separately, it seems logical to study the two systems concurrently. According to Bauer et al. (2002), to obtain an optimal adaptation to stress, a coordinated response of the two stress systems is necessary. Thus, an uncoordinated response could mean a maladaptive response related to health or behavior problems. Studies examining this relationship in children and adolescents have suggested its value in predicting individual differences in behavioral adjustments to stress (Gordis et al., 2006, 2008; El-Sheikh et al., 2008; Vigil et al., 2010; Allwood et al., 2011). Recently, a few studies have focused on the effects of stress on cognitive functioning and even academic achievement (Berry et al., 2012; Keller et al., 2012); however, as mentioned above, these interactions, and specifically their potential effects on cognitive performance, have not been studied in young and older people.

With all this in mind, the purpose of the present study is to investigate age-related differences in memory performance in response to acute psychosocial stress, taking into account the sex and the relationship between the two stress systems, the HPA-axis and the SNS. No previous studies have been

published on the influence of an acute laboratory social stressor on declarative memory in young and older people of both sexes. Previously, we reported stress effects on declarative memory in older people, especially in post-menopausal women (Almela et al., 2011a), but not in young people (Hidalgo et al., 2012). Based on these results, in the present study we have directly compared two different age samples employing the same protocol, a statistically different approach, and both stress markers (cortisol and sAA), in order to examine the different effects of stress on declarative memory depending on age or sex. The present study compares sixty-seven healthy participants divided into two age groups, 35 young adults and 32 older adults, with a similar number of men and women in each group. All the older women were postmenopausal, and all the young women were in the early follicular phase of their menstrual cycle, that is, the period with lower sex hormone levels. In a crossover design, the participants were exposed to both psychosocial stress (Trier Social Stress Test, TSST; Kirschbaum et al., 1993) and a control condition. In each condition, declarative memory performance was measured after the task. Previous studies employing a limited age range (41-49 vs. 61-69 years) and a 4-hour mental stressor in women (Bohnen et al., 1990) or cortisol administration in men (Wolf et al., 2001a) did not find age-related differences in stress/cortisol effects on declarative memory. However, we think that with a broader age range and a psychosocial stress task as the stressor, age differences would appear in the stress effects on declarative memory. To test this, we directly compared two age groups (18-35 years vs. 54-78 years) containing men and women, and we employed the TSST, which provokes both HPA-axis and SNS activation. In addition, we investigated stress reactivity by combining the two main stress physiological systems, considering that the imbalance between the two systems (an uncoordinated response) could prejudice memory performance. Finally, since sex differences have been reported in the effects of stress on memory in older people, greater negative stress effects were expected in older women.

3.2. METHOD

3.2.1. Participants

This study is part of extensive research on the moderating role of age and sex in the effects of acute stress on memory. Partial results from the older (Almela et al., 2011a) and young (Hidalgo et al., 2012; Espin et al., 2013) participants have been previously published. Here, we employed a subsample to directly compare the stress effects on declarative memory, taking into account the age and sex factors.

The final sample employed was composed of sixty-seven participants divided into two age groups (older adults: N = 32; 16 men and 16 women; young adults N = 35; 18 men and 17 women). There were no differences between the two age groups with regard to sex, in subjective socioeconomic status (SES) or educational level, but there were differences in body mass index (BMI), with young men showing a higher BMI than young women (p = 0.047) (see Table III.1). SES was measured using the MacArthur Scale of Subjective Social Status (Adler et al., 2000). Subjects were asked to rate themselves according to their subjective socioeconomic status and compared to other people in Spain, on a scale ranging from 1 (people with the lowest education, income and worst jobs) to 10 points (people with the best education, income and jobs).

	Young			Older		
	Total	Men	Women	Total	Men	Women
Age	21.1 (0.7)	22.1 (1.2)	20.0 (0.7)	62.1 (0.8)	60.5 (1.2)	63.7 (1.1)
BMI	23.0 (0.5)	23.9 (0.7)	21.9 (0.7)	26.5 (0.5)	27.0 (0.5)	26.0 (1.0)
SES*	6.3 (0.1)	6.4 (0.2)	6.1 (0.2)	6.0 (0.2)	6.1 (0.3)	5.9 (0.3)
E. Level**	2.3 (0.1)	2.5 (0.2)	2.2 (0.1)	2.8 (0.2)	2.7 (0.3)	2.9 (0.2)

Table III.1. Descriptive statistics (mean ± SEM of younger (N = 30) and older groups (N = 30). *SES: Subjective Socio-Economic Status Scale, ranging from 1 (lowest SES) to 10 (highest SES) (Adler et al., 2000). **Range: 0 = no studies, 1 = primary school, 2 = secondary education, 3 = university and higher education, 4 = postgraduate (Master, PhD).

The older participants belonged to a study program at the University of Valencia for people over 50 years of age (NAU GRAN). We chose this University Program to increase the homogeneity of the sample and the likelihood of getting healthy volunteers to compare with young people. Most of the young people were college students from different areas. The sample was recruited using informative talks and posters at the faculties of the University campus. Two hundred and seventy-two volunteers (113 older and 159 young subjects) were interviewed by phone and completed a general questionnaire to check whether they met the study prerequisites. The criteria for exclusion were: smoking more than 5 cigarettes a day, alcohol or other drug abuse, dental, visual or hearing problems, presence of cardiovascular, endocrine, neurological or psychiatric disease, and the presence of a stressful life event during the past year. Participants were excluded if they were using any medication directly related to emotional or cognitive function, or one that was able to influence hormonal and sAA levels, such as glucocorticoids, β-blockers, antidepressants, benzodiazepines, asthma medication, thyroid therapies, psychotropic substances or contraceptives. Two hundred and five volunteers (81 older and 124 young volunteers) were eliminated for two reasons: (i) meeting the exclusion criteria, and/or (ii) incompatibility with the experiment's schedules.

All the older women were postmenopausal, having had their last menstrual period at least four years before, and none of them were receiving estrogen replacement therapy. All the young women were regular free-cycling and in the early follicular phase (2-5 days) of their menstrual cycle. The menstrual cycle phase was determined using a questionnaire (included in the general questionnaire) about the regularity and length of the menstrual cycle as well as the bleeding during the last year. Then, taking the day of onset of the last menstruation and the average length of the cycles as the reference, we estimated the day of onset of the next menstruation, and this was also verified by phone. Thus, we established the day of the appointment at the laboratory as the second to the fifth day after the onset of the new menstrual cycle.

The participants meeting the criteria were contacted by telephone and asked to attend two sessions that took place in a laboratory at the Faculty of Psychology. 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 session. Additionally, they were instructed to drink only water, refrain from eating, smoking or taking any stimulants, such as coffee, cola, caffeine, tea or chocolate, two hours prior to the session, and not brush their teeth at least one hour prior to the session. The study was conducted in accordance with the Declaration of Helsinki, and the protocol and conduct were approved by the Ethics Research Committee of the University of Valencia. All the participants received verbal and written information about the study and signed an informed consent form.

3.2.2. Procedure

This study used a within-subject design with two completely randomized and counterbalanced conditions, a stress condition and a control condition, in two separate sessions with less than 10 days between them. The sessions consisted of several phases of equal durations in both conditions. Sessions took 1 hour and 50 minutes to complete, and they were always held between 16.00 and 20.00 hours. Each participant started his or her two sessions at the same hour. Upon arrival at the laboratory, the weight and height of the participants were measured (first session), and the experimenter checked to see whether they had followed the instructions given previously (both sessions).

Stress Condition. To produce stress, we subjected the participants to the TSST. The stress tasks consisted of 5 min of free speech (job interview) and a 5 min arithmetic task, performed in front of a committee composed of a man and a woman. The participants remained standing at a distance of 1.5 meters from the committee. Additionally, a video camera and a microphone were clearly visible. Both the speech and arithmetic tasks were filmed.

The protocol started with a habituation phase of 15 min to allow the participants to adapt to the laboratory setting. During this phase, the participants remained seated. After the habituation phase, the introduction phase started (duration 5 min). In this phase, the participants were informed about the procedure for the stress task. They received the instructions in front of the committee in the same room where the task took place. Next, the participants had 10 min to prepare for the task at hand. Following the preparation phase, the stress task was carried out. Then, subjects had 20 min to recover after the stress task. Each participant performed a standardized memory test consisting of 8 trials (Rey Auditory Verbal Learning Test, RAVLT), in order to measure declarative memory. The participants completed the first six trials between 30 to 40 min after the beginning of the TSST. After trial 6, they waited 30 min (delay period) before continuing with the memory test. After the delay period, they finished the memory test with trials 7 and 8 and, finally, were debriefed.

Taking into account the different time courses of the cortisol and sAA responses to stress induction, we collected the saliva samples for each of them at different moments. To measure cortisol, we collected four saliva samples, two before the stress task and two after the stress task. Specifically, the first saliva sample to measure cortisol was taken during the habituation phase, 10 minutes after the participant's arrival at the laboratory (-20 min pre-stress), and the second cortisol sample was taken during the preparation phase (-5 min pre-stress). The third and fourth cortisol samples were collected 15 (+15 min post-stress) and 40 (+40 min post-stress) minutes, respectively, after the onset of the stress task. To measure sAA, we collected five saliva samples, two before the task and three after it. Thus, the first saliva sample was collected 10 minutes before the onset of the stress task (-10 min pre-stress), and the second one was taken immediately before the onset of the speech (0 min). The third, fourth and fifth saliva samples were collected 5, 10 and 14 minutes after the onset of the stress

task (after speech, +5 min; after arithmetic task, +10 min; +14 min post-stress, respectively).

Control Condition. The control condition was similar to the experimental condition, except that the stressful task was replaced by a control task. This task was designed to be similar to the stress task in mental workload and global physical activity (Het et al., 2009), but without the main components capable of provoking stress, such as evaluative threat and uncontrollability (Dickerson and Kemeny, 2004). The control task was composed of 5 min of reading aloud and 5 min of counting. In the preparation phase, the participants read a book with neutral content. The timing of the saliva samples and the phase durations were the same for the two conditions.

3.2.3. Memory

Declarative memory. To measure declarative memory, the Spanish version of Rey's Auditory-Verbal Learning Test (RAVLT) was used (Miranda and Valencia, 1997). This test has several versions, and for each participant a different version of the RAVLT was used in the second session to avoid learning effects. The order of the two versions was randomized and counter-balanced. The RAVLT is composed of different trials. In the first five trials the experimenter read aloud a target list of 15 neutral words, and each participant had to repeat as many words as possible in each of the five trials. The performance on these first five trials reflects the rate of learning (Trials 1 to 5: Learning curve). After trial 5, the experimenter read aloud an interference list of 15 words and tested the retention of these new words. Following this step, participants were asked to recall the words from the target list (Trial 6: Recall after interference); after a delay of 30 min, they had to recall them a second time (Trial 7: Delayed recall). In trial 8 (Recognition), participants had to recognize the memorized words from a verbally-presented list containing 15 new and 15 previously learned words. Trial 8 was divided into two different scores: Hits, the number of words correctly

recognized as being on the target list; and False alarms, the number of words incorrectly recognized as being on the target list. To analyze the effects on recognition (trial 8), we used d-prime (d´), which is the difference between the standardized proportion of correct hits and the standardized proportion of false alarms. One older woman (due to problems in the application of the memory test) and one young man (an outlier for memory outcomes) were removed from the statistical analyses for memory.

3.2.4. Biochemical Analyses

Cortisol. Participants provided four saliva samples by depositing 5 ml of saliva in plastic vials. They took no more than 5 minutes to fill each vial. The samples were frozen at -80ºC until the analyses were performed. The samples were analyzed by a competitive solid phase radioimmunoassay (tube coated), using the commercial kit Coat-A-Count C (DPC, Siemens Medical Solutions Diagnostics). Assay sensitivity was 0.5 ng/ml. For each subject, all the samples were analyzed in the same trial. The within and inter assay variation coefficients were all below 8%. Five people (one older man, two young men and two young women) were excluded from the statistical analyses for cortisol because they were multivariate outliers on the basis of the $p < 0.001$ criteria for the Mahalanobis distance in cortisol samples.

Alpha-amylase (sAA). Saliva was collected using salivettes (Sarstedt, Nümbrecht, Germany). Participants were instructed to introduce the cotton swab into their mouths for exactly 1 min, not chew the cotton, and move the swab around in a circular pattern to collect saliva from all the salivary glands (Rohleder and Nater, 2009). The samples were frozen at -20º C after the completion of the session, until the analyses took place. The samples were shipped to Dresden and analyzed at the Kirschbaum lab, Technical University of Dresden. Concentration of alpha-amylase in saliva was measured by an enzyme kinetic method, according to the protocol specified in Rohleder et al. (2006). The

lowest detectable concentration in our assay was 1.56 U/ml. Inter- and intra-assay variation was below 10%. Analyses of sAA failed to detect the sAA concentrations in the samples of two men, one young and one older, and one older woman; therefore, these subjects were eliminated from the sAA statistical analyses.

3.2.5. Statistical Analyses

Data were checked for normal distribution and homogeneity of variance using Kolmogorov-Smirnov and Levene's tests before the statistical procedures were applied. Since neither the cortisol nor the sAA data had a normal distribution, they were square root transformed. Student's *t*-tests were used to investigate age and sex differences in the demographic variables.

We used linear mixed modeling to assess the salivary cortisol and sAA responses in both the stress and control conditions. As an estimation method, we used the restricted maximum likelihood procedure, since this procedure deals with outliers better (Diggle, 1998). As the dependent variable, we included either sAA or cortisol levels. To allow for differences in patterns between and within participants, we included random components for moment (cortisol: 4 saliva samples, sAA: 5 saliva samples) and for each subject. To analyze salivary cortisol and sAA levels, we added the following factors: (i) Time (for cortisol: -20 min, -5 min, +15 min, +40, and for sAA: -10 min, 0 min, +5 min, +10 min, +14 min), (ii) Condition (control, stress), (iii) Sex (man, woman), and (iv) Age (old, young).

We also used linear mixed modeling to assess memory performance. We performed separate analyses for the following indices: (i) learning curve, (ii) total learning, (iii) recall after interference or retroactive interference, (iv) delayed recall performance, and (v) recognition. As the dependent variable, we included the number of words remembered. We included random components for trial

(trial 1-5) and for each subject. Furthermore, we included the following factors: (i) Trial (learning curve: trial 1 to trial 5, total learning: ∑trial 1 to trial 5, recall after interference: trial 6, delayed recall performance: trial 7, and recognition: trial 8), (ii) Condition (control; stress), (iii) Sex (man, woman), and (iv) Age (old, young).

For all linear mixed models, we started with the most complex model containing all possible interactions, and then progressively removed non-significant effects, starting with the most complex effects. After removing a factor, we investigated whether the model's fit improved according to Akaike's Information Criterion (AIC) and Schwarz's Bayesian Information Criterion (BIC). To calculate AIC and BIC, the maximum likelihood procedure in SPSS was used because it gives more reliable estimates than the restricted maximum likelihood procedure. A lower value of at least 2 on one or both criteria was considered a better model (Burnham and Anderson, 2004).

In order to find out the possible order effects of session (whether the stress or control condition was first), we included this variable in each linear mixed model described above. Results did not show order effects in any model (all $p > 0.113$).

We calculated the cortisol reactivity and sAA reactivity to stress by subtracting the baseline levels from the sample taken immediately after stress, and then we obtained the ratio variable of cortisol over sAA by dividing the cortisol reactivity to stress by the sAA reactivity to stress (RCA). Furthermore, the ratio of sAA over cortisol was calculated by dividing the sAA reactivity to stress by the cortisol reactivity to stress (RAC). Pearson's correlations were performed to assess the relationships between cortisol reactivity and sAA reactivity and the two ratios (RCA and RAC) with memory performance (Trial 6 outcome). In addition, Fisher's Z tests were used to test significant differences between correlation coefficients.

For *post hoc* planned comparisons, we employed the Bonferroni correction. All *p*-values reported are two-tailed, and the level of significance was marked at <0.05. When not otherwise specified, results shown are means ± standard error of means (SEM). We used SPSS 17.0 to perform the statistical analyses. For an easy interpretation of the figures, the values in the figures represent raw values and not square root transformed values.

3.3. RESULTS

3.3.1. Stress Response

Salivary cortisol. The model predicting cortisol levels showed main effects for Condition ($F_{1, 174.490}$= 87.842, p < 0.001), Time ($F_{3, 132.927}$= 8.225, p < 0.001) and their interaction Condition×Time ($F_{3, 132.890}$= 36.480, p < 0.001). There were no baseline differences in cortisol levels between conditions (p = 0.856). In the stress condition, cortisol levels increased, reaching their peak immediately after the stress task (p < 0.001), and then starting to decrease but without recovering baseline levels in the last saliva sample (p = 0.001). In the control condition, cortisol levels decreased across time, but the differences were only significant between the -5 min and +15 min samples and the -20 min and +40 min samples (both $p \leq 0.001$). Cortisol levels were higher in the stress condition than in the control condition in both samples provided after the task (both p < 0.001).

The main effect of Age was not significant (p = 0.486), but the Condition×Age ($F_{1, 216.607}$= 9.404, p = 0.002) interaction was significant. Both age groups had higher cortisol levels in the stress condition than in the control condition (both $p \leq 0.001$). In addition, in the stress condition both age groups had similar cortisol levels (p = 0.680), but, as a trend, the older group had lower cortisol levels than the younger group in the control condition (p = 0.057). The interaction between Time and Age was also significant ($F_{3, 111.853}$= 13.868, p <

0.001), with older participants showing lower baseline cortisol levels than young participants (p = 0.018).

Finally, the factor Sex ($F_{1,\ 67.246}$= 9.056, p = 0.004) and the interactions Condition×Sex ($F_{1,\ 216.607}$= 13.894, p < 0.001) and Time×Age×Sex ($F_{3,\ 111.853}$= 2.856, p = 0.040) were also significant. Men showed higher cortisol levels than women in the experimental condition (p < 0.001), but not in the control condition (p = 0.110). With respect to Time, we observed that in older people, men only showed higher pre-task levels of cortisol than women in the -5 min sample (p < 0.015). However, in young people, men presented significantly higher cortisol levels than women in the +15 min and +40 min samples (both p < 0.020), and as a trend, in the -5 min sample (p = 0.053). There were no age differences between men and women in any of the four samples (all p > 0.080) (see Figure III.1). Model fit did not improve when adding other main effects or interaction effects (see Table III.2).

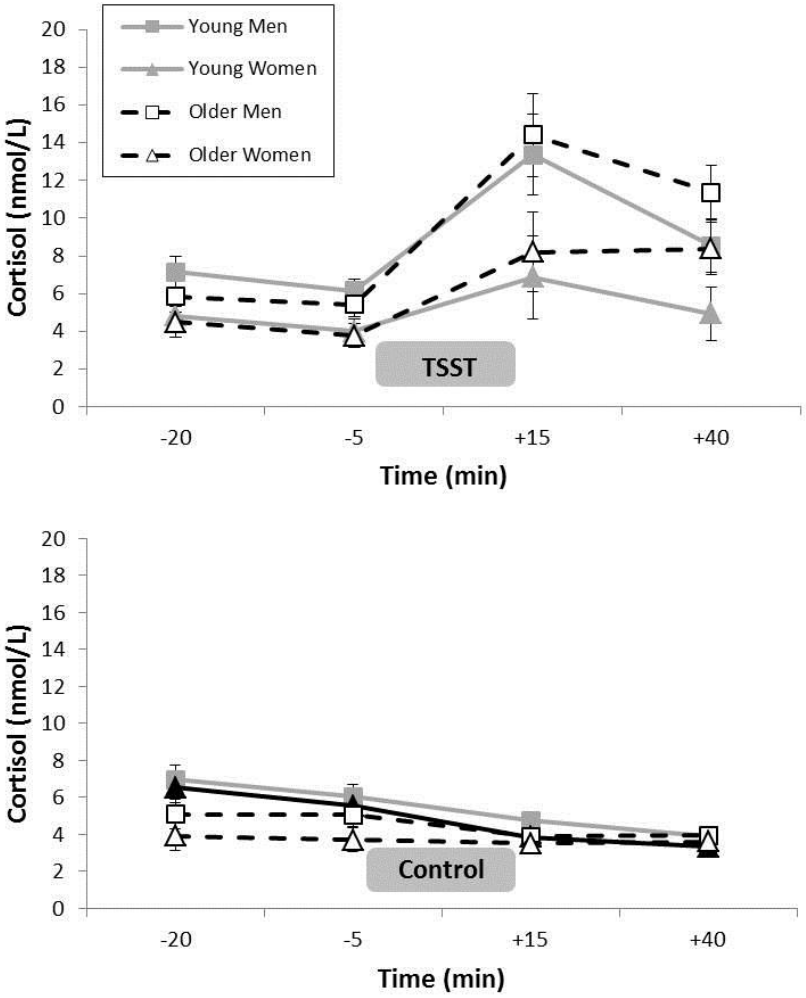

Figure III.1. Means of salivary cortisol concentrations (± SEM) in the TSST (up) and the control (down) conditions in both age groups (young: N = 31, older: N = 31). In the stress condition, all participants increased their cortisol levels immediately after the stress task ($p < 0.001$), with men having higher cortisol levels than women ($p < 0.001$). In the control condition, all participants decreased their cortisol levels across time, according to the normal cortisol circadian rhythm.

Model	Removed variable	AIC	BIC
Complete model	None	589.301	761.770
Removal of ns.4-way interactions	All	583.611	743.461
Removal of ns. 3-way interactions	ConditionxTimexAge Condition×TimexSex	578.229	712.840
Removal of ns. 2-way interactions	None	-	-
Removal of ns. main effects	None	-	-

Table III.2. Fit of the various models predicting cortisol levels.

Salivary Alpha-amylase (sAA). The model predicting sAA levels showed main effects of Condition ($F_{1, 453.476}$= 64.348, p < 0.001), Time ($F_{4, 206.281}$= 35.838, p < 0.001), and their interaction, Condition×Time ($F_{4, , 206.034}$= 4.940, p = 0.001). There were no baseline differences between conditions (p = 0.942); however, the sAA concentrations were higher in the stress condition than in the control condition in the rest of the samples (all $p \leq 0.001$). In the stress condition, sAA levels were similar to baseline in the 0 min sample (p = 0.123), higher in the + 5 min and +10 min samples (both $p \leq 0.001$), and decreased until reaching baseline levels in the last sAA sample (+14 min) (p = 0.423). In the control condition, a similar sAA profile was found.

The factor Age (F1, 61.431= 3.239, p = 0.077) and the interaction Condition×Age ($F_{1, 458.180}$= 3.503, p = 0.062) were marginally significant, whereas the interaction Time×Age ($F_{4, 173.901}$= 3.164, p = 0.015) was significant. Older adults had higher sAA concentrations than younger adults, with this difference being significant in the control condition (p = 0.035), but not in the stress condition (p = 0.172). Both age groups had higher sAA concentrations in the stress condition than in the control condition (both $p \leq 0.001$). Comparing the two age-groups, the older participants showed higher sAA levels than the younger participants in the +5 min (p = 0.036) and +10 min (p = 0.008) samples, but not in the rest of the samples (all p > 0.190). Finally, the factor Sex and its

interactions were not significant (all $p > 0.116$) (see Figure III.2). Model fit did not improve when adding other main effects or interaction effects (see Table III.3).

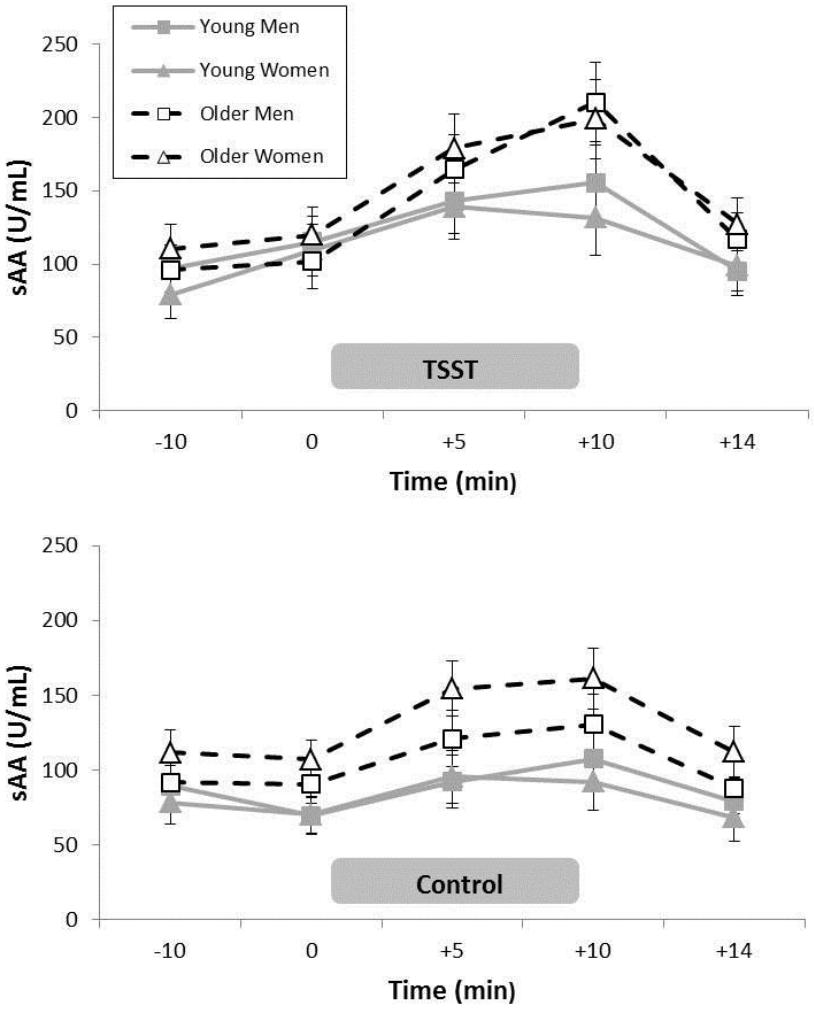

Figure III.2. Means of salivary alpha-amylase (sAA) concentrations (± SEM) in the TSST (up) and control (down) conditions in both age groups (young: N = 34, older: N = 30). Except on baseline sAA concentrations ($p = 0.942$), all participants had higher sAA concentrations in the stress condition than in the control condition (all $p < 0.001$). In addition, the older group had higher sAA concentrations than the young adults, although this difference was only significant in the control condition ($p = 0.035$).

Model	Removed variable	AIC	BIC
Complete model	None	2905.681	3133.215
Removal of ns. 4-way interaction	All	2899.627	3109.316
Removal of ns. 3-way interactions	All	2879.794	3031.484
Removal of ns. 2-way interactions	Age×Sex Time×Sex Condition×Sex	2873.824	2998.745
Removal of ns. main effects	None	-	-

Table III.3. Fit of the various models predicting sAA concentrations.

3.3.2. Memory performance

Learning curve (Trials 1 to 5). The model predicting the learning curve showed that there was a main effect of Trial ($F_{4, , 211.477}$=336.876, $p < 0.001$) and Age ($F_{1, 62.296}$=31.178, $p < 0.001$). All the participants showed a positive learning curve across the first five trials. In every consecutive trial, more words were remembered (all $p < 0.002$). Moreover, older participants had lower performance across the learning curve than young participants (see Figure 3). Model fit did not improve when adding other main effects or interaction effects (see Table III.4).

Model	Removed variable	AIC	BIC
Complete model	None	2577.870	2806.196
Removal of ns. 4-way interaction	All	2572.740	2783.158
Removal of ns. 3-way interactions	All	2559.030	2711.247
Removal of ns. 2-way interactions	All	2541.284	2626.347
Removal of ns. main effects	Condition Sex	2538.214	2614.323

Table III.4. Fit of the various models predicting Learning Curve (Trials 1 to 5).

Total Learning (∑Trial 1 to Trial 5). The model predicting total learning showed that there was only a main effect of Age ($F_{1, 63}$=31.775, p < 0.001); older people had worse total learning performance than young people. Model fit did not improve when adding other main effects or interaction effects (see Table III.5).

Model	**Removed variable**	**AIC**	**BIC**
Complete model	None	906.110	937.653
Removal of ns. 3-way interactions	All	905.217	933.892
Removal of ns. 2-way interactions	All	899.924	919.997
Removal of ns. main effects	Condition Sex	896.596	910.933

Table III.5. Fit of the various models predicting Total Learning (ΣT1 to T5) performance.

Recall after interference (Trial 6). The model predicting immediate recall performance showed that there was a main effect of Age ($F_{1, 61.995}$= 21.103, p < 0.001). Older participants recalled fewer words than young participants. Although the main effect of Condition was not significant (p = 0.319), the Condition×Age **(**$F_{1, 63}$= 4.935, p = 0.030, Cohen's d = 0.32) interaction was significant. Older participants recalled fewer words after the stress condition than in the control condition (p = 0.029); therefore, the stressor only impaired older participants' performance. However, young participants had a similar performance in both conditions (p = 0.382), and their performance was better than that of the older participants in both conditions (both p <0.002) (see Figure III.3). Model fit did not improve when adding other main effects or interaction effects (see Table III.6).

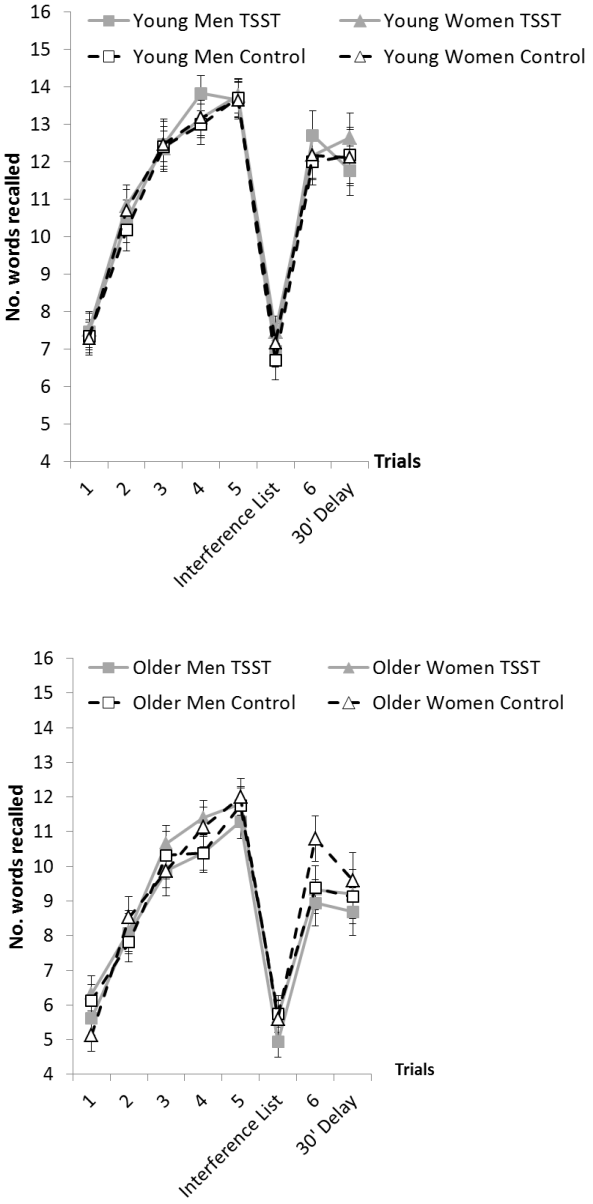

Figure III.3. Number of words recalled in each trial of the RAVLT by (left) young (N = 34) and (right) older (N = 31) groups, divided into men and women in the TSST and control conditions. Among the young participants, no stress effects were found on memory; however, we found an interaction between Condition and Age in the trial 6 outcome. Older people have poorer performance on this trial in the stress condition than in the control condition. Depicted values are means, and error bars represent the SEM.

Model	Removed variable	AIC	BIC
Complete model	None	602.724	634.266
Removal of ns. 3-way interactions	All	600.807	629.482
Removal of ns. 2-way interactions	All except Condition×Age	600.077	623.017
Removal of ns. main effects	Sex	598.516	618.589

Table III.6. Fit of the various models predicting Recall after Interference (Trial 6) performance.

Delayed recall (Trial 7). The model predicting short-term delayed recall performance showed that there was only a main effect of Age ($F_{1, 126.314}$=37.284, $p < 0.001$). Thus, older participants recalled fewer words than young participants (see Figure 3). Model fit did not improve when adding other main effects or interaction effects (see Table III.7).

Model	Removed variable	AIC	BIC
Complete model	None	658.546	687.222
Removal of ns. 3-way interactions	All	656.753	682.560
Removal of ns. 2-way interactions	All	651.247	668.452
Removal of ns. main effects	Condition Sex	648.306	659.776

Table III.7. Fit of the various models predicting Delayed Recall (Trial 7) performance.

Recognition (Trial 8). The model predicting recognition performance did not show main effects for condition, age or sex, nor were there interactions among these factors (all $p > 0.377$).

3.3.3. The relationship between the stress response and memory performance

The correlations among biomarker indexes and memory performance were analyzed only for trial 6, due to the significant effect found in the older group. In this group, no significant correlations were found between recall after interference and cortisol reactivity, sAA reactivity or RCA (ratio of cortisol over sAA) (all $p > 0.167$). However, a negative relationship was observed between recall after interference and the RAC (ratio of sAA over cortisol) ($r = -0.507$, $p = 0.006$). Therefore, the older people who had a predominance of sAA response over cortisol response had poorer memory performance.

In the young group, no significant correlations were found between the trial 6 outcome and cortisol reactivity, sAA reactivity, RCA or RAC (all $p > 0.337$). Significance testing using Fisher's Z tests revealed marginal differences between the older and young groups in the correlation between RAC and trial 6 outcome ($z = 1.6$, $p = 0.054$).

3.4. DISCUSSION

The purpose of this study was to examine the role of age and sex in the relationship between stress and memory performance. To do so, we compared the effect of acute stress on memory in young and older, healthy and non-stressed adults. In a crossover design in which each subject participated in a stress condition and a control condition, we induced stress in the participants by exposing them to an acute psychological stressor (TSST). After both the stress and control tasks, we evaluated their declarative memory performance. Our results confirm that the experimental procedure induced stress, since the TSST provoked an increase in cortisol and sAA responses in the total sample. Although we failed to find stress-induced changes in learning, delayed recall or recognition, the exposure to the TSST impaired immediate recall after interference, but only in older people. In addition, among older people, this

effect was negatively related to the ratio of sAA over cortisol. No sex differences were found in the stress effects on memory performance.

The experimental procedure was indeed able to induce stress, since both stress systems (i.e. HPA-axis and SNS) were activated, as reflected in the cortisol and sAA responses (see Figures III.1 and III.2, respectively). However, when we studied the role of age and sex in the stress response, we observed that they each had a different role in the response of each stress biomarker. Thus, we found sex differences in the cortisol response, but not in the sAA response. Men had a higher cortisol response to the TSST than women, regardless of the age. This result coincides with previous studies in young (Kirschbaum et al., 1999; Childs et al., 2010) and older people (Kudielka et al., 1998, 2004). In contrast, we failed to find age differences in the cortisol response, but we found that older adults had higher sAA concentrations than younger adults, and these differences were significant in the control condition, but not in the stress condition. This result confirms the idea that there is increased basal sympathoneural activity among older people (Seals and Dinenno, 2004).

To our knowledge, this is the first study to compare acute stress effects on the memory performance of young and older men and women. The results show that, in general, older people had poorer declarative memory performance than young people, as they recalled fewer words than young participants on all trials of the RAVLT, except the recognition task (see Figure III.3). This result agrees with a previous review on this topic (Park et al., 2003). According to these authors, there is an age-related decline in some types of memory, including declarative and working memory; however, non-declarative and recognition memory performance were maintained, or even improved, across the lifespan.

It is worth noting that the exposition to an acute stressful event tends to enhance learning of new information in adult male animals (for a review on this topic see: Shors, 2006). It is important to note that the direction of stress effects on memory depends on several factors, such as the memory phase assessed (i.e.

acquisition, consolidation or retrieval), the type of memory studied, the magnitude of the stress-induced cortisol reactivity, and the sex of the subjects.

We found a very specific, negative effect of the stressor on memory. Specifically, the stressor impaired immediate recall (trial 6) only in older people. Why did the stressor selectively affect the memory performance of older people? One explanation could be that, although the RAVLT assesses declarative memory, the effect obtained on trial 6 may fall under the domain of working memory. On this trial the participants had to recall, after an interference list, as many words as possible from the target list, but without its previous presentation as occurred in the first five trials. This new word list interferes with the recall of the previously-learned target list, resulting in retroactive interference (Dewar et al., 2007). According to Hedden and Park (2001), older people show greater retroactive interference effects compared to young adults, so that they seem to be more vulnerable to this interference than young people. Difficulties in deleting irrelevant information from the working memory could hinder their performance. Moreover, both working memory (Galloway et al., 2008) and retroactive interference (Dewar et al., 2007) may be related to prefrontal cortex functioning. In addition to the hippocampus, this brain area seems to be sensitive to glucocorticoid effects during human aging. Several studies suggest that stress exacerbates the aging process (Lupien et al., 2007; Piazza et al., 2010) and, consequently, age-related changes such as memory impairment.

Previous studies by our group and others have suggested that older people may be less sensitive to the effects of acute stress on long-term memory retrieval (Pulopulos et al., 2013) and to the effects of pharmacologically-induced acute cortisol increases on working memory tasks involving the maintenance and manipulation of information (i.e. Digit Span and Letter-Number Sequencing tasks) (Wolf et al., 2001a; Yehuda et al., 2007). An age-related dysregulation of the HPA-axis activity (Mizoguchi et al 2009) and functional changes in the

amygdala and hippocampus (Mather, 2006; St. Jacques et al., 2009; Murty et al., 2010) have been proposed as possible explanations for the lack of cortisol effects on the performance of these kinds of tasks. Together with our results, these studies indicate that older people may be sensitive to the effect of stress on retroactive interference, a cognitive ability that involves the activation of the prefrontal cortex to control irrelevant information and that has shown a greater age-related decline (Hedden and Park 2001), but not on other kinds of memory abilities, such as long-term memory retrieval or working memory tasks, which involve maintenance and manipulation of information. However, it should be noted that previous studies investigating the effects of cortisol on working memory in older people have used a pharmacological approach (Wolf et al., 2001a; Yehuda et al., 2007); therefore, the lack of SNS activation in these studies may also account for the absence of cortisol effects observed. Thus, more research is needed to investigate the effects of acute stress on other kind of tasks that specifically measure working memory. Moreover, we found a negative relationship between the ratio of sAA over cortisol and recall after interference only in older people. It should be pointed out that even after considering the Bonferroni correction for multiple analyses, the critical α level would be 0.00625 (0.05/8); therefore, this correlation would remain significant. As we outlined above, the immediate recall of wordlists not only reflects declarative memory processes, but also working memory functions (Lezak et al., 2004; Tops et al., 2004). On the one hand, it has been well established that declarative memory depends on hippocampal functioning (Scoville and Milner, 2000), and working memory depends on prefrontal cortex functioning (Galloway et al., 2008). On the other hand, these two brain structures are affected by the glucocorticoid action and noradrenergic activation in response to stress, respectively (Patel et al., 2000; Schoofs et al., 2008). Therefore, this trial will be affected by the activation of both stress systems related to each type of memory. Taking this into account, we considered it appropriate to examine whether the impairing effects found in the recall after interference were related not only to the HPA-axis or SNS action

separately, but also to the relationship between them, expressed as the ratio of one biomarker over the other and vice versa. The hormonal ratio method has been widely implemented in research as a reliable index for a variety of health and behavioral outcomes (Ostroff et al., 1982, 1985; Adlercreutz et al., 1986; Terburg et al., 2009). Recently, the sAA over cortisol ratio has been suggested as a good marker of stress system dysregulation, positively related to subjective indexes of stress and depression (Ali and Pruessner, 2012). We tried to extend this relationship into the cognitive domain, as has been initiated in other stages of the life span (Berry et al., 2012).

Sex differences have previously been reported among older people (Almela et al., 2011a), showing impaired declarative memory, but only related to higher cortisol response to stressors in older women. However, we failed to find sex differences in the relationship between acute stress and memory performance. The small sample size may be the underlying explanation for this lack of significant effects. Further studies are needed to investigate whether the sex affects the relationship between acute stress and retroactive interference, specifically in older people.

Some other limitations have to be considered in the current study. We collected a homogeneous and cognitively and physically healthy sample, using exclusion criteria that have contributed to obtaining a very restricted sample. This fact may limit the ability to detect effects and generalize the results. Further studies are needed to extend this research to a more general population, including older people with age-related diseases and medication use, young women in other phases of the menstrual cycle, and oral contraceptive users. In this study, several outcomes were examined (e.g. different dependent variables from the same memory task), which can lead to an increase in the type I error. However, we found a correlation between the trial 6 outcome and the RAC in older people and, although as a trend, differences between the correlations in older and young people, in line with the results shown with linear mixed

modeling. Taken together, these consistent results do not seem to be due to chance, but they must be considered tentative and confirmed in further studies with other and more extensive samples.

In conclusion, we have studied the role of age in the effects of acute psychosocial stress on declarative memory, considering sex. Our results show a very specific effect associated with the worse consequence of the interference derived from very similar and neutral stimuli in healthy, non-stressed older people. They confirm that age moderates this specific stress-induced effect on memory, providing new knowledge about the importance of studying both physiological systems involved in the stress response together.

CHAPTER IV

STUDY 3

STUDY 3

The impact of stress prior retrieval on memory performance: the role of age and sex[4]

[4] The main results of this study are under review in *Behavioural Brain Research*: Hidalgo, V., Pulopulos, M.M., Puig-Perez, S., Espin, L., Gomez-Amor, J., and Salvador, A. Acute stress affects free recall and recognition of pictures differently depending on age and sex.

4.1. INTRODUCTION

A large body of research in animals and humans shows that stress affects memory. Stress involves the release of glucocorticoids (corticosterone in rodents, cortisol in humans) and catecholamines due to the activation of the hypothalamus-pituitary-adrenal axis (HPA-axis) and the sympathetic nervous system (SNS), respectively. While glucocorticoids can cross the blood-brain barrier and bind to receptors (i.e. mineralcorticoid and glucocorticoid receptors) located in the hippocampus, prefrontal cortex and amygdala, brain areas related to memory processes (Lupien and Lepage, 2001; Lupien et al., 2007; Roozendaal et al., 2009), the catecholamines do not have this property. Thus, the latter exert their action on memory by activating the β-adrenergic receptors on vagal afferents projecting to the nucleus of the solitary tract in the brainstem (McGaugh, 2000), and these noradrenergic projections influence the neuronal activity of the amygdala (Packard et al., 1995). Nevertheless, memory can be enhanced, impaired or even unaffected by stress, since factors such as the memory phase tested (i.e. learning, consolidation or retrieval), the emotional valence of the material to be remembered (i.e. emotional or neutral), or the age and sex of the individuals can modulate this relationship.

In line with animal studies, a pharmacologically-induced (de Quervain et al., 2000; 2003; Kuhlmann and Wolf, 2005; Kuhlmann et al., 2005a) or stress-induced (Domes et al., 2004; Kuhlmann et al., 2005b; Buchanan et al., 2006; Oei et al., 2006; Buchanan and Tranel, 2008; Smeets et al., 2008; Smeets, 2011) cortisol increase impairs retrieval performance in young people. The effect of stress on long-term memory (24h at least) retrieval seems to be rather consistent, since impairing effects have been observed when stress triggers high (Kuhlmann et al., 2005b; Oei et al., 2006; Buchanan and Tranel, 2008) and moderate (Buchanan et al., 2006; Smeets et al., 2008; Smeets, 2011) cortisol responses. In these studies different types of memory tasks with different levels of difficulty have been employed, such as lists of words (with 30 in Kuhlmann et al. (2005b) and Smeets (2011), 80 words in Buchanan et al. (2006) and 100 words

in Smeets et al. (2008)), pictures (20 in Buchanan and Tranel, 2008) and paragraphs (Oei et al., 2006). A few studies have shown a lack of a stress effect on long-term memory retrieval in young women in the luteal phase of the menstrual cycle (Schoofs and Wolf, 2009) and when the memory retrieval was performed two or more days after learning (Wolf et al., 2002; Beckner et al., 2006).

One modulatory factor in the relationship between cortisol and memory seems to be the emotional valence of the material to be remembered (i.e. emotional or neutral). Emotional material induces a greater noradrenergic activation of the amygdala than neutral material, and, as has been described, the interactions between the amygdala and hippocampus are crucial in finding cortisol effects on hippocampus-dependent memory performance (Roozendaal et al., 2009). Thus, the majority of studies carried out in young people showed a stronger impact of cortisol or stress on memory for emotionally arousing material than for neutral material (for a review see: Wolf et al., 2004).

Most of the studies on the effects of cortisol administration or stress-induced cortisol increases on memory have been conducted in young people**.** However, some age-related changes may affect the relationship between stress-induced cortisol response and memory performance in the older population. Previous studies have suggested that older people show (in comparison with young people) changes in the functional connectivity between the amygdala and hippocampus and decreases in amygdala activation for negative stimulus (Mather and Carstensen, 2005; Mather, 2006; Murty et al., 2009; St Jacques et al., 2009). Thus, given that interactions between the amygdala and hippocampus seem to be essential to observe cortisol effects on hippocampus-dependent memory performance (Roozendaal et al., 2009), it is possible that this age-related change may affect the effects of stress and cortisol on long-term memory retrieval in older people. Another change that can be observed in the aging brain is a reduction in the number of mineralcorticoid and glucocorticoid receptors

located in the hippocampus, prefrontal cortex and amygdala (Giordano et al., 2005; Perlman et al., 2007; Mizoguchi et al., 2009), which could make older people's memory less sensitive to being affected by cortisol increases (Newcomer et al., 1995; Heffelfinger and Newcomer, 2001).

In spite of evidence suggesting an age-related change in stress and cortisol effects on memory performance, only a few studies have been reported in older people. Previous studies that have investigated the effects of stress on memory in older people have mainly shown that cortisol increases before learning (i.e. without differentiating stress effects on the learning, consolidation or retrieval phases) impair memory performance (Lupien et al., 1997; Wolf et al., 2001a; but see Domes et al., 2002; Almela et al., 2011a; Hidalgo et al., 2014), an effect that seems to be due to the detrimental effect of cortisol on retroactive interference in older people, but not in young adults (Hidalgo et al., 2014). By contrast, studies in animals and humans have shown a lack of stress and cortisol effects on working memory, spatial memory and declarative and non-declarative memory (Wolf et al., 2001a; Porter et al., 2002; Yehuda et al., 2007; Beuchel et al., 2014; Pulopulos et al., in press). To our knowledge, only one study investigated the effects of acute stress on long-term memory retrieval in a sample of older people, finding no effects of stress (Pulopulos et al., 2013). However, although some previous studies have used both older and young samples to investigate the effects of cortisol increases on learning (Hidalgo et al., 2014), and a short-time after learning (Wolf et al., 2001a), there are no studies that have directly compared the effect of a stress-induced cortisol increase on long-term memory retrieval in young and older people.

In the present study we have compared, for the first time, the effects of a stress-induced cortisol increase on long-term memory retrieval of pictures in older and young people. To this end, two age groups of participants (older and young) were exposed to the Trier Social Stress Test (TSST) or a control task. After the stress or control task, free recall and recognition of pictures learned one day

before were assessed. Moreover, in order to investigate whether the emotional arousal of the memory material plays a crucial role in the acute stress effects on memory retrieval, we used positive, negative and neutral pictures. Finally, we also tested whether the participants' sex influenced the stress effects on retrieval, due to the existence of sex differences in the stress response and their effects on this type of memory. Based on the literature, we expected stress to impair long-term memory retrieval in young people, but not in older people (e.g. Kuhlmann et al., 2005b; Buchanan et al., 2006; Oei et al., 2006; Buchanan and Tranel, 2008; Smeets et al., 2008; Smeets, 2011; Pulopulos et al., 2013), and sex-related differences in young people (e.g. Wolf et al., 2001b; Andreano and Cahill, 2006; Espin et al., 2013). Thus, we hypothesized that there would be a stronger impairing effect in young men, due to their expected higher cortisol response to the stressor (Kirschbaum et al., 1999; Kudielka and Kirschbaum, 2005; Kudielka et al., 2009) and the protective effects of estrogen in women (Wolf et al., 2006).

4.2. METHOD

4.2.1. Participants

The current study is part of an extensive on-going project (Mneme Project) aimed to investigate the effects of psychosocial stress on memory performance, taking into account different moderating factors (including age and sex) through separate and consecutive studies in healthy people. Here, we studied a sample composed of 102 subjects divided into a group of older people (from 56 to 76 years of age) and a group of young people (from 18 to 27 years of age). Participants were submitted to one of two different conditions (stress or control). The older group (N = 52) was composed of 27 men (stress = 12, control = 15) and 25 women (stress = 13, control = 12). The young group (N = 50) consisted of 26 men (stress = 14, control = 12) and 24 women (stress = 12, control = 12), all undergraduate students. The older group belonged to a study program at the University for people over 55 years of age, and they had an

educational level beyond high school. There were no significant differences between the two (stress vs control) conditions on age, educational level or body mass index (BMI) ($p > 0.286$). Partial results from the older subsample have been previously reported. In the current study we added a group of young participants to our previous sample (Pulopulos et al., 2013) in order to test whether the same experimental design may show stress effects on long-term memory retrieval of pictures in young adults.

All the participants completed a general questionnaire to check whether they met the study prerequisites. In order to obtain an optimal comparison of the two age cohorts and eliminate a number of possible confounding factors that could interfere with the aim of the study, we applied very restrictive criteria. The exclusion criteria were: smoking more than 10 cigarettes a day; alcohol or other drug abuse; dental, visual or hearing problems; presence of cardiovascular, endocrine, neurological, or psychiatric disease; 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 (volunteers were asked whether they had experienced any situation that would affect them negatively). The presence of a stressful life event was considered as an exclusion criterion because of its effects on both cognitive performance and HPA-axis functioning (Sapolsky and Plotsky, 1990; Sauro et al., 2003; Lupien et al., 2007; Peavy et al., 2009). The participants were excluded if they were using any medication directly related to emotional or cognitive function, or one that was able to influence hormonal and salivary alpha-amylase (sAA) levels, such as glucocorticoids, β-blockers, antidepressants, benzodiazepines, asthma medication, thyroid therapies or psychotropic substances. All the older women were postmenopausal, having had their last menstrual period more than 3 years before the testing time, and none of them were receiving estrogen replacement therapy. All the young women were regular, free-cycling and nulliparous, and none of them had taken oral contraceptives. All the participants in the older group 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 them 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).

The participants who met the criteria were contacted by telephone and asked to attend two sessions that took place in a laboratory at the University. Previously, 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 session. Additionally, they were instructed to drink only water, not eat, smoke or take any stimulants such as coffee, cola, caffeine, tea or chocolate two hours prior to the session, and not brush their teeth at least one hour prior to the session. The study was conducted in accordance with the Declaration of Helsinki, and the protocol was approved by the Ethics Research Committee of the University of Valencia. All the participants received verbal and written information about the study and signed an informed consent form.

4.2.2. Procedure

This study consisted of two individual sessions: acquisition and retrieval. In the first session (acquisition session), which was similar for all subjects, after the participants' arrival, the experimenter checked whether they had followed the instructions given previously, and he/she noted their weight and height. After a period of habituation to the laboratory, participants were shown 30 color pictures consisting of 10 unpleasant (e.g. mutilated bodies), 10 pleasant (e.g. baby smiling) and 10 neutral (e.g. glass of water on a table) pictures extracted from the Spanish version (Vila et al., 2001) of the International Affective Picture System (IAPS; Lang et al., 2005). Pictures were presented individually for 5 s on a screen, followed by a black screen for 15 s, during which participants rated the pictures using the Self-Assessment Manikin (SAM) scales (Lang, 1980). No

mention of a memory test was made in order to ensure incidental encoding of stimuli. Participants were asked to return the next day, and they were not informed about the procedure. The second session (retrieval session) was carried out the next day. In it, half the participants were randomly assigned to the stress condition, and the other half were assigned to the control condition.

Stress condition. To produce stress we used the Trier Social Stress Test (TSST, Kirschbaum et al., 1993). The stress task consisted of 5 min of free speech (job interview) and a 5 min arithmetic task, performed in front of a committee composed of a man and a woman. The participants remained standing and were filmed throughout both tasks. Immediately after the TSST, subjects filled out a questionnaire about some aspects of the task: stress, difficulty, frustration and effort (Situational Appraisal). These questions were created based on previous studies on this topic (Bagget et al., 1996; Gonzalez-Bono et al., 2002). Participants responded to each question on a 5-point Likert scale ranging from 1 (not at all) to 5 (extremely). Finally, 15 min after the end of the TSST, participants performed the free recall and recognition memory tasks with the pictures they had seen the previous day. We collected four saliva samples to measure sAA and cortisol. Specifically, 15 min before the TSST (habituation phase), the first saliva sample was taken (-15 min pre-stress). The second saliva sample was collected immediately after the TSST (+10 min post-stress) at the onset of the recovery phase. Before the free recall, participants contributed the third saliva sample (+25 min post-stress). Finally, the last saliva sample was taken after the recognition memory test (+45 min post-stress).

Control condition. Both the stress and control conditions had the same schedules, but participants in this condition performed the control task instead of the stressful task. This control task consisted of 5 min of presenting non-emotional information and 5 min of counting, as in previous studies (Almela et al., 2011b; Espin et al., 2013). In order to avoid evaluative threat and uncontrollability, the main components capable of provoking stress (Dickerson

and Kemeny, 2004), during the control task participants were left alone in the room, and there was no video or committee present. The two conditions were identical (same timing of the saliva samples, phase durations and questionnaires applied), and only the task differed (TSST vs. Control).

4.2.3. Memory

Free recall task. To assess the free recall, in the second session (retrieval session) participants were instructed to recall as many pictures as possible from the set they had seen in the first session (acquisition session). To do so, participants wrote a brief description of the pictures for 10 min. Free recall was scored by two independent judges who were blind to the group to which each participant belonged, and who determined which picture (if any) was being described. Agreement between judges was 91.5%, and discrepancies were discussed until a consensus was reached.

Recognition task. Participants viewed 60 pictures (30 new and 30 previously-viewed pictures) individually on a screen for 5 min. Each of the two sets of pictures was composed of 10 negative, 10 positive and 10 neutral pictures. Participants had to recognize the pictures they had seen before (in session 1). Thus, they verbally responded “yes” or “no” after seeing each picture on the recognition test. Recognition received two different scores: *Hits*, the number of pictures correctly recognized as being in the target presentation; and *False alarms*, the number of pictures incorrectly recognized as being in the target presentation. The difference between the percentage of hits and the percentage of false alarms was calculated to analyze the effects on recognition (Cornelisse et al., 2011).

4.2.4. Biochemical analyses

Saliva samples were collected using salivettes (Sarstedt, Nümbrecht, Germany) for cortisol and sAA. Participants were instructed to keep the cotton swab in their mouth for exactly 2 min, 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 15 min, resulting in a clear supernatant with 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). Salivary cortisol and sAA levels were measured in duplicate, and each participant's sample was analyzed in the same trial.

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

Alpha-amylase (sAA). The concentration of sAA was measured by using an enzyme kinetic method with the commercial salivary α-amylase assay kit from Salimetrics (USA). Assay sensitivity was 0.4 U/mL. Inter- and intra-assay variation coefficients were all below 10%. Analyses of sAA failed to detect the sAA concentrations in the samples of three participants in the stress condition (one young man and two young women) and one in the control condition (one young woman). Therefore, these participants were removed from the statistical analyses for sAA.

4.2.5. Statistical analyses

Cortisol and sAA values were logarithmic transformed because they did not have a normal distribution after Kolmogorov-Smirnov and Levene's tests were applied.

Student's *t*-tests were conducted to evaluate differences in the demographic variables by condition (stress vs. control). Three-way ANOVAs were used to study condition, age (older vs. young) and sex (men vs. women) differences in situational appraisal. ANOVAs for repeated measures were performed to investigate the physiological response, ratings of picture material and memory performance. Finally, bivariate Pearson's correlations were conducted between the free recall or recognition outcomes and cortisol or sAA responses to stress, calculated as the percentage increase from baseline to peak (Cornelisse et al., 2011).

One outlier in the cortisol data (one older woman in the control condition) and two outliers in the sAA data (one older man and one young man in the stress condition) were removed from the analyses because their concentrations differed by more than 3 S.D. from the total sample mean. Four outliers in the recognition data (one older woman and one young woman in the stress condition, and one older man and one older women in the control condition) were removed from the recognition analysis because their scores differed by more than 3 S.D.

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 taken as < 0.05. When not otherwise specified, the results shown are means ± SEM. We used SPSS 19.0 to perform the statistical analyses. In order to provide an easy interpretation of the figures, the values in the figures represent raw values and not logarithmic-transformed values.

4.3. RESULTS

4.3.1. Situational appraisal

Participants in the stress condition perceived the stress task as more stressful (F1, 93) = 43.399, $p < 0.001$), difficult (F (1, 93) = 42.577, $p < 0.001$), frustrating (F (1, 93) = 25.882, $p < 0.001$) and requiring more effort (F (1, 93) = 40.430, $p < 0.001$) than participants in the control condition. Older participants (2.181 ± 0.168) perceived the stress task as less frustrating than young participants (2.729 ± 0.169); however, no age differences were found for stress, difficulty or effort (for all $p > 0.226$). No sex differences were found on any of the variables evaluated (for all $p > 0.392$).

4.3.2. Physiological response

Salivary cortisol. The repeated-measures ANOVA with Time (-15, +10, +25, +45 min) as a within-subject factor and Condition, Age and Sex as between-subject factors showed main effects for Condition (F (1, 93) = 32.960, $p < 0.001$), Time (F (1.942, 180.652) = 21.580, $p < 0.001$) and the Condition×Time interaction (F (1.942, 180.652) = 50.381, $p < 0.001$). Baseline cortisol concentrations were similar between conditions ($p = 0.773$). In the stress condition, cortisol levels increased immediately after the TSST ($p < 0.001$), reaching their peak 25 min after the onset of the stress task ($p < 0.001$). Although cortisol concentrations decreased in the last saliva sample, participants did not recover their baseline levels ($p < 0.001$). In the control condition, there were no differences in the cortisol concentrations between the -15, +10 and +25 min saliva samples (both $p > 0.99$), reflecting a lack of cortisol response to the control task. In addition, in the last saliva sample (+45 min), the cortisol concentrations decreased ($p < 0.001$), reaching lower levels than in the first sample (-15 min) ($p < 0.001$), in accordance with the cortisol circadian rhythm.

The Age factor was significant (F (1, 93) = 18.487, p < 0.001), as was the Condition×Time×Age interaction (F (1.942, 180.652) = 8.214, p < 0.001). In both age groups, baseline cortisol did not differ between conditions (both p > 0.126). Higher cortisol concentrations were found in the stress condition than in the control condition in the rest of the salivary samples in both age groups (all p < 0.008). In the stress condition, older participants had significantly lower cortisol concentrations than young participants in the +10, +25 and +45 min saliva samples (all p < 0.007) and, as a trend, in their baseline levels (p = 0.068). However, in the control condition, older participants had lower baseline cortisol levels (p < 0.001) and, as a trend, in the +10 min saliva sample (p = 0.058), with similar levels found in the rest of the samples (both p = 0.128) (see Figure IV.1).

Finally, the Sex factor was significant (F (1, 93) = 8.790, p = 0.004), with men showing higher cortisol concentrations than women. None of the interactions between Sex and the other factors were significant (all p > 0.187).

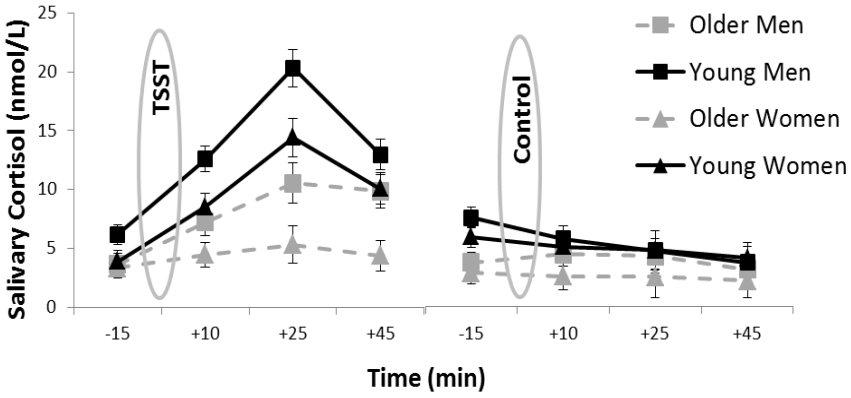

Figure IV.1. Means (±SEM) of salivary cortisol concentrations in the TSST (left) and control (right) conditions for older men (N = 26), young men (N = 25), older women (N = 24) and young women (N = 24).

Salivary alpha-amylase (sAA). The repeated-measures ANOVA with Time as a within-subject factor and Condition, Age and Sex as between-subject factors indicated that the factor Condition was not significant (F (1, 87) = 0.011, p = 0.918), but the factor Time (F (2.653, 230.827) = 14.649, p < 0.001) and the Time×Condition interaction (F (2.653, 230.827) = 4.375, p = 0.007) were significant. There were no baseline sAA concentration differences between the stress and control conditions (p = 0.328). In the stress condition, the sAA concentrations increased immediately after the TSST (p = 0.015), decreasing 25 min after the onset of the stress task (p = 0.002), and recovering baseline concentrations in the last saliva sample (p > 0.99). In the control condition, the sAA concentrations were similar to baseline after the control task (p > 0.99), and they decreased over time (all p < 0.033). There were no differences between conditions in sAA concentrations in any sample (all p > 0.111).

The Age factor was significant (F (1, 87) = 7.160, p = 0.009), as the older participants had higher sAA concentrations. However, the Sex factor was not significant (F (1, 87) = 1.339, p = 0.250), nor were its interactions with other factors (all p > 0.99) (see Figure IV.2).

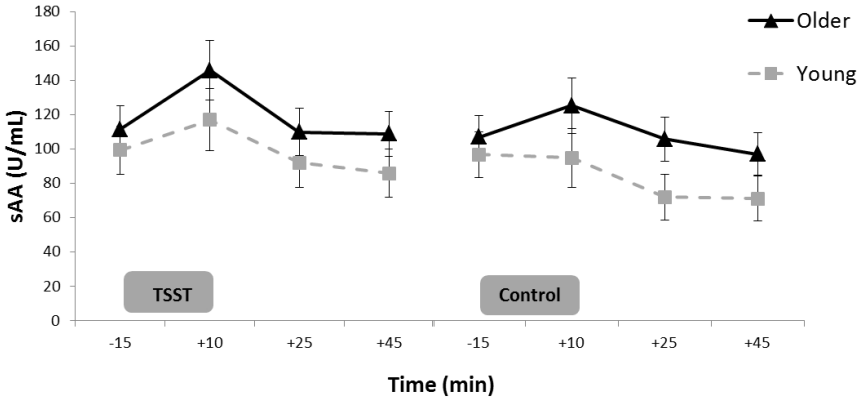

Figure IV.2. Means (±SEM) of salivary alpha-amylase concentrations in the TSST (left) and control (right) conditions for older (N = 50) and young (N = 45) participants.

4.3.3. Ratings of picture material

A repeated-measures ANOVA with Valence (positive, negative, neutral) as a within-subject factor and Condition, Age and Sex as between-subject factors was used to analyze the classification of the valence and arousal of the pictures to-be-remembered.

Valence. Results confirmed the a priori classification, so that the negative pictures (M = 1.709, SEM = 0.089) were rated lower than the neutral (M = 5.117, SEM = 0.081) and positive pictures (M = 7.045, SEM = 0.082) (for all $p < 0.001$). Neutral pictures were rated lower than positive pictures ($p < 0.001$). There were no significant differences based on condition, age or sex (all $p < 0.217$).

Arousal. Results revealed that the neutral pictures (M = 3.711, SEM = 0.122) were significantly scored as less arousing than the negative pictures (M = 7.605, SEM = 0.104) ($p < 0.001$) and, as a trend, less than the positive pictures (M = 4.007, SEM = 0.147) ($p = 0.064$). Older participants (M = 5.5833, SEM = 0.136) scored all the pictures as more arousing than the younger participants (M = 4.633, SEM = 0.137) ($p < 0.001$). There were no significant differences based on condition or sex (both $p > 0.203$).

4.3.4. Memory Performance[5]

Free Recall. A repeated-measures ANOVA with Valence as a within-subject factor and Condition, Age and Sex as between-subject factors was used to measure the effect of stress on free recall of pictures. The results showed the main effects for Valence (F (2, 174) = 62.032, $p < 0.001$), Age (F (1, 87) = 99.698, p

[5] Because older participants rated all pictures as more arousing than young participants, the effect of the arousal rating on the relationship between stress and memory performance was assessed. However, the inclusion of the arousal rating as a covariate in the ANOVAs analyses does not substantially change the statistical conclusion of the memory performance analyses, except the recognition analysis, in which the Valence factor loses its significance, Valence (F (1.724, 141.382) = 0.875, $p = 0.405$).

< 0.001), and the Valence×Age interaction (F (2, 174) = 4.762, p < 0.010). *Post hoc* analyses revealed that in all participants the negative pictures were recalled more than the positive and neutral pictures (both p < 0.001), and positive pictures were recalled more than neutral pictures (both p < 0.001). With respect to Age, older participants recalled fewer positive, negative and neutral pictures than young participants (all p < 0.001). In addition, in both age groups, negative pictures were recalled more than positive (both p < 0.007) and neutral pictures (both p < 0.001), and positive pictures were recalled significantly more than neutral pictures in older (p < 0.001) and, as a trend, young participants (p = 0.063).

The factors Condition (F (1, 87) = 0.154, p = 0.696) and Sex (F (1, 87) = 0.122, p = 0.727) were not significant, but the Condition×Sex×Age interaction (F (1, 87) = 6.219, p = 0.015) was significant. Older participants showed significantly worse free recall performance than young participants in both conditions and both sex groups (all p < 0.005). Among older people, there were no condition differences in men or women (both p > 0.476). By contrast, among young people, condition differences were found, so that the young men in the stress condition recalled fewer pictures than the young men in the control condition (p = 0.025). This result was not found in young women (p = 0.185). Moreover, in the stress condition young men recalled fewer pictures than young women (p = 0.012). This significant difference was not observed in young people in the control condition (p = 0.316), or in older people in either of the two conditions (both p > 0.260) (see Figure IV.3).

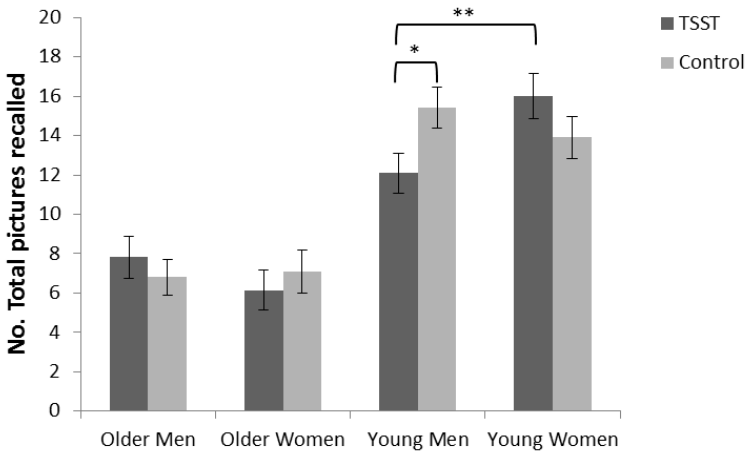

Figure IV.3. Means (±SEM) of total pictures recalled for older and young participants in both conditions (TSST vs. Control). Stress had impairing effects on memory retrieval only in young men. Young men in the TSST condition showed lower recall than young men in the control condition (*p = 0.025). Moreover, in the stress condition, young men recalled fewer pictures than young women (*p = 0.012).

Recognition. Repeated-measures ANOVA with Valence as a within-subject factor and Condition, Age and Sex as between-subject factors was used to measure the effect of stress on the recognition task. Results revealed a main effect for Valence (F (1.719, 142.711) = 7.008, p = 0.002), but not for Condition (F (1, 83) = 1.591, p = 0.211). The Valence×Condition interaction was significant (F (1.719, 142.711) = 4.807, p = 0.013), but not the ValencexAge interaction (F (1.719, 142.711) = 0.025, p = 0.962. *Post hoc* analyses revealed that the positive pictures were recognized less in the stress condition than in the control condition (p = 0.004). No condition differences were found in negative and neutral picture recognition (both p > 0.435). Age and Sex were not significant (both p > 0.455), nor were their interactions with other factors (all p > 0.1) (see Figure IV.4).

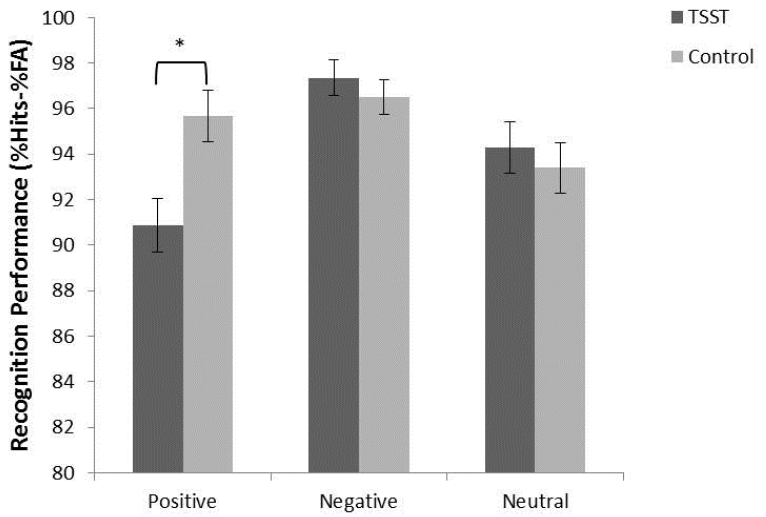

Figure IV.4. Means (±SEM) of recognition performance in % Hits - % False Alarms for older and young participants in both conditions (TSST vs. Control). The positive pictures were recognized less in the stress condition than in the control condition (*p = 0.004).

4.3.5. The relationship between the stress response and retrieval performance

To minimize Type I error rates, the correlations between the physiological response to the TSST and memory performance were analyzed only in young men in the stress condition for free recall data, and in participants in the stress condition for recognition of positive pictures data, based on the significant effects found.

Free Recall. Results showed that cortisol was negatively related to free recall performance (p > 0.158), although it only reached statistical significance in relation to the negative pictures, showing that young men who reacted to the stressor with large cortisol responses recalled fewer negative pictures (r = -0.584, p = 0.046). However, this relationship was not significant for positive or neutral pictures (both p > 0.367). Moreover, sAA response did not show any significant correlations (all p > 0.360).

Recognition. Neither cortisol nor the sAA responses to stress were associated with recognition performance on positive pictures when correlations were performed with young and older participants together (both $p < 0.410$).

4.4. DISCUSSION

The present study intended to parse the effects of an acute psychosocial stressor on long-term memory retrieval in different age groups in order to better understand the importance of age-related changes. No significant stress effects were found on memory retrieval for positive, negative and neutral pictures in the older group. Conversely, in young people the stressor diminished memory retrieval, but only in men. Additionally, this impairment was negatively associated with the cortisol response to stress in young men and, although in a very tentative way, especially in negative pictures. Regardless of age and sex, stress impaired recognition memory for positive pictures, but this effect was not correlated with the cortisol or sAA response.

The task used as the stressor, the TSST, was able to induce stress at both psychological and physiological levels. At the psychological level, the stress task was perceived as more stressful, difficult, frustrating and requiring more effort than the control task. At the physiological level, the stress task provoked greater cortisol and sAA responses than the control task. Age had a modulating effect on the stress-induced cortisol response; thus, older participants had a lower cortisol response to the stressor than young participants. Several studies reported no age differences (Nicolson et al., 1997; Kudielka et al., 1999, 2000; Hidalgo et al., 2014), although others described different cortisol responses in young and older people (Kudielka et al., 2009; Foley and Kirschbaum, 2010). However, no age differences were found in the sAA response to stress, but the sAA global output was higher in older people than in young people in both conditions, supporting the hypothesis established by Seals and Dinenno (2004) about increased basal sympathoneural activity due to aging. Regarding the role of sex, our results agree

with previous findings showing a higher cortisol response in men than in women (for a review see: Kudielka et al., 2009), but no sex differences were found in the sAA response to the TSST, which agrees with previous studies (Kivlighan and Granger, 2006; Takai et al., 2007; Almela et al., 2011b).

As expected, older people performed worse on free recall than young people. When we compared the stress effects on the retrieval performance of older and young participants, stress effects were observed only in young men. Thus, young men in the stress condition had lower free recall performance when they were compared with: (i) young men in the control condition and (ii) young women in the stress condition. Our results coincide with previous studies that have shown a detrimental effect of stress on memory retrieval in young men (Wolf et al., 2001a; Kuhlmann et al., 2005b; Oei et al., 2006). Therefore, our suggestions about the role of age in explaining the results obtained (Pulopulos et al., 2013) are now confirmed, since the same experimental design impairs memory retrieval in young men, but not in older people. These results support the idea that, as shown for working memory, spatial memory, declarative and non-declarative memory in older animals and humans (Wolf et al., 2001a; Porter et al., 2002; Yehuda et al., 2007; Beuchel et al., 2014; Pulopulos et al., in press), older people may be less sensitive to stress effects on long-term memory retrieval than young people.

One explanation for this lack of stress effects on memory performance in older people could be an age-related reduction in the sensitivity and density of the glucocorticoid receptors (GRs or Type II) in the hippocampus (Bhatnagar et al., 1997; Mizoguchi et al., 2009), which might decrease cortisol's direct effect on the hippocampus. Furthermore, although we did not observe a positive effect on memory performance in our participants, a decrease in the functional interconnectivity between the amygdala and hippocampus has been observed (Mather, 2006; Murty et al., 2009; St Jacques et al., 2009). This age-related change might also reduce the effect of the noradrenergic activation of the

amygdala, which has been shown to be necessary to observe stress effects on memory (Roozendaal et al., 2009; Schwabe et al., 2009). Taken together, these facts may contribute to reducing stress effects on memory retrieval in older people. It is conceivable that the lack of stress effects on free recall reported in the older group might also be due to an attenuated cortisol response, as the older people in our study showed a lower stress-induced cortisol response. However, previous studies carried out with young individuals have shown impairing stress effects on memory retrieval performance with similar cortisol response magnitudes to those of our older group (e.g. Smeets et al., 2008; Smeets, 2011).

Interestingly, while the present study found that a stress-induced cortisol increase affects memory retrieval in young men, but not in older people, previous studies directly comparing older and young individuals have shown a different pattern of results. In a previous study, we showed that stress impaired a very specific aspect of declarative memory, immediate recall after interference (i. e. retroactive interference) in older but not young individuals (Hidalgo et al., 2014). Similarly, Wolf et al. (2001a) showed impairing effects of a hydrocortisone injection on memory retrieval of a word list learned 75 min before cortisol administration in both young and older men. In this study, the word-list recall was measured after other memory tasks were performed, and so it is possible that the effect observed was also due to the effect of cortisol on retroactive interference. One explanation for these contradictory effects could be that the pattern of sensitivity to the effects of acute stress on memory in older people differs depending on the type of memory, with retroactive interference being more affected by stress and cortisol than other memory processes (e.g., working memory, declarative and non-declarative memory, long-term memory retrieval). This may be due to age-related changes in the sensitivity to cortisol's effects on memory performance. Roozendaal (2002) proposed that stress blocks long-term memory retrieval to facilitate consolidation of new information in young people. It has been suggested that this mechanism would diminish retroactive

interference, allowing the brain to learn important new information to be used in the future (Roozendaal, 2002; Joëls et al., 2006). Thus, the increase in retroactive interference after stress observed in previous studies may be due to the fact that stress and cortisol do not block the memory retrieval of previously learned material in older people, as observed in our results.

Sex differences were only found among young people. In fact, the stressor only impaired young men's retrieval performance, while this effect was not found in young women. This result found in men coincides with previous studies performed solely in men after both pharmacological treatment (de Quervain et al., 2003) and acute stress (Kuhlmann et al., 2005b; Oei et al., 2006). At the same time, the lack of a stressor effect in women agrees with findings reported in a study conducted to investigate the effects of stress in luteal women (Schoofs and Wolf, 2009). It is important to note that most of the studies with both sexes did not report sex-related differences, and they did not control the phase of the menstrual cycle of the women (de Quervain et al., 2000; Beckner et al., 2006; Buchanan et al., 2006; Buchanan and Tranel, 2008; Smeets et al., 2008). In our opinion, an explanation for this discrepancy with previous literature might be that, as we did not register the menstrual cycle phase of the young women, it is impossible to know whether the null effect of the stressor on free recall performance in young women is related to the sex factor or, on the contrary, to sex hormone levels. Taking into account the results found by Schoofs and Wolf (2009), it is possible that most of the young women in our sample were in the luteal phase of their menstrual cycle, which would explain the lack of effects on them. However, other studies have reported no differences between men and women in the luteal or follicular phases (Smeets, 2011), or between men and women in the luteal phase of their menstrual cycle (Wolf et al., 2002). Therefore, further research is needed to examine the role of sex and sex hormone levels, as well as the use of oral contraceptives, in the relationship between acute stress and memory retrieval in young people.

Unlike on the free recall task, we found similar recognition performance in older and young individuals. Regardless of age and sex, stress impaired recognition memory of positive pictures. This result is in line with Domes et al. (2004), who found an impairing stress effect on the recognition of positive words, but only in young men. An explanation for this result could be that the positive pictures were rated as moderately arousing in comparison to the negative (highly arousing) and neutral pictures (less arousing). Both negative and neutral pictures would be less sensitive to stress effects because the former would have strong traces (Buchanan et al., 2006), and the latter would have no traces. However, the traces of positive pictures were weaker and more vulnerable to stress.

Interestingly, stress selectively impaired free recall in young men, but it impaired recognition in the entire sample (i.e. older and young men and women). Numerous studies have shown that recognition memory consists of two components, recollection (i.e. remembering details about previously learned material) and familiarity (i.e. knowing whether the material has been previously presented or not) (Mandler, 1980; Squire et al., 2007), which seem to be dependent on the hippocampus and the adjacent perirhinal cortex, respectively (Brown and Aggleton, 2001; but see Squire et al., 2007). Along these lines, we can only speculate that the effect observed in recognition may be related to the adjacent perirhinal cortex, since subjects were asked to answer whether they had seen the pictures before by saying “yes” or “no”, and the task could be a recognition task with a stronger familiarity component. Given that recognition is a cognitive function that does not seem to suffer an age-related decline (Park et al., 2003), it is possible that both young and older people are sensitive to the detrimental effect of stress on this type of memory task. However, it should be noted that correlation analyses did not show a significant relationship between the stress-induced cortisol or sAA response and memory recognition, suggesting that cortisol and sAA are not the main contributors to this effect. Thus, it is possible that other factors not addressed in this study might account for the

results observed; therefore, further research is clearly needed to investigate this hypothesis.

A limitation of the current study is that in order to avoid introducing confounding factors and obtain the best comparison of old and young people, we made an effort to get a very healthy sample by applying restrictive exclusion criteria. This strategy allowed us to obtain two cognitively and physically homogeneous age groups. However, at the same time, it makes it difficult to generalize our results to the older population with age-related diseases (e.g. diabetes or hypertension). Future studies with a more general population should be carried out. Another limitation of our study is the sample size. Despite having a large number of participants (i.e. 102 participants), dividing the sample according to the condition, age and sex factors caused the sample size of each subgroup to be reduced. Finally, our study coincides with previous studies that have observed stress effects on long-term memory retrieval when testing 10 or fewer items for each emotional category in young people (Kuhlmann et al., 2005b; Buchanan and tranel, 2008; Schwabe et al., 2009). However, future studies could explore whether stress effects are observed in older people when more items to-be-recalled and/or more difficult memory tasks are used.

In conclusion, our study is unique in examining, for the first time, age differences in acute stress effects on memory retrieval in men and women, although further research should consider more age ranges in order to better understand the role of age in stress effects on memory retrieval across the lifespan. Moreover, this study adds evidence to the issue of sex differences in stress effects on memory retrieval among young people.

CHAPTER V

STUDY 4

STUDY 4

The diurnal cortisol cycle and memory performance in older people[6]

[6] The main results of this study are being prepared for submission: Hidalgo, V., Almela, M., Pulopulos, M.M., Salvador, A. Memory performance is related to the cortisol awakening response in older people, but not to the diurnal cortisol slope.

5.1. INTRODUCTION

Cognitive decline stands out among the main negative changes associated with aging. However, there is great variability in the way people experience these age-related changes in cognition. While there are some people who maintain their cognitive abilities intact or show few changes, others experience important cognitive problems that can even lead to dementia (Christensen et al., 1999). It has been suggested that hypothalamus-pituitary-adrenal axis (HPA-axis) functioning can explain these differences, at least in part. Along these lines, an HPA-axis dysregulation has been related to poorer cognitive performance (Lupien et al., 2007, 2009). The HPA-axis would exert its effects on cognitive performance through the action of cortisol, the main glucocorticoid in humans, which binds to receptors (i.e., mineralocorticoid and glucocorticoid receptors) especially distributed in the hippocampus, prefrontal cortex and amygdala. In this vein, memory is one of the main cognitive processes that have been related to HPA-axis functioning because these brain structures are key brain areas for learning and memory processes (for review see: Lupien et al., 2007). However, most studies that relate the diurnal cortisol cycle and memory have included limited sets of memory tests. Thus, more research is clearly needed to elucidate the relationship between diurnal HPA-functioning and different kinds of memory processes.

The dynamic nature of the cortisol cycle makes the study of HPA-axis functioning difficult. In basal conditions (i.e. non-stress), the secretion of cortisol follows a circadian pattern characterized by higher cortisol levels in the morning and lower cortisol levels in the last hours of the day. Thus, two components are clearly distinguished in the diurnal cortisol cycle: (i) the cortisol awakening response (CAR; a sharp rise in cortisol that occurs between 30 to 45 minutes after awakening), and (ii) a steeper decrease in cortisol levels secreted throughout the rest of the day. It seems that the regulatory mechanism underlying the CAR is independent from the rest of the diurnal cycle (Edwards et al., 2001). Given that these are considered two independent components of

HPA-axis activity, it has been indicated that they deserve to be analyzed independently (Fries at al., 2009; Clow et al., 2010a,2010b). Importantly, most of the cross-sectional studies investigating the relationship between HPA-axis functioning and cognitive performance in healthy older people that have included memory tasks have not considered these different discrete components of diurnal HPA-axis activity (Seeman et al., 1997; MacLullich et al., 2005; Li et al., 2006; Kuningas et al., 2007; Lee et al., 2007, 2008; Comijs et al., 2010; Souza-Talarico et al., 2010; Pulopulos et al., 2014). To our knowledge, only a few studies have investigated the specific contribution of these two components of the diurnal cortisol cycle to memory performance in older people. Among them, two only studied the CAR (Almela et al., 2012; Evans et al., 2012), others only studied the cortisol secreted during the rest of the day (Abercrombie et al., 2004; Fiocco et al., 2006; O'Hara et al., 2007; Beluche et al., 2010; Gerritsen et al., 2011), and a few investigated these two components in the same sample (Evans et al., 2011; Franz et al., 2011; Stawski et al., 2011; Singh-Manoux et al., 2014).

Regarding the CAR, most of the studies failed to find an association with memory performance (Evans et al., 2011; 2012; Stawski et al., 2011; Singh-Manoux et al., 2014). Specifically, Evans et al. (2011) showed that, in 50 older participants (60-91 years old) of both sexes, the CAR was positively related to overall cognitive performance, and more specifically to executive function and verbal fluency tasks, but not to memory performance. In a second analysis of the same sample, the authors showed a positive association between CAR and executive function (Evans et al., 2012), but again no relationship with memory performance was observed. Accordingly, Stawski et al. (2011) found that, in middle and older adults (33-84 years old), the CAR was not related to cognitive function, assessed, among other cognitive domains, by episodic verbal and working memory. Similarly, Franz et al. (2011) found that, in older men (51-60 years old), the CAR was negatively related to visual spatial memory and working memory. However, when they controlled for several covariates, these associations disappeared. Finally, Singh-Manoux et al. (2014) did not report any

associations between CAR and short-term verbal memory, inductive reasoning or verbal fluency performance in older men and women (mean age 61 years). By contrast, in a more recent study carried out by our group, a different relationship between the CAR and memory performance depending on the type of memory was reported. Thus, while higher CAR was negatively related to verbal memory, only among men, it was positively related to spatial working memory (Almela et al., 2012).

For the cortisol secreted during the rest of the day, a flatter diurnal cortisol slope (DCS, cortisol index, which reflects the decline in cortisol levels during the day) has been associated with poorer memory performance (Abercrombie et al., 2004; Evans et al., 2011; Gerritsen et al., 2011), although steeper DCS has also been associated with poorer declarative memory (O'Hara et al., 2007), and other studies did not report any relationship with memory performance (Fiocco et al., 2006; Beluche et al., 2010; Singh-Manoux, 2014). On the other hand, when other indices were calculated, mixed results were also reported. For example, the diurnal cortisol decline was positively related to overall cognitive performance, executive function and verbal fluency tasks (Evans et al., 2011), while the cortisol AUCg (i.e. considered an index of total hormonal output throughout the day) was negatively related to visual spatial memory, executive function, and processing speed (Franz et al., 2011). Therefore, in light of the inconclusive results, the need to obtain more evidence about this issue seems clear.

It is worth noting that, among the studies that have investigated the specific contribution of the CAR and the cortisol secreted during the rest of the day to differences in cognitive performance in healthy older people, most of them only assessed one type of memory: visual (Beluche et al., 2010) or verbal memory (Abercrombie et al., 2004; O'Hara et al., 2007; Evans et al., 2011, 2012; Gerritsen et al., 2011; Singh-Manoux et al., 2014), while others only assessed two types of memory: working and declarative memory (Fiocco et al., 2006; Stawski

et al., 2011; Almela et al., 2012). To our knowledge, only Franz et al. (2011) used several working memory tasks (two tests for verbal working memory and one test for spatial working memory) and short and delayed recall (two tests for verbal memory and one test for visual memory). However, this study focused on a younger sample (mean age of 55.9; range from 51 to 61 years old), which could explain the lack of association between cortisol and most of the memory tasks used in this study. Thus, more research is needed to investigate whether the two components of HPA-axis activity may be related to different types of memory tasks in healthy older people.

With this in mind, the aim of the present study was to investigate whether the two different components of the cortisol diurnal cycle (i.e. the CAR and the diurnal cortisol slope) were related to different types of memory performance (i.e. declarative and working memory) assessed with several tasks in older men and women. To do so, we tested cognitive performance on different memory tests in 64 older people. Moreover, the participants provided fourteen saliva samples on two consecutive weekdays in order to obtain the CAR and the diurnal cortisol slope. Based on previous studies, we expected the CAR to be associated with poorer performance on memory tasks that are dependent on hippocampal functioning (Almela et al., 2012) and, at the same time, with better performance on memory tasks that are dependent on prefrontal cortex functioning (Almela, et al., 2012; Evans et al., 2012). Moreover, higher diurnal cortisol levels or flatter DCS would be associated with poorer memory performance (Abercrombie et al., 2004; Evans et al., 2011; Franz et al., 2011; Gerritsen et al., 2011). Finally, as in older people the sex factor has been shown to play a modulatory role in the relationship between stress-induced cortisol and memory performance (Seeman et al., 1997; Wolf et al., 1998; Almela et al., 2011a), and in the relationship between CAR and working memory (Almela et al., 2012), the current study included men and women in order to investigate possible sex differences in the relationship between the diurnal cortisol cycle and memory performance.

5.2. METHOD

5.2.1. Participants

The sample was composed of 64 participants (32 men and 32 women) from 57 to 76 years old (Men: *M* = 64.47, *SD* = 4.295; Women: *M* = 64.84, *SD* = 3.886). There were no sex differences in age or educational level (both $p > 0.586$), but men had a higher body mass index (Men: *M* = 28.35, *SD* = 3.79; Women: *M* = 25.73, *SD* = 4.18, $p = 0.011$) and reported slightly higher subjective socioeconomic status (SES; Adler et al., 2000) than women (Men: *M* = 6.63, *SD* = 1.24; Women: *M* = 5.97, *SD* = 1.09, $p = 0.028$). All women were postmenopausal, having had their last menstrual period more than two years before the testing time, and none of them were receiving estrogen replacement therapy.

Participants belonged to a study program at the University of Valencia for people over 55 years of age. They completed a general questionnaire to check whether they met the study prerequisites. The criteria for exclusion were as follows: smoking more than 5 cigarettes a day; alcohol or other drug abuse; dental, visual or hearing problems; presence of cardiovascular, endocrine, neurological, or psychiatric disease. Participants who were using any medication directly related to emotional or cognitive functioning or able to influence cortisol levels (e.g. glucocorticoids, anti-diabetic medication, antidepressants, benzodiazepines, and psychotropic substances) were excluded from participation. None of the participants met the criteria for dementia, as defined by the NINCDS-ADRDA criteria for Alzheimer's disease. Vitamins and sporadic use of painkillers were allowed.

5.2.2. Procedure and neuropsychological assessment

Participants meeting the criteria were contacted by telephone and asked to attend a neuropsychological assessment, which took place in a laboratory at the Faculty of Psychology (University of Valencia). 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 to eat, smoke or take any stimulants (e.g. coffee, cola caffeine, tea or chocolate) two hours prior to the session, or brush their teeth at least one hour prior to the session. All the participants received verbal and written information about the study and signed an informed consent form. The study was conducted in accordance with the Declaration of Helsinki, and the protocol and conduct were approved by the Ethics Research Committee of the University of Valencia.

The neuropsychological assessment was conducted between 10.00 h and 12.00 h and lasted no more than 1.5 h. Participants performed a total of 6 tests that assessed different memory domains: verbal memory, visual memory and working memory. All the tests were extracted from the Spanish version of the Wechsler Memory Scale III (Pereña et al., 2004).

Verbal memory was assessed with the Logical Memory and the Verbal Paired Associates tests. For Logical Memory, participants had to recall as many memory units or "ideas" as possible from two brief narratives, immediately after the experimenter had read them. After a 30 min delay, participants were again asked to recall as many "ideas" as possible from the two narratives. Participants' answers were audio recorded and later corrected by an expert who followed the instructions provided in the test manual. From this test, two outcomes were used in the analyses: (i) Immediate Recall: total "ideas" recalled from the two narratives immediately after having heard them, and (ii) Delayed Recall: total "ideas" recalled from the two narratives after a 30 min delay. For the Verbal Paired Associates, the experimenter read aloud eight word pairs (e.g. horse-glass) across four trials. The word pairs list was the same across the different trials, but it was presented in a different order in each trial. In each trial, after reading the eight word pairs, the experimenter read the first word in the pair (e.g. horse), and participants had to recall the other word in the pair (e.g. glass).

After a 30 min delay, the experimenter again read the first word in each pair and participants had to recall the second word. We calculated two outcomes from this test: (i) Immediate Recall: total number of words recalled on the first four trials and (ii) Delayed Recall: total number of words recalled after a 30 min delay.

Visual memory was assessed with the Family Pictures test. Participants were shown 4 pictures presented consecutively and for 10 seconds each, and then they were asked to recall as much information as possible about them. Each picture represented a different familiar scene with different family members appearing on it. Once the pictures were presented, participants were asked (i) which family member appeared in each picture, (ii) where they were situated in the picture and (iii) what they were doing. After a delay of 30 min, participants had to answer the same questions again. Participants' answers were audio recorded and later corrected by an expert who followed the instructions provided in the test manual. The outcomes used in the analyses were: (i) Immediate Recall: total number of correct answers from the 4 pictures immediately after having seen them, and (ii) Delayed Recall: total number of correct answers from the 4 pictures after the 30 min delay.

Working memory was evaluated with two verbal tests: Letter-Number Sequencing (LNS) and Digit Span (DS) and a spatial test: Spatial Span (SS). For the LNS, participants listened to a sequence of alternating digits (from 0 to 9) and letters (from A to Z) of increasing length. Immediately after that, they first had to repeat the digits in numerical order and then the letters in alphabetical order. The length of the sequences increased from two to eight items, and for each set length, three attempts were given to solve it. One point was assigned for each correctly recalled attempt, and the task ended only when the participant had failed the three attempts for the same set length. As an outcome measure, we used the total correctly recalled attempts. On the DS, which had two parts, the Digit Span Forward (DS Forward) and the Digit Span Backward (DS Backward), participants were read a series of numbers (from 0 to 9) with increasing length

(from two to nine digits). Participants had to repeat the numbers in the same (DS Forward) or the reverse (DS Backward) order as their presentation. For each set length, two attempts were given to solve it, and the task ended only when the participant had failed the two attempts of the same set length. Two outcomes were obtained: (i) DS Forward: total correctly recalled attempts in the same order, and (ii) DS Backward: total correctly recalled attempts in the reverse order. Finally, for the SS, which had two parts, the Spatial Span Forward (SS Forward) and the Spatial Span Backward (SS Backward), participants were presented with a set of 10 cubes on a board. The experimenter touched the cubes in a specific order and the participants had to repeat the sequence in the same (SS Forward) or reverse (SS Backward) order. The length of the sequences increased from two to nine cubes, and for each sequence length two attempts were given to solve it. One point was assigned for each correctly recalled attempt, and the task ended only when the participant had failed the two attempts of the same sequence length. Two outcomes were obtained: (i) SS Forward: total correctly recalled attempts in the same order, and (ii) SS Backward: total correctly recalled attempts in the reverse order.

5.2.3. Salivary cortisol

To measure the diurnal cortisol cycle, participants provided 7 saliva samples per day for 2 consecutive weekdays using salivettes (Sarstedt, Nümbrecht, Germany) at their home. To check for adherence to the sampling times, we stored the salivettes in MEMS TrackCap containers (MEMS 6 TrackCap Monitor, Aardex Ltd. Switzerland), which recorded the exact time the participants provided each sample. Additionally, the participants wrote down the exact sampling times in a diary. After a demonstration given by the experimenter in the lab about how to provide the saliva sample, participants received written instructions and were advised to drink only water, and not to eat, smoke or brush their teeth at least 1 h prior to each saliva sample. The saliva samples were

provided immediately after awakening, 30 and 45 min post-awakening, and at 12.00 h, 16.00 h, and 20.00 h and immediately before bedtime. Participants were instructed to store their samples in their fridge and bring them to the University as soon as possible, never exceeding more than three days after completion. In order to control the cortisol concentrations in the neuropsychological assessment, participants provided two additional saliva samples (at the beginning and at the end of the neuropsychological session).

In the lab, the samples were centrifuged at 4000 rpm for 15 min. Cortisol concentrations were determined by radioimmunoassay using the commercial kit Spectria Cortisol RIA from Orion Diagnostica (Espoo, Finland). Assay sensitivity was 0.8 nmol/L., and the within- and inter-assay variation coefficients were all below 8 %. Each subject's samples were analyzed in the same trial.

5.2.4. Statistical analyses and data management

As salivary cortisol values did not have a normal distribution, they were square root transformed. Student's *t*-tests were used to investigate sex differences in the demographic variables. An ANCOVA for repeated measures with Time (Awakening, 30', 45', 12 h., 16 h., 20 h., Bed) as within-subject factors, and Sex (men, women) as between-subject factor was performed to investigate differences across days and between men and women in cortisol levels at home. Because sex differences were observed in SES and BMI, these variables were included as covariates in this analysis. We used Greenhouse-Geisser because 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. According to Franz et al. (2011), DS Backward and LNS were adjusted for DS Forward, and SS Backward was adjusted for SS Forward. To calculate these adjusted indexes, we save the standardized residual scores from regression analyses using the forward condition as a predictor (i.e., DS Forward

or SS Forward) and the backward condition (i.e., DS Backward or SS Backward) or LNS as dependent variable.

For the cortisol levels during the neuropsychological assessment, the mean of the two saliva samples collected (pre and post) was calculated. The equivalent cortisol samples measuring the diurnal cortisol cycle were averaged across days because the concentrations were correlated (r between 0.3 and 0.5, for all $p \leq 0.019$), and no significant differences between salivary cortisol levels across days were observed ($p = 0.377$). Two cortisol indices were used from the cortisol samples taken at home: (i) the CAR, to reflect the post-awakening measure of cortisol secretion (Clow et al., 2010) and calculated by the cortisol area under the curve with respect to the increase (AUCi; see Pruessner et al., 2003) from the 0, +30 and +45 min cortisol samples, and (ii) the diurnal cortisol slope (DCS): to reflect the decline in cortisol levels during the day, and calculated by regressing cortisol values (except +30 min and +45 min samples to avoid biasing the slope by CAR) on each sample collection time individually for each participant (Sephton et al., 2009; Smeets et al., 2007; Singh-Manoux et al., 2014). A larger β value was interpreted as a flatter slope, reflecting a slower cortisol decline, while a smaller β value was interpreted as a steeper slope, reflecting a rapid diurnal decline.

Regression analyses were performed to investigate the relationship between CAR, DCS and memory performance. In addition, moderator regression analyses were conducted to investigate whether sex was a moderator in these relationships, according to Aiken and West (1991). Scatterplots were checked to investigate linear or curvilinear relationships.

Two participants were excluded from the analyses, one woman because her cortisol concentrations differed by more than 3 SD from the CAR mean and DCS mean samples, and one woman who had three missing values for the DCS samples from day 1. All p values reported here are two-tailed. When not otherwise specified, the results shown are means ± standard error of mean

(SEM). We used SPSS 22.0 to perform the statistical analyses. In order to provide an easy interpretation of figures, the values represented are raw values and not square root transformed values.

5.3. RESULTS

5.3.1. Preliminary analyses: adherence to the salivary sampling protocol

It has been indicated that if the first saliva sample is not collected immediately after awakening, the reliability of the measurement of the CAR is compromised. Thus, based on Thorn et al. (2006), and in line with previous data from our group (Almela et al., 2012), we explored the cortisol profile of the participants to identify any participants who might be suspected of non-adherence to protocol. In order to control this issue, participants were divided into two groups, according to the method suggested by Thorn et al. (2006): (i) those who had a positive CAR on both days (2 Day-CAR group), and (ii) those who had a positive CAR on only one day or none, (1 or 0 Day-CAR group). Of the total sample, 57.8% of the participants showed a positive CAR on both days (15 men and 22 women), 35.9% of the participants showed a positive CAR on only one day (13 men and 10 women), and the other 6.3% of the participants did not show a positive CAR on either of the two days (4 men). No differences in age, educational level, SES or BMI were found between the 2 Day-CAR and the 1 or 0 Day-CAR subgroups (all $p > 0.699$).

Figure V.1 represents the differences in the CAR profiles between the 2 Day-CAR and the 1 or 0 Day-CAR subgroups (TimexCAR groups: F (2, 116) = 51.411, $p < 0.001$). While the 2 Day-CAR subgroup showed a steeper rise from awakening to 30 min later ($p < 0.001$), the 1 or 0 Day-CAR subgroup showed a flatter rise, given that cortisol levels were higher in the awakening sample (Awakening: 2 Day-CAR vs. 1 or 0 Day-CAR, $p = 0.004$). Forty-five min later, cortisol concentrations started to decrease; however, only in the 1 or 0 Day-CAR

subgroup, they were similar to awakening levels (Awakening vs. +45 min: p = 0.999). The Sex factor was not significant (p = 0.520), nor were its interactions with other factors (all p > 0.114).

As proposed by Thorn et al. (2006), and as performed in our previous study (Almela et al., 2012), we repeated all the analyses, excluding those participants who were suspected of being non-adherent to the protocol (0 or 1 Day-CAR).

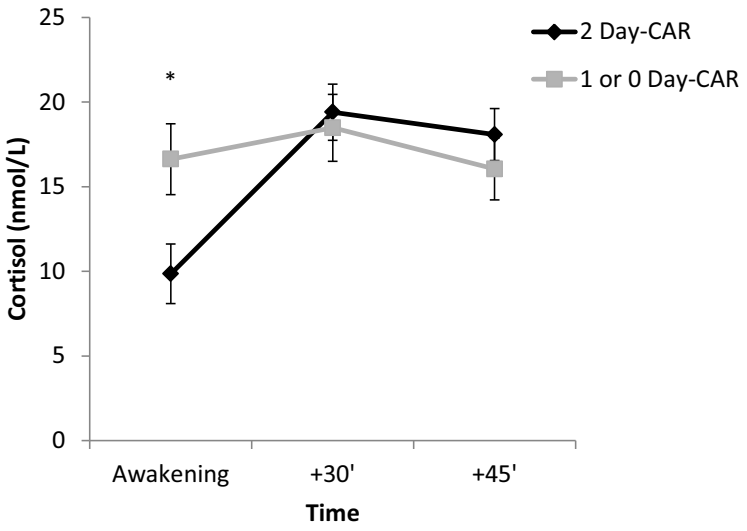

Figure V.1. CAR profiles for the 2 Day-CAR and for 1 or 0 Day-CAR subgroups. In the first saliva sample (awakening), participants suspected of non-adherence (1 or 0 Day-CAR group) had higher cortisol concentrations than participants suspected of being adherent (2 Day-CAR)(*p = 0.004). Depicted values are means, and error bars represent the SEM.

5.3.2. Sex differences in the diurnal cortisol cycle

The repeated-measures ANCOVA showed the main effect of Time (F (3.462, 200.812) = 4.202, p = 0.004). As expected, participants presented the CAR because their cortisol levels increased from awakening to 30 min later (p < 0.001), and then decreased over time, reaching the lowest levels in the last two saliva samples (20 h. vs Bed, p > 0.999). None of the interactions were significant, but the interaction between Time and Sex was marginally significant (F (3.462, 202.812) = 2.383, p = 0.062). *Post-hoc* analyses showed that men had slightly higher cortisol levels in the awakening sample (p = 0.070) and in the 16 h. sample (p = 0.086).

When the same analyses were performed only with the 2 Day-CAR group, the same results were observed for Time (F (3.265, 107.758) = 6.552, p < 0.001), but not for the TimexSex interaction, which was not significant (F (3.265, 107.758) = 0.545, p = 0.667) (see Figure V.2).

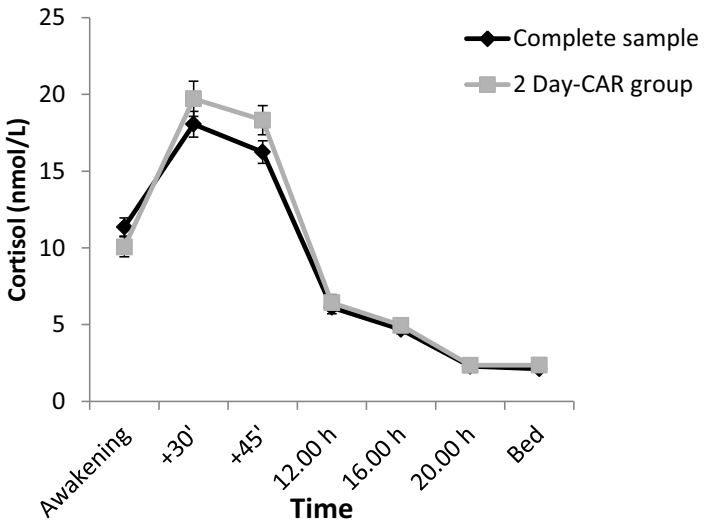

Figure V.2. Diurnal cortisol cycle for complete sample and for 2 Day-CAR group. Depicted values are means, and error bars represent the SEM.

5.3.3. Relationship between diurnal cortisol cycle and memory performance

Hierarchical regression analyses were performed to investigate the relationship between the two components of the diurnal cortisol cycle (CAR and DCS) and memory performance. Separate analyses were conducted for each memory outcome. To control for possible confounder effects, the following covariates were included in the analyses: we included Age and BMI because they affect cognitive and HPA-axis functioning (Cournout et al., 2006; Dettenborn et al., 2012; Silver et al., 2012; Stalder and Kirschbaum, 2012). SES was included due to its relationship with HPA-axis activity (Wright and Steptoe, 2005; Cohen et al., 2006) and health status (Adler et al., 2000; Singh-Manoux et al., 2005; Demakakos et al., 2008). Finally, the mean of the cortisol levels during the neuropsychological assessment was included as a covariate to control the stressfulness of the testing situation (Sindi et al., 2013). To do so, in step 1, we included these control variables and sex (0 = women, 1 = men). In step 2, we included the CAR or DCS. In step 3, we included the square of the CAR or DCS. The significant curvilinear relationships were interpreted as a concave upward relationship (U-shaped form), where the value of β is positive, and a concave downward relationship (inverted U-shaped form), where the value of β is negative. In order to reduce multicollinearity, all predictors included in the regression analyses were previously standardized. Results of the analyses performed are shown in Table V.1 (verbal and visual memory) and Table V.2 (Working Memory). Because none of the associations were moderated by Sex ($p > 0.1$), only the linear and curvilinear associations for men and women together are shown in the tables.

CAR and memory performance. Results for the complete sample showed that higher CAR was associated with worse performance on the immediate recall trial of Verbal Paired Associates (i.e., negative linear relationship) (p=0.042)(Table 1). Additionally, there were significant curvilinear relationships (i.e., inverted U-shaped) between the CAR and performance on the Logical

Memory test: immediate (p = 0.040) and delayed recall (marginally, p = 0.054)(Table V.1), the Family Pictures test: immediate (p = 0.014) and delayed recall trials (p = 0.010)(Table V.1), and Digit Span Backward Adjusted (p = 0.037) (Table V.2). These curvilinear associations indicated that a larger and lower CAR was related to worse performance on these tests, while a moderate CAR was related to better performance. None of the others associations were significant (all p > 0.181).

If the analyses are performed only with the 2 Day-CAR group, results show a significant negative linear relationship between the CAR and performance on the Logical Memory test: immediate (p = 0.033) and delayed recall (p = 0.005) (Table V.1), the Verbal Paired Associates test: immediate recall (marginally, p = 0.079) (Table V.1), and the Family Pictures test: immediate (marginally, p = 0.063) and delayed recall (p = 0.040) (Table V.1). None of the others associations were significant (all p > 0.142). Table V.3 summarizes the main results of the regression analyses for the complete sample and only for the 2 Day-CAR group.

Table V.1. Regression analyses with CAR as predictor and verbal and visual memory outcomes as dependent variables

Total sample		Logical Memory		Verbal Paired Associates		Family Pictures	
		Immediate recall	Delayed recall	Immediate recall	Delayed recall	Immediate recall	Delayed recall
Linear associations	Adj$^c R^2$	-0.044	-0.064	0.140	0.086	0.086	0.084
	β	-0.007	-0.035	-0.268	-0.163	0.037	0.046
	p	0.962	0.810	**0.042**	0.226	0.780	0.733
Curvilinear associations	Adj$^c R^2$	0.017	-0.011	0.139	0.090	0.168	0.175
	β	-1.362	-1.292	-0.589	-0.682	-1.508	-1.576
	p	**0.040**	**0.054**	0.335	0.278	**0.014**	**0.010**
2 Day-CAR group		Logical Memory		Verbal Paired Associates		Family Pictures	
		Immediate recall	Delayed recall	Immediate recall	Delayed recall	Immediate recall	Delayed recall
Linear associations	Adj$^c R^2$	0.096	0.178	0.107	0.069	0.085	0.073
	β	-0.393	-0.317	-0.317	-0.269	-0.342	-0.381
	p	**0.033**	**0.005**	**0.079**	0.142	**0.063**	**0.040**
Curvilinear associations	Adj$^c R^2$	0.075	0.162	0.078	0.104	0.068	0.065
	β	-0.925	-1.011	-0.280	-2.376	-1.128	-1.428
	p	0.576	0.521	0.865	0.150	0.497	0.392

Table V.2. Regression analyses with CAR as predictor and working memory outcomes as dependent variables

Total sample		Letter-Number Sequency		Digit Span		Spatial Span	
		NAdj	Adjusted	DSForward	DSAdjusted	SSForward	SSAdjusted
Linear associations	Adj$^c R^2$	0.096	0.075	-0.055	-0.030	-0.026	-0.020
	β	0.015	-0.017	0.091	0.117	0.137	-0.152
	p	0.913	0.898	0.526	0.409	0.336	0.282
Curvilinear associations	Adj$^c R^2$	0.081	0.062	-0.071	0.032	-0.024	-0.005
	β	-0.214	-0.337	0.307	-1.372	-0.687	-0.887
	p	0.734	0.596	0.651	**0.037**	0.303	0.181
2 Day-CAR group		Letter-Number Sequency		Digit Span		Spatial Span	
		NAdj	Adjusted	DSForward	DSAdjusted	SSForward	SSAdjusted
Linear associations	Adj$^c R^2$	0.290	0.283	-0.103	-0.012	0.006	0.009
	β	-0.169	-0.061	0.074	-0.253	-0.083	-0.217
	p	0.867	0.701	0.707	0.183	0.657	0.248
Curvilinear associations	Adj$^c R^2$	0.272	0.278	-0.126	-0.046	0.035	-0.022
	β	-0.729	-1.309	1.153	0.013	2.307	0.548
	p	0.619	0.372	0.527	0.994	0.177	0.752

NAdj: Non adjusted

Table V.3. Summary of the regression analyses between memory test outcomes and CAR for complete sample and only for the 2 Day-CAR group

Memory Domain and Tests	Outcome	Total sample	2 Day-Car group
Verbal Memory			
Logical Memory	Immediate Recall	Inverted U-shaped	Negative linear
	Delayed Recall	Inverted U-shaped*	Negative linear
Verbal Paired Associated	Immediate Recall	Negative linear	Negative linear**
	Delayed Recall	-	-
Visual Memory			
Family Pictures	Immediate Recall	Inverted U-shaped	Negative linear***
	Delayed Recall	Inverted U-shaped	Negative linear
Working Memory			
LN Sequencing	LNS non-adjusted	-	-
	LNS Adjusted	-	-
Digit Span	DS Forward	-	-
	DS Adjusted	Inverted U-shaped	-
Spatial Span	SS Forward	-	-
	SS Adjusted	-	-

$^*p = 0.054$; $^{**}p = 0.079$; $^{***}p = 0.053$

DCS and memory performance. None of the associations between the diurnal cortisol slope and memory performance were significant ($p > 0.085$). Sex did not moderate any of these relationships ($p > 0.1$) (See Table V.4).

Table V.4. Regression analyses with DCS as predictor and verbal, visual and working memory outcomes as dependent variables

		Logical Memory		Verbal Paired Associates		Family Pictures	
		Immediate recall	Delayed recall	Immediate recall	Delayed recall	Immediate recall	Delayed recall
Linear associations	Adj$^c R^2$	-0.037	-0.047	0.075	0.073	0.090	0.089
	β	0.087	0.137	0.053	-0.110	0.076	0.086
	p	0.538	0.336	0.694	0.412	0.566	0.516
Curvilinear associations	Adj$^c R^2$	-0.034	-0.038	0.063	0.058	0.097	0.081
	β	-10.963	-12.499	5.276	3.742	11.225	6.906
	p	0.287	0.226	0.589	0.702	0.244	0.475
		Letter-Number Sequency		Digit Span		Spatial Span	
		NAdj	Adjusted	DSForward	DSAdjusted	SSForward	SSAdjusted
Linear associations	Adj$^c R^2$	0.105	0.087	-0.063	-0.043	-0.035	0.014
	β	0.102	0.115	-0.017	0.016	0.095	0.241
	p	0.439	0.390	0.903	0.912	0.503	0.085
Curvilinear associations	Adj$^c R^2$	0.100	0.079	-0.080	-0.062	-0.054	-0.002
	β	7.763	6.895	3.747	-1.923	-0.194	-4.166
	p	0.418	0.477	0.720	0.853	0.985	0.679

NAdj: Non adjusted

5.4. DISCUSSION

The present study investigated whether the CAR and the DCS were related to different memory domains in older men and women. For the complete sample, the CAR was negatively associated with tasks that are dependent on hippocampal functioning (in a linear form: Verbal Paired Associates test; in a curvilinear form: Logical Memory and Family Pictures tests) and with only one outcome that is dependent on prefrontal cortex functioning (i.e. DS Backward Adjusted). When the same analyses were performed with only those participants who showed CAR on both days (2 Day-CAR group), the CAR was only related to

tasks that are dependent on hippocampal functioning in a negative linear form (Logical Memory, Verbal Paired Associates and Family Pictures), but not to tasks that are dependent on prefrontal cortex functioning (i.e. Letter and Number Sequencing, Digit Span and Spatial Span). With regard to the DCS, no relationship was found with memory performance on any task used in the complete sample or the 2 Day-CAR group. Finally, the sex factor did not moderate the relationships found between the CAR and memory performance in the complete sample or the 2 Day-CAR group.

In the present study, we assessed cognitive performance through six different memory tasks measuring verbal, visual and working memory (verbal and spatial). Memory performance is one of the main cognitive domains related to HPA-axis functioning in response to acute stress (for a review see: Schwabe et al., 2013). However, few studies have investigated the specific association between the CAR and DCS and memory performance assessed by several memory tasks. As expected, the CAR was negatively related to memory performance on verbal and visual memory tasks only in those participants who showed CAR on both days (2 Day-CAR group). Specifically, higher CAR was associated with worse performance on immediate and delayed recall on the Logical Memory test and marginally with immediate recall on the Verbal Paired Associates test (p = 0.079). Moreover, higher CAR was also associated with worse performance on delayed recall on the Family Pictures test and marginally with immediate recall on this same task (p = 0.063). It is important to note that for the complete sample, the relationships with the Logical Memory and Family Pictures tests were quadratic (inverted U-shaped form). As previously discussed, the fact that some people in the total sample had a shifted CAR measurement, and the possibility that these people were on the left side of the inverted U curve, could explain these different results (Almela et al., 2012). In the same study, we showed, as in the present findings, that a greater CAR was related to poorer verbal memory performance assessed with paragraph recall (Almela et al., 2012). The present study confirms this previous result and extends these findings to

memory performance on other verbal (i.e. association task) and visual tasks. The performance on these tasks is especially related to hippocampal functioning; thus, we consider that there is a consistent negative relationship between the CAR and performance on memory tasks related to hippocampal functioning. However, other studies report no relationship (Evans et al., 2011; 2012; Franz et al., 2011; Sigh-Manoux et al., 2014). Methodological differences could explain the contradictory results. It is possible that this absence of the link reported by Franz et al. (2011) and Singh-Manoux et al. (2014) is due to the fact that to calculate the CAR, these authors used only two saliva samples, the first at awakening and the second 30 minutes after awakening. Thus, despite the fact that the CAR has been calculated as the change, as has been suggested (Clow et al., 2010b), there are individual differences in the exact moment of the maximum peak, which can take place between 30 and 45 min post awakening (Pruessner et al., 1997; Wilhelm et al., 2007). Therefore, it is not strange that the maximum peak for the complete sample was not collected, leading to a weak CAR measurement. By contrast, in our previous (Almela et al., 2012) and present studies, we included a third saliva sample 45 min after awakening. This has allowed us to calculate the CAR as the AUCi, a more confident measurement. Another explanation could be the type of memory task used. Specifically, Evans et al. assessed verbal memory performance using a word list learning test (Evans et al., 2011; 2012); however, we found the CAR-verbal memory link on paragraph recall and word associated tests. Supporting this argument, Almela et al. (2012) found that the CAR was negatively related to a paragraph recall test, but not to a word list learning test. These results suggest that this test may be less sensitive than others to associations with the CAR.

To measure the working memory domain, three different tests were used: Letter-Number Sequencing, Spatial Span and Digit Span. When we performed the analysis for the total sample, we found a negative curvilinear relationship between the CAR and Digit Span Backward. However, when we replicated the analysis for the 2 Day-CAR subgroup, this result did not remain

significant. Therefore, as observed in Franz et al. (2011), we cannot conclude that there is a relationship between CAR and working memory. By contrast, two previous studies reported an association between CAR and working memory performance. Thus, Almela et al. (2012) showed that a greater CAR was related to better spatial working memory only in men, but this result was observed only when the analyses were performed with the sample suspected of being adherent to the protocol on both days of CAR sampling. Moriarty et al. (2014), in 19 young men, showed a U-shaped relationship between the CAR and spatial working memory performance. Although it is still not well understood, it has been indicated that the frontal cortex may play a part in the dynamics and magnitude of the CAR (for a review see: Evans et al., 2010). Then, a possible relationship between working memory and CAR could be expected. However, for the time being, the results observed in our studies and others do not offer a clear explanation of what the direction is, or even if there is a relationship between them. Further studies are needed to investigate this association more in-depth, using other kinds of working memory tasks that might be more sensitive to changes in CAR and diurnal cortisol levels.

Regarding the association between the DCS and cognitive performance, our results showed that this index of the diurnal cortisol cycle was not related to any of the six memory tasks. This lack of association agrees with previous studies in older people that failed to find a relationship between the DCS and declarative memory in a small sample (N=42, Fiocco et al., 2006), verbal and visual memory in a medium sample (N=197, Beluche et al., 2010), and short-term memory in a large sample (N=3229, Singh-Manoux, 2014). By contrast, most of the studies that investigated the link between this cortisol index and memory reported a negative association. Thus, both a flatter DCS (Abercrombie et al., 2004; Evans et al., 2011; Gerritsen et al., 2011) and a steeper DCS were associated with poorer declarative memory (O'Hara et al., 2007). Methodological differences, such as the number of participants studied, the cognitive domains assessed, the method used to calculate the DCS, or a combination of them, can explain these

inconsistent findings. Taken together, a systematic examination of the implications of these factors in the relationship between this component of the diurnal cortisol cycle and memory performance will help to better understand this link.

Some limitations should be considered. Since participants were selected based on having good physical and psychological health, our results may not be representative of the general older population. In this stage of life, the presence of several diseases and, consequently, the use of several medications are very common. For this reason, replication studies that include participants with age-related diseases (i.e. diabetic or hypertensive older people) are clearly warranted. Moreover, it is important to note the correlational nature of our results, which means that we cannot endorse causal relationships. Despite this limitation, our results are consistent across different memory tasks. Finally, although we used devices to check for adherence to the sampling times, we still do not know the exact time at which the participants awoke and, therefore, if the first saliva sample (i.e. awakening) was correctly collected. To control this, we performed the same statistical analysis, first for the complete sample and then for the 2 Day-CAR subgroup. Thus, for the complete sample, most of the associations followed an inverted U-pattern, while for the 2 Day-CAR subgroup, these associations changed to a negative linear form. These results highlight the importance of paying attention to protocol adherence in order to avoid confounding conclusions.

On the whole, the present study confirms, in healthy older people, the negative relationship between the CAR and memory performance dependent on hippocampal functioning. In addition, it provides further evidence about the relationship between memory performance and the DCS, another component of the diurnal cortisol cycle.

CHAPTER VI

GENERAL DISCUSSION

The previous chapters have described the main results of several studies on the effects of acute stress on memory performance in healthy adults, considering some factors related to memory (i.e. phase and type of memory assessed and type of material to be remembered) and others related to individuals (i.e. age and sex). Moreover, the link between the HPA-axis functioning in basal situations (non-stress) and different memory domains in older people has also been investigated. As these studies showed, under stress, both the SNS and, especially, the HPA-axis activity are related to memory performance. However, depending on the factors mentioned above, the direction of these relationships will be different. Under non-stressful situations, the HPA-axis activity is also related to memory performance, depending on the type of memory assessed. In this final chapter, a summary of the main findings of these studies and the conclusions of this thesis are presented.

6.1. MAIN FINDINGS

6.1.1. Study 1

The aim of this study was to determine the direction of the impact of an acute psychosocial stress on memory performance in healthy young adults. To do so, the two stress systems' (i.e. SNS and HPA-axis) reactivity to the stressor was measured. After the stress and control tasks, the non-declarative memory was assessed with a priming test and declarative memory was assessed with the RAVLT. Thus, we investigated the effects of an acute psychological stressor on these two types of memory when the stressor was applied before learning. Moreover, we studied the moderating role of sex and the level of sexual hormones in this relationship. Then, 18 men and 34 women (17 women in the early follicular phase and 17 women using oral contraceptives) were exposed to the TSST and a control condition in a crossover design. The task used as the stressor provoked larger sAA and cortisol responses than the control condition in all participants.

Sex differences were found in the cortisol response, but not in the sAA response. Men had higher cortisol response to the TSST than both groups of women. Moreover, regardless of sex, the acute stressor was associated with an enhancement of non-declarative memory performance, and this enhancement was greater in participants who had a larger sAA response to the TSST. By contrast, the stressor did not affect declarative memory performance. Taken together, our results confirm an effect of acute stress only on non-declarative memory performance (i.e. priming), and they suggest a different relationship between the two stress biomarkers and the different types of memory in healthy young adults.

6.1.2. Study 2

The aim of this study was to explore the role of age in the impact of the magnitude of the stress-induced sAA and cortisol responses on memory performance, considering the sex of the participants. To this end, in a crossover design, 32 older (16 men and 16 women) and 35 young adults (18 men and 17 women) were exposed to both the stress and control conditions. Afterwards, declarative memory performance was assessed using the RAVLT test. The TSST was shown to induce stress because an increase in sAA and cortisol responses was observed for the total sample. Sex differences were found in the cortisol response, but not in the sAA response. Regardless of age, men had a higher cortisol response to the stressor than women. However, no age differences were found in the cortisol response, but in the sAA response older participants had higher sAA concentrations than younger ones. In addition, as expected, older people had poorer declarative memory performance than young people. However, although no stress-induced changes in learning, delayed recall or recognition were reported, only in older people, stress impaired immediate recall after interference (i.e. retroactive interference). Moreover, this effect was negatively related to the ratio of sAA over cortisol. In conclusion, these findings

confirm a moderating role of age in the effects of stress-prior learning on retroactive interference, a very important sub-process of declarative memory associated with aging. At the same time, they highlight the importance of considering the response of the two stress systems together.

6.1.3. Study 3

In line with study 2, the aim of this third study was to investigate the role of age in the effects of stress on memory performance, considering the sex of the participants. Unlike in studies 1 and 2, here the stressor was applied before the retrieval phase, and the material to be remembered consisted of positive, negative and neutral pictures. After learning (day 1), 52 older (27 men and 25 women) and 50 young (26 men and 24 women) adults were randomly assigned to the stress or control condition in a counterbalanced way, following a between-subjects design. After the stress or control tasks, the retrieval of previously-presented pictures was measured. As in previous studies, there was a significant response to the stressor used. As expected, older people performed worse on free recall than young people, but no age-related differences were found on the recognition task. With regard to free recall, stress only impaired memory retrieval in young men, which was negatively related to the cortisol response to the stressor, especially for negative pictures. Regarding recognition, regardless of age and sex, a poorer performance was found for positive pictures. However, this impairment was not related to the sAA or cortisol response. To our knowledge, this is the first study to investigate age differences in acute stress effects on memory retrieval in both sexes, and it adds evidence about the role of sex in the stress-memory link in young people.

6.1.4. Study 4

In the final study, we investigated whether the HPA-axis activity in basal conditions (non-stress) was also related to memory performance in healthy older people. Specifically, two different components of the diurnal cortisol cycle were measured: the CAR and the DCS. To study so, we tested three cognitive domains in 64 (32 men and 32 women) healthy older people using a neuropsychological assessment. Two of the domains were related to declarative memory (verbal memory: logical memory and verbal paired associates tests, and visual memory: pictures tests) and the other to working memory (verbal memory: letter-number sequencing and digit span tests, and spatial memory: spatial span test). Moreover, participants had to provide 14 saliva samples on two consecutive weekdays to obtain the diurnal cortisol cycle collected in their homes. Results showed a negative linear association between the CAR and performance on memory tasks related to hippocampal functioning. The sex factor did not moderate these associations. By contrast, we failed to find a link between the CAR and working memory performance. No relationship was found between the DCS and the three memory domains evaluated.

6.2. LIMITATIONS AND STRENGTHS

In the previous empirical chapters, the specific limitations of each study have already been mentioned. Thus, this section presents some general comments to consider when interpreting the main findings of this thesis. Among them, the most important is the fact that both older and young participants were mainly selected because they were cognitively and physically healthy, which makes it difficult to generalize these results to people in these age ranges in the general population. It is important to note that, in older people, it is typical to find several age-related diseases and the subsequent medication use. Therefore, future research on this topic should consider replicating these studies in other clinical populations (i.e. diabetic or hypertensive older people). Moreover, all the

participants in these studies were cognitively active people because they were university students, and so bias related to socio-economic status and education could have been introduced. Hence, it might be of interest to explore the aims of this thesis in other populations with different educational levels or without active aging.

Another weak point of this thesis could be the low number of different age groups considered. Here, we only studied two age ranges, young (from 18 to 35 years old) and older (from 54 to 76 years old) people. However, there are other stages across the lifespan. Thus, in order to provide a more complete picture of the role of age in stress effects on memory performance, the study of other age groups is clearly warranted.

Finally, this thesis did not compare the effects of the stressor on priming or the consolidation phase of memory in older and young people, or the CAR/DCS-memory performance association. Studies designed to answer these questions will help to obtain a broader vision of the reality and fill existing gaps in the literature.

Despite these limitations, this thesis also has a number of strengths that allow us to be confident about our results. The strongest point in all the studies collected here is the rigor in the sample selection. We applied numerous and very restrictive exclusion criteria. This fact, apart from being an impediment to obtaining a larger sample size, can also be interpreted as an effort to collect a very homogeneous sample. Thus, we have avoided the introduction of several confounding factors, which was especially important when we compared the two different age groups, due to the large differences between them. We were also strict about the collection of the saliva samples. In order to obtain mainly unbiased cortisol and sAA concentrations in the four studies, participants were asked to follow a set of recommendations prior to the experimental sessions.

Moreover, and in contrast to most of the previous studies about this topic, we made a concerted effort to study the role of sex. To do so, a similar

number of participants was included in each sex group. It is well known that the sexual hormone levels interfere in the relationship between stress and memory, which means it is easier to only include men in the samples. Therefore, addressing this factor in this relationship extends previous findings in men.

Finally, to our knowledge, most previous studies addressing this issue have been carried out with young people. However, few have studied older people, and none have included both age groups. Hence, for the first time, we provide findings from a direct comparison of older and young populations about stress effects on memory performance (studies 2 and 3), which contributes to better understanding the mechanisms underlying the stress-memory link.

6.3. FUTURE

In addition to trying to design studies that address the limitations mentioned in each study in particular, and in the thesis in general, there are still many unresolved issues. In this thesis we have only focused on the study of memory function; however, there are other cognitive processes that are susceptible to being affected by stress. Future studies should investigate these other cognitive operations, from attention to executive function, in order to have more comprehensive knowledge about the link between stress and cognition.

On the other hand, we have not considered factors related to the stressor that can also mediate in the stress effects on cognitive function, in addition to those related to memory function and individual differences already mentioned. Among them, the magnitude, the origin (endogenous vs. exogenous) and/or the duration of the stressor (acute vs. chronic) stand out. However, we have only explored the effects of an acute stressor. Therefore, investigating the effects of other types of stress will be of interest, above all chronic stressors, due to their direct relationship with the decline associated with aging. Therefore, longitudinal studies are clearly needed.

Finally, drawing on the findings found in the fourth study, we strongly recommend a more comprehensive study of the CAR. We think it is important to focus on aspects that can determine the magnitude of the CAR, such as adherence to the protocol and health-related quality of life.

CHAPTER VII

MAIN CONCLUSIONS

Taken together, the results found in the empirical studies point out that in an acute stress situation, SNS and HPA-axis activation affect memory performance. This is a complex relationship, given that several factors can modulate the direction of these effects. Moreover, the diurnal cortisol cycle, is also associated with memory performance in healthy older people. A more specific and detailed discussion of the results has been presented in the different empirical chapters. In this section, the most important conclusions stemming from the objectives of this thesis are described below:

- An acute psychosocial stressor applied before learning a neutral word list enhances non-declarative memory performance in healthy young people, while it does not affect declarative memory performance. These findings are consistent with the hypothesis that acute stress leads to a shift between memory systems, favoring less cognitively demanding learning strategies over those that require awareness (Schawabe et al., 2007). Moreover, this enhancement is positively related to the SNS activity. This finding, not reported previously, reflects the importance of this system activation in the positive effects of stress on non-declarative memory.

- A direct comparison of people of different ages but other similar characteristics has shown that older age is associated with poorer declarative memory performance, while the recognition performance remains invariable. However, there are no age-related differences in the stress effects on declarative memory, except in one aspect of this type of memory. Thus, the stressor applied before learning impairs the immediate recall after interference, a memory aspect involved in retroactive interference. This result coincides with the fact that older people seem to be more vulnerable to this interference than young people (Hedden and Park, 2001). In addition, this negative effect of the stressor on memory seems to be mediated by the response of both physiological systems involved in the stress response.

- Despite sex-related differences found in the cortisol response to the stressor, the sex of the individuals does not seem to be crucial in the effects of stress-prior learning on non-declarative memory (young people) and declarative memory (older and young people).

- Acute psychosocial stress applied before retrieval memory impairs free recall and recognition of emotional and neutral pictures differently, depending on age and sex. Specifically, stress impairs free recall of positive, negative and neutral pictures, only among young men, and recognition memory of positive pictures in older and young people of both sexes. Only the cortisol response is negatively related to the effect found on free recall.

- Overall, successful aging can cushion the effects of acute psychosocial stress on memory performance in older people, given that only retroactive interference was affected by stress.

- There is a different relationship between stress biomarkers and the different memory systems depending on age. Thus, among young adults, only the sAA response is related to the enhancing stress effects on priming, and only the cortisol response is related to the impairing stress effects on free recall performance. However, among older people, the ratio of sAA over cortisol is related to impairing stress effects on retroactive interference.

- The diurnal HPA-axis functioning is also related to memory performance in healthy older people. In both sexes, the cortisol awakening response (CAR) is negatively related to declarative memory performance, which is more dependent on hippocampal functioning, but not to working memory performance, which is more dependent on the prefrontal cortex.

However, the magnitude of the diurnal cortisol slope (DCS) is not related to the declarative and working memory performance of healthy older people.

- The reliability of the measurement of the CAR is an issue that should be considered. The association between the CAR and memory performance is different depending on the adherence to the salivary sampling protocol. For a complete sample, this relationship is mainly curvilinear (i.e. inverted U-shaped), but only for those people suspected of being adherent, the association is linear and negative.

CHAPTER VIII

GENERAL SUMMARY IN SPANISH

En nuestra vida diaria estamos constantemente expuestos a diferentes fuentes de estrés, principalmente, de tipo psicológico y/o social. Nuestra reacción al mismo es considerada una respuesta adaptativa debido a su papel en la facilitación de la supervivencia de los individuos. Sin embargo, el estrés puede tener también consecuencias perjudiciales a corto y largo plazo en la salud, tanto es así que es considerado uno de los problemas de salud más significativos del siglo XXI, según la Organización Mundial de la Salud (2001).

Su impacto se extiende a la mayoría de los sistemas fisiológicos (cardiovascular, digestivo, inmune, neuroendocrino o nervioso) dando como resultado numerosas enfermedades. Entre los efectos del estrés relacionados con el sistema nervioso destacan los problemas cognitivos. Dado el gran impacto que estos problemas pueden tener sobre la sociedad en general y, sobre el individuo y sus familias en particular, parece clara la necesidad de entender más esta relación. Esta es una de las razones del creciente interés en investigar los principales mecanismos que subyacen al impacto del estrés en diferentes procesos cognitivos, tales como la memoria, la atención y las funciones ejecutivas. Sin embargo, diferentes factores relacionados con las características del estresor, del individuo y del proceso cognitivo estudiado puede jugar un papel importante en determinar la dirección de estos efectos del estrés. Por tanto, esta tesis se centra en cómo el estrés afecta a la cognición, concretamente al rendimiento en memoria, en adultos sanos, considerando el papel de algunos de estos factores implicados.

8.1. OBJETIVOS E HIPÓTESIS

Realizamos cuatro estudios con el fin de aclarar aquellos resultados contradictorios, además de proporcionar evidencia en aquellos aspectos sobre los que no hay trabajos previos. Los objetivos e hipótesis de esta tesis se presentan a continuación:

Objetivo general 1. Determinar el impacto del estrés agudo aplicado antes del aprendizaje sobre el rendimiento en memoria de material neutro en adultos sanos.

- *Objetivo específico 1.1:* Estudiar los efectos del estrés sobre el rendimiento en la memoria no declarativa y declarativa en adultos jóvenes.

- *Objetivo específico 1.2*: Comparar los efectos del estrés sobre el rendimiento en la memoria declarativa entre adultos mayores y jóvenes sanos.

- *Objetivo específico 1.3*: Investigar el papel del sexo en los objetivos específicos anteriormente mencionados.

Debido a los pocos estudios llevados a cabo en memoria no declarativa y a los resultados contradictorios en la literatura sobre los efectos que tiene el estrés agudo en la memoria declarativa, no tenemos hipótesis específicas sobre los efectos del estrés antes del aprendizaje en estos dos tipos de memoria en adultos jóvenes. Sin embargo, se esperan diferencias de edad en los efectos del estrés sobre la memoria declarativa. Además, basándonos en hallazgos previos de nuestro grupo (Almela et al., 2011a), esperamos mayores efectos negativos del estrés en mujeres mayores.

Intentaremos responder al objetivo específico 1.1. en el estudio 1 donde ponemos a prueba la hipótesis de que las respuestas de ambos biomarcadores del estrés al TSST están relacionadas con el rendimiento de la memoria no declarativa y declarativa. Además, en este mismo estudio, se aborda también el objetivo específico 1.3. El estudio 2 tiene como objetivos responder a los objetivos específicos 1.2 y 1.3 ya que en este trabajo comparamos directamente

los efectos del estrés sobre el rendimiento de memoria en dos grupos diferentes de edad con similar número de participantes de ambos sexos

Objetivo general 2. Determinar el impacto del estrés agudo aplicado antes del recuerdo sobre el rendimiento en la memoria de material positivo, negativo y neutro en adultos jóvenes.

- *Objetivo específico 2.1*: Comparar los efectos del estrés sobre el recuerdo entre adultos mayores y jóvenes.

- *Objetivo específico 2.2*: Investigar el papel del sexo en el objetivo específico anterior.

El hecho de que no existan estudios previos que hayan investigado directamente los efectos del estrés sobre el recuerdo comparando adultos mayores y jóvenes nos hace muy difícil proponer una hipótesis sobre este objetivo general. Sin embargo, teniendo en cuenta los hallazgos de estudios previos con un diseño similar que investigan estos grupos de edad por separado, nosotros esperamos un efecto negativo sólo en el grupo de jóvenes y no en el grupo de mayores. Además, este efecto será más fuerte para el material emocional que para el neutro (Wolf et al., 2004). Finalmente, aparecerán diferencias de sexo dado que la magnitud de la respuesta de estrés depende del nivel de hormonas sexuales.

El propósito del estudio 3 es contestar estos objetivos.

Objetivo general 3. Examinar la relación entre la actividad diurna del eje hipotálamo-hipofiso-adrenal (eje HHA) (situación basal, no estrés) y el rendimiento en memoria en adultos mayores sanos.

- *Objetivo específico 3.1*: Estudiar la asociación entre el CAR y el rendimiento en memoria en adultos mayores sanos.

- *Objetivo específico 3.2*: Explorar la relación entre el DCS y el rendimiento en memoria en adultos mayores sanos.

- *Objetivo específico 3.3*: Investigar el papel que juega el sexo en estos objetivos.

Esperamos que el funcionamiento diurno del eje HHA estará relacionado con el rendimiento en memoria de personas mayores, pero esta relación será diferente dependiendo del componente del ciclo diurno de cortisol estudiado. Además, aunque no han sido reportadas diferencias de sexo en los perfiles de cortisol diurno, es posible encontrar diferencias de sexo en la relación entre el ciclo diurno de cortisol y el rendimiento en memoria dado el papel crucial que este factor juega en la relación entre la actividad del eje HHA y la función cognitiva en personas mayores (Seeman et al., 1997; Almela et al., 2011a).

Estos objetivos serán abordados en el estudio 4.

8.2. METODOLOGÍA

Para proporcionar una visión global de la metodología utilizada en los cuatro estudios empíricos presentados, en esta sección se realiza un breve resumen sobre los sujetos que participaron, el procedimiento utilizado así como las variables estudiadas.

8.2.1. Participantes

Con el fin de asegurar la homogeneidad de la muestra y la comparación entre los participantes mayores y jóvenes, la muestra utilizada en la presente tesis está compuesta por adultos sanos que son cognitivamente activos. Los participantes eran estudiantes de un programa de estudio de la Universitat de València para personas mayores de 50 años (NAU GRAN) en el caso de los participantes mayores o estudiantes universitarios de diferentes áreas de conocimientos en el caso de los participantes mayores. Dependiendo del objetivo de cada estudio, nosotros estudiamos sólo al grupo joven (estudio 1) o al grupo mayor (estudio 4) o ambos grupos de edad (estudios 2 y 3). El rango de edad fue similar dentro de cada grupo de edad en todos los estudios aunque diferían ligeramente. En el grupo de mayores el rango general de edad fue desde 54 hasta 76 años (estudio 2: 54-72, estudio 3: 56-76 y estudio 4: 57-76). En el grupo de jóvenes el rango general de edad fue desde 18 hasta 35 años (estudios 1 y 2: 18-35, estudio 3: 18-27).

Además, en todos los estudios el factor sexo se tuvo en cuenta. Así, las muestras estuvieron compuestas por un número similar de hombres y mujeres de cada grupo de edad, excepto en el estudio 1 que el objetivo fue investigar, además del sexo, el uso de anticonceptivos.

8.2.2. Procedimiento

El procedimiento llevado a cabo en esta tesis fue diferente dependiendo del objetivo de cada estudio. Hay tres procedimientos diferentes.

En los estudios 1 y 2 se utilizó un diseño intra-sujetos con dos condiciones (estrés y control) aleatorias y contrabalanceadas en dos sesiones separadas (menos de 10 días). Las sesiones consistieron en las mismas fases con igual tiempo de duración, empezaron a la misma hora y las muestras de saliva fueron

tomadas en los mismos tiempos dentro de la fase experimental. Ambas condiciones difirieron sólo en la tarea a realizar (tarea estrés o control). Mientras que en la condición de estrés a los participantes se les pidió realizar una tarea estresante (TSST, esta tarea será explicada con más detalle en la sección siguiente), en la condición control los participantes tuvieron que realizar una tarea control (tarea con similar carga de trabajo mental y actividad física global pero sin las amenaza de evaluación e incontrolabilidad). Además, en ambas condiciones, después de las tareas estrés o control, los participantes realizaron las pruebas de memoria objeto de estudio (estudio 1: tareas de memoria no declarativa y declarativa, estudio 2: tarea de memoria declarativa). Por lo tanto, el estresor fue aplicado antes del aprendizaje.

El procedimiento del estudio 3 consistió en dos sesiones consecutivas diferentes. En la primera sesión (sesión de adquisición), a los participantes se les presentó el material a recordar (imágenes). Esta sesión fue igual para todos los participantes. En la segunda sesión (sesión recuerdo), los participantes fueron asignados de forma aleatoria a la condición estrés o control. Las tareas de estas dos condiciones fueron similares a las tareas de estrés o control explicadas anteriormente. Cabe destacar una diferencia importante entre este diseño y el diseño utilizado en los estudios 1 y 2, esta es que aquí, en el estudio 3, el estresor fue aplicado antes del recuerdo.

Finalmente, el procedimiento utilizado en el estudio 4 fue muy diferente a los anteriores ya que nuestro objetivo fue investigar la relación entre el funcionamiento en condiciones basales (no estrés) del eje HHA y el rendimiento en memoria en personas mayores sanas. Para ello, analizamos dos componentes del ciclo diurno de cortisol: la respuesta de cortisol matutina y la pendiente diurna del cortisol. Así, los participantes proporcionaron siete muestras de saliva de dos días entre semana consecutivos recogidas en su casa. Además, realizaron en el laboratorio una sesión neuropsicológica con diferentes pruebas de memoria para evaluar el rendimiento cognitivo.

8.2.3. Variables estudiadas

A continuación, se presentan las diferentes variables estudiadas en los estudios empíricos.

8.2.3.1. Tarea de estrés

Para provocar estrés, utilizamos un estresor psicosocial agudo de laboratorio, el Trier Social Stress Test (TSST; Kirschbaum et al., 1993). Es una herramienta ampliamente utilizada por los investigadores en el campo de la psiconeuroendocrinología ya que es capaz de provocar una respuesta de estrés similar a aquella que ocurre en una situación real. Después de una fase de introducción, donde a los participantes se les dio las instrucciones del TSST y una fase de preparación, donde tuvieron tiempo para preparar la tarea (discurso libre, se explica a continuación), los sujetos realizaron dos tareas: (i) discurso libre: los participantes tuvieron que realizar un discurso libre con el fin de convencer a un comité de que ellos eran el mejor candidato para una posición interesante para ellos, y (ii) tarea aritmética: los participantes tuvieron que realizar una tarea mental aritmética (restar). Ambas tareas tuvieron una duración de 5 minutos y fueron realizadas frente a un comité de supuestos experto. De acuerdo con Dickerson y Kemeny (2009), la efectividad del TSST está explicada por sus características de tarea que escapa al control de la situación por parte del sujeto y la evaluación social.

8.2.3.2.Variables antropométricas, demográficas y psicológica

Para asegurar la homogeneidad de las muestras estudiadas y las comparaciones entre ellas, y evitar todos los posibles factores confundentes que podrían interferir tanto con la respuesta de estrés como con el rendimiento en

memoria, medimos en todos los estudios de esta tesis las mismas variables demográficas y antropométricas, a continuación:

- *Índice de masa corporal (IMC)*. Una vez que los participantes llegaban al laboratorio fueron pesados y medidos para luego, poder calcular el IMC (Kg/m^2). Este índice ha sido asociado con el funcionamiento del eje HHA (Dettenborn et al., 2012) y el rendimiento cognitivo (Cournout et al., 2006).

- *Estatus socioeconómico subjetivo (ESS)*. El ESS fue evaluado usando la escala de MacArthur de Estatus Social Subjetivo (Adler et al., 2000). Los participantes tuvieron que valorar su ESS en comparación con otros españoles. La escala va desde 1 (personas que tienen un bajo ESS ya que tienen menos dinero, educación y trabajo respetable o incluso no tienen trabajo) hasta 10 (personas que tienen un alto ESS ya que tienen mayor cantidad de dinero, educación y trabajo respetable). Esta variable ha sido relacionada con la actividad del eje HHA (Cohen et al., 2006).

- *Nivel educativo (NE)*. El NE fue determinado preguntando a los participantes qué nivel educativo tenían completado. Éstos tuvieron que contestar una de las diferentes opciones: 0=no estudios, 1=primaria, 2=educación secundaria, 3= universidad y educación superior y, 4=estudios postgraduado (máster o doctorado).

En el estudio 3 también incluimos una variable psicológica para evaluar la respuesta de estrés a nivel psicológico.

- *Evaluación de la situación*. Medimos cómo los participantes percibieron las tareas estrés (TSST) o control utilizando 5 preguntas sobre aspectos concretos de la tarea. Concretamente, las preguntas estuvieron relacionadas con el grado de estrés, dificultad, frustración, esfuerzo y

motivación que la tarea había supuesto a los participantes. Estas preguntas fueron previamente creadas por nuestro grupo (Gonzalez-Bono, 2002) a partir de la evidencia previa sobre este tema (Baggett et al., 1996).

8.2.3.3.Variables enzimática y hormonal

Para estudiar el funcionamiento del Sistema Nervioso Simpático (SNS) y del eje HHA, analizamos los niveles salivares de alfa-amilasa y cortisol, pero, dependiendo del objetivo que queríamos abordar, éstos fueron analizados en situaciones diferentes: en respuesta al estresor/control y/o en situación basal. Es importante destacar que estos biomarcadores fueron obtenidos a partir de muestras de saliva, ya que es una técnica no invasiva de obtención y acceso fácil. Además, medir los niveles de cortisol en saliva nos permite determinar sólo la fracción libre de la hormona que está biológicamente activa (Foley y Kirschbaum, 2010).

- *Alfa-amilasa salivar (AAs)*. Medimos la actividad del SNS a través de los niveles de AAs. Para ello, los participantes proporcionaron (en salivettes, Sarstedt, Nümbrecht, Alemania) varias muestras de saliva en diferentes momentos, de acuerdo con el procedimiento de cada estudio. Durante un minuto, los participantes tuvieron que mantener el algodón en su boca, moviéndolo de forma circular para recoger saliva de todas las glándulas salivares (Rohleder y Nater, 2009). Después de que las muestras fueron congeladas, los niveles de AAs se obtuvieron mediante un método cinético enzimático. Concretamente, el AAs fue estudiado en respuesta a la tarea de estrés o control en los estudios 1, 2 y 3.

- *Cortisol salivar*. La actividad del eje HHA fue medida a través de los niveles de cortisol en saliva. De acuerdo con el objetivo de cada estudio, las muestras de saliva fueron proporcionadas por los participantes

usando salivettes o depositando 5 ml de saliva en viales de plástico durante no más de 5 minutos. Las concentraciones de cortisol fueron analizadas por radioinmunoensayo (RIA). Este biomarcador fue estudiado en diferentes situaciones: en respuesta al estrés o control en los estudios 1, 2 y 3 y en situación basal en el estudio 4. En este estudio se recogieron diferentes muestras de cortisol a lo largo de 2 días consecutivos. De esta forma, recogimos el ciclo diurno de cortisol de los participantes. También, en este mismo estudio, los niveles de cortisol antes y después de la evaluación neuropsicológica fueron recogidos.

8.2.3.4. Variables cognitivas

El proceso cognitivo investigado en esta tesis ha sido el rendimiento en memoria, y, en más profundidad, el rendimiento en memoria declarativa. Sin embargo, otros tipos de memoria también han sido considerados.

- *Memoria no declarativa*. La memoria implícita o no declarativa representa el efecto de la experiencia previa inconsciente en el posterior comportamiento (Graf et al., 1984) e incluye al condicionamiento clásico, el aprendizaje no asociativo, la adquisición de habilidades motoras, perceptivas y cognitivas así como el efecto priming (Daum y Ackerman, 1997). En la presente tesis, estudiamos este tipo de memoria a través del estudio 1. El efecto priming se refiere al cambio en la velocidad, sesgo o precisión del procesamiento del estímulo, seguido de la experiencia previa con el mismo estímulo u otro relacionado (Henson, 2003). Para evaluarlo, usamos una tarea de completar raíces de palabras.

- *Memoria declarativa*. De forma diferente a la memoria no declarativa, la memoria explícita o declarativa requiere el recuerdo consciente de experiencias previas (Milner et al., 1998) e incluye la memoria semántica

y episódica. En este tipo de memoria podemos distinguir tres fases: adquisición, consolidación y recuerdo. En esta tesis, este tipo de memoria ha sido tratada en los 4 estudios. Para evaluarla, hemos utilizado listas de palabras (estudios 1 y 2), imágenes (estudio 3), y textos, listas de parejas de palabras, imágenes y caras (estudio 4).

- *Memoria de trabajo*. Tipo de memoria que nos permite almacenar y manipular una cantidad limitada de información a corto plazo. En nuestra investigación, se ha abordado en el estudio 4 a través de tres tipos diferentes de tareas: tarea de letras y dígitos, tarea sólo de dígitos y tarea espacial.

8.2.3.5. Variables moderadoras

Como hemos resumido en este capítulo de introducción, la dirección de los efectos del estrés sobre el rendimiento en memoria depende de varios factores, unos relacionados con las características del individuo (edad y sexo) y otros relacionados con aspectos de la tarea de memoria (el arousal y la valencia del material a recordar), entre otros. De este modo, consideramos estos factores en nuestra investigación.

- *Sexo*. Este factor ha sido considerado en los 4 estudios. Así, comparamos sistemáticamente hombres y mujeres. Dado que el papel moderador del sexo podría estar explicado por los diferentes niveles de hormonas sexuales, en el estudio 1, la fase del ciclo menstrual y la toma de anticonceptivos fueron tenidos en cuenta. Además, todas las mujeres mayores eran post-menopáusicas y ninguna de ellas estaba recibiendo terapia hormonal sustitutiva (estudios 2, 3 y 4).

- *Edad*. Con respecto a este factor, comparamos si además de hombres y mujeres, los efectos del estrés sobre el rendimiento en memoria entre

mayores y jóvenes (estudios 2 y 3). Sólo adultos jóvenes o mayores fueron incluidos en los estudios 1 y 4, respectivamente. El rango de edad fue en mayores (54-76 años) y en jóvenes (18-35 años).

- *Tipo de material a recordar*. Atendimos a este factor, investigando sólo material neutro en los estudios 1, 2 y 4. En el estudio 3, los participantes tuvieron que aprender material neutro y emocional (positivo y negativo).

8.3. CONCLUSIONES

Los resultados encontrados en los estudios empíricos señalan que en una situación de estrés agudo, la activación del SNS y el eje HHA afecta al rendimiento de la memoria. Ésta es una relación compleja dado que existen diferentes factores que pueden modular la dirección de estos efectos. Además, el ciclo diurno del cortisol está también asociado al rendimiento en memoria de las personas mayores sanas. Una discusión más específica y detallada de los resultados ha sido presentada en los diferentes capítulos empíricos. En este apartado, las conclusiones más importantes a partir de los objetivos de esta tesis son descritos a continuación:

- Un estresor psicosocial agudo aplicado antes del aprendizaje de una lista de palabras neutras mejora el rendimiento de la memoria no declarativa en jóvenes sanos, mientras que no afecta al rendimiento de la memoria declarativa. Estos hallazgos son consistentes con la hipótesis de que el estrés lleva a un cambio entre los sistemas de memoria, favoreciendo las estrategias de aprendizaje menos demandantes cognitivamente sobre aquellas que requieren conciencia (Schwabe et al., 2007). Además, esta mejora está relacionada de forma positiva con la actividad del SNS. Este resultado, no reportado previamente, refleja la importancia de la activación de este sistema en los efectos positivos del estrés sobre la memoria no declarativa.

- Una comparación directa de personas de diferente edad pero con otras características similares ha mostrado que el envejecimiento está asociado con peor rendimiento en memoria declarativa mientras que el reconocimiento permanece invariable. Sin embargo, no hay diferencias relacionadas con la edad en los efectos del estrés sobre la memoria declarativa, excepto en un aspecto de este tipo de memoria. El estresor aplicado antes del aprendizaje deteriora le recuerdo inmediato, aspecto de memoria implicado en la interferencia retroactiva. Este resultado está en línea con el hecho de que las personas mayores parecen ser más vulnerables a esta interferencia que los más jóvenes (Hedden y Park, 2001). Además, este efecto negativo del estresor sobre la memoria parece estar mediado por la respuesta de ambos sistemas fisiológicos implicados en la respuesta de estrés.

- A pesar de las diferencias relacionadas con el sexo encontradas en la respuesta de cortisol al estresor, el sexo de los participantes no parece ser crucial en los efectos del estrés aplicado antes del aprendizaje sobre la memoria no declarativa (jóvenes) y la memoria declarativa (mayores y jóvenes).

- Un estresor psicosocial agudo aplicado antes de la fase de recuerdo deteriora el recuerdo libre y el reconocimiento de imágenes emocionales y neutras de forma diferente dependiendo de la edad y el sexo. Así, el estrés deteriora tanto el recuerdo libre de imágenes positivas, negativas y neutras, sólo en los hombres jóvenes, así como el reconocimiento de imágenes positivas en personas mayores y jóvenes de ambos sexos. Sólo la respuesta de cortisol está relacionada negativamente con el efecto encontrado en el recuerdo libre.

- En general, el envejecimiento con éxito puede amortiguar los efectos de un estresor psicosocial agudo sobre el rendimiento en memoria en personas mayores, dado que sólo la interferencia retroactiva se vio afectada por el estrés.

- Existe una relación diferente entre los biomarcadores del estrés y los diferentes sistemas de memoria dependiendo de la edad. Así, entre los adultos jóvenes, sólo la respuesta de AAs está relacionada con los efectos de mejora del estrés sobre el priming y sólo la respuesta de cortisol está relacionada con los efectos perjudiciales del estrés sobre el recuerdo libre. Sin embargo, entre los adultos mayores, la ratio Aas/cortisol está relacionada con los efectos perjudiciales del estrés sobre la interferencia retroactiva.

- El funcionamiento diario del eje HHA está también relacionado con el rendimiento en memoria en personas mayores sanas. En ambos sexos, el CAR está negativamente relacionado con el rendimiento en la memoria declarativa, la cual es más dependiente del funcionamiento del hipocampo, pero no con la memoria de trabajo, la cual es más dependiente del funcionamiento de la corteza prefrontal. Sin embargo, la magnitud del DCS no está relacionada ni con el rendimiento en memoria declarativa y memoria de trabajo de personas mayores sanas.

- La fiabilidad de la medida del CAR es un tema que debe ser considerado. La asociación entre el CAR y el rendimiento en memoria es diferente dependiendo de la adherencia al protocolo de las muestras de saliva. Para la muestra completa, esta relación es, principalmente, curvilínea (patrón de U-invertida), pero sólo para aquellos participantes sospechosos de ser adherentes al protocolo, la asociación es lineal y negativa.

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