The cortisol awakening response: neuropsychological correlates and implications for function in healthy humans

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Abstract

In healthy individuals, cortisol secretory activity shows a marked circadian rhythm. This rhythm is characterised by a declining pattern across the day, reaching a nadir in the late evening and early part of sleep, before gradually increasing during late sleep to reach peak levels upon subsequent morning awakening. The focus of the present thesis is a distinct aspect of this circadian rhythm: the rapid increase in cortisol concentrations to reach the diurnal acrophase within the first 45 minutes after awakening, known as the cortisol awakening response (CAR).

The CAR is a much studied but poorly understood aspect of the circadian pattern of cortisol secretion. Despite being considered a biomarker of health status in psychophysiological and neuroendocrine research, its precise function within the healthy cortisol circadian rhythm has yet to be elucidated, and results from CAR studies in clinical populations have often been inconsistent. The focus of this programme of research was to seek a role for the CAR in healthy functioning young adults, and to address methodological issues that may have contributed to the inconsistent and contradictory findings from earlier studies.

The data presented here from healthy young adult participants demonstrate for the first time that the CAR predicts better performance on an index of executive function (EF) in the same day. This is first demonstrated in a detailed case study of a healthy young adult male, showing that state variation in the CAR predicts EF at 45-min post-awakening, and then in a sample of healthy young adults, demonstrating that CAR predicts EF in the afternoon of the same day. In a

further study of healthy young adults, the magnitude of the CAR was found to positively predict the capacity to induce synaptic plasticity in the motor cortex on that same day by Transcranial Magnetic Stimulation (TMS). This study demonstrates, for the first time, that the daily magnitude of the CAR may be responsible for some daily variation in synaptic plasticity, presenting a possible mechanism by which the CAR might influence cognitive function.

The unique contribution of this thesis lies in its progression from previous studies of elderly and clinical populations by demonstrating a possible role for the CAR in healthy young adults, as well as the detailed examination of state variation in the CAR and the use of strict methodologies to ensure accuracy of measurement. This has the potential to lead toward a clearer understanding of the role of the CAR within the healthy cortisol circadian cycle, and therefore the consequences of dysregulation in a range of conditions. The findings are discussed with regard to their contribution to understanding the relationship between the CAR and cognitive function, and the implications for methodology and interpretation of the broad range of studies using the CAR as a trait biomarker.

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Peer-reviewed Publications:

Law, R., Evans, P., Thorn, L., Hucklebridge, F., Clow, A. (2015). The cortisol awakening response predicts same morning executive function: Results from a 50-day case study. *Stress, 18*(6), 616-621.

Clow, A., **Law, R.**, Evans, P., Vallence, A.M., Hodyl, N.A., Goldsworthy, M.R., Rothwell, J.R., Ridding, M.C. (2014). Day differences in the cortisol awakening response predict day differences in synaptic plasticity in the brain. *Stress, 17*(3), 219-223.

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Presentations:

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50-day case study. *International Society of Psychoneuroendocrinology (ISPNE) Annual conference*, Edinburgh, UK, September.

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Law, R., Zacharia, H., Mandzukic-Kanlic, L. (2015). Use of CANTAB cognitive assessment for research, learning and teaching. *Association of Technical Staff in Psychology (ATSiP) Annual conference*, Stoke-on-Trent, June.

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Law, R., Hucklebridge, F., Thorn, L., & Clow, A. (2014). Day differences in the cortisol awakening response predict day differences in motor cortex plasticity. *University of Westminster Postgraduate Fair*, London, April.

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Law, R. (2013). Intra-Individual variation in the cortisol awakening response and motor cortex plasticity. *University of Westminster Department of Psychology PhD Students' mini-conference*, London, February.

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Author’s Declaration

I declare that all the material contained in this thesis is my own work. Assistance with the data collection process of study II was provided by colleagues from the Robinson Institute, University of Adelaide, Australia.

List of Abbreviations

ACTH	Adrenocorticotrophic hormone
AST	Attention switching task
AUCg	Area under the curve with respect to ground
AUCi	Area under the curve with respect to increase
CAR	Cortisol awakeing response
COSHH	Control of substances hazardous to health
CRH	Corticotrophin-releasing hormone
CSF	Cerebrospinal fluid
ELISA	Enzyme linked immunosorbent assay
FC	Frontal Cortex
GR	Glucocorticoid receptors
HC	Hippocampus
HPA	Hypothalamic-pituitary-adrenal
MnInc	Mean increase
MR	Mineralocorticoid receptors
OTS	One-touch stockings
PVN	Paraventricular nuclei
RPS	Research participation scheme

S1	Sample 1
SAM	Sympathetic adrenomedullary
SCN	Suprachiasmatic nucleus
SD	Standard deviation
TMS	Transcranial magnetic stimulation
TMT	Trail making task

1 Introduction I

Cortisol, the Cortisol Awakening Response, and Cognition

1.1 Overview

This chapter examines the nature of psychosocial stress, the function of the stress response system and the neuroendocrine systems involved in mediating a stress response. This is followed by examination of a major stress response system, the hypothalamic-pituitary-adrenal (HPA) axis, activation of which stimulates release of the glucocorticoid hormone cortisol. Biosynthesis of cortisol in the adrenal cortex is described, along with discussion of the actions of cortisol throughout the body. Cortisol secretion follows a pronounced circadian rhythm, the acrophase of which is the cortisol awakening response (CAR). This neuroendocrine response to awakening is the primary focus of this thesis. Amongst the various roles that have been proposed for the CAR is an influence upon cognitive performance. This association between awakening, neuroendocrine activation, and cognitive processes forms the major rationale of the present work. Circadian rhythms in cognitive performance are therefore described, followed by discussion of the associations between cortisol and cognition.

There is evidence that disruption in the circadian rhythm of cortisol is associated with psychosocial state and trait variables and implicated in ill health. It is thought that cortisol may be one modulator in the relationship between psychological variables and somatic illness, and also with mental health (e.g. McEwen, 2008). The cortisol awakening response (CAR) (first identified by Pruessner et al., 1997) is a distinct component of the circadian rhythm, and subject to both inter- and intra-individual variation. The CAR is frequently used as a biomarker of health status in psychophysiological and neuroendocrine research (see Clow et al., 2010b) but despite this its precise function within the healthy cortisol circadian rhythm has yet to be elucidated. Within this chapter, the associations between the CAR and cognitive/neurological function will be described, with the consideration that such associations may provide insight into the function of the CAR in healthy humans.

1.2 Psychoneuroendocrinology

The studies within this thesis are concerned with psychoneuroendocrinology, an interdisciplinary research area incorporating the interrelated disciplines psychology, neurobiology, endocrinology, immunology, neurology, psychiatry and medicine. The general aim of this research area is to integrate these disciplines to provide evidence of links between the mind/brain and health. Considerable progress has been made in this area in recent years, and it is now well-established that psychosocial factors such as stress and depression are linked with adverse health outcomes and increased mortality risk (Juster,

McEwen, & Lupien, 2010). Self-reported stress has been shown to predict a reduced rate of wound healing in healthy adult males (Ebrecht et al., 2004), and chronic stress exposure has been shown to predict increased susceptibility to disease, inflammation, and both susceptibility to and intensity of cold symptoms (Cohen et al., 2012). Chronic stress is also the most commonly implicated mechanism by which adverse work environments contribute to disease progression (Taylor, Repetti, & Seeman, 1997). Perhaps the best known examples of research on stress in the workplace and longer-term health outcomes are the Whitehall studies (Kuper & Marmot, 2003; Marmot et al., 1991), which have provided strong support for the association between psychosocial factors and disease progression. These longitudinal studies explored the effects of social disadvantage, health behaviours and psychosocial factors on longer term health outcomes in a large, cross-sectional sample of British civil servants. The results of these studies have consistently shown that factors such as increased job strain, perceived effort-reward imbalance and injustice in the workplace all independently predict later occurrence of heart disease. A recent meta-analysis of the stress-cardiovascular function research has indicated that increased cardiovascular morbidity in adulthood is predicted by exposure to early-life stressors, such as childhood abuse and early socioeconomic adversity, and also independently predicted by experience of social isolation during adulthood (Steptoe, A., & Kivimäki, M.,2013). Depressive symptoms are also known to be a risk factor for incidence of heart disease, and also significantly increase the risk of mortality in heart disease patients (Barth, Schumacher, & Herrmann-Lingen, 2004; Suls & Bunde, 2005). In addition, a growing body of evidence suggests that psychosocial factors have potentially powerful modulating effects on cancer progression (Reiche, Nunes, & Morimoto,

2004; Sephton & Spiegel, 2003), including emerging evidence that chronic stress may enhance tumor growth (Thaker, Lutgendorf, & Sood, 2007). Evidence such as that presented here implicates psychoneuroendocrinology as having potentially great contribution to our understanding and treatment of human health disorders.

Within the peripheral nervous system, the body's physiological response to stress is mediated by the neuroendocrine system, that is, the interaction between the endocrine and nervous system. Neuroendocrine signalling facilitates communication between nerve cells and organs, allowing a coordination of the homeostatic response. The endocrine system entails secretion of biologically active substances (hormones) into the circulating blood, which interact with receptors on target cells to influence cellular function. Both the pituitary and adrenal glands are responsive to neural stimulation, and the primary neuroendocrine interface is that of the hypothalamus of the brain and the anterior portion of the pituitary gland. The distinction between the nervous system and endocrine system is complicated, as the same or related ligands (substances which can bind to target cell receptors) can act as neurotransmitters or hormones depending upon whether they are acting within the nervous system or in the general circulation. However, ligands released into the circulation are generally considered hormones while ligands released from nerve terminals to act across synaptic clefts are classified as neurotransmitters.

Appraisal of a stimulus as stressful results in the activation of the two primary neuroendocrine systems: the sympathetic adrenomedullary (SAM) system and the HPA axis. Both of these systems are activated by neurosecretory cells in

the hypothalamus, though they serve separate roles in the homeostatic response to stress. Activation of the SAM system is responsible for the fight-or-flight response, which occurs within seconds of stress appraisal, and results in the activation of the adrenal medulla to secrete the catecholamines epinephrine and norepinephrine into the bloodstream. These catecholamines encourage increased blood pressure, heart rate, muscle contraction, respiration rate, glycogenolysis, and sweat release from eccrine glands. Separately, activation of the HPA-axis results in cortisol secretion approximately 20-30 minutes after the onset of the stressor, with most of the physiological actions of cortisol not manifesting until approximately 1 hour post-stress appraisal (Dickerson & Kemeny, 2004; Kudielka & Kirschbaum, 2005; Sapolsky, Romero, & Munck, 2000). HPA activation is therefore a slower response, and serves to assist the organism in responding to sustained challenge or threat. The HPA-axis is the focus of the present thesis; the system is described in detail in section 1.3, and the end-product, cortisol, is considered in detail in section 1.4.

1.3 The Hypothalamic-pituitary-adrenal axis

Cortisol secretion is regulated by the hypothalamic-pituitary-adrenal (HPA) axis, a neuroendocrine system consisting of the hypothalamus, the anterior part of the pituitary gland, and the adrenal cortex. Psychological stress is considered to be the most prominent trigger for activation of the HPA-axis in humans, though

other causes include awakening, pain, smoking, immune system activation, food consumption, vigorous exercise, marked changes in cardiovascular tone, and respiratory distress. The HPA-axis regulates cortisol secretion by way of a physiological cascade. This cascade begins with stimulation of the parvocellular neurons in the paraventricular nuclei (PVN) of the hypothalamus to produce corticotrophin-releasing hormone (CRH), which is transported to the anterior pituitary in the hypophyseal portal blood supply. Adrenocorticotropic hormone (ACTH) is then synthesised and released from corticotropic cells in the anterior pituitary for general circulation in the blood. Circulating ACTH subsequently reaches the adrenal glands, located above the kidneys. ACTH acts as a secretagogue for glucocorticoids, which are consequently produced by the adrenal cortex. Cortisol is the primary glucocorticoid in humans, and is produced predominantly by the zona fasciculata (located directly below the zona glomerulosa), but also to a lesser extent by the zona reticularis (the innermost layer of the cortex).

HPA-axis activation is also regulated by negative feedback inhibition which ensures that in healthy functioning humans, glucocorticoid secretion does not reach excessive levels. As illustrated in Figure 1.1, circulating cortisol is detected by cortisol receptors located throughout several brain regions including the hippocampus and hypothalamus, and also in the pituitary gland itself. This detection results in down-regulation of CRH and ACTH and in turn, decreased HPA-axis activation, resulting in reduced cortisol production. In healthy functioning humans, this negative feedback cycle maintains cortisol levels within an appropriate range, enabling the individual to adapt to and recover from stress (Huizenga et al., 1998).

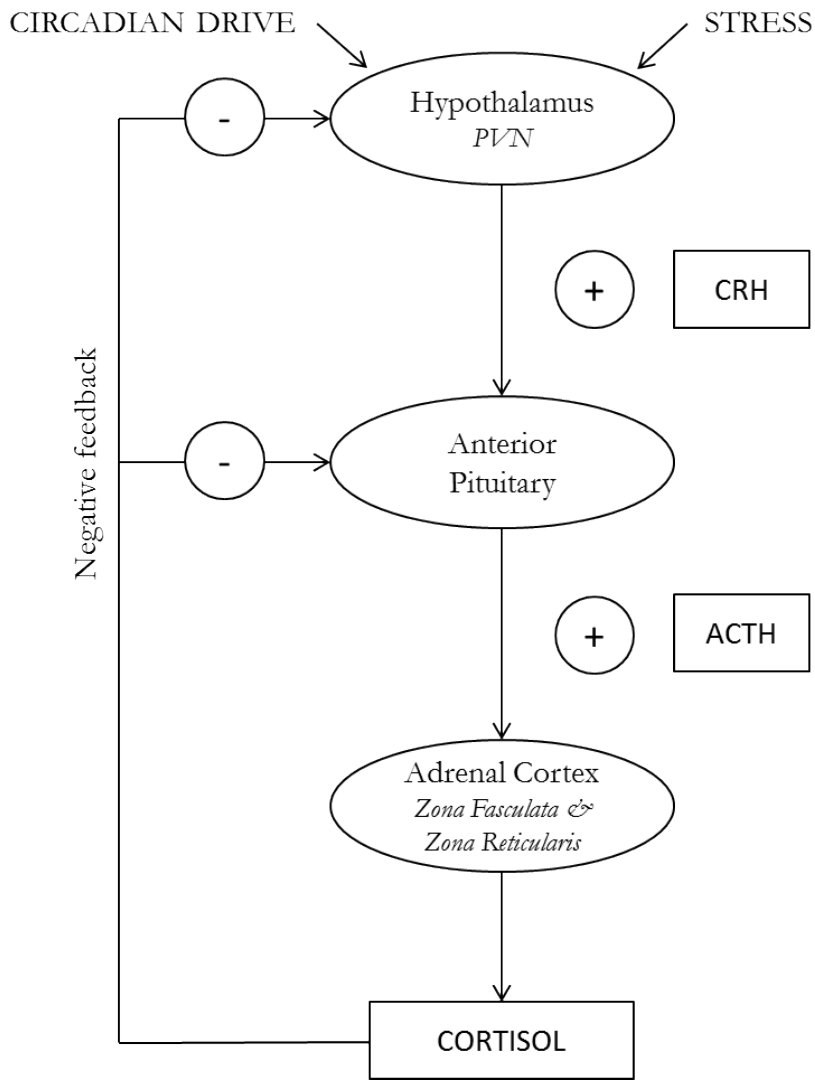


Figure 1.1 The physiological cascade involved in the hypothalamic-pituitary-adrenal (HPA) Axis in humans. Corticotrophin-releasing hormone (CRH) is transported to the anterior pituitary via the hypothalamic-pituitary portal blood supply. Adrenocorticotrophic hormone (ACTH) is released into the general circulation. The '+' and '-' symbols indicate activation and inhibition respectively; cortisol exerts negative feedback as indicated to regulate the on-going cascade

1.4 Actions of cortisol

While prominently known for its role within the context of the stress response, cortisol also plays an essential role in maintaining normal physiological function and is one of the few hormones that are crucial for life (Baxter, Frohman, & Felig, 1995). Cortisol can passively diffuse into tissues and cells, and virtually all of the body's cells act as potential targets. This allows cortisol to have a broad and diverse range of physiological effects throughout the body. It has a regulatory effect for normal functioning but can also have deleterious effects in cases of exposure to irregularly high or low circulating quantities. A primary role of cortisol is in energy mobilization, through the maintenance and regulation of blood glucose. Cortisol increases circulating glucose concentrations through several mechanisms: permitting the hyperglycaemic actions of glucagon and epinephrine, inhibiting glucose uptake in peripheral tissues, and stimulating gluconeogenesis in the liver. This process is physiologically beneficial for responding to a host of challenges when cortisol levels are within a healthy range, but can have the adverse effect of draining protein stores when cortisol concentrations are excessively high (White, 2009).

Beyond its role in glucose-regulation, cortisol has many other physiological actions within the body that are both complex and diverse in nature. Some of the principle functions of cortisol are the regulation of blood pressure, cardiovascular function, carbohydrate metabolism, body-fat distribution, serum calcium concentrations, and immune function. Cortisol also infleunces appetite and caloric intake. Both chronic hypercortisolism and chronic hypocortisolism can have deleterious effects on physical and psychological health. Such extreme cortisol secretory profiles are observed in Cushing's disease and Addison's

disease respectively, and such disorders therefore offer some insight into the harmful effects of cortisol secretion in either extreme. Hypercortisolism leads to obesity typified by of abnormally high distribution of fat in the abdomen and wasting in the arms and legs, and can lead to carbohydrate intolerance, hyperglycaemia and insulin resistance. Whereas hypocortisolism leads to gastrointestinal symptoms such as nausea, vomiting, diarrhoea, and abdominal pain, in addition to weight loss, metabolic symptoms including hypoglycaemia and increased insulin sensitivity and cardiovascular symptoms including hypotension.

Cortisol also influences neural activity, mood and behaviour. Again, cases of extreme high or low secretion offer some insight into the actions of cortisol with regard to psychological state. In the case of chronic hypercortisolism, this can lead to labile affect and depression. While hypocortisolism has similarly deleterious consequences, in the form of apathy, irritability, and depression. Both extremes have been shown to be associated with psychosis, with risk for this in Cushing's and Addison 's disease sufferers alike (Porterfield, 2001).

The relationship between cortisol secretion and cognition too reflects the many and varied actions of cortisol within the body. This relationship is also somewhat complex, as the effects of cortisol can either result in enhancement or impairment of cognitive performance, depending on the quantity of cortisol secreted (Kirschbaum et al., 1996; Domes et al., 2005; Schilling et al., 2003; Yehuda et al., 2007). The differing extent of the influence of cortisol on different functions is in part a reflection of the number, and type, of cortisol receptors found in these different regions. Two separate intracellular receptors are

expressed within the body that bind with cortisol: Glucocorticoid receptors (GR) and mineralocorticoid receptors (MR). GR are found in most tissues, wheras MR are found in the kidney, colon, heart, sweat glands and the brain (Joels et al., 2008). The MR shows a similar intrinsic affinity for aldosterone, cortisol and corticosterone, while the GR has reduced intrinsic affinity for glucocorticoids in comparison (Gomez-Sanchez, 2010). Within the cell, MR and GR activation interacts in either a complementary or opposing fashion depending on the cell type, and this interaction is crucial in determining cell function (Gomez-Sanchez, 2014). The hippocampus, pre-frontal cortex, and amygdala all show high levels of both MR and GR receptors compared to other brain regions (De Kloet et al., 2000; Herbert et al., 2006). The effects of cortisol on the functioning of these regions is determined by the balance of the MR binding relative to GR binding. For example, basal levels of cortisol binding to MR receptors in the hippocampus (HC) in the temporal lobe enhance excitability, and elevated levels of cortisol binding to the GR during stress responding have the opposing effect, manifesting in a U-shaped relationship (De Kloet, Oitzl, & Joëls, 1999; Herbert et al., 2006). Longer-term regulation of brain structure and function by prolonged exposure to cortisol is also achieved by alteration of the density of neurons through apoptosis and inhibited neurogenesis (Pittenger and Duman, 2008), and by influencing dendritic complexity (McEwen, 2008). Glucocorticoids play an important role in mediating the remodelling of HC neurons during repeated stress, likely due to the regulatory effects on glutamate (McEwen, 1999), as glutamate is important for differentiation, migration and survival of neurons in the developing brain, and is thought to have an influence on neurodegenerative processes (Meldrum, 2000).This might explain why elevated glucocorticoids have been shown to play a role in the remodelling of the dendrites of neurons

not just in the HC, but also in the frontal cortex (FC) and amygdala, whereby excessive glucocorticoid exposure causes simplification of dendritic structure in the HC and FC (McEwen, 2008), but dendritic growth in neurons in the amygdala (Vyas et al., 2002). In studies of animals (rats and primates) the HC, cornu ammonis (CA3) pyramidal cells (involved in memory formation/consolidation) are particularly vulnerable to the deleterious effects of prolonged and excessive glucocorticoid exposure (Sapolsky, 2000). Such actions make glucocorticoids, along with other mediators such as serotonin, GABA, and excitatory amino acids, very important modulators of brain plasticity, with implications for cognitive function (McEwen, 1999; McEwen, 2008).

1.5 Circadian rhythm of cortisol secretion

All life on earth depends on the presence of the sun, and the evolution of circadian rhythms may be traced to an early dependence on the sun as an energy source. These rhythms are seen in nearly all organisms, from simple bacteria to human beings. Having evolved to adapt to the 24-hour cyclic fluctuations of this energy source, many of the cells of organisms have developed a temporal organisation. Cellular clock mechanisms have evolved that are capable of maintaining approximate 24-hour cycles, but are sensitive to light signals for their entrainment. These rhythms have been observed in a wide range of behaviours, from processes at the level of individual cells to information processing, feeding, and mood, and serve to ensure that the

activities important for survival are temporally organised to match the optimum times within the 24-hour day (Saper, Scammell, & Lu, 2005). In humans, as a diurnal species, circadian rhythms are arranged so that alertness and physiological performance will peak during the daylight hours and sleep pressure will peak during the dark hours of the night.

In healthy individuals cortisol secretion shows a marked circadian rhythm. This rhythm is characterised by peak levels in the morning following awakening (the cortisol awakening response: CAR) and a declining pattern across the day, reaching nadir in the late evening and early part of sleep, before gradually increasing during late sleep prior to subsequent morning awakening (Weitzman, Fukushima, Nogeire et al., 1971; Linkowski, Mendlewicz, Leclercq, et al., 1985).

1.5.1 The Suprachiasmatic nucleus

Although the physiological mechanisms regulating the circadian rhythm of cortisol secretion are not yet fully understood, it is thought to be responsible for synchronising function to appropriate times of day around the sleep-wake and the light-dark cycles. The brain region which has been implicated in this process is the suprachiasmatic nucleus (SCN) of the hypothalamus. The SCN is the circadian pacemaker in humans, and is located dorsal to the optic chiasm. The SCN receives light information from the eye, though this photic input requires neither rods nor cones (the only previously known retinal photoreceptors), but instead a novel class of intrinsically photoreceptive retinal ganglion cells (Berson, Dunn, & Takao, 2002; Berson, 2007). These photosensitive cells project directly to the SCN via the retinohypathalamic tract (Berson, 2007; Herbert et

al., 2006). Light is the primary zeitgeber ('time giver') for human circadian rhythms (Aschoff, 1965), and bright light exposure stimulates a cascade of molecular events in the SCN involving clock gene expression, which facilitates the entrainment of endogenous circadian rhythms to the light-dark cycle and allows for adaptation to seasonal changes in day length (Bunney and Bunney, 2000). Animal studies have shown that if the SCN is removed, the duration of sleep and wake remain similar, but that these behaviours no longer show a regular cycle (Buijs et al., 2003). As such it is clear that the SCN does not directly control these behaviours, but simply plays a role in synchronising them with the external environment.

With regards to cortisol secretion, the SCN is connected to the PVN of the hypothalamus both directly by axonal projections from SCN neurons, and also indirectly via the SCN output pathway to the dorsomedial hypothalamus (Herbert et al., 2006). Additionally the SCN exerts influence over cortisol secretion via a direct autonomic pathway (Clow et al., 2010a), given there exists direct sympathetic innervation of the zona fasciculata of the adrenal gland (Charlton et al., 1992), and this direct input has been shown to be involved in modulation of cortisol at specific times of day (as discussed in section 1.5.2). One of the results of SCN signalling is stimulation of the PVN to release CRH, in turn activating the HPA-axis, and resulting in the secretion of cortisol. Thus, the SCN's light-responsive mediation of the HPA-axis helps to ensure cortisol secretion is appropriately synchronised such that it is stimulated during the daytime and inhibited during the night (Benarroch, 2011). The SCN itself does not express GR, and therefore acts as a cortisol output signaller only (Herbert et al., 2006).

1.5.2 The CAR

The CAR is defined as the rapid increase in cortisol concentrations within the first hour after awakening from night-time sleep (Clow et al., 2004; Pruessner et al., 1997). Cortisol concentrations typically increase between 50% and 160% during this hour, with the peak of the CAR typically occurring between 30- and 45-min post-awakening (Clow et al., 2004). The CAR is seen from early infancy to older adulthood (Fries et al., 2009; Stalder et al., 2013), and initial studies indicated that the CAR is observed in around 77% of the healthy adult population (Wüst et al., 2000b), though this figure appears far higher when appropriate study methodology is employed (e.g. Oskis et el., 2009; Smyth et al., 2016). Within the circadian rhythm of cortisol secretion the CAR is a relatively discrete aspect, it is initiated in response to morning awakening and superimposed upon the circadian rhythm (Clow et al., 2004, 2010a; Wilhelm et al., 2007). This has been demonstrated by investigation of measures of pre- and post-awakening levels of cortisol and ACTH in blood and saliva by Wilhelm et al. (2007), showing that steep increases in both hormones are seen in response to awakening and that this is distinct from baseline circadian secretion (Wilhelm et al., 2007). The CAR is also relatively independent of cortisol secretion across the rest of the day (Edwards et al., 2001a; Schmidt-Reinwald et al., 1999; Maina et al., 2009). This is thought to be because regulation of the CAR involves additional direct modulation of the adrenal cortex by the SCN (via the pathway described in section 1.5.1), which enhances adrenal sensitivity in the immediate post-awakening period (Clow et al., 2010a).

Since first identification of the CAR in humans by Pruessner et al. (1997), it has been researched extensively in the context of understanding its regulation and its implications as a biomarker of health status (Clow et al., 2004; 2010b; Fries et al., 2009). From this research it has been established that the magnitude of the CAR is associated with a range of physiological and psychological factors, at both an inter- and intra-individual level. With regard to physiological factors, it has been established that CAR magnitude shows a negative association with age (Clow et al., 2010b) and also differs between sexes (Wright & Steptoe, 2005; Wüst et al., 2000b). Notably gender differences are observed in the timing of the CAR peak, with the peak concentrations typically seen at 30-min post-awakening in males and at 45-min post-awakening in females (Oskis et al., 2009; Stalder et al., 2009). Females also show a delayed decline in cortisol post-CAR peak resulting in greater overall cortisol secretion in the post-awakening hour (Wüst et al., 2000b). Genetic factors appear to play an important role in determining interindividual differences in the CAR; Wüst et al. (2000a) reported results from a twin study revealing a heritiability index (HI) estimate of .40 for dynamic increase in cortisol levels post-awakening. With regard to psychological factors, it has been shown that greater CAR magnitude is seen both in response to negative prior-day experiences and increased anticipation of challenge in the day ahead (Adam et al., 2006; Clow et al., 2010b; Doane & Adam, 2010; Stalder et al., 2010a,b).

Beyond studies of the nature and function of the CAR, research to date has primarily focused on its application as a biomarker in association with a range of psychosocial and physical variables including health outcomes (Chida & Steptoe, 2009). Abnormal CAR profiles are seen in a range of disorders,

including cardiovascular, autoimmune, allergic, and psychiatric diseases (Wüst et al., 2000b; Clow et al., 2004; Fries et al., 2009). Results of such comparative studies in clinical populations have often been inconsistent, perhaps due to participant non-adherence to the CAR sampling protocol, differences in experimental design, and differences in sample demographics such as age, gender and genotype (Clow et al., 2010b). However, from the numerous studies in this field it is noteworthy that higher awakening levels of cortisol and an attenuated CAR are seen in a range of conditions, including cardiovascular disease, clinical depression, mild cognitive impairment, Alzheimers disease, and autoimmune conditions. Whereas attenuated CAR and a lower awakening cortisol level is seen in post-traumatic stress disorder (Clow et al., 2010b).

The usefulness of the CAR as a biomarker results in it typically being used as a trait measure, however the CAR is also characterised by substantial state variation. As described earlier, state variation in the CAR is associated with psychosocial factors such as anticipations of challenge (Adam et al., 2006; Stalder et al., 2010a), and situational factors such as light exposure upon awakening (Scheer & Buijs, 1999, Thorn et al., 2004). The accumulating evidence describing marked intraindividual variation in the CAR is described in detail in chapter 2 of this thesis. The positive association between CAR magnitude and ambient light exposure (Scheer & Buijs, 1999, Thorn et al., 2004) is of relevance here however, as it provides some indication of the important regulatory influence of the SCN.

The SCN regulates the CAR via two separate pathways; the previously described input to the PVN and the HPA axis cascade (CRH and ACTH), and also a direct

autonomic input to the adrenal cortex via the splanchnic nerve of the sympathetic nervous system (Clow et al., 2010b). This dual-pathway SCN input plays an important role in determining CAR magnitude by a combination of pre- and post-awakening influences. While the HPA axis encourages cortisol secretion by increasing circulating ACTH in anticipation of awakening, the direct SCN-adrenal innervation pathway is implicated in the fine-tuning of the CAR by encouraging reduced adrenal sensitivity to ACTH in the immediate pre-awakening period (Bornstein et al., 2008; Buijs et al., 2003), followed by a reversal of this pattern upon awakening such that adrenal sensitivity to ACTH is increased in the immediate post-awakening period (Bornstein et al., 2008; Buijs et al., 1997, 2003; Fehm et al., 1984). Direct innervation from SCN to adrenal gland is also implicated in CAR regulation due to the increased adrenal sensitivity to light in this period, as in rats this has been demonstrated to be both independent of circulating ACTH and dependent on the integrity of the SCN (Buijs et al., 1999).

In summary, the CAR is fine-tuned by the SCN via a dual-pathway system, and as such should be considered as a relatively discrete aspect of the cortisol circadian rhythm. Unlike diurnal cortisol or acute stress-induced cortisol secretion, the CAR is sensitive to complex pre- and post-awakening influences which may influence it at both an inter- and intra-individual level. The dual-pathway SCN-mediated regulation of the CAR is represented in the diagram in figure 1.2.

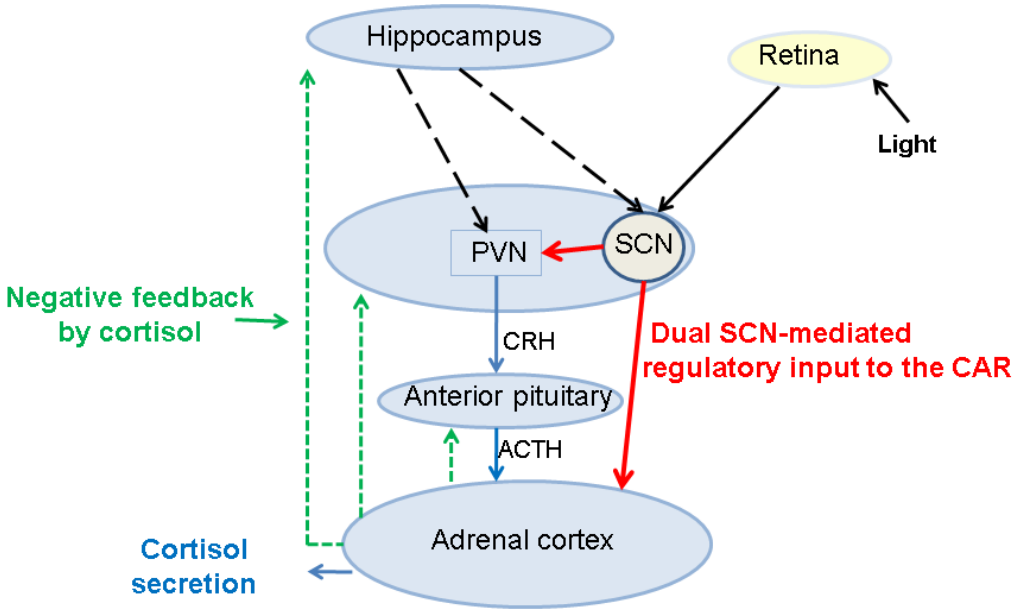


Figure 1.2 A simplified diagrammatic representation of the proposed regulatory inputs to the CAR (adapted from Clow et al., 2010b).

1.5.3 Peripheral clocks of the circadian system

While the SCN acts as the circadian pacemaker, there are 'peripheral clocks' in various regions throughout the body, including the liver, pancreas, heart, and the brain. These peripheral clocks show 'free-running' circadian rhythms in isolation, but are synchronised by indirect signalling from the SCN. It has been established that the SCN entrains the rhythms of endocrine and behavioural rhythms (e.g. glucocorticoids, melatonin; sleep-wake, and body temperature) via a range of signalling methods, which include direct influence by neuronal

input and indirect influence via regulation of paracrine signals. In turn, these rhythms act to synchronise peripheral clocks, including those in the brain (Antle & Silver, 2005; Menet and Rosbash, 2011). Recently, and for the first time, it has been demonstrated that human peripheral clocks are entrained by glucocorticoids (Cuesta, Cermakian, and Boivin, 2015). Importantly for the present PhD, this implicates a role for the circadian cortisol rhythm, and perhaps the CAR, in entrainment of the peripheral circadian system in the body and brain.

1.5.4 The circadian system and psychopathology

The importance of the circadian rhythm of cortisol secretion is highlighted by evidence that disruption to this rhythm is associated with a number of psychological and somatic disorders. For example, disruption of the cortisol circadian rhythm is evident in individuals suffering post-traumatic stress disorder (Yehuda et al., 1996), chronic stress (Miller, Chen, & Zhou, 2007), burnout (Pruessner, Hellhammer, & Kirschbaum, 1999), chronic fatigue syndrome (Nater et al., 2008), and major depressive disorder (Linkowski, 2003; Yehuda et al., 1996). Chronic exposure to elevated levels of cortisol results in physiological stress response systems becoming dysregulated. This can result in an excessively high and flat circadian profile, as mechanisms such as cortisol negative feedback are no longer able to regulate secretion. Given the importance of the cortisol circadian rhythm for such a wide range of bodily functions, including immune function, this type of disruption to the rhythm is associated with increased disease incidence and disease progression (Sephton & Spiegel, 2003). This includes not only diseases like the common cold (Cohen

et al., 2012), but in the longer term, increased susceptibility for diseases such as cancer (Abercrombie et al., 2004, Cohen et al., 2012; Mormont et al., 1998; Sephton & Spiegel, 2003; Touitou et al., 1996), further emphasising the importance of understanding the circadian rhythm of cortisol secretion for the elucidation and potential prevention and treatment of physiological and psychological disorders.

In recent years, the role that circadian rhythms play in the development of psychiatric conditions has become a pertinent topic in psychoneuroendocrinology, psychology and medicine. It has long been known that human mood disorders are often associated with sleep disruption, which can be associated with disruption to circadian rhythms. In attempts to understand the genetic basis of circadian rhythm disorders, genetic linkage and association studies have identified candidate genes, including the S662G mutation and T3111C polymorphism in the 3'UTR of the CLOCK gene corresponding with advanced and delayed sleep onset disorders, respectively (Takahashi et al., 2008). The findings regarding the specific genes involved should be received with caution at this early stage (in the absence of appropriately powered genome wide association studies), but nonetheless it is clear that individuals with mutations to these genes can also exhibit behavioural dysfunctions associated with addiction and human mood disorders (Takahashi et al., 2008). Disruption to the sleep-wake cycle is a frequently reported problem in patients with depression, bipolar disorder, obsessive-compulsive disorder, and schizophrenia (Jagannath et al., 2013; Karatsoreos, 2014). It has previously been thought that these sleep problems are symptomatic of the disorders, but in recent years it has been proposed that abnormal circadian

rhythms might in fact be a contributing causal factor (e.g. Menet and Rosbash, 2011, Karatsoreos, 2014). This is supported by the accumulating evidence implicating the SCN and circadian rhythms in influencing mood and cognitive performance, including numerous demonstrations that impairment of the SCN in animal models has downstream consequences on various brain regions involved in cognition and mood (see Menet and Rosbash, 2011). For example, it is known that signalling from the SCN is vital to the function of the hippocampus, a primary structure for episodic memory storage, consolidation, and retrieval (Aggleton and Brown, 1999; Nadel et al., 2000; Ryan et al., 2001; Squire and Zola, 1998). Studies using animals have shown that removing or interfering with the function of the SCN causes impairments of hippocampus-dependent memory (Ruby et al., 2008; Stephan & Kovacevic, 1978). Crucially however, such impairments of hippocampus-dependent memory can also be induced by simply changing the pattern of the light/dark cycle (Devan et al., 2001). It has been proposed therefore that the CAR, under the influence of the SCN, may serve as a hormonal time-of-day marker for regulation of peripheral clocks in the circadian system (Clow et al., 2010a). This theory is supported by the recent finding that glucocorticoids entrain peripheral clocks in humans (Pezük, Mohawk, Wang, & Menaker, 2012; Cuesta et al., 2015), suggesting that the circadian cortisol rhythm may influence the temporal programmes of gene expression in diverse brain regions, complementing their neural regulation by circadian inputs (Herbert et al., 2006). As such, it is apparent that mismatch between the circadian cortisol and neural patterns may contribute to localised malfunction of these brain regions (Herbert et al., 2006). This theory is addressed in further detail in chapter 2 of the present thesis.

1.6 Circadian rhythms in cognitive performance

Circadian rhythms have been observed in many measures of cognitive performance in humans (Dijk, Duffy & Czeisler, 1992; Schmidt et al., 2007; Valdez et al., 2008). For most tasks, performance tends to peak around the middle of the day, and be worst in the early morning and late evening (Valdez et al., 2008). Many of these rhythms in cognitive performance are related to both the endogenous temperature rhythm and the arousal rhythm (Monk et al., 1983; Wright et al., 2002), though there are some exceptions to this rule. There are also inter-individual factors which may influence these associations. An example of this would be the individual differences in performance and preference for time of day between the morning and evening. This preference for 'morningness' or 'eveningness' is known as as 'chronotype', and is normally assessed using the morningness-eveningness questionnaire (Horne and Östberg, 1976). There is substantial evidence to suggest that differences in chronotype are associated with differences in biological rhythms, including the circadian rhythms of core body temperature, cortisol secretion, melatonin secretion, and the sleep-wake cycle (Adan et al., 2012; Baehr et al., 2000; Duffy et al., 1999; Gibertini et al., 1999), and in turn, have been shown to influence several of the circadian effects on cognition. Another important inter-individual factor here is age. It has been suggested that during adolescence, time of day preferences tend to shift towards the evening, while in older adulthood from around 50 years onwards there is a shift towards morningness (Horne & Östberg, 1976; Schmidt et al., 2007), which is potentially important when comparing cognitive associations between-subjects as the timing of circadian

peaks in cognitive performance have been observed to vary by chronotype (Schmidt et al., 2007).

Speed of motor task performance has been observed to increase over the day, and seems to quite closely match the core body temperature rhythm (Folkard and Tucker, 2003). Accuracy of performance on a simple motor task has also been shown to be related to the body temperature rhythm, and with wake duration (Edwards et al., 2007). With regard to short-term (working) memory, this has also been shown to vary according to time-of-day. It appears that this too is closely associated with the core body temperature rhythm (Wright et al., 2002). However, for long-term memory, there are known to be individual differences in the circadian rhythm in performance, and these effects also vary depending on the type of memory observed. Declarative memory recall has been reported to increase across the day for evening types but decrease for morning types (Petros et al., 1990), though research in this area has focused almost entirely on episodic rather than semantic memory (memory for events, rather than knowledge). Procedural memory performance is worse at night than during the day, and this is seen even after controlling for the amount of time spent awake (Schmidt et al., 2007).

Several studies have reported time of day effects for components of executive functions (Valdez et al., 2008). For example, when measuring inhibitory control using a Stroop-type task, the performance nadir is seen approximately 1–2 h after habitual wake time, with the peak appearing at about 9pm in the evening (Burke et al., 2015). Further support for this is provided by Allen et al. (2008), who explored a range of cognitive performance measures in a sample of 56 US

college students, in the morning, afternoon and evening. These students showed improved performance in two executive function measures (fluency and digit symbol task performance) in the afternoon and evening compared to their morning performance.

There are also very well established time-of-day effects for tasks involving attention and arousal (Schmidt et al., 2007; Valdez et al., 2008). Attention can be considered a multidimensional construct, being made up of several components including tonic, phasic, selective and sustained attention. It has been demonstrated that all of these separate components show a performance nadir at around 4 am -7 am in the typical circadian human rhythm (Valdez et al., 2005). Sustained attention (or 'vigilance') tends to remain quite stable throughout the day, but begins to decline after the individual has been awake for over 16 hours, probably reflecting the effects of fatigue (Schmidt et al., 2007). Indeed, it is often a challenge in this area of research to tease out the relative contribution of the circadian rhythm from that of progressive fatigue during the day, not least because the two factors are often co-related. For instance, it has been suggested that fluctuations in working memory performance throughout the day may in fact be driven by the circadian fluctuations in attention (Schmidt et al., 2007). According to this account, the time-of-day effects for attention and executive function may be caused by fatigue, but that this process may involve a cascade of effects. This cascade may begin with impairment of tonic alertness (the most basic component of attention, comprising arousal and general alertness), and this in turn is manifested in the increase in errors observed in these other cognitive tasks (Valdez et al., 2008).

Although the research indicates a general peak in cognitive performance throughout the middle of the day, it is possible that this may be complicated somewhat by a dip in performance at around 12 noon in the 24-hour cycle. This is often informally referred to as the 'post-lunch dip', and has been observed in studies using a range of measures of attention and vigilance (Monk, 2005). There is some evidence to suggest that this 'post-lunch' dip occurs even in absence of food consumption, and when participants are kept unaware of the time of day (Monk, 2005). A dip around this time of day can also clearly be seen in patterns of workplace efficiency (Folkard and Tucker, 2003). However, it is important to note that the evidence for this dip in performance has been inconsistent, with some studies failing to find any reduction in cognitive performance at this time (Van Dongen and Dinges, 2005). It is possible therefore that there may be individual differences in terms of the occurrence of a post-lunch dip in cognitive performance, but the specific circadian system responsible for regulating this is yet to be identified.

In summary, there are well established time-of-day effects for most cognitive functions, and this is considered an important variable to control in laboratory studies. When comparing cognitive performance data within- or between-individuals, it is important to control for potentially confounding effects of circadian rhythms in cognition by testing participants at approximately the same time of day (e.g. early morning or late afternoon) in order to minimise possible biases related to circadian phase.

1.7 Cognitive Performance in the Immediate Post-Awakening Period

An additional hypothesis regarding the possible role of the CAR, initially proposed by Clow et al. (2010a), is that the CAR might assist in the recovery of cognitive functions in the post-awakening period; a process known in the sleep research literature as 'sleep inertia' (SI). Electroencephalograph (EEG) and brain imaging studies indicate that although awakening from sleep comprises rapid reestablishment of consciousness, the attainment of alertness is relatively slow (e.g. Ferrara et al., 2006). SI is considered to be the time lag between these two states, and has been shown to typically last anywhere between 1 and 30 minutes post-awakening (Ferrara et al., 2006, Ikeda and Hayashi, 2008). Although some studies have reported detectable performance impairment for up to 4 hours after waking in cases of major sleep deprivation (Tassi & Muzet, 2000). SI is therefore temporally associated with the CAR, as the substantial increase in cortisol has been shown to begin approximately 10 minutes post-awakening (Smyth et al., 2013a) and peak at around 30-45 minutes (Clow et al., 2004).

Evidence for SI comes from a range of studies, mostly using cognitive tasks such as attention switching and reaction times, but also often using arithmetic tasks, memory tests, visual-perceptual tasks, and even visual evoked potentials, which have decreased amplitude and increased latency immediately after awakening relative to the pre-sleep waking state (e.g. Brück and Pisani (1999); Ferrara et al., 2006; Tassi et al., 2006; Ikeda and Hayashi, 2008). It has been

suggested that SI mainly affects accuracy of performance in these tasks, while speed is less impaired (Marzano et al., 2011). In addition, studies of regional cerebral blood flow during the transition from sleep to full alertness show that although reactivation in the brainstem, thalamus and basal ganglia is rapid, reactivation of the frontal cortex (associated with EF) takes 20-30 mins following awakening (Balkin et al., 2002). Further evidence to suggest that cortisol or the CAR might influence the recovery from SI is provided by the finding that acute bursts of cortisol have a stimulatory influence on psychological arousal and reduce fatigue. This effect has been confirmed using self-report measures (Tops et al., 2006), arousal ratings (Abercrombie et al., 2005), as well as electroencephalographic (EEG) indicators of central alertness (Chapotot et al., 1998).

It has been quite clearly established that SI is influenced by circadian phase and sleep stage upon awakening (Tassi and Muzet, 2000). A possible cause of SI is the delay in blood flow reaching the anterior cortical regions of the brain after awakening (Balkin et al., 2002). Other researchers have suggested that it may be caused by increased levels of the arousal supressing neuromodulator adenosine in the brain during non-REM sleep, which may temporarily continue after abrupt awakening, causing reduced vigilance and increased sleep pressure (Van Dongen et al., 2001). In order to establish whether the effects of SI vary with the phase of the individuals' circadian cycle, Scheer et al. (2008) conducted a study using a 'forced desynchrony' protocol, which requires participants to adjust to sleeping and waking at all stages of the circadian cycle. Using body temperature measurements to establish the circadian phase at the time of waking, Scheer et al. found that the worst SI impairment of cognition

occurred when participants were woken-up during their 'biological night' (approximately between 2300 and 0300 hours of the circadian cycle). Notably, it has also been demonstrated that the CAR is blunted in cases of night-time awakening (Dettenborn, Rosenloecher, & Kirschbaum, 2007), again indicating the potential for an association between these two processes. Finally, it has also been shown that exposure to light during this immediate post-waking period is an effective countermeasure for the symptoms of SI (Ferrara, De Gennaro, & Bertini, 2000). This might again implicate the CAR in assisting with the recovery from SI, given that cortisol and the CAR are stimulated by light exposure (Scheer and Buijs, 1999; Thorn et al., 2004).

In summary, there is a temporal association between the SI and the CAR, a known influence of bursts of cortisol secretion on increased arousal and recovery from fatigue. Further, there is an association between recovery from SI and re-establishment of FC function; a brain region known to also be influenced by stress-responding and likely a result of glucocorticoid secretion (McEwen, 2012). Therefore there may be good reason to hypothesise that a larger CAR may be related to increased arousal in the post-awakening period, and also associated with a more rapid recovery from SI.

1.8 The CAR and Cognition

1.8.1 Relationship of the CAR with brain structural and functional integrity

The CAR is considered a key link between mind and body due to its sensitivity to psychosocial factors such as negative affect and anticipation of workload in the day ahead (Clow et al., 2010a; Fries et al., 2009). There is much evidence to show that the CAR is dependent upon the integrity of brain regions including the hippocampus (HC) and frontal cortex (FC) (de Kloet et al., 2005, Sullivan and Gratton, 2002, Buchanan et al., 2004, Wulff et al., 2010). Though the precise function of the CAR remains unknown, numerous studies have indicated relationships between the CAR and indices of cognition including declarative memory (Rimmele et al., 2010; Wolf et al., 2005), prospective memory (Baumler et al., 2014), working memory (Moriarty et al., 2014), and executive function (EF) (Evans et al., 2012). A summary of the studies exploring the relationship between the CAR and cognition is presented in Table 1.1.

Table 1.1 Summary of CAR-cognition studies

Memory Only					
Author	**Sample**	**CAR Measure**	**Cog. Test**	**Findings**	**Notes**
Pruessner et al. (2007)	13 healthy males (age 19-32, mean =23.85)	AUC of 0-30-60	DM (Cued recall)	No significant relationship	Unclear if AUCg or AUCi used
Baumler,	97 children	0-30	Prospective	CAR (+)	

Stalder et al. (2014)	(47 f) age 37-87 months	delta	memory (ball sorting)	Prospective memory (+)	
Almela et al. (2012)	88 healthy middle aged adults, 1:1 m/f (ages 55-77)	AUCi & AUCg	DM and WM	AUCi, AUCg (+) DM (-) *in men only,* CAR (+) WM (+)	Those with negative CAR also showed poorer DM (inverted-U?). n.b. Issues with adherence.
Rimmele et al. (2010)	16 healthy males (mean age 22.3, SD 3.89)	'Morning cortisol' 7:00-8:30 am (15 min ints.)	DM (Free recall)	Inverted U-shaped relationship: Extreme high or low CAR, DM (-)	
Moriarty et al. (2014)	19 male age 30-60 (mean 40.6, SD 5.8)	AUCg & AUCi	Spatial working memory (Newcastle 2D SM test) and Attention networks (broadly EF).	U-shaped relationship, extreme high or low AUCg, SWM (-)	No relationship with AUCi
Hinkelmann et al. (2013)	41 depressed patients, 41 controls (ages 18-70)	AUCg & AUCi	Verbal learning & visuospatial memory	In depressives, AUCi & AUCg (+) both memory scores (-) In healthy P's, CAR (+) Verbal mem (+)	Broad age range
Hodyl et al. (2016)	39 healthy adults (mean age 22, SD 4)	AUCi	Serial sequence reaction time task	CAR (+), learning (-), speed of performance (-)	

EF Only					
Author	**Sample**	**CAR Measure**	**Cog. Test**	**Findings**	**Notes**
Evans et al. (2012)	50 Older adults (ages 60-91)	MnInc, Av. peak	Trail making	Early peak + CAR (+) EF (+)	
Zhang et al. (2015)	63 healthy young males	AUCi (two days's. sum)	Go/no go task	CAR (+) error-related negativity latency and post-error miss rate	Did not electronically monitor adherence to protocol
Various Cognitive Measures					
Author	**Sample**	**CAR Measure**	**Cog. Test**	**Findings**	**Add. Notes**
Maldonado et al. (2008)	116 children, ages 9-12, defined as either low or high stress	0-30 delta	Cognitive drug research assessment system	CAR (+) speed of memory (-)	
Stawski et al.(2011)	1,500 midlife adults (mean age 57, SD 12)	0-30 min 'Morning rise'	Overall measure inc. word recall, working mem, reasoning & proc speed.	No relationship with dynamic measure, but overall 0-30 cortisol (+) Cog (+)	No age differences in cortisol-cog associations
Evans et al. (2011)	50 older adults, ages 60-91 (mean 74)	Mean 0-45, and 0-30 delta	Overall cog perf (OCP)	CAR (+) Cog (+)	
Franz et al. (2011)	795 male twins ages 51-	0-30 delta	Range including	CAR (+) visual-spatial memory	Report that the effect

	60 (mean 55.9, SD 2.6)		spatial abilities & memory, STM, EF, verbal fluency, reasoning, & processing speed.	(-)	was entirely driven by overall diurnal cortisol
Aas et al. (2011)	30 Patients with first episode psychosis, 26 controls (ages 18-65)	AUCi	Range including OCP, verbal mem, spatial abilities, processing speed, EF and WM.	In patients, CAR (+) Verbal memory, processing speed (+)	No relationship between CAR and cog in healthy controls
Cullen et al. (2014)	Children at risk for psychosis, & healthy control group (age range 11-14)	AUCi	Wide range of memory and EF tests	In at risk groups, CAR (+) letter fluency, verbal memory (+)	No CAR-cog relationship in healthy controls
Oosterholt et al. (2016)	85 adults (31 with clinical burnout, 27 non-clinical burnout, 27 healthy controls)	AUCg & 0-30 delta	EF (prepotent response inhibition, irrelevant information inhibition, & task switching), verbal mem, & cognitive failures (self-report)	Burnout patients CAR (-) compared to controls, and updating (-) (measured by the 2-back task), and cognitive failures (+).	Did not explore cortisol & cognition directly, and did not monitor adherence to CAR protocol

Ennis et al. (2016)	56 healthy adults, ages 23-79 (mean = 53, SD = 16.9)	0-30 delta divided by 0-30 sample-time delta	Episodic mem, Working mem, & processing speed. All measured 8-38 months after CAR.	CAR (+) episodic memory, but no association with working mem or processing speed.	Did not electronically monitor adherence to protocol.
Hidalgo et al. (2016)	64 healthy adults, ages 57-76 (mean = 64.7, SD = 4.1)	AUCi	Logical memory, verbal paired associates, family pictures test, letter-number sequencing, digit span, and spatial span.	CAR (-) immediate verbal and visual recall, but no association with any other measures	No significant effects with appropriate controls applied. Electronically monitored sampling time but not awakening time.
Labad et al. (2016)	60 patients with early psychosis, ages 18-35	AUCi	Range including OCP, WM, verbal & visual mem, reasoning & problem solving, & social cognition.	CAR magnitude (-) OCP, and flatness of CAR (-) spatial memory	Did not monitor adherence to protocol, and only used one day's CAR (different day to cognitive test)
Salvat-Pujol et al. (2017)	97 medicated patients with MDD (mean age = 59.8,	AUCi	Range of tests, including Hopkins	CAR (+) TMTB, processing speed and Rey complex figure	Did not monitor adherence to protocol,

	SD = 11.7), and 97 healthy controls (mean age = 56.6, SD = 11.9)		verbal learning test, TMT, Rey complex figure test, Stroop, working memory span	test (copy) in MDD patients in remission, but no relationship with any other measure in this group. No relationship with any cognitive measure in overall sample.	and only used one day's CAR

AUCi = Area under the curve with respect to increase, AUCg = Area under the curve with respect to ground, OCP = Overall cognitive performance, WM = Working memory, DM = Declarative memory, EF = Executive function, Mem = Memory, MDD = Major depressive disorder, SD = Standard deviation, (+) = Significantly associated increase, (-) = Significantly associated decrease

1.8.2 The CAR and memory function

It is well established that cortisol secretory activity is associated with integrity of the HC (as described in section 1.4). The HC has been implicated in the regulation of hormonal responses to physical and psychological challenge, primarily in an inhibitory capacity, contributing to the negative feedback cycle (Fries et al., 2009; Herman et al., 2005). The CAR too has been shown to be associated with HC integrity, volume, and associated function (Almela et al., 2012; Bruehl, Wolf, & Convit, 2009; Buchanan et al., 20014; Pruessner et al., 2007; Wolf et al., 2005). For example, neither is the CAR observed in individuals with unilateral and bilateral HC lesions, nor in those with severe global amnesia (Buchanan et al., 2004; Wolf et al., 2005). Further, it has been demonstrated that a larger CAR is associated with increased HC volume in healthy young men (Pruessner et al., 2007), and impaired declarative memory performance in older adults (Almela et al., 2012). However, this association may be bi-directional,

such that the HC may regulate cortisol secretion during the CAR period, but so too may the increase in cortisol secretion during the CAR period influence hippocampal functioning. This is because not only does an abnormal CAR occur in the presence of lesions to these brain regions, but in the reverse direction it has been shown that changes in cortisol secretion and the CAR can influence performance on cognitive measures of HC function. For example, Rimmele et al. (2010) demonstrated that pharmacologic suppression of the CAR using metyrapone (a cortisol synthesis inhibitor) impaired memory for prior-day learnt text and imagery in a free recall task, and separate studies have shown that elevated circulating cortisol during sleep has a negative effect on simultaneous HC dependent memory consolidation (Born and Wagner, 2009). Therefore this suggests a bi-directional relationship between the CAR and the structural and functional integrity of the HC and FC. In a sample of elderly participants without dementia, Geerlings et al. (2015) demonstrated that higher levels of cortisol at 45-min post-awakening are associated with better processing speed and executive functioning, and slightly increased white matter, despite a lack of any association with general memory performance or gray matter volume. Though this study did not assess the CAR directly, the results implicate a possible role of the CAR due to the timing of the cortisol assessment (i.e. at the same time as one would normally expect the CAR peak), and as such, adds to the accumulating evidence for a relationship between cortisol secretion in the post-awakening period and HC function.

The importance of HC function upon awakening is apparent; besides its importance for episodic memory function, the HC is also involved in space and time orientation (O'Keefe and Nadel, 1978; Burgess, Maguire, & O'Keefe, 2002).

It has therefore been hypothesised that the CAR may play a role in activation of prospective memory function upon-awakening, encouraging the orientation of self in terms of space and time, and anticipation of the demands of the coming day in the post-awakening period (Baumler et al., 2014; Fries et al., 2009). This hypothesis was tested by Baumler and colleagues in a sample of young children. The study involved completion of a game-like task assessing prospective memory, performance on which was found to be positively associated with CAR magnitude (Baumler et al., 2014). These findings provide support for the previously discussed CAR and HC-associated memory function relationship, but further suggest that the CAR-cognition relationship may be subtly different at different times within the waking day.

The CAR has also been investigated in relation to working memory. It has been demonstrated that a larger trait CAR (measured across 2 days) is associated with increased working memory performance in a sample of older adult males, though this relationship was not seen in females of similar age (Almela et al., 2012). Moriarty et al. (2014) have also reported a strong relationship between overall levels of cortisol secretion within the CAR period and spatial working memory using the Newcastle 2D Spatial Memory Test (which involves target detection over a fixed series of points of appearance on a computer screen) in healthy older adults, including both males and females. This study suggested a U-shaped relationship, such that an extreme high or low level of total cortisol secretion in the CAR period was associated with decreased memory performance, which the authors interpret as being consistent with the previously reported positive relationship. However, these significant results were limited to total cortisol secretion, and the authors did not find any

relationship between the CAR itself and spatial working memory. A particular limitation of this study is the lack of adequate controls for participant non-adherence, known to be a major confound in CAR studies (discussed in detail in chapters 2 & 3; also see Clow et al., 2010b; Smyth et al., 2013a; Stalder et al., 2016). Moreover, a broad measure of total secretory activity is considered to be a limited method of illuminating the CAR, given that it is defined not by total secretory activity, but by the dynamic increase in cortisol secretion in the post-awakening period, (Clow et al., 2010b). This would suggest that the reported relationship with working memory observed here may not be a valid or reliable finding. The inconsistency in this particular area suggests a need for further research exploring this relationship, with appropriate application of CAR measures, and careful control for the numerous potential confounds that can influence CAR research (See Clow et al., 2010b; Stalder et al., 2016).

1.8.3 The CAR and executive function

Like the HC, the frontal cortex (FC) is a brain region with a high density of cortisol receptors, and the structure and function of the FC is also associated with circulating levels of glucocorticoids (e.g. Evans et al., 2011). The FC is considered the most prominent structure responsible for executive functions (EF). EF can be understood as a range of functions including inhibition and interference control, working memory, and cognitive flexibility (Diamond, 2013; Miyake et al., 2000). One of the aspects of cognitive flexibility is the ability to switch between task demands, often assessed using attention switching paradigms (for review, see Diamond, 2013). Evans et al. (2012) indicated that

better performance on one of these tasks, form B of the Trail Making task (Arbuthnott & Frank, 2000), is associated with a larger CAR in older adults.

This finding of a trait relationship between the CAR and EF is supported by similar trait studies of clinical samples. For example, Oosterholt et al. (2016) showed that in a sample of patients with clinical burnout, an attenuated CAR was associated with poorer performance on the updating component of the '2-back' task, and an increased frequency of cognitive failures. Though it is important to note that the trait CAR-EF relationship was not observed in a recent study of healthy adults (Ennis et al., 2016), which may suggest that this relationship only emerges in older age, or in cases of dysfunction in healthy adults.

While a relationship between individual differences in CAR magnitude and EF has been demonstrated in between-subjects studies, the impact of daily variation in the CAR on EF has not been explored. There may be good reason to hypothesise that such a relationship may exist, as EF tends to be impaired upon awakening, but recover across the post-waking hour (Tassi and Muzet, 2000; Ikeda and Hayashi, 2008), and is therefore temporally associated with the CAR (Clow et al., 2010a). The importance of these functions in the post-waking period, and the associations between the CAR and these brain regions has therefore led to the proposal that the role of the CAR may be to stimulate waking (Fries, Dettenborn, & Kirschbaum, 2009; Clow et al., 2010a). It has been proposed that this may be achieved by regulation of blood flow to these brain regions (Clow et al., 2010a), a theory supported by the evidence from brain scanning studies which show that cerebral blood flow (CBF) to anterior cortical

regions increases across the first 15-minutes post-awakening (Balkin et al., 2002). Indeed, it has been suggested that the dissipation of sleep inertia effects is effected by reactivation, and perhaps functional reorganization, of these regions (Balkin et al., 2002), indicating a potential role for the CAR in this process. The association with negative prior day experience and anticipated demand (e.g. Adam et al., 2006; Stalder et al., 2010a) would therefore suggest that this 'boost', and the respective recovery of brain function, would be increased on days when the individual anticipates increased challenge (Adam et al., 2006; Fries et al., 2009; Clow et al., 2010a). This is a novel hypothesis, and one which is explored in study I of the present thesis (Chapter 4).

In summary, there is a growing body of research exploring the relationship between the CAR and cognition. As noted in table 1.1, the growth of interest in this topic has resulted in at least 13 published studies since 2012 which have specifically explored CAR-cognition associations, with positive but not entirely consistent findings. Within this literature, much of the focus has been on memory/HC function, while there has been very little exploration of EF/frontal function. Further, despite several demonstrations of a relationship in older adults or abnormal functioning samples, not one study to date has explored the association between the CAR and EF in healthy young adults, and there has been no dedicated effort to explore the mechanisms that might underpin the relationship between the CAR and cognition. As such, there are many important questions in this field that remained unanswered, and which present exciting challenges for further research.

2 Introduction II

State Variation in the CAR

2.1 Overview

Despite a large number of studies the role of the CAR remains poorly understood. The purpose of this chapter is to discuss state variation in the CAR and the associated factors, and further, how an understanding of CAR state variation may inform its role and regulation within the healthy circadian pattern of cortisol secretion. One of the primary considerations for CAR research in recent years has been to address methodological issues that may have contributed to the inconsistent and contradictory findings from earlier studies (Smyth et al., 2013a; Stalder et al., 2016). One of these methodological issues

may have been the tendency to conceptualise the CAR as a trait measure, and overlook its considerable degree of state variation (Almeida et al., 2009; Hellhammer et al., 2007; Ross et al., 2014; Stalder et al., 2009). Indeed in a recent review, Ross et al. (2014) reported that most of the variance in the CAR (and indeed other aspects of the diurnal cortisol rhythm) in fact reflects state fluctuation; whereas there is little evidence for trait influences. This has important implications for the field, as the majority of CAR research to date has primarily focused on its application as a trait biomarker in association with a range of psychosocial and physical variables, including health outcomes (Chida & Steptoe, 2009). This chapter puts forward the argument that understanding such variation within healthy populations has the potential to lead toward a clearer understanding of the role of the CAR within the healthy cortisol circadian cycle, and therefore the consequences of dysregulation in a range of conditions. In addition, it is proposed that this increased understanding of CAR state variation should be informative for interpretation of the broad range of results from studies using the CAR as a trait biomarker.

2.2 Background: use of the CAR as a trait biomarker

The first report of the CAR, by Pruessner et al. (1997), suggested relative intra-individual stability in a sample of both male and female participants across a broad age range (see Table 2.1 for a summary of the study details). In this initial study, it was reported that the CAR was not affected by participants' time of awakening, menstrual cycle phase or alcohol consumption in any of the three groups. This finding was supported by several further demonstrations of

intraindividual stability (e.g. Edwards et al., 2001b; Wüst et al., 2000b). These results proved to be particularly influential in recommending the use of the CAR (measured across 1 to 2 days only) as a reliable trait biomarker for HPA-axis status. Subsequently, a series of studies emerged in which contradictory outcomes were observed when using the CAR as a trait measure. At the same time, further within-subjects studies of the CAR demonstrated state differences across sampling days, bringing the reliability of the CAR as a trait biomarker into question. One of the earliest state associations to emerge was the weekend–weekday difference (Kunz-Ebrecht et al., 2004; Schlotz et al., 2004) (see Table 2.1 for details). These early studies also provided an indication that variation in the CAR may be associated with the individual's psychological state and anticipation of the day ahead.

It has since been established that the CAR is prone to significant state variation which is greater than trait variation (Almeida et al., 2009; Hellhammer et al., 2007; Stalder et al., 2009, 2010b) (see Table 2.1 for a summary of studies). Such studies have been generally consistent, with Hellhammer et al. (2007) reporting that state factors can account for between 61% and 82% of the variation in the CAR. Almeida et al. (2009) suggested that these factors can account for 78% of CAR variation, and Stalder et al. (2010b) suggested a figure of 64% for a smaller sample of female participants. The first longitudinal case study of the CAR in a healthy participant (Stalder et al., 2009) employed a researcher–participant design to avoid participant non-adherence to the protocol. Again, this approach provided clear evidence of state variation in the CAR, which ranged from 3.6 to 39.0 nmol/l on any one day. Indeed, recent research by Mikolajczak et al. (2010) (see Table 2.1 for details) found that

factors known to be protective of health such as high happiness, low stress and low neuroticism, were found to be associated not with the size of the CAR but rather greater day-to-day variation in the CAR. This hinted that CAR flexibility (e.g. a greater difference between days) rather than CAR magnitude may be a better way to understand the relationship between the CAR and positive psychosocial status.

Intra-individual studies in healthy participants have provided evidence (sometimes from a single study) that the CAR can be associated with a range of situational and psychosocial state variables. These include time of awakening, ambient light, prior day experiences, anticipation of the day ahead, ovulation, jet lag and alcohol consumption (Adam et al., 2006; Doane et al., 2010; Edwards et al., 2001b; Stalder et al., 2009, 2010a; Wolfram et al., 2011). These reports provide clues about the role of the CAR in healthy functioning which can hopefully be replicated and extended.

Table 2.1 Summary of studies that have examined state variation of the CAR in healthy participants.

Author(s)	Participants	HPA Measurement	Findings
Pruessner, Wolf, Hellhammer, Buske-Kirschbaum, Von Auer, Jobst, Kaspers & Kirschbaum (1997)	Healthy. N=152 (1:1 male-female), 11, 26 & 70 (3 groups).	Group 1 – 0, 10, 20, 30 on 3 consecutive days. Group 2 – 0, 15, 30, 60 on 3 days at one week intervals. Group 3 – 0, 15, 30 & 60 on two consecutive days.	CAR in all three studies reported as stable within subjects. Sleep duration, time of awakening and alcohol also appeared unrelated to the CAR.
Scheer & Buijs (1999)	Healthy males. N=14, ages 24-41 (Mean 32.5)	0, 20, 40, 60 & 120 mins	Bright light (800 lux) for the hour following awakening produced a significant increase in CAR magnitude at 20 and 40 minutes post-awakening.
Wüst, Wolf, Hellhammer, Federenko, Schommer & Kirschbaum (2000b)	Healthy. N=509 (319 Female, 190 Male), ages 18-71 (Mean 37)	0, 30, 45 & 60 mins on two consecutive days	Observed a MnInc of about 50% within the first 30 minutes. Intraindividual stability was shown to be high with correlations up to r=.63
Edwards, Evans, Hucklebridge & Clow (2001b)	Healthy. N=40 (31 female, 9 male), ages 23-	0, 15, 30 & 45 mins on two consecutive days	Moderate stability of the CAR across two

	53 (Mean age 35)		sampling days, with early waking participants showing a larger CAR.
Federenko, Wüst, Hellhammer, Dechoux, Kumsta & Kirschbaum (2004)	Healthy shift workers. N=86, 55 female (24 nurses & 31 students). Nurses ages 29-52 (Mean 40.3). Students ages 20-31 (Mean 25)	0, 30, 45 & 60 mins on two consecutive days	In the sample of nurses, the CAR was larger in the early shift than in the late and night shifts.
Kunz-Ebrecht, Kirschbaum, Marmot & Steptoe (2004)	Healthy. N=196, ages 47-59	0 & 30 mins, on two days	The cortisol awakening response (defined as the difference between waking and 30 min later) was greater on work than weekend days.
Schlotz, Hellhammer, Schulz & Stone (2004)	Healthy. N=219, ages 24-83 (Mean 48.6)	0, 30, 45 & 60 mins on 6 consecutive days starting on Saturday.	Clear weekend-weekday difference in the CAR, and this difference was associated with chronic work overload and worry. This was independent of

			sex weekend-weekday differences in time of awakening and sleep duration.
Thorn, Hucklebridge, Esgate, Evans & Clow (2004)	Healthy. N=12 (5 Female, 7 Male), ages 24-54 (Mean age 39)	0, 15, 30 & 45 mins, on 4 consecutive days	Increased AUCg magnitude over 45 mins when participants were exposed to approximately 250 lux light for 30 mins on awakening.
Williams, Magid & Steptoe (2005)	Healthy shift workers. N=32 (17 male, 15 female) no age data	2, 30 & 60 mins on 6 days, including two early shift days, two day shift days, and two control/ leisure days	Observed a greater CAR on early-shift days. However, respondents were more stressed over the hour after waking and reported more sleep disturbance on early-shift days; when these factors were taken into account, the CAR-early shift relationship was no longer significant.

Adam, Hawkley, Kudielka & Cacioppo (2006)	Healthy older adults. N=156, ages 50 to 68 (mean 57)	0 mins, 30 mins and at bedtime each day for 3 days	Prior-day feelings of loneliness, sadness, threat, and lack of control were associated with a higher cortisol awakening response the next day, but morning awakening responses did not predict experiences of these states later the same day
Clow, Edwards, Owen, Evans, Evans, Hucklebridge & Casey (2006)	Healthy military sample. N=20 ages 17-24	0, 15, 30 mins on 5 days throughout a military training course	Reduction in CAR (AUC) during the middle of an intense training course, and a return to baseline CAR following completion.
Thorn, Hucklebridge, Evans & Clow (2006)	Healthy students. N=48 (4:1 female to male), ages 18-36 (Mean 20)	0, 15, 30 & 45 mins on two consecutive weekdays, and two consecutive weekend days.	Steeper rise in the CAR on weekdays, not accounted for by differences in waking time, state stress or perceived stress over the previous

			month. AUC over 4 days also negatively correlated with the measure of longer term stress and awakening time. Mean increase also negatively correlated with waking time, not mediated by stress or vice versa, since both independently predicted cortisol.
Hellhammer, Fries, Schweisthal, Schlotz, Stone & Hagemann (2007)	Healthy. N=193, ages 28-88	0, 30, 45 & 60 mins on 6 consecutive days	Concluded that the CAR of a single day is largely determined by situational factors, and only a small proportion is determined by trait factors.
Wilhelm, Born, Kudielka, Schlotz & Wüst (2007)	Healthy males. N=16, ages 20-33 (Mean 25)	Saliva at 0, 30, 45 & 60 mins. Blood sampling every 15 minutes	Demonstrated that the CAR is a response to waking, and did not differ in lab

			conditions from that observed at home.
Dettenborn, Rosenloecher & Kirschbaum (2007)	Healthy females. N=13, ages 20-32 (Mean 24)	0 mins & 15 mins following 3 night awakenings, and again at 0 & 15 mins following morning awakening on 3 nights. Also taken at 0 &15 after 3 undisturbed nights.	Significant difference between night time and morning CAR. No CAR after waking during the first half of the night, but some reactivity during the early hours of the morning, and pronounced CAR in the morning before getting out of bed.
Almeida, Piazza & Stawski (2009)	Healthy. N=1143, ages 33-84 (Mean 57)	0 mins, 30 mins, before lunch and at bedtime for 4 days	Substantial day-to-day variability in the CAR. State factors reported to account for 78% of CAR variation.
Stalder, Hucklebridge, Evans & Clow (2009)	Healthy male. N=1 (Case study), age 27	0, 15, 30 & 45 mins on 50 measurement days, at 3 day intervals.	Considerable day-to-day variability in the CAR. Waking time also strongly related to first waking sample (Stronger

			relationship on days when awakening time was earlier). Preliminary indication of inverse association between alcohol consumption.
Doane & Adam (2010)	Healthy. N=108 (27 male), mean age 19.	0 mins & 30 mins	Prior day feelings of loneliness were associated with an increased CAR. Prior day feelings of nervousness/stress were associated with lower average wake-up levels of cortisol.
Doane, Kremen, Eaves, Eisen, Hauger, Hellhammer, Levine, Lupien, Lyons, Mendoza, Prom-Wormely, Xian, York, Franz & Jacobson (2010)	Healthy males. N=786, middle-aged, no specific age data given on full sample	0 & 30 mins, then 10am	Evidence that travelling across time zones is associated with diurnal cortisol regulation. Reduced peak of post-travel CAR following Eastward or Westward travel, and also a

			steeper decline after travelling Eastward.
Mikolajczak, Quoidbach, Vanootighem, Lambert, Lahaye, Fillée & De Timary (2010)	Healthy males. N=42, ages 30-50 (Mean 38)	0, 15, 30, 45 & 60 mins on 3 consecutive measurement days (Sunday, Monday, Tuesday).	Protective factors (i.e., high happiness, low stress, and low neuroticism) are associated with a flexible CAR (i.e. greater difference between Sunday and Monday CARs).
Stalder, Evans, Hucklebridge & Clow (2010a)	Healthy male. N=1 (Case study), age 27	0, 15, 30 & 45 mins on 50 measurement days, at 3 day intervals.	Relationships found between psychosocial state variables and the AUCI, including an inverse relationship with the level of prior-day happiness and a positive relationship with study-day anticipations of the level of obligations/no leisure.
Stalder, Evans,	Healthy females.	0, 15, 30, and	CAR variability

Hucklebridge & Clow (2010b)	N=12, ages: 22 - 41 (Mean 29)	45 mins, on 12 days at 3-day intervals.	(AUC_I) was found to be inversely related to simultaneous variability in awakening time, and positively related to variability in adverse psychosocial states of stress and tension 45 min post-awakening.
Strahler, Ehrlenspiel, Heene & Brand (2010)	Healthy (martial arts practitioners). N=20, ages 16-30 (Mean 23)	0, 15, 30, 45, 60 mins on 3 days prior to competition.	No change observed in the CAR in the build up to competition.
Thorn, Evans, Cannon, Hucklebridge, Evans and Clow (2011)	Self-reported SAD and control (healthy) sample. N=52 (26 SAD sample, 26 Control), ages 26 – 75 (mean 50)	0, 15, 30, 45 mins on two days in summer, and two days in winter.	In winter the CAR was significantly attenuated in SAD participants in comparison to the healthy control participants, but no difference in summer.
Wolfram, Bellingrath & Kudielka (2011)	Healthy females. N=29, ages 20-34 (Mean 26.3)	0,30,45 &60 mins on 4 measurement	The CAR is elevated during ovulation. But no

		days, representing 4 phases across the complete menstrual cycle.	differences in mood states across the four cycle phases.

2.3 Factors associated with state variation in the CAR

2.3.1 Light and the regulatory influence of the suprachiasmatic nucleus

The first report of a relationship between ambient light and state variation in the CAR was provided by Scheer & Buijs (1999), with the demonstration from a repeated measures study design that awakening in darkness (induced by blindfold) leads to an attenuated CAR AUCg compared to awakening in moderate ambient light (800 lux). These effects of light on the CAR AUCg have since been replicated with relatively low light levels (250 lux). In this repeated measures study exposure to dawn simulation in winter (i.e. increasing light intensity to a maximum of 250 lux in the 30 min immediately prior to awakening) resulted in both significantly higher cortisol levels for participants during the first 45-min post-awakening and an associated significant increase in selfreported levels of arousal (Thorn et al., 2004). Surprisingly, only these two studies have directly examined the impact of light on the CAR, with both studies suggesting an influence of ambient light on the CAR. In so doing they implicate a role for the hypothalamic suprachiasmatic nucleus (SCN) in regulation of the CAR, as previous studies in rodents have demonstrated the dependence of the

SCN for the effects of light on basal cortisol levels (Buijs et al., 1999; see Clow et al., 2010a for a more detailed discussion of this issue).

In self-assessed seasonal affective disorder (SAD) participants have been found to show a significant decrease in the CAR in winter when waking before sunrise in comparison to both their CAR in the summer, and the CAR of non-SAD participants. Furthermore, a general dysphoria construct correlated inversely with the CAR in the winter, indicating that participants reporting greater depression, stress and anxiety and lower arousal exhibited lower CARs. It was noteworthy that participants who lacked marked seasonality in terms of affect did not show seasonal variation in the CAR (Thorn et al., 2011) (see Table 2.1 for study details). These data suggest that the effects of light on the CAR may differ between individuals. In separate studies it has been demonstrated that individuals who report symptoms of SAD show reduced retinal sensitivity to light during the winter months in comparison to healthy controls (Hebert et al., 2002; Terman & Terman, 1999). It is possible (although not yet tested) that these two factors (retinal sensitivity and the CAR) are linked via light detection-SCN-neuroendocrine pathways. This may be something that can be investigated in future studies, but is beyond the scope of the present PhD research.

Contrary to these findings, it has also been reported (again in a repeated measures study design) that awakening in darkness in a sleep laboratory environment was associated with the same size of CAR as awakening in the domestic setting with typical ambient light (Wilhelm et al., 2007). However, as the authors acknowledge this study was not designed to study the effect of light on the CAR as there was no standardization of other potentially confounding

state factors. For example, it is possible that awakening in the dark in a strange environment with experimenters present might impact on the CAR, since it has been demonstrated that anticipation of the day ahead can influence the magnitude of the CAR (Stalder et al., 2010a).

In summary, it is possible, and deserves further investigation, that there is not only variation in the CAR in response to ambient light but also individual differences in that variation, potentially dependent on retinal sensitivity. This added complexity suggests that although it is important to take ambient light into account when using the CAR as a biomarker it is not usually possible to make allowances for individual differences in sensitivity to that light. Notwithstanding this limitation, greater variation in pre- and post-awakening light exposure between participants increases the potential for a confounding influence when comparing CAR data across individuals. Indeed, it has been demonstrated that variation between light levels of 0–800 lux can account for a not insubstantial 35% of state variation in the CAR (Scheer & Buijs, 1999).

It is possible that attenuated CAR magnitude when awakening in the dark may prevent unnecessary sleep disturbance in response to accidental nocturnal awakening. If the CAR does assist in the re-establishment of cognitive function (as proposed by Fries et al., 2009) then in theory, light-responsive systems may allow for optimally enhanced cognitive function when awakening at dawn, in preparation for the day ahead. Indeed, preliminary support for this theory is provided by studies using functional magnetic resonance imaging techniques which indicate that exposure to light during the morning period results in increased activation of the subcortical regions associated with alertness and

working memory (Vandewalle et al., 2006, 2010). To date, however, there have been no studies which have explored the effects of light on the CAR in combination with the activation of these brain regions. This presents another gap in the scientific literature, as the underlying mechanisms which may regulate the transition from consciousness to alertness upon awakening are yet to be determind. It is quite possible that the CAR plays a role in this process, as it is well established that the SCN utilizes a range of signaling methods, including the neuroendocrine system, to entrain the peripheral clocks in different regions of the body including the brain (Menet & Rosbash, 2011). Of particular relevance here is that signaling from the SCN is vital to the functional integrity of the hippocampus with impairments of hippocampus-dependent memory demonstrated in rats with SCN lesions or changes in the light/dark cycle (Devan et al., 2001; Ruby et al., 2008; Stephan & Kovacevic, 1978). Physical and functional integrity of the hippocampus is also related to circadian cortisol secretion and the CAR (Buchanan et al., 2004; Pruessner et al., 2007; Rimmele et al., 2010; Sapolsky, 2001; Wolf et al., 2005). This raises the question of whether a role of the CAR may be to signify the start of the awakening phase and maximize optimal capacity for appropriate daytime activity.

2.3.2 Sleep variables

Although not yet fully understood, there is evidence for between and within participant associations between the time of awakening and the magnitude of the CAR, first demonstrated between participants by Edwards et al. (2001b) and later confirmed by several further studies within participants (e.g. Almeida et

al., 2009; Dettenborn et al., 2007; Federenko et al., 2004; Stalder et al., 2009, 2010a, Zeiders et al., 2011). Later awakening has been associated with higher cortisol levels immediately upon awakening and in multiple regression analyses this factor was able to account for the reduced CAR (Stalder et al., 2009). However, not all studies have reported an association between time of awakening and CAR magnitude (Brooke-Wavell et al., 2002; Kunz-Ebrecht et al., 2004; Pruessner et al., 1997; Wüst et al., 2000b) and this inconsistency may be attributable to a range of factors. For example, in studies where the range of awakening times is small there is less likely to be an association. There is also a potential confound in terms of participant age, as aging is associated with earlier waking and yet an attenuated CAR (Kudielka & Kirschbaum, 2003), and intra-individual variability of the CAR has also been shown to increase with age among men (Almeida et al., 2009). Another important factor in large epidemiological studies that have not found this relationship may be related to participant non-adherence (Smyth et al., 2013a; Thorn et al., 2006). Participants may be worse at completing the CAR sampling procedure when in a compromised situation (such as when suffering the effects of a change of sleep patterns) and much of the research into the effects of changes to the sleep–wake cycle on the CAR has relied solely upon participants' self-reported awakening and sampling times, despite the fact that these have consistently been shown to be inaccurate (Broderick et al., 2004; Smyth et al., 2013a; Stalder et al., 2016). Notably, whilst earlier studies had suggested that delays of up to 15 min between awakening and collection of the first sample did not affect the accuracy of the CAR measurement (DeSantis et al., 2010; Dockray et al., 2008; Okun et al., 2010), more recent research has shown that delays of only 8 min do in fact lead to inaccurate assessment of the CAR (Smyth et al., 2013a). It

would seem therefore, that this is very likely to have been a serious confounding factor in many of these studies.

Interestingly, as observed for light, there may be other trait factors that interact with this state association. It has been reported that the relationship between time of awakening and the magnitude of the CAR is moderated by depressive symptoms, such that the relationship was reduced in mild to moderate depression (Stetler & Miller, 2005). In addition, in the case of night awakenings, the magnitude of the CAR upon forced awakening appears to increase as the night goes on, with the most pronounced CAR upon normal morning awakening (Dettenborn et al., 2007).

Evidence to demonstrate that the CAR adapts to changes in awakening times is provided by studies exploring the CAR during shift work and altered sleeping patterns (e.g. Griefahn & Robens, 2008, 2010; Harris et al., 2010; Kudielka et al., 2007; Williams et al., 2005). When individuals begin working night shifts (i.e. begin to sleep during the daylight hours) the CAR is initially blunted, but then gradually increases following consecutive night shifts until it returns to baseline magnitude (Griefahn & Robens, 2010; Harris et al., 2010; Kudielka et al., 2007). Additionally, there appears to be a sex difference in this return to baseline CAR magnitude in night shift workers, with the CAR typically reaching baseline levels after 3 days in men, and 4 days in women (Griefahn & Robens, 2010). Griefahn & Robens (2010) note that the adjustment of the CAR across consecutive night shift work occurs faster than the other aspects of the circadian system, suggesting that circadian functions are not the sole cause of the adjustment of the CAR. It has also been demonstrated that the process of cortisol circadian

rhythm adaptation in night shift workers is facilitated by the presence of appropriate cues for waking. For instance, the extent to which the circadian rhythm of cortisol adapts following changes in shift work patterns is increased in individuals exposed to bright light during a night-shift and protected from light exposure during the day-sleep period (James, Walker, & Boivin, 2004). The evidence provided by these studies may therefore implicate a regulatory influence of the SCN that can be moderated by external zeitgeibers. It also provides some indication that although the CAR can adapt to waking during nocturnal hours, this adaptation may not be complete without facilitative alterations to the light/dark cycle.

The findings from shift-work studies may also illuminate the phenomenon of ‘‘jet lag’’ experienced by individuals travelling across several time zones and subsequently changing their sleep and waking times. Indeed, the re-adjustment of the body clock following travel is similar to that experienced in shift work, perhaps only differing in the natural external cues to waking (i.e. timing of the natural light cycle). Recent research by Doane et al. (2010) has confirmed that jet-lag when travelling across three or fewer time zones does result in an attenuated CAR, with both Eastward and Westward travel showing an association with a lower peak in the CAR on the following morning (see Table 2.1 for study details). This study reported an effect on the CAR (difference between awakening and 30-min samples) of nearly twice the magnitude for Eastward travel than for Westward travel. The extent to which these results can be generalized is limited as there has only been this one study to date. Additionally the authors recognized that there was an imbalance in the study conditions, with the majority of the eastward travelling sample (66%) crossing

only one time zone, and the majority of the Westward travelling sample (80%) crossing two to three time zones (Doane et al., 2010). It is clear that further research is needed in order to properly explore the association between these two variables. Nonetheless, this study does provide some preliminary evidence of a relationship with travel across time zones and associated jet lag.

2.3.3 Prior day experiences and anticipation of challenge

An increased CAR has been reported in response to subjective feelings of prior day threat, lack of control, loneliness and other negative feelings (Adam et al., 2006; Doane & Adam, 2010; Stalder et al., 2010a,b). This prior day association suggests that the CAR may play a preparatory (homeostatic) role in healthy functioning (Adam et al., 2006). This may be achieved by a pre-emptive increase in CAR magnitude in response to prior-day negative mood, in order to help to prepare the individual for a challenging day (Adam et al., 2006). Increased CARs have also been reported in response to anticipation of challenge or workload in the day ahead (Stalder et al., 2010a,b). Differences in anticipations of the day ahead have also been proposed as an explanation of reported weekend versus weekday differences in the CAR across the typical working week (Kunz-Ebrecht et al., 2004; Schlotz et al., 2004). Although it should be noted that Thorn et al. (2006) found the weekend/weekday difference in their data was no longer significant in students when suspected non-adherence was controlled for, suggesting that this particular association may be

a product of participants failing to comply with the sampling protocol on weekends. Alternatively, it may reflect the nature of the student lifestyle in which weekday/weekend differences may not be as marked as in the typical working population. In another study, a progressive decrease in CAR magnitude over several weeks has been demonstrated in a military sample participating in an intense training program (Clow et al., 2006). This finding is likely representative of a response to exhaustion, although this was not measured directly it would also be in agreement with trait studies of burnout and fatigue (see Adam et al., 2006; Fries et al., 2009 for further discussion). In another study, no change in the CAR was observed during the build-up to athletic competition (Strahler et al., 2010) (see Table 2.1 for study details). The authors noted that this was unexpected and attributed the lack of variation in the CAR to psychological and neuroendocrine habituation to competition. Nonetheless, these inconsistent findings emphasize the need for further research in this area, in order to clarify the complex relationship between the CAR, psychological anticipation and prior-day experience.

Evidence of a relationship with prior negative experience and same day anticipated challenge has led to the prominent theory that the CAR plays a preparatory (homeostatic) role in healthy functioning activating and preparing the individual for the challenges of the day ahead (Adam et al., 2006; Fries et al., 2009). The means by which the CAR may prepare the individual are unknown. However, the results of studies looking at between participant variation in the CAR show that an increased CAR is associated with lower fatigue, increased alertness and energy (Adam et al., 2006; Fries et al., 2009; Thorn et al., 2011). Whether an increased CAR also enhances cognitive or

physical functioning in the day ahead are both strong possibilities. Evidence from trait studies does indicate that in older people a greater CAR magnitude is associated with enhanced overall cognitive performance (Evans et al., 2011), but it is yet to be explored whether state variation in the CAR results in state variation in cognitive function.

2.3.4 The menstrual cycle

Early work by Pruessner et al. (1997) indicated that the CAR is stable across the female menstrual cycle, and this was replicated by Kudielka & Kirschbaum (2003) in a study that compared females in the luteal and follicular phases of the menstrual cycle. A more recent study by Wolfram et al. (2011) again reported no difference in the CAR between the phases of the menstrual cycle, although there was significant elevation in the CAR during the 2 days of ovulation. The findings of this study showed not only an interaction between CAR magnitude and ovulation within participants, but also a later peak during ovulation (mean peak of CAR at 45 min during ovulation, as opposed to 30 min during all other phases). Although Wolfram et al. (2011) did measure mood states in this study (using the Profile of Mood States Questionnaire), no difference was observed in participants' moods between the phases of the menstrual cycle.

Future research in this area could take into account the status of other hormones known to affect cortisol levels, such as estrogen, as well as disruption of sleep across the menstrual cycle (particularly pronounced during the luteal phase) (Shechter & Boivin, 2010). Consistent sleep disturbance may produce

confounding variables in CAR studies not only by affecting participants' mood states, but also by complicating the measurement of initial wake time. Another factor to examine in such studies is cognitive function as evidence suggests that visuo-spatial memory is enhanced during ovulation, whilst verbal fluency is decreased (Solı´s-Ortiz & Corsi-Cabrera, 2008). If the CAR plays a role in orientation, context recollection and general cognitive activation upon awakening, as proposed by Fries et al. (2009), then it may be that the CAR is related to this enhanced visuo-spatial memory activation during ovulation.

2.3.5 Alcohol consumption

There has only been one previous state CAR study exploring alcohol consumption, in which the association between the two variables was not a primary aim of the study, and only a very simple method of measurement was used which did not take account of units or volume of alcohol (Stalder et al., 2009). Nonetheless, the results of this case study indicated a negative correlation between the CAR and the number of alcoholic drinks consumed during the previous evening (Stalder et al., 2009). This finding was observed in a case study of a healthy young male conducted over 50 study days (accounting for awakening time). This was interpreted as evidence of the acute effects of alcohol consumption in a healthy participant as opposed to the chronic effects of general alcohol consumption (associated with an elevated CAR in trait CAR research) (Adam et al., 2006; Badrick et al., 2008). However, this is the only previous exploration of the effect of alcohol on state variation in the CAR and therefore may not necessarily be considered a reliable finding if taken on its

own, and therefore this relationship was explored in the present PhD, in studies I and III (Chapters 4 and 6, respectively).

2.3.6 Other potentially related factors

There are a number of further state factors which may be related to the CAR, but which have not yet been explored in any depth. For instance, exercise (both aerobic and anaerobic) causes acute increases in serum cortisol (Kindermann et al., 1982), and increased basal levels of serum cortisol during the waking day are demonstrated in athletes during periods of intense activity (Tsai et al., 1991). Notably, these elevated basal cortisol levels were recorded at 1- to 1.5-h post-awakening, and therefore temporally proximal to the CAR period. Whilst it is common practice to discourage participants from exercising during the CAR testing period, so as to avoid a potential confound in studies, there has not been any research to investigate the effects of acute exercise during the prior day or the first hour post-awakening on the CAR. Although Stalder et al. (2009) provided evidence that motility was not associated with the dynamic of the CAR within a single case study, this was only a measure of motility within a range of resting states as the participant was instructed to avoid exercising during the CAR testing period. Likewise, the mode of awakening (alarm or natural awakening) does not appear to impact on the CAR (Stalder et al., 2009).

A further potential factor contributing to CAR state variation is the nature of cortisol secretion from the adrenal cortex. It is important to note that this secretion is not maintained at a steady rate, but rather follows a pulsatile pattern. The pulses vary in both amplitude and frequency, and the circadian

pattern is the result of changes in pulse frequency across the 24-hour period (Young, Abelson, & Lightman, 2004). The pulsatility of cortisol secretion has potentially important implications, as this means that the effects of psychological or environmental stimuli such as stress or bright light on cortisol will involve an interaction between the stimulus and the prevailing level of cortisol, which at least partially explains the state and trait variation in magnitude of cortisol stress responses (Young et al., 2004). It may be reasonable to hypothesise that the pulsatile secretory activity can explain some portion of the state and trait variation observed in the CAR, though this has not been investigated in previous research.

2.4 Aims and Thesis Summary

It has been argued that state-related increases in the magnitude of the CAR in healthy participants are a homeostatic response allowing for a physical or psychological ‘‘boost’’, either in anticipation of the day ahead or as a compensatory increase following negative experiences of the previous day (Adam et al., 2006). Support for this theory is provided by the finding that increased intraindividual variation in the CAR is associated with protective psychological factors for human health (Mikolajczak et al., 2010). Whilst further evidence is required in order for this account of the CAR to be truly tested, the possibility that appropriate CAR flexibility in response to coincident state factors may be a feature of healthy HPA axis responding certainly intensifies the difficulty in interpreting trait CAR research. It is arguable that future research should seek to establish whether a lack of flexibility in the CAR may be a

vulnerability factor which results in negative mood states, or is instead a product of repeated negative life events (see Lupien et al., 2009). In such a scenario, an interaction between state and trait factors would be apparent, with negative state factors at sensitive stages of the life course giving rise to sustained or ‘‘trait’’ characteristics in the CAR. It follows that longitudinal or comparative research exploring state variation in the CAR between individuals and across age groups may help to answer this question.

The present chapter highlights the need for further research into state variation to help establish the role of the CAR within the cortisol circadian rhythm. Since cortisol and the CAR are associated with hippocampal and frontal lobe function (see Fries et al., 2009 for review), it has been suggested (Clow et al., 2010a) that a rational direction for future research would be the measurement of the relationship between the CAR and neuropsychological processes underlying awakening (e.g. changes in regional cerebral blood flow and the activation of the frontal cortex in particular). Furthermore, the function of these brain regions should also be measured later in the day, to establish whether the CAR in fact prepares the individual for a day of enhanced cognitive function, in order to respond to anticipated challenge. In this way, research could establish whether the flexibility of the CAR serves the purpose of anticipatory cognitive preparation for challenge during the waking day. Exploring state changes in the CAR in relation to workload could perhaps help toward establishing whether a reduced CAR magnitude on days of rest is beneficial in terms of physical or psychological rest and recovery. The exploration of the association between the CAR and recovery from SI also presents an exciting opportunity, given the temporal associations between the two processes and many common

associations described in section 1.7. Such research might help to identify some of the causes of SI, and in turn may have important implications both for understanding human psychology and physiology, and potential for applications in a range of professions.

It is now well established that in healthy individuals, the CAR shows a large degree of variability across days, supporting a regulatory role within the healthy circadian pattern of cortisol secretion (Ross et al., 2014). Although limited in number, the studies reviewed here offer a relatively coherent emerging story about state factors that influence the CAR and the impact of the CAR on daily functioning. There is evidence that the magnitude of the CAR in healthy individuals is positively correlated with increased levels of ambient light in the morning, earlier morning waking times, anticipation of significant workload or challenge during the day ahead, negative experiences during the previous day, and also with the ovulatory period of the menstrual cycle in females. Although this literature is relatively small it appears generally more consistent than the literature on trait variation in the CAR. Indeed, since the degree of state variation is so large (e.g. Hellhammer et al., 2007; Ross et al., 2014; Stalder et al., 2009) this may well account for the lack of consistency in the trait variation literature. While the utility of the CAR as a trait biomarker is evident, it has been recommended that for a reliable assessment it is sampled over 6 consecutive days (Hellhammer et al., 2007) and it is notable that this recommendation is rarely implemented.

The overall aim of this PhD therefore is to further our understanding of the role of the CAR in the healthy human circadian cycle by exploring both psychological

and physiological correlates of state and trait variation. The design of the studies seeks to achieve this by studying both patterns of the CAR within-participants (correlates of daily variation) and between-participants (correlates of between-participant average CAR variation) in order to explore cause and effect relationships between the CAR and cognitive function. It is proposed that the examination of daily co-variation allows for stronger support for causality, and the application of an appropriate CAR sampling methodology will also alleviate the risk of the many potential confounds observed in previous studies, aiding the interpretation of these relationships and the implications for understanding the role and regulation of the CAR. It is evident from the research discussed here that understanding state variation in the CAR may be key to understanding its role in healthy functioning, and in turn, may shed light on the implications of abnormal CAR functioning. The present PhD therefore has potentially broad implications for illuminating the utility of the CAR as a promising biomarker in psychophysiological and epidemiological research. In the longer term, it is perhaps somewhat speculative but nonetheless worthy of consideration that better understanding the CAR might present the possibility of interventions in pathological illness, such as depression, to assist adaptation to shift work for those required to work at night, or even for the preservation of cognitive function in older age. Certainly this research should help to better establish the role of the CAR is in healthy functioning, which is a question that has existed in the field of psychophysiology for almost two decades (since first identified by Pruessner et al., 1997). In turn, this can provide insight into the factors which underlie everyday psychological and physiological functioning, and also influence the methodology of future studies using this biomarker.

3 General Methods

3.1 Ethical considerations

Protocols for all the studies were in accordance with the British Psychological Society guidelines for research with human participants and the Declaration of Helsinki. Ethical approval for studies I and III was provided by the Psychology Departmental Ethics Committee of the University of Westminster, UK, and Study II was approved by the Human Ethics Committee of the University of Adelaide,

Australia. A Control of Substances Hazardous to Health (COSHH) form was prepared and signed by researcher and supervisor, and was kept visible in the wet laboratory throughout cortisol assessment, and all analysis, storage, and disposal of cortisol samples was conducted in accordance with the Human Tissue Act (UK) (2004).

In studies II and III, participants attended a 30-min 1-to-1 briefing with the researcher upon recruitment, and in both the briefing and in the participant information sheet, it was made clear that participation was voluntary and all data collected would be kept confidential and anonymous. Participants read the information sheets and provided informed consent (Appendices 1, 2, & 3), and asked any necessary questions. Contact details of both the researcher and the Director of PhD Studies (Prof. Angela Clow) were provided in the information sheet and reiterated in the verbal debrief, should participants wish to ask questions or express any concerns or issues during or following participation. These details were also included with the study listing on the Research Participation Scheme (RPS) website where this was employed in Study III. This scheme is organised by the psychology department of the University of Westminster, with the aim of engaging undergraduate psychology students in research as a participant for a period of three hours in their first year of study. Participants were encouraged to participate only if they felt comfortable to meet the requests of the study protocol, and were made aware of their right to withdraw at any time without giving a reason. Where study credits were gained for participation (using the RPS) participants were made aware that equal credit was available from other studies listed on the RPS website, or alternatively, for

writing of a short essay detailing the ethical considerations for conducting research.

Due to the nature of these studies there was no requirement for major deception or withholding of information from participants. Minor deception was required with regards to specific content of the cognitive tests, for example, participants in Study III were informed that they would be required to take a memory test, but were not informed that there would be a follow-up test at session 2, but this was necessary in order to control for the risk of deliberate practice in the memory consolidation task. This is standard practice when conducting these common cognitive tests and was not considered to present any ethical issue beyond minor deception. Aside from these small details, participants were fully informed of the aims, rationale and procedure of these studies when consenting to participate. A reiteration of the study aims, including the details about the content of the cognitive tests, was provided the verbal debriefing, which took around 5-min. At which time the participants were also offered another opportunity to ask questions or raise any concerns they might have.

In all of the present studies, the participants were healthy adults without any impairments in communication or understanding. As such, informed consent could be ensured. Although, some participants in Study III were students of the researcher, who was therefore in a position of potential influence, participants were not coerced either to participate or to remain in the study. Participation in the studies did not increase participants' exposure to risk or harm greater than that which they might be expected to encounter in their normal daily routines.

Further, all efforts were made to ensure participants felt at ease throughout the study, including giving participants the opportunity to practice saliva sampling using a salivette at the time of the study briefing. In some cases, participants reported disliking the taste of the saliva-sampling swab. However, they were reassured by the researcher that these cotton swabs are entirely safe and harmless for saliva sampling. Completing the morning and evening diaries (described in section 3.6) was identified by the researcher as a potential source of discomfort, as it included disclosure of alcohol consumption, exercise, and reporting of psychosocial states. Therefore, participants were assured during the study briefing that they were under no obligation to disclose any information they did not desire. A confident and respectful relationship with participants was maintained at all times. All reasonable steps were taken to ensure that participants understood the nature of the studies, including provision of both verbal and written information about the studies and appropriate opportunities to ask any questions.

In all studies, participants' data were de-identified using a participant numbering system identifiable by only the researcher, and all necessary steps were taken to ensure data-security. Electronic copies of this data were stored in files on password-protected computers accessible only to the researcher, while a hard-copy record was kept in a locked draw accessible only by the researcher, in secure offices within the Psychology Department at the Universities of Westminster (Studies I and III) and Adelaide (Study II). All data collection, analysis and dissemination was conducted using these participant numbers to ensure anonymity. The de-identified participant data files were viewable only to

the researcher and supervisory team, all of whom were aware of procedures regarding anonymity and confidentiality.

In Studies II and III, on completion of each study, participants were given a 1-to-1 verbal debrief with the researcher, of around 5-min, and the opportunity to discuss with the researcher their experiences of partaking in the study and any feedback, questions or concerns they may have had. This debrief occurred immediately upon finishing the study. This allowed the researcher to monitor any unforeseen negative outcomes or misconceptions, such these could be addressed with regards to the participants concerned and also taken into account before any testing subsequent participants. This did not apply to Study I as this was a researcher-participant case study in which the researcher monitored his own experience and health throughout the study and communicated this to the Director of Studies (Prof. Clow). Finally, participants were informed that, voluntarily and separate from the 1-to-1 debrief, they could contact the investigator via email if they wished to receive news of the findings from the study with regards to group results. During the 1-to-1 debrief it was made clear that, in all cases, both in the 1-to-1 debrief and any email correspondence, participants would only be able to access group results and not individual results. This was to preclude unnecessary anxiety caused to participants should they for any reason compare these relatively sensitive data in an unfavourable way to published norms, or where particular CAR profiles may have been proposed as biomarkers of future ill-health.

3.2 Cortisol assessment and assay

3.2.1 Salivary cortisol

Salivary assessment is the most widely used method in modern psychobiological research, and all of the studies presented in this thesis make use of salivary cortisol assessments. It is well-established that the circadian rhythm of plasma free cortisol secretion is mirrored in salivary free cortisol (Aardal & Holm, 1995; Aardal-Eriksson, Karlberg, & Holm, 1998; Kirschbaum & Hellhammer, 1994).While cortisol can also be measured in serum, cerebrospinal fluid (CSF), urine and lachrymal secretions, saliva sampling has clear advantages over other measurement methods as it is simple and non-invasive, and allows the participant to collect multiple samples independently outside of the laboratory as they go about their normal daily routines (Umeda et al., 1981; Vining et al., 1983). Finally, the introduction of commercially available, reliable assays for evaluating salivary cortisol concentrations presents another clear advantage of this method.

In all of the studies presented in this thesis, saliva samples were obtained using 'Salivettes' (Sarstedt Ltd., Leicester, UK). These Salivettes consist of a plain cotton swab, contained within a polypropylene saliva examination tube with push cap (see Figure 3.1). The sampling method involved participants chewing on a cotton swab for 1-2 mins, until wet, and then returning the swab to the suspended insert within the Salivette and then firmly sealing the cap for storage. Recovery of the saliva sample from the cotton swab is achieved by centrifuging the Salivette, during which the saliva passes through a small hole in the suspended insert, and gathers in the centrifuge vessel. Participants were

instructed to continue with their normal routine in the morning, apart from those activities that would impair the CAR assessment; upon awakening, and throughout the saliva collection period, protocol instructions were to take nil by mouth other than water, and to refrain from brushing teeth to avoid abrasion and microvascular leakage. After taking the final sample, participants were free return to their usual routines.

Figure 3.1 The Salivette (Sarstedt Ltd., UK), consisting of (left to right) push cap, small cotton swab, suspended insert, centrifuge vessel.

3.2.2 Cortisol assay

The sampling and assay protocol was consistent across all of the studies described in this thesis. The assay procedure for studies I and III were carried out by the researcher in the PSRG laboratory at the University of Westminster, and the assay for Study II was carried out at the Robinson Institute of the University of Adelaide. Samples were frozen at -20 C within 1 day of collection and stored at this temperature until analysis. Samples were thawed and centrifuged at 1,500 g (at 3,000 rpm) for 10 min, after which cortisol

concentrations were determined by Enzyme Linked ImmunoSorbent Assay (ELISA; Salimetrics, State College, PA).

In all cases, the procedure provided by Salimetrics was carefully followed. Further, samples were assayed in duplicate and when comparing multiple participants (in studies II and III) it was ensured that each participants' samples were assayed within the same day. High and low cortisol controls, (27.6 nmol/l and 2.76 nmol/l, respectively) provided by Salimetrics were used to determine inter-assay variability, and this was observed as being below 10% for all studies presented in this thesis. In the, very rare, event that percentage variation between any duplicate samples exceeded 10%, these samples were re-assayed on another plate. It was therefore ensured that both intra- and inter-assay coefficients of variation were below 10% in all cases. The limit of detection of the assay was 0.33 nmol/L. Undetectable samples were treated as missing data, and all other cortisol measures were included in the final analysis.

3.3 Adherence to the CAR protocol

All of the studies presented in this thesis meet the criteria provided by Stalder et al. (2016) and Smyth et al. (2013a) for the strict control of adherence to the CAR sampling protocol. Self-collection of saliva samples for CAR assessment is typically undertaken in the domestic setting, and performed by the participant independent of any assistance from researchers. In all of the present studies, the CAR protocol involved sampling upon awakening, and then across at least the first 45-min post-awakening, at 15-min intervals, as displayed in figure 3.2.

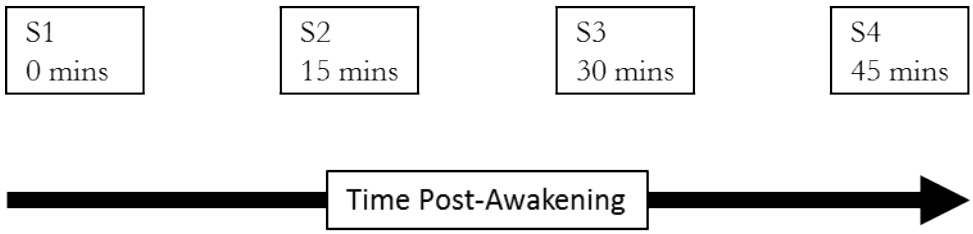


Figure 3.2 Schedule of saliva sampling

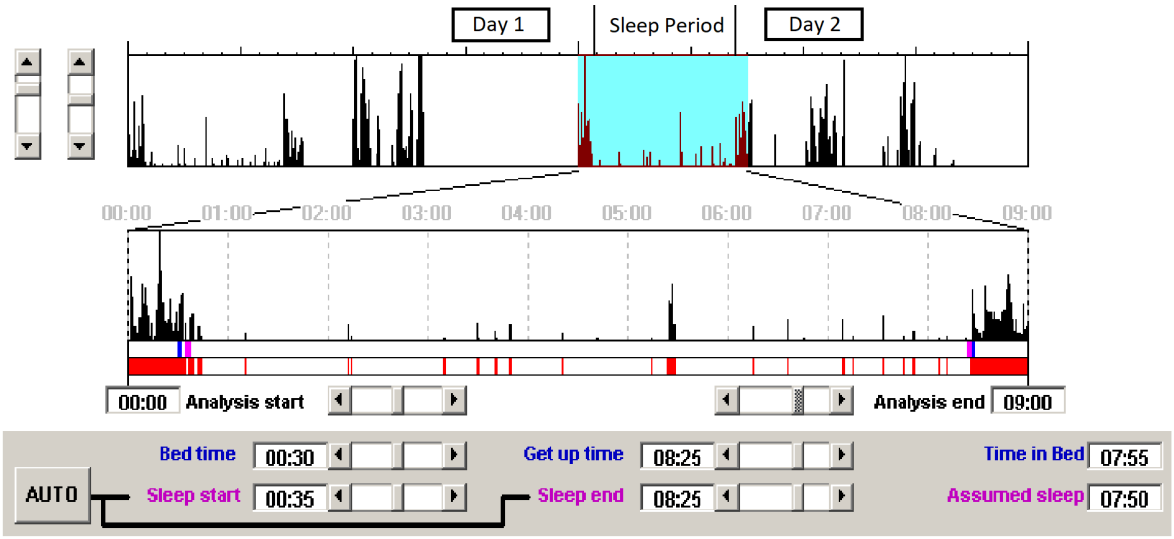
Given that the rapid increase in cortisol secretion at the beginning of the CAR period begins just 8-min post-awakening (Smyth et al., 2013a), CAR assessment is critically dependent upon accuracy of sampling times relative to awakening (Smyth et al., 2013a; Smyth et al., 2016; Stalder et al., 2016). Indeed, studies have indicated that delays between awakening and sampling result in inaccurate calculation of the CAR (Kudielka et al., 2003; Broderick et al., 2004; Kupper et al., 2005; Dockray et al., 2008; DeSantis et al., 2010; Okun et al., 2010; Griefahn and Robens, 2011; Smyth et al., 2013a; Golden et al., 2014; Smyth et al., 2016), and as such, the importance of strict adherence controls has been very emphatically stated in a recent expert consensus guidelines paper (Stalder et al., 2016). However, despite these many warnings, the great majority of CAR literature to date has failed to apply strict controls on participant adherence, instead relying on self-report alone. It has been estimated that only around 6% of studies conducted in the domestic setting employed electronic monitoring of awakening and sampling times (Smyth et al., 2016), and recent evidence suggests such widespread sample-timing inaccuracy may at least partially explain some of the inconsistencies reported in the CAR literature (Clow et al., 2010a; Smyth et al., 2016).

3.3.1 Electronic methods of monitoring adherence

Following the expert consensus guidelines of Stalder et al. (2016), and the recommendations of Smyth et al. (2013a; 2016), both the awakening times and sampling times of participants were monitored electronically. Precision in identifying delay is paramount, as failure to detect delay, or incorrect categorisation of data as delayed or non-delayed may confound interpretation (Smyth et al., 2016), therefore the controls applied in the present studies were very carefully applied. Awakening times and sleep efficiency were determined using Actiwatch-Score (Phillips Respironics, Surrey, UK; see Figure 3.3). These are wrist-worn, piezoelectric motion sensors that distinguish sleep and awakening periods by respective reduction or increase in activity. In line with the recommendations of Boyne et al. (2013), actigraphy-recorded awakening times were scored by the human eye, rather than relying on the (sometimes imprecise) computer algorithm. At least 10% of these researcher-scored Actiwatch-measured awakening times were further checked by members of the supervisory team, to ensure accuracy. Saliva sampling times were verified using Medical Event Monitoring (MEMS) (The Aardex Group, Sion, Switzerland) caps as described by Smyth et al. (2013a). Participants were also required to complete a sample time self-report sheet on each morning of the study for verification of self-report with electronic measures (see appendix 5). The times recorded by each device were carefully checked alongside the times recorded by the device with which the participants would record their self-reported wake and sample-times (e.g. a watch or mobile phone), to ensure any inconsistency in these

measures would not confound later comparisons between electronic monitoring and self-report.

(a)



(b)

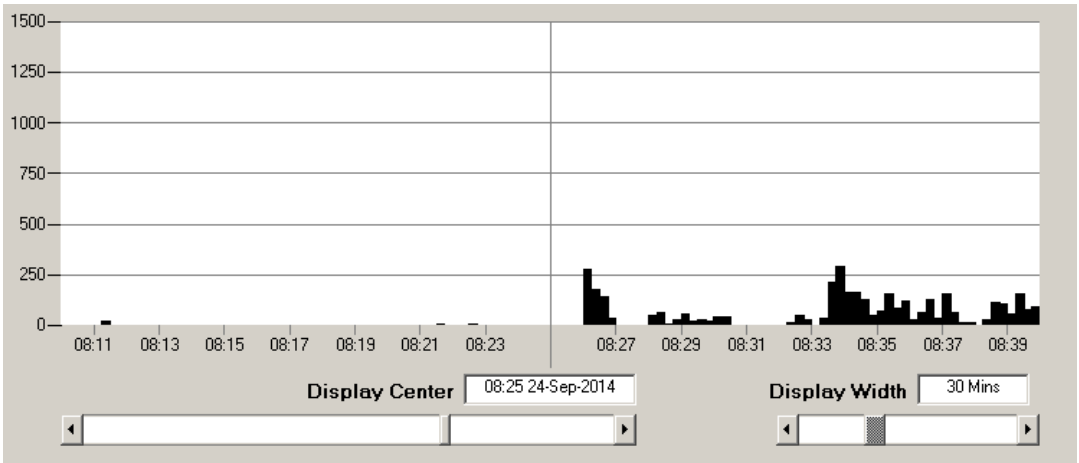


Figure 3.3 Actigraph analysis software used to determine the precise time of awakening (a) and detailed disaply of actigraphy data in the awakening period (b).

In all studies, data were treated as adherent where the difference between electronically measured wake time and timing of the first sample was ≤4 min, as delays greater than this have been shown to result in overestimated CARs (Smyth et al., 2016). However, where delay was >4 min but ≤15 min, these data were included in the analyses, and analysed in comparison to accurate data, as per the recommendations of Smyth et al. (2016). This was the case for the analysis of study III, in which sample time accuracy was divided into two groups (≤8 min, and 9-15 min) as, despite the potential importance of the small increase in CAR from 0-4 and 5-8 min in other contexts, for the purpose of the present thesis controlling for the clearly established larger decrease in CAR effect after 8-min was the primary concern. Any cases of electronically measured delay >15 min were excluded from the analysis, as the typical post-awakening growth-curve is not evident in such data, therefore rendering it unsuitable even for real-time modelling (Stalder et al., 2016; Smyth et al., 2016).

3.4 Defining the CAR

The CAR can be quantified in various ways. In each of the following formulae it is assumed that s1 is taken immediately upon awakening, s2 collected at 15-min, s3 at 30-min, and s4 at 45-min post-awakening. It is further assumed that

these are measured at equal time intervals, arbitrarily denoted at unity, for all samples. For example, the ‘‘area under the curve with respect to increase’’ (AUCi: s2 + s3 + [(s4 - s1)/2] - 2s1) (see Edwards et al., 2001b; Pruessner et al., 2003; Smyth et al., 2013b) for a discussion of this calculation); the ‘‘mean increase’’ (MnInc: (s2 + s3 + s4)/ 3 - s1) (see Wüst et al., 2000b) or a simple delta score, e.g. cortisol concentration at 30 min (or peak concentration) minus that on awakening (see Steptoe & Usher, 2006). Over the years, the CAR has also been assessed in relation to absolute levels of cortisol secretion post-awakening using measures such as the AUCg: s1 + s2 + s3 + [(s4 - s1)/2] (see Pruessner et al., 2003). Although the AUCg could be an interesting measure in some contexts, interpretation is limited as it gives no indication of the dynamic increase post-awakening, which is the defining feature of the CAR. It has been recommended (Clow et al., 2010a) that the best practice is to report the first waking sample plus the dynamic increase, from which the total secretion can be deduced. In the studies presented within this thesis, samples were taken immediately upon awakening and at 15-, 30- and 45-min post-awakening (s1–4, respectively) on each study day (notably with this inclusion of a 60-min, s5, in stuy I only), and wherever a composite CAR measure is used, this is calculated using the MnInc formula.

3.5 Computerised cognitive measures

3.5.1 Test selection

Cognitive function was primarily assessed using the Cambridge Neuropsychological Test Automated Battery (CANTAB®) (Cambridge Cognition, Cambridge, UK). From this battery, two CANTAB Eclipse version 5.0.0 tests were used in these studies; the 'Attention Switching Task' (AST) and 'One-Touch Stockings of Cambridge' (OTS), described in sections 3.6.2 and 3.6.3, respectively. While EF and cognitive flexibility can be assessed using a broad range of tasks, many of these are subject to practice effects and, therefore, unsuitable for repeated-measures assessment (Basso et al., 1999; Rabbitt, 1997). Therefore the selection of the AST as the primary EF measure in the present studies was in relation to it being specifically designed for this purpose (CANTAB, 2017). All CANTAB tests were administered using Windows operating system on a 15.6 inch touch-screen tablet computer. Participants' responses to the AST were recorded using a 2-button press-pad connected to the computer, while responses to the OTS were recorded using the touch-screen. All tests were administered by the researcher, after training in administration of CANTAB tests, and with strict adherence to version 5.0.0 of the CANTAB Eclipse 5 test administration guide (Cambridge Cognition Limited, 2012).

The various algorithms for producing outcomes from these tests include both mean and median modes for calculating overall performance across trials (for example, when calculating a participant's mean reaction latency, this is measured across the full 160 individual arrow presentations in the AST). The mean is the default option. The median can be the more useful measure of the central point when scores are influenced by outliers, for example, in a clinical sample. However, the studies in this thesis explore cognition in healthy functioning young adults, all of whom were familiar with the testing procedure

after completing the practice trials.. Such samples are less prone to abnormal responses, viz., outliers. In such a case, the mean is the more accurate measure of the central point (Wilcox, 2010). The mean score was therefore used in the algorithm to compute all performance scores from the CANTAB tests.

Aside from the CANTAB tests, cognitive function was also assessed using the Trail Making Test (TMT) and a word-recall and recognition, long-term memory (LTM) test. However, these measures were only employed in Study III, and are therefore described in detail separately in chapter 6, section 6.3.3.

3.5.2 The AST

The AST is a test of EF that provides a measure of attentional set-shifting; processes involving the prefrontal cortex (Cambridge Cognition, 2017). This is calculated from response times recorded from 160 trials, with the complete test taking around 6-8 min to administer. The task consists of repeated presentation of an arrow that can appear on either side of the screen (left or right) and can point in either direction (left or right). The presentation of the arrow is accompanied by a cue in the form of a response instruction at the top of the screen. This instruction either requires the participant to respond to the spatial location (“side on which the arrow appeared”) or the direction of the arrow (“direction in which the arrow was pointing”) by pressing the right or left button on the response-pad. Figure 3.4 shows an example screenshot from the AST. The presentation sequence of these trials is randomised so as to prevent sequence learning. The participant is asked to ignore task-irrelevant information or distracting events, and to respond to the presentation of the stimuli by

pressing the appropriate key on the response pad (left or right) as quickly as they can.

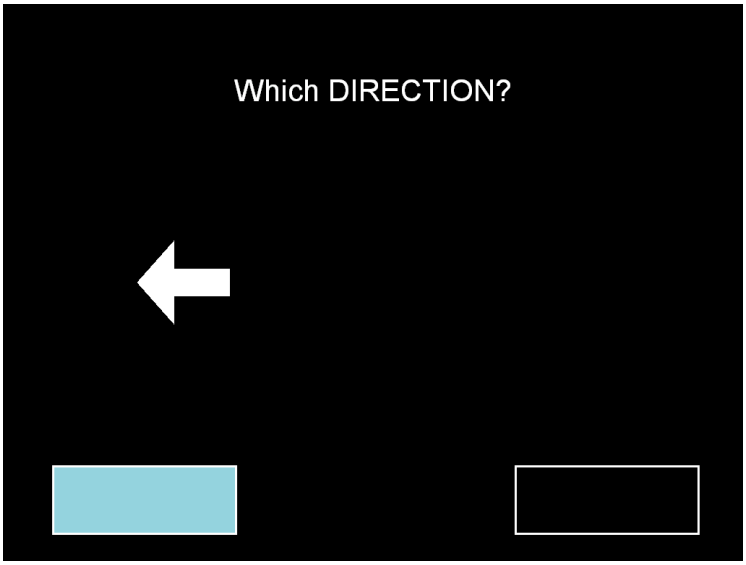


Figure 3.4 Screenshot from the CANTAB AST, showing a trial and response instruction to a direction-based problem. Adapted from Cambridge Cognition (2017).

Outcome measures of the AST include simple reaction time latency (ms), percent incorrect trials, total omission errors (no response within the trial window), total commission errors (a response prior to the appearance of the stimulus or the end of the pre-empt window), the difference in response latency for trials where direction and spatial location are congruent and incongruent (congruency cost), and the difference in response latency for switched and non-switched trials (switch cost). The switch cost measure is an index of mental flexibility as it is influenced by the interference of incongruent task-irrelevant information (i.e. a Stroop-like effect). This calculation included all switched trials, meaning any case where the trial type was ‘direction’ but the previous

trial type was ‘side’, and vice versa. Switch cost is calculated as the difference in mean reaction latency for switched trials compared to the mean reaction latency for non-switched trials and, as such, a smaller switch cost score is representative of better EF performance. However, for the purposes of presentation, switch cost scores were inverted in the present analysis such that the switch cost variable, ‘‘EF’’, is positively scored.

3.5.3 The OTS

The OTS is based on the Tower of London task and assesses spatial planning and working memory components of EF, as an index of frontal lobe function (Cambridge Cognition, 2017). The CANTAB Eclipse 5 battery includes five versions of this test that differ by the number and difficulty of trials. The version used in the present study was ‘7-choice-15’, and was selected as the most appropriate for the sample population of healthy functioning, young adults, of at least undergraduate level education, as it is designed to be very challenging even for able subjects (Cambridge Cognition, 2012). EF in this version of the test is calculated in this version of the test from performance across 15 problems, with the complete test typically taking around 7-10 min to administer.

In this task, the participant is presented on-screen with two sets of three coloured balls suspended in a row of three stockings, each able to contain up to three stacked balls. One set is displayed at the top of the screen and the other at the bottom. The participant is read a standardised set of instructions explaining that the goal is to rearrange the balls in the bottom display to match the layout of the top display. These instructions further ensure the participant

understands that balls will sit at the bottom of the stocking, or on top of another ball, and that they cannot move a ball that is beneath another ball. Prior to commencing the task, the test administrator first demonstrates the touch-screen method. The participant is then required to solve three practice problems of two, three, and four moves, presented in ascending difficulty. After this, the participant is instructed that for the remainder of the test they will not be required to move the balls, but instead should calculate in their head the minimum number of moves required to complete the task and touch the appropriate box at the far bottom of the screen to indicate their answer. These problems are of varying difficulty but are presented in randomised order to prevent sequence learning. The maximum number of moves required to solve the most difficult problems is always one fewer than the number of options across the bottom of the screen, to prevent participants from simply guessing the largest number of moves if a problem looks difficult. Therefore, for the 7-choice version of the test, 6 moves is the maximum number required in any one problem (in such case, requiring a 6-item spatial working memory span to complete), and 1 move is the minimum number as required by the simplest problems. Figure 3.5 shows an example screenshot from the OTS with a participant’s correct answer selected.

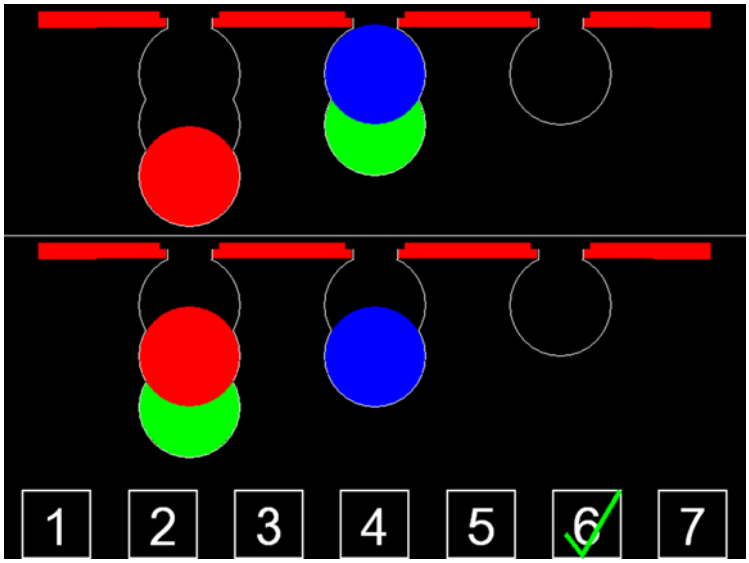


Figure 3.5 Screenshot from the CANTAB OTS, showing a trial and response instruction to a 6-move problem. Adapted from Cambridge Cognition (2017).

Outcome measures of the OTS include the number of problems solved on first choice, mean choices to correct, mean response latency (ms) to first choice, and mean response latency (ms) to correct choice. These outcome measures can be adjusted to include all trials or restricted to include only those of a particular number of moves. For the present study, the selected outcome measure of interest from the OTS was the mean response latency (ms) to correct choice. This is calculated as the mean latency (from all trials) from the appearance of balls on the screen until the participant touches the box indicating the correct number of moves required. A lower score on this response latency measure is, therefore, representative of better EF performance. Of the various outcome measures, latency to correct answer was selected because it provided the most appropriate and complete index of EF. For example, the latency to first choice measures latency, but is limited in usefulness as it does not account for

correctness of solution, and the choices to correct measure accounts for correctness, but not for latency. Unlike the other measures, latency to correct incorporates both processing speed and the time added by any errors.

3.6 Psychosocial measures

To measure psychosocial variables, evening (pre-test) and morning self-report 'diaries' were constructed. These diaries were used in all of the studies presented in the present thesis, with only minor adaptations between studies, as described here. Copies of these diaries are presented in appendices 6, 7, and 8.

Participants were instructed to complete the evening diary upon going to bed. This diary consisted of an adapted version of the Pittsburgh Sleep Diary (Monk et al., 1994); measuring levels of perceived obligation and mood throughout the day, and anticipated sleep quality, followed by the Stress/Arousal Check List (SACL; described in section 3.2.1). The adapated version of the Pittsburgh Sleep Diary was based on that of Stalder et al. (2010a), and included a series of pre-validate 10cm visual analogue scale measures for participants to self-report on their psychosocial state. These VAS measures include arousal, alertness, stress, tension, happiness, obligations, leisure, and anticipated sleep quality, and reflected both the present state, and the retrospective assessment of state throughout the day. The evening diaries were also used to record self-reported alcohol consumption and hours of exercise on each day prior to CAR sampling.

Evening alcohol consumption was recorded in estimated units of 10ml alcohol, and exercise recorded as sustained moderate to hard exercise of both aerobic and anaerobic nature (both self-reported, and based upon activities during that particular day only; see appendix 8). In study II, these criteria for calculating prior evening alcohol consumption and prior day exercise duration were explained in the study briefing. The self-reporting of pre-study day exercise and alcohol consumption in Study III was aided by an attached guide to 10ml units of alcohol in commonly consumed alcoholic drinks, and self-reporting of exercise was aided by an adapted version of the perceived exertion scale (Borg, 1982) to identify appropriate grades of exercise to include in the calculation (see appendix 8).

The morning diary consisted of the Pittsburgh Sleep Diary and SACL only. The VASs included in these morning diaries were adjusted to provide measures of present psychosocial states and anticipations of states during the coming day. A retrospective self-report of the number of awakenings during the night was also included in the morning diary.

3.6.1 The Stress/Arousal Checklist

The SACL provides a self-report measure of state arousal and stress the participant is experiencing at that moment in time, and was developed by Mackay, Cox, Burrows and Lazzerini (1978). The SACL was developed by factor analysis of participant responses to a checklist of 45 mood adjectives, indicating two orthogonal factors, which were identified by Mackay et al. (1978) as stress and arousal. Positively loaded onto the stress factor were adjectives such as

tense, bothered and worried, and loading negatively onto this factor were adjectives such as peaceful, pleasant and calm. For the arousal factor, positively loaded adjectives included lively, energetic and alert, while negatively loaded adjectives included sleepy, drowsy and sluggish. The 45-item SACL was validated by Mackay et al. (1978) for use in UK population samples. Scoring of responses is indicated on a 4-point scale ("definitely feel", "slightly feel", "cannot decide" and "definitely do not feel"), with reverse scoring applied to the negatively loaded adjectives. The standard scoring method is to score positive items such that "definitely feel" or "slightly feel" are assigned a score of one, and a zero is assigned for either of the other two responses. Negative items are scored such that "cannot decide" or "definitely do not feel" are assigned a score of one, and a zero is assigned otherwise. In sum, the range of possible scores are 0-19 for stress and 0-15 for arousal, with higher scores indicating greater stress and arousal.

The full, 45-item SACL was used in Studies I and II, where the participants were researchers, and a revised version was used in Study III. This revised version was the same as that used by Thorn (2007), who omitted 11 adjectives with factor loadings below 0.40, which included less commonly-used adjectives, such as "somnolent". The revised SACL consisted of 34 items, of which 19 were stress adjectives and 15 arousal adjectives.

3.7 Approach to statistical analysis

In all studies, undetectable samples from the cortisol assay were treated as missing data, and all other cortisol measures were included in the final analysis. These data were tested for normality of sample distribution, as the assumption of normality is required for the use of parametric statistical tests. Cortisol data are often positively skewed (e.g. Edwards et al., 2001b), and where tests of normality revealed this to be the case, appropriate (square root or log10) transformations of the raw data are used to ensure normalised sample distributions for each study. In the present studies, raw data were either normally distributed (studies I and II), or so few of the samples were skewed as to render such transformations detrimental to the normality of the data (study III). However, where CAR composites were produced, these were calculated for each participant for each study day and appropriate transformations were applied to the composites to ensure normally distributed data.

Data were analysed using mixed regression modelling. Although, the traditional statistical approaches to cortisol data have followed the general linear model, the use of mixed regression modelling allows for a more flexible and efficient analysis of the data, without the requirement for crude artificial correction for sphericity violation. Such modelling is more suitable for data patterns involving multiple repeated measures and nested data (Twisk, 2006), as is the case in CAR studies in which cortisol data is measured across several time-points and is nested within individuals. A clear advantage of mixed regression modelling is its robustness to missing data in repeated-measures analysis, as is often the case in cortisol research, due to, for example, participant errors or samples falling below detection of the assay. That these models allow for missing data (assuming it is ‘randomly’ missing) without excluding the entire case prevents

the needless exclusion of participants from such studies, and is therefore more efficient as it increases the overall statistical power provided by the analysis. This is also a more ethical and cost-effective approach to cortisol research, as it means that participants' time collecting samples and the funds spent on the samples and assay, are not wasted by unnecessary data exclusion.

Mixed regression modelling can be considered a compromise between frequentist and Bayesian approaches to statistical analysis. For example, the model is structured hierarchically with the assumption that parameters are random, however, hyperparameters are estimated from the data (Demidenko, 2004). Such models further allow for simultaneous consideration of both within- and between-participant associations, and are therefore considered more appropriate for designs incorporating multiple repeated measures over time with both fixed and random parameters (Blackwell, de Leon, & Miller, 2006), in addition to modelling of dynamic aspects of the diurnal cortisol cycle (Adam & Kumari, 2009) rather than being restricted to over-simplified linear relationships (Smyth et al., 2016).

In all cases, the selection of the best model was based upon lowering of Schwarz's Bayesian Information Criterion (BIC) (Schwartz, 1978). As compared to other closely related information criterions, such as the Akaike Information Criterion (AIC) (Akaike, 1974), the BIC is the more conservative choice as it applies a greater penalty to the number of parameters in the model (Burnham and Anderson, 2002), and therefore favours simpler models.

The final stage in each analysis was the modelling of extraneous and potentially confounding variables, for example, participants' time of awakening, cortisol levels upon awakening, and sampling delay. These were explored by way of creating secondary models, including the respective variables and testing both for associations or interactions with these variables as influencing the dependent variable, and observing whether any previously significant effects remained robust to the inclusion of these variables in the model.

Other statistical analyses included Pearson's tests of correlation to explore zero-order relationships between cortisol measures other variables, and exploratory factor analysis to explore components underlying psychosocial variables and related situational measures. For all correlations, scattergrams were produced to check that any significant associations were valid and not unduly influenced by outliers. All given values of p are two-tailed. All statistical analysis was performed using SPSS for Windows version 23, and all visualisations of data in the present thesis were performed using Prism (GraphPad Software, CA, USA).

4 Study I

Case Study of the CAR and Cognitive Performance in a Healthy Young Adult

4.1 Overview

A relationship between individual differences in trait estimates of the cortisol-awakening response (CAR) and indices of executive function (EF) has been reported. However, it is difficult to determine causality from such studies. Moreover, the reliance on data from abnormally functioning or older adults means that previous research offers limited insight into this association within the context of healthy functioning young adults.. The aim of the present study was to capitalise upon state variation in both variables to seek stronger support for causality by examining daily co-variation. A 50 days researcher–participant case study was employed, ensuring careful adherence to the sampling protocol, in-keeping with the recommendations of Stalder et al. (2016). A 24-year-old healthy male collected saliva samples and completed an attention-switching index of EF on the morning of each study day. Subsidiary control measures included wake time, sleep duration, morning fatigue, and amount of prior day exercise and alcohol consumption. As the CAR preceded daily measurement of EF, it was hypothesised that, over time, a greater than average CAR would predict better than average EF. Results of mixed regression modelling of variation in cortisol concentrations indicated that a larger increase from 0 to 30 min post-awakening predicted better EF at 45-min post-awakening ($t = 2.29$, $p = 0.024$), independent of a range of statistical controls for potentially confounding variables. These findings are discussed in terms of implications for the understanding of the relationship between the CAR and cognitive function, and the previously suggested role of the CAR in “boosting” an individual’s performance for the day ahead.

4.2 Introduction

Although the precise function of the CAR remains unknown, numerous studies have indicated relationships between the CAR and indices of cognition including declarative memory (Rimmele et al., 2010; Wolf et al., 2005), prospective memory (Bäumler et al., 2014), working memory (Moriarty et al., 2014), and executive function (EF) (Evans et al., 2012). While a relationship between individual differences in CAR magnitude and executive function has been demonstrated in between-subjects studies (e.g. Aas et al., 2011; Cullen et al., 2014; Evans et al., 2012), the impact of daily variation in the CAR on EF has not been explored. The aim of the present study was to use an individual case study approach to explore associations between daily variations in the CAR and a measure of EF.

EF can be understood as a range of functions including inhibition and interference control, working memory, and cognitive flexibility (Diamond, 2013; Miyake et al., 2000). One of the aspects of cognitive flexibility is the ability to switch between task demands, often assessed using attention switching paradigms (for review, see Diamond, 2013). Evans et al. (2012) indicated that better performance on one of these tasks, form B of the Trail Making task (TMT; Arbuthnott & Frank, 2000), is associated with a larger CAR in older adults.

Current theories for the role of the CAR within the human circadian rhythm suggest that it serves to provide an unspecified physiological or psychological

‘‘boost’’ upon awakening (Adam et al., 2006; Clow et al., 2010a; Fries et al., 2009). These theories are supported by studies reporting state associations between the CAR and both negative prior day psychological experience and anticipation of demand in the day ahead (e.g., Adam et al., 2006; Stalder et al., 2010a). Such theories might support the idea of a state association between the CAR and EF, as optimising EF could serve as a homeostatic response suitable for tackling the expected challenges of the waking day.

Associations between CAR and psychological state also present a possible potential mediator or moderator for a relationship between CAR and EF. High levels of sleepiness, for example, have been shown to be associated with lower levels of cortisol 15 min after awakening in healthy participants (Dahlgren et al., 2009). Further, studies by Stalder et al. (2009) and Adam et al. (2006) have demonstrated that psychosocial state predicts CAR magnitude. However, this appears limited to states of anticipation or arousal, but not stress. Recent studies have shown that measures of neuroticism, stress and negative mood are not related to the CAR itself, but are related to overall post-awakening cortisol secretion and elevated cortisol in the post-CAR period (Weik and Deinzer, 2010; Garcia-Banda et al., 2014). It is apparent, therefore, that studies of CAR-cognition associations should consider the potential moderating or mediating influence of psychosocial state variables, and that measurement across a slightly longer period post-awakening (e.g. 60 min, rather than the usual 45 min) would be best suited to observe such associations accurately.

4.2.1 Aims

The aim of the present study was to use a researcher– participant case study design to investigate in detail whether daily variation in the CAR predicted daily variation in EF at the end of the CAR period. The primary hypothesis was that the magnitude of increase in cortisol secretion post-awakening (CAR) would be predictive of subsequently better EF performance in the same morning.

4.3 Method

4.3.1 Design

Ethical approval was provided by the Institutional Ethics Committee. The study employed a 50 days researcher– participant case study design based on the novel study of Stalder et al. (2009, 2010a). Use of a researcher–participant provides a novel and convenient method for reducing the reliance on participant adherence to the protocol. Non-adherence to the requested saliva sampling protocol, of as little as 5 min between awakening and collection of the first sample, can lead to inaccurate CAR assessment (see Clow et al., 2004; Kudielka et al., 2003; Smyth et al., 2013a; Thorn et al., 2006). Intensive testing over 50 days is exceptionally demanding for participants and it has been reported that participant adherence decreases over a period of just 7 days (Broderick et al., 2004). The researcher–participant design ensures sustained motivation and commitment to the study, maximising adherence (which is checked by electronic monitoring) and reducing data wastage. In order to establish whether cognitive testing itself might influence the CAR, two separate cognitive testing protocols were produced. These were designed to entail a ‘heavy cognitive load on waking’, and a ‘light cognitive load on waking’ (labelled ‘Type 1’ and ‘Type 2’, respectively). These two types of cognitive testing protocols were then used

on alternate days throughout the study, allowing for comparison of cortisol data between ‘heavy’ and ‘light’ testing schedules. The possibility of introducing bias was avoided as all data were logged and analyzed at the end of the study. The participant could not be aware of daily CAR magnitude, avoiding the possibility of biasing the results, consciously or unconsciously.

4.3.2 The studied case

The researcher–participant (RL) was a 24-year old non-smoking male in postgraduate education, who described himself as healthy and free from medication. Similarly to the previous CAR case study participant, TS (Stalder et al., 2009; 2010a), RL reported very little difference in perceived workload or challenge between weekdays and weekends, given his status as a postgraduate student.

4.3.3 Materials

The CANTAB AST was used to measure switch cost and simple reaction time (ms), as described in chapter 3, section 3.5. To alleviate the risk of an AST learning effect, the participant completed a short pilot prior to study commencement. A practice effect was observed, such that mean reaction times on the AST task decreased with successive trials. However, a relatively

consistent level of performance was achieved prior to commencement of the case study reported here.

To objectively assess fatigue, critical flicker frequency (FF) was measured using a flicker fusion system (Lafayette Instrumentation, Lafayette, IN). This is a measure of the frequency at which rapid flickering light becomes impossible to detect to the human eye. FF is subject to within-individual variation and is a well validated and widely used, objective measure of central fatigue (e.g. Davranche & Pichon, 2005; Simonson & Brozek, 1952). The equipment set-up for the morning testing sessions, including CANTAB computer and FF system, is displayed in figure 4.1. Evening and morning diaries were used to record psychosocial state variables (as described in section 3.6), and these are presented in Appendix 6. The evening diary was also used to record self-reported alcohol consumption and hours of exercise on each day prior to CAR sampling (Alcohol was recorded in estimated 10 ml units of alcoholic content, and exercise was recorded in the number of hours of self-assessed high-intensity, sustained aerobic, or anaerobic exercise during the day).

Figure 4.1 Photograph of study equipment set-up in participant's home environment in preparation for morning testing. Including FF system (far left) and CANTAB computer (far right).

The study was designed such that data from the flicker fusion upon awakening and at 45 min post-awakening, and also the AST at 45-min post-awakening, would be available for all study days, while data from the AST at 15 min post-awakening and the PAL would only available for half of the study days. This was achieved by implementation of an alternating morning protocol (type I and type II days, described below in section 4.3.4; Procedure). This alternating protocol was included in order, first, to explore CAR magnitude subsequent to manipulation of 'cognitive load' in the post–awakening period, and second, to provide some limited estimates of association between EF performance earlier in the post-awakening period and later EF performance. This also allowed for exploration of whether inclusion of cognitive testing earlier in the post-

awakening period might influence any CAR-EF association at 45 min post-awakening.

Ambient light was recorded using a HOBO Pendant Light Data Logger UA-002-64 (Onset Computer, Bourne, Massachusetts, USA) which was worn on a lanyard by RL throughout testing, and kept by the bedside throughout the night – thereby accompanying the participant throughout the case study. This procedure has been successfully implemented in previous research using the same device (Martinez-Nicolas et al., 2011), and was intended to allow for measurement of ambient light both pre- and post-awakening to be included in the analysis here.

To provide an objective measure for validation of self-reported sleep onset and awakening times, actigraphical recordings were collected using a wrist-worn Actiwatch (Philips Respironics, Surrey, UK).

4.3.4 Procedure

Data were collected on a total of 50 days, at 3-day intervals, so as to match the procedure of the previously mentioned CAR case study (Stalder et al., 2009). Fifty study days provided statistical power to test the hypothesis and collection at regular intervals, 3-days apart, standardised the protocol and minimised study fatigue in the participant (which might happen with daily testing). Data collection took place in the United Kingdom between the months of January and May. Salivary cortisol was assessed at 5 time points across the post-awakening hour: immediately on awakening (0 min), and then at 15, 30, 45, and 60 min post-awakening. All samples were collected and handled as per the standard

protocol described in section 3.2. Wrist actigraphy was used throughout the study to check self- reported sleep onset and awakening time with an objective measure of awakening time and sleep duration, as described in section 3.3.1.

On each evening prior to morning cortisol sampling, the participant recorded the duration of exercise and total units of alcohol consumption for the pre-study day. On type I days, shortly after awakening, and immediately after collecting the first cortisol sample, the participant completed the flicker fusion task, followed immediately by the AST. This was followed by a short break before taking the second saliva sample at 15 min post-awakening. The AST was then completed again at approximately 45 min post-awakening (after collection of the fourth saliva sample) and followed immediately by the PAL task (prior to collecting the 60 min post-awakening sample). On all mornings, although required to be seated for the psychological testing, the participant was otherwise free to move around throughout the sampling period.

On type II study days (alternating with type I described above), the procedure was adapted such that the first AST, and the PAL task, were both excluded, allowing the participant more time to rest during the sampling period. On type II days, the participant completed flicker fusion at both times, but the post-45-min AST only.

In-line with the previously published researcher participant studies (Stalder et al., 2009, 2010a), all data handling and analysis was carried out after completion of data collection.

4.3.5 Treatment of data

Data were analysed using mixed regression modelling (Blackwell et al., 2006) of variation in cortisol concentrations in the first hour after awakening. In healthy males, the first 0–30 min period typically characterises the CAR, and the 30–60 min period is typically characterised by decline in cortisol concentrations. Each period was addressed in independent analyses comprising the running of two consecutive models. In model A (within day), time of sampling (0, 15, and 30 min) was entered as a fixed covariate with day of study (0– 50) as the subject variable. In model B (within day + between day) the covariate of z-scored EF was added to the model together with its interaction with time of sampling. As noted in section 3.5.2, for the purposes of presentation, the EF variable was produced by inverting the switch cost variable, such that high scores represent better EF performance. At each modelling point, three ways of modelling residual covariance were compared: random intercept only (equivalent of compound symmetry for repeated measure covariate), random intercept + random time, and finally a first level autoregressive (AR1) covariance structure. In all cases, AR1 provided the best fit of the data as indicated by minimising of Schwarz's Bayesian Criterion (BIC), and all models presented here adopted an AR1 covariance structure for the repeated measure of cortisol sampling time. Finally, further modelling was undertaken to check that any findings from the principal model were not compromised by extraneous and potentially confounding variables, including the passage of time (5 months) over the course of the trial.

The identical psychosocial measures taken at the two morning time-points (i.e. the 15 minute and 45 minutes) were highly correlated in all cases and were therefore combined to produce single variables accounting for the mean response to each item. A principal component analysis was then performed on the amalgamated psychosocial data and FF scores, with varimax rotation. The various indicators of factorability were good, and the residuals indicate that the solution was a good one. Two components with an eigenvalue of greater than 1.0 were found, and the scree plot also indicated two components. The components can be thought of as representing the extent to which participant RL was reporting stress/unhappiness and arousal/reduced fatigue: component 1 – stress; component 2 – arousal. The components and the variables that load on them are shown in table 4.1. The Stress and Arousal factors produced from this analysis were then explored in terms of the possible mediation or modulation of associations between the CAR and EF.

Table 4.1 Components found by principal component analysis, and the variables that load on to them(n.b. All variables represent Visual Analogue Scales, apart from 'SACL' and FF measures described previously).

Component 1	Component 2
SACL Stress	SACL Arousal
Poor Sleep Quality	FF Score
Tension	Alertness
Obligations	
Reduced Leisure	
Reduced Happiness	
Stress	

With regard to measurement of ambient light, data from the light logger were minimal in quantity, and on many days the device appeared to have failed to detect ambient light despite RL reporting bright ambient light during data collection. Initial analyses also indicated there was no relationship between the CAR or any cortisol measures and the very limited ambient light data available. The light logger data were therefore excluded from the remaining analyses.

As with the 50-day case study design of Stalder et al. (2009) and Stalder et al. (2010a) the data of the present study represent a time series (repeated measurements from the same subject), and are therefore subject to the same potential for time trends influencing the analyses (Gujarati, 2003; Stalder et al., 2009). In order to control this possibility, the same precaution was taken as in Stalder et al. (2009); a variable was created representing the linear sequence of measurement days, and the bivariate correlations between this and all of other study variables were assessed so as to indicate the presence of any time trends.

4.4 Results

4.4.1 General descriptives

The cortisol data showed a similar pattern of state variation across the 50 days period to that previously observed in participant TS (Stalder et al., 2009). Figure 4.2 displays the mean (SD) salivary cortisol concentrations (nmol/l) across the full 50-day study and Table 4.2 presents descriptive data and linear time trends for all relevant variables. As expected the peak in cortisol is found at 30-min post-awakening, followed by a decline until 60-min post awakening.

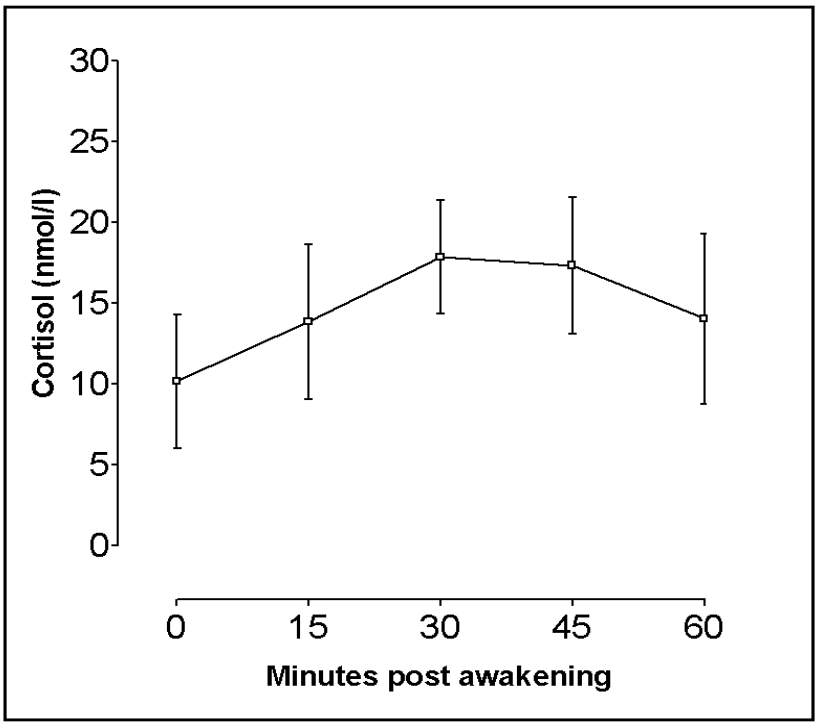


Figure 4.2 Mean (S.D.) salivary cortisol concentrations (nmol/l) for participant RL across 50 days.

Table 4.2 Descriptive statistics and linear time trends for cortisol samples (0-60min), sleep variables, reaction time, EF, flicker fusion, prior day alcohol consumption, prior day exercise, stress and arousal, and CAR measures.

	Mean	SD	Linear Time

			Trend
Cortisol 1 (0 min)	10.17	4.14	.072
Cortisol 2 (15 min)	13.85	4.81	.128
Cortisol 3 (30 min)	17.86	3.50	.210
Cortisol 4 (45 min)	17.33	4.26	.296*
Cortisol 5 (60 min)	14.05	5.27	.389*
Wake time (hh:mm)	8:16	1:03	.033
Sleep duration (hh:mm)	6:21	0:52	-.100
Reaction latency mean (ms)	282.46	20.15	-.767**
Task switching at 15 min	6.81	14.71	.391
EF (task switching) (ms)	9.18	11.51	-.006
Fatigue (flicker frequency) (Hz)	35.22	1.65	-.408**
Prior day alcohol (units)	0.94	1.91	.123
Prior day exercise (approx. hrs)	0.39	0.65	-.163
Stress component	n/a	n/a	.247
Arousal component	n/a	n/a	-.043
MnInc	6.18	3.97	.056
AUCg (45-min)	45.46	9.89	.263**
AUCg (60-min)	61.15	11.86	.365**

MnInc = Mean Increase (0-45-min post-awakening),

AUCg (45-min) = AUCg measured across 45-min post-awakening,

AUCg (60-min) = AUCg measured across 60-min post-awakening,

* Significant at .05 level, ** Significant at .01 level.

4.4.2 Modelling of data

4.4.2.1 Cortisol from 0 to 30 min post-awakening

Model A analysis of the rise in cortisol secretion from 0 to 30 min post-awakening showed the expected association with sampling time ($t = 13.39$, $p =<0.001$). The intercept coefficient (10.15) indicates a predicted concentration of cortisol of 10.15 nmol/l at awakening. The slope coefficient of 0.26 indicates a predicted rise of 0.26 nmol/l per minute over the 30 min period, giving an estimated rise (CAR value) of 7.8 nmol/l.

Model B of the 0–30 min data (introducing main and interactive terms for EF) indicated the same rise in cortisol (i.e. the CAR) as observed in model A with regard to intercept for slope (10.15) and slope coefficient for sample time (0.26). Further, with regard to the overall effect on cortisol of day differences in EF, no difference was observed in prevailing starting values ($t = -1.66$, $p = 0.101$). However, the slope interaction between sample time and z-scored EF (ZEF) is significant ($t = 2.29$, $p = 0.024$), indicating that the slope of the mean line varies with EF performance. For every standard deviation above or below mean EF, there is a 1.2 nmol/l increase or decrease in the CAR. Therefore, the model predicts that on days when EF is +1 SD, the slope is 9 nmol/l, and on days when, EF is 1 SD, the slope is 6.6 nmol/l. Table 4.3 shows coefficient estimates and significance (p) values for the parameters in the modelled data.

Table 4.3 Cortisol increase from 0-30 min associated with better than average Executive Function (EF). Comparison between simple Model A including only sample time and cortisol, with Model B including z-scored EF (ZEF).

	Model A		Model B	
	Coefficent (SE)	*P*	Coefficent (SE)	*P*
Fixed effects				
Intercept	10.15 (.57)	<.001	10.15 (.57)	<.001
Sample time	0.26 (.02)	<.001	0.26 (.02)	<.001
ZEF			–0.95 (.57)	.101
Sample time * ZEF			0.04 (.02)	.024
	Variance (SE)	*P*	Variance (SE)	*P*
AR Diagonal				
AR1 diagonal	16.36 (2.49)	<.001	16.19 (2.50)	<.001
AR1 rho	0.70 (0.05)	<.001	0.71 (0.05)	<.001

SE = standard error

Further modelling examined whether the effects observed within the 0–30 min period were confounded by the covariates of time (day 1 through day 50), wake time, sleep duration, reaction time scores, prior day exercise, prior day alcohol consumption, fatigue on awakening (FF), or presence of the AST at 15-min post-awakening. These variables were entered separately into the model, so as to achieve a suitable ratio of variables to cases and conserve degrees of freedom.

The results of these analyses indicated that the interaction between CAR-EF proved robust to each of these controls, remaining similarly significant with a similar effect size in all cases. However, as a subsidiary finding, a non-hypothesised post hoc finding emerged such that increased prior-day exercise predicted a smaller 0–30 CAR increase ($t = -2.03$, $p = 0.044$), but this was independent of the previously reported CAR-EF association (for which $t = 2.55$, $p = 0.012$ when exercise was included in Model B).

4.4.2.2 Cortisol from 30 to 60 min post-awakening

Model A of the cortisol data from the 30 to 60 min period indicated the expected post-CAR general decline in cortisol secretion ($t = -5.85$, $p = <0.001$), but Model B did not indicate any significant interaction with EF in this period ($t = 0.64$, $p = 0.527$), suggesting that the observed cortisol-EF interaction is unique to the 0–30 min rise (CAR).

4.4.2.3 Cortisol and earlier AST performance

In addition to these analyses, the association between the earlier AST measure and CAR (performed on half of the study days) was examined. The effect size was substantially lower than for the association between the CAR and EF at 45-min post-awakening (performed on all study days), and did not reach significance (noting the reduced sample size for this analysis) ($t = 1.13$, $p = 0.26$). Finally, there was only a very weak and non-significant relationship between EF functioning measured at 15-min post-awakening and EF measured 45-min post-awakening ($r = 0.154$, $p = 0.464$, two-tailed).

4.4.2.4 Exploration of moderation/mediation by psychosocial factors

Further modelling examined whether the CAR-EF association was influenced by the psychosocial factors of stress and arousal (components described in 4.2.5). First, to inform this analysis by indication of common variance, zero order associations between these factors and both the cortisol data and EF were explored (see table 4.4). Associations were observed between arousal and both the dynamic CAR measures and waking cortisol, while stress was associated with greater overall cortisol secretion (including higher cortisol at 15- and 60-min post-awakening) but not with the dynamic of the CAR. As neither factor was significantly associated with EF, these variables were only included for modelling of potential moderating/mediating influence on the CAR-EF relationship.

Table 4.4 Zero-order associations (Pearson correlations) between psychosocial factors and cortisol, CAR measures, and EF.

	Sample (mins post-awakening)					CAR measure				EF
	0	15	30	45	60	MnInc	Delta 0-30	AUCg	Peak value	
Arousal	.334*	.218	.103	-.188	-.253	-.297*	-.287*	.172	-.058	-.206
Stress	.169	.289*	.217	.199	.312*	.075	-.007	.295*	.290*	-.107

N=50 in all cases

*Significant at .05 level

The stress and arousal variables were entered into model B, and the results of these analyses indicated that the interaction between CAR-EF remained similarly significant with a similar effect size ($t = 2.301$, $p = 0.023$), thereby demonstrating that this effect was not mediated/modulated by the influence of these psychosocial factors on the CAR.

4.5 Discussion

This study explored daily variation in the CAR and an index of EF in a healthy adult male over 50 days. Results indicated that a larger increase in cortisol concentrations from 0 to 30 min post-awakening predicted better EF (attention switching performance) at 45-min post-awakening, independent of day order effects, awakening time, sleep duration, reaction time, prior day exercise, prior day alcohol consumption, earlier cognitive demands (a 15-min postawakening AST), and level of fatigue on awakening. This observed relationship between the CAR and EF is thus temporally predictive (given the temporal order of CAR and EF measures). The design is nonetheless correlational and the phrase ‘‘predictive’’ should be interpreted with caution with regard to strong inferences of causation.

The results of the present study build on the work of Evans et al. (2012) which showed an association between trait estimates of CAR magnitude and integrity of EF in older adults, with the present data suggesting that the same direction of significant relationship between the CAR and EF also exists in the pattern of daily co-variation for at least one normal healthy young adult male. This accumulating evidence suggests a potentially important relationship between

the CAR and pre-frontal functions. It has been suggested (e.g. see, Law et al., 2013) that a possible role of the CAR may be to regulate peripheral circadian rhythms under the influence of the suprachiasmatic nucleus (SCN) of the hypothalamus, acting as a time-of-day marker to optimise cognitive function appropriately. This is a feasible explanation of the CAR-EF relationship, as circadian organisation of brain functions via the SCN has been suggested to be essential for normal cognitive functioning (Cohen & Albers, 1991; Karatsoreos et al., 2010). Moreover, the recent demonstration of an association between daily variation in CAR magnitude and capacity for neuroplasticity (Clow et al., 2014) provides a possible mechanism which could underpin this relationship. The importance of EF for dealing with challenge in the typical waking day is evident (e.g. Manly et al., 2002), and the direction of this relationship is certainly in line with the prominent theories that the CAR plays a role in preparing or ‘‘boosting’’ the individual for the day ahead (Adam et al., 2006). Furthermore, if this predictive ‘‘state’’ relationship between the CAR and EF is in fact causal, then given the known progressive decline in both CAR magnitude and EF performance with advancing age (e.g. Huizinga et al., 2006; Kudielka & Kirschbaum, 2003; Zelazo et al., 2004), this in turn could inform the trait relationship observed in older adults (Evans et al., 2012).

There is good evidence that sustained exposure to high levels of glucocorticoids evokes neuronal cell damage and impairs synaptic plasticity and cognitive function (Joels & Baram, 2009; Sapolsky et al., 1990, 2000; Suri & Vaidya, 2013). However, it has recently become evident that the circadian rhythm of glucocorticoid secretion may promote internal homeostasis and optimal brain function (Nader et al., 2010). For example, animal studies indicate that healthy

circadian glucocorticoid oscillations boost learning-dependent synaptic formation and maintenance (Liston et al., 2013). It is clear that disrupted circadian patterns (not just sustained high levels) of glucocorticoid secretion are associated with cognitive deficits (Cho et al., 2000; Evans et al., 2011; Gibson et al., 2010) as well as a wide range of neuropsychiatric diseases (Jagannath et al., 2013; Menet & Rosbash, 2011; Wulff et al., 2010). Results from this study are consistent with these findings and, whilst other aspects of the circadian pattern of cortisol were not examined, suggest a role for the CAR in cognitive function.

A limitation of all case study research is generalisability of the results to the wider population; hence the importance of establishing convergence, refinement, and replication of effects across studies of differing design, with differing strengths and weaknesses. For instance, sex differences exist in both CAR magnitude and timing of the peak (Pruessner et al., 1997) and the CAR is also associated with menstrual cycle phase (Wolfram et al., 2011); therefore, the need for replication of these effects in a female sample is evident. In addition, specific characteristics of the participant might limit generalisability. For example, given the influence of sleep on cortisol secretion the reported average sleep duration of 6:21 h per night (below the recommended average of 7–9 h for his age) may have been a factor in the results obtained. While the generalisability of these results might also be limited by the dependence on a single EF measure, these findings of temporal covariation in a single participant-researcher are directionally in agreement with the between-participants covariation findings of Evans et al. (2012), who used a very different EF task (TMT part B).

That the CAR-EF association remained robust to the inclusion of potentially confounding factors, including measures of arousal and stress, provides support for the validity of this finding and suggests that psychosocial state does not mediate or modulate the observed relationship. However, the independent correlations between psychological arousal and cortisol levels upon awakening, and CAR MnInc, as well as the correlations between stress and cortisol levels at 60-min post-awakening and the AUCg, are generally supportive of previous findings regarding CAR-psychosocial state and trait associations (e.g. Stalder et al., 2010a; Weik and Deinzer, 2010; Garcia-Banda et al., 2014). Although not the primary focus of this study, these subsidiary findings, combined with previous evidence that stress and negative mood are related to overall post-awakening cortisol secretion and elevated cortisol in the post-CAR period is deserving of further investigation.

The subsidiary finding of increased prior day exercise predicting an attenuated 0–30 min CAR, although novel, was an unexpected and, therefore, post-hoc finding. As such, it is necessary to exercise caution against over interpretation of what it might mean. From the perspective of this thesis, it should perhaps only be emphasised that controlling for exercise did not influence the principal interactive effect of EF on cortisol rise from 0 to 30 min post-awakening.

4.5.1 Conclusions

The results of the present case study are novel and deserving of further research to confirm generalisability. They provide an exciting first indication of a within-subject association between the 0 to 30 min CAR and a measure of EF

determined after the CAR period. When combined with the earlier demonstration of a trait-like CAR-EF relationship (Evans et al., 2012), it is apparent that the relationship between the CAR and EF is a highly promising area of investigation, with potentially important implications for the role of the CAR within the healthy circadian rhythm in humans.

5 Study II

The CAR & Plasticity of the Human Motor Cortex (M1)

5.1 Overview

The cortisol awakening response (CAR) is the most prominent, dynamic and variable part of the circadian pattern of cortisol secretion. Despite this its precise purpose is unknown. Aberrant patterns of the CAR are associated with impaired physical and mental health and reduced cognitive function, suggesting that it may have a pervasive role or roles. It has been suggested that the CAR primes the brain for the expected demands of the day but the mechanisms underlying this process are unknown. The present study examined temporal covariation of the CAR and rapid transcranial magnetic stimulation (rTMS)-induced long term depression (LTD)-like responses in the motor cortex. Plasticity was evaluated across 180 measures from 5 time points on 4 sessions across 9 healthy researcher participants. Plasticity estimates were obtained in the afternoon after measurement of the CAR on 4 days, at least 3 days apart. As

both CAR magnitude and rTMS-induced responses are variable across days it was hypothesised that days with larger than individual average CARs would be associated with a greater than individual average plasticity response. This was confirmed by mixed regression modelling where variation in the CAR predicted variation in rTMS-induced responses. This study demonstrates, for the first time, that the daily magnitude of the CAR may be responsible for some daily variation in synaptic plasticity, presenting a possible mechanism by which the CAR might influence cognitive function. As the magnitude of the CAR is regulated by the 'master' circadian CLOCK, and synaptic plasticity is known to be modulated by peripheral 'slave' CLOCK genes, it is suggested here that the CAR may be a mediator between the master and peripheral circadian systems to entrain daily levels of synaptic plasticity.

5.2 Introduction

There is good evidence that sustained exposure to high levels of glucocorticoids evokes neuronal cell damage and impairs synaptic plasticity (Joëls, 2008; Sapolsky et al., 1990; Suri & Vaidya, 2013). However, it has recently become evident that the circadian rhythm of glucocorticoid secretion may promote internal homeostasis and optimal brain function (Nader et al., 2010). For example, animal studies indicate that healthy circadian glucocorticoid oscillations boost learning dependent synaptic formation and maintenance (Liston et al., 2013). The actions of cortisol within the brain are mediated by two types of receptors found in the nuclei of cells: high affinity (CR1) or ‘mineralocorticoid’ (MR) receptors, and low affinity (CR2) or ‘glucocorticoid’ (GR)

receptors (De Kloet et al., 2005). The difference in affinity of MRs and GRs for cortisol (the natural ligand) results in MRs being constantly in a state of almost full occupation, while extensive occupation of GRs occurs only at the peaks of the ultradian rhythm or in response to stress (Conway-Campbell et al., 2007). Moderate cortisol exposure therefore preferentially activates MRs. It has been suggested, based upon data from several studies, that occupation of MRs in HC neurons is necessary for maintaining neuronal integrity and a stable excitatory tone (Joëls et al., 2008), and is associated with the cognitive functions of the HC facilitation of memory acquisition and cognitive processes such as the appraisal of novel situations (Oitzl et al., 1994; De Kloet et al., 1999). In the context of the stress response, saturation of MRs and occupancy of GRs at high cortisol exposure tends to have opposing negative effects on these cognitive processes. While, following a stressful event, a primary function of GRs in the brain is to restore normal function and promote consolidation of the event related memories (De Kloet et al., 2005).

It is clear that disrupted circadian patterns (not just sustained high levels) of glucocorticoid secretion are associated with cognitive deficits (Cho et al., 2000; Evans et al., 2011; Gibson et al., 2010) as well as a wide range of neuropsychiatric diseases (Jagannath et al., 2013; Menet & Rosbash, 2011; Wulff et al., 2010). In healthy animals, including humans, glucocorticoid hormones have a marked underlying circadian pattern with characteristically low levels during sleep, peak levels soon after awakening, followed by a gradual decline (Edwards et al., 2001a). The circadian pattern of cortisol secretion is regulated by the central master CLOCK: the hypothalamic suprachiasmatic nucleus (SCN) (Perreau-Lenz et al., 2003). Furthermore, it has increasingly

become recognized that glucocorticoids adjust the circadian rhythm and function of the ubiquitous peripheral CLOCKs. Dysfunction or dysregulation in either circadian system alters internal homeostasis and causes pathologic changes virtually in all tissues, including the brain (Nader et al., 2010).

The mechanism underlying the storage and processing of information in the brain is synaptic plasticity, that is, the relative adaptation of neuronal synapses in response to recent activation to either decrease or increase in firing threshold; known as 'Long-term Potentiation' (LTP) and 'Long-term Depression' (LTD), respectively (Bailey, Kandel, & Si, 2004). As elevated levels of circulating glucocorticoids impair memory function and LTP (Dubrovsky et al., 1987; De Quervain et al., 2000; Grillon et al., 2004), and also impair neuroplasticity in the M1 region of the motor cortex (Yoo et al., 2007; Sale et al., 2008), it has been proposed that cortisol may be a key neuromodulator of circadian effects on neuroplasticity (Sale et al., 2010). As described in Chapters 1 and 2, although it has been suggested that the CAR primes the brain for the expected demands of the day (e.g. Fries et al., 2009), the specific mechanisms underlying this process are unknown. One of the possible mechanisms involved may, therefore, be the modulation of neuroplasticity.

One non-invasive method of investigating synaptic plasticity of the cortex is Transcranial Magnetic Stimulation (TMS), whereby a magnetic field generator is used to create a small charge resulting in electromagnetic induction of localised cell firing in an adjacent area of the cortex. TMS can have a diverse range of effects on descending activity depending on the type, intensity, and location of

stimulation over the cortex (Hallett, 2000; Di Lazzaro et al., 2004), which have been shown to include both LTP-like and LTD-like responses (Fitzgerald et al., 2006; Müller-Dahlhaus et al., 2008). A common application is in Rapid TMS (rTMS) protocols, which are considered to be an effective non-invasive measure for induction of synaptic plasticity, able to induce long term depression (LTD)-like responses in the human brain (Huang et al., 2005). A study by Sale et al. (2008) used a Paired Associative Stimulation (PAS) paradigm (an rTMS protocol) to demonstrate that basal cortisol levels at the time of testing could predict these LTD-like responses in the human motor cortex, and more so, that such apparent neuroplasticity also shows significant state (day-to-day) variation. Research from this laboratory has also indicated that a greater propensity for neuroplasticity is observed in the evening than in the morning (Sale et al., 2007; 2008). While previous studies had demonstrated this in a between-subjects context, showing cortical excitability measured by TMS and EEG is affected by the duration of wakefulness and modulated by circadian phase (Ly et al., 2016), a study by Sale et al. (2008) crucially demonstrated that this variation in TMS-induced LTD-like neuroplasticity within the same group of subjects tested in the morning and evening of the same day. In addition, using a similar rTMS protocol, Pitcher et al. (2012) have recently demonstrated that reduced neuroplasticity of the motor cortex in adolescents born preterm is associated with reduced basal cortisol.

TMS is used as a therapeutic intervention in a range of neurological and psychiatric conditions, including Parkinson's disease, Obsessive-compulsive disorder, and (most notably) depression (for review, see: Wassermann & Lisanby, 2001; Couturier, 2005; Loo & Mitchell, 2005). Such time of day variation presents a challenge for non-invasive stimulation therapy (Fratello et

al., 2006; Sale et al., 2007), and for its usefulness for investigating pathophysiological changes in the brain (Pitcher et al., 2012), but might simultaneously offer insight into the potential circadian modulators (Sale et al., 2008). For example, Abnormal functioning of the cortex plays a critical role in the motor, cognitive, and sensory impairment observed in individuals born preterm. Pitcher et al. (2012) explored the association between TMS-induced neuroplasticity and cortisol secretion in a sample of 28 preterm-born adolescents (15 females & 13 males, aged 13.8 ± 0.5). This experiment used a Continuous theta burst stimulation (cTBS) protocol, which involves rTMS-induced lasting LTD-like neuroplasticity in M1 of the left motor cortex hand area. The results indicated that when compared with term-born adolescents, both early and late preterm adolescents showed reduced LTD-like neuroplasticity in response to the cTBS, and that this was further associated with lower levels of salivary cortisol.

The evidence presented therefore suggests that LTD-like plasticity of the human motor cortex is inhibited by cortisol secretion, and is further subject to circadian variation. Since this circadian variation shows an inverse pattern to that of the circadian rhythm of cortisol secretion, this presents a novel hypothesis: that the circadian rhythm of cortisol modulates TMS-induced neuroplasticity, and that a relationship will therefore be observed between intra-individual variation in features of the cortisol circadian rhythm and same-day propensity for TMS-induced neuroplasticity. A neurotransmitter which may play a role in this association is Brain-derived Neurotropic Factor (BDNF), which plays a role in determining the susceptibility of synapses to undergo the processes of long-term potentiation (LTP) and long-term depression (LTD) (Cheeran et al., 2008).

The role of BDNF in neuroplastic change further supports the cortisol-synaptic plasticity hypothesis suggested here as diurnal BDNF and cortisol levels show synchrony and are thought to be physiologically co-regulated (Begliuomini et al., 2008), with cortisol modulating the gene which determines BDNF production (also called BDNF) and regulating the effects of BDNF on the brain (Suri and Vaidya, 2013).

Previous research has indicated that the CAR is associated with anticipations of obligations/increased challenge in the same day (Adam et al., 2006; Stalder et al., 2010a). Although the findings of these studies are consistent with the hypothesis that a role of the CAR is to prime the brain for the demands of the day ahead (Fries et al., 2009), the mechanisms underlying this process have not been elucidated. As described in chapter 2 of the present thesis, it has been proposed that the CAR, under the influence of the SCN, may serve as a hormonal time-of-day marker for regulation of peripheral clocks in the circadian system (Clow et al., 2010a). This theory is supported by the recent finding that glucocorticoids entrain peripheral clocks in humans (Pezük, Mohawk, Wang, & Menaker, 2012; Cuesta et al., 2015), suggesting that the circadian cortisol rhythm may influence the temporal programmes of gene expression in diverse brain regions, complementing their neural regulation by circadian inputs (Herbert et al., 2006). As such, it is apparent that mismatch between the circadian cortisol and neural patterns may contribute to localised malfunction of these brain regions (Herbert et al., 2006).

The present study examined the CAR in relation to levels of synaptic plasticity measured in motor cortex (M1) some 6–7 h after awakening. Although not a region frequently explored in cortisol or CAR research (compared to, say, the HC or FC), the proximity of the motor cortex to the scalp makes it an ideal target for TMS procedures, and the association between this region and cortisol secretion has now been demonstrated in both the Sale et al. (2008) and Pitcher et al., (2012). For consistency (and thus, ease of comparison) with previous studies, a model of synaptic plasticity previously used to explore associations with levels of cortisol in healthy intact participants was selected: rapid transcranial magnetic stimulation (rTMS)-induced long term depression (LTD)-like responses in the motor cortex (Pitcher et al., 2012; Sale et al., 2008). This measure was used as a representative indicator of effects caused by any factor that affects the whole brain. It was hypothesized that one function of the CAR is to regulate the sensitivity of synaptic plasticity, known to be modulated by peripheral CLOCK genes (Wang et al., 2009) during the coming day and that this could be one mechanism whereby the CAR could influence a wide range of behaviours. It was therefore predicted that day-to-day variation in the CAR would correlate with day-to-day variation in rTMS-induced synaptic plasticity of the motor cortex.

5.3 Method

5.3.1 Design

The present study was designed to explore the relationship of temporal variation within a large sample (N = 180) of plasticity estimates with temporal variation of the magnitude of the CAR. The design was entirely within-participant with plasticity estimates collected at five fixed time points repeated over four sessions, and was replicated between participants.

5.3.2 Participants

9 participants completed the study, all of whom were young research staff from the Universities of Adelaide and South Australia, chosen to ensure rigorous adherence to the demanding protocol. Participants were also selected on the basis of being predominantly right-handed and also free from any medication (notably corticosteroids) known to affect cortisol status. All participants were screened for any conditions that would contraindicate TMS (see screening form in appendix 2; Rossi et al. 2009), and all were in good health, with normal BMI. One participant reported smoking cigarettes, but agreed to refrain from smoking over the course of the study. All other participants were non-smokers. There were 7 female and 2 male participants in total, and the mean age of participants was 25 (SD 2.5) years.

5.3.3 Materials

Self-reporting of awakening and sampling times for verification against electronic monitoring was obtained using the standard sampling self-report sheets described in Chapter 3 (see appendix 5). Single-pulse TMS was delivered using a Magstim-200 stimulator (Magstim Co., Whitland, UK), and rTMS was

delivered using a figure of eight-shaped Double-Cooled-Coil-System coil (70 mm, Magstim Co., Whitland, UK). Muscle contractions in the hand in response to TMS stimulation were measured using electromyographic (EMG) data from surface electrodes.

5.3.4 Procedure

Participants gave written informed consent prior to testing and were screened for any conditions that would contraindicate TMS (Rossi et al., 2009). Each participant completed 4 separate testing sessions, with a minimum interval of 2 days between sessions. Prior to going to sleep, participants completed the evening diary to measure a range of psychosocial variables, very similar to that used in Study 1 (see appendix 7). Wake times were established using both self-report and actigraphical recordings (in-line with the recommendations of Smyth et al., 2013a). Actiwatch and MEMS data analysis indicated that all wake times and sampling times were highly accurate and well within the acceptable ranges proposed in Smyth et al. (2013a; 2016) and Stalder et al. (2016). The morning diary (appendix 7) was completed immediately after taking the final cortisol sample. In the afternoon of each study day, participants visited the laboratory to complete the TMS session. All TMS sessions were completed at either 2 or 3 pm in order to minimize time of day influences (Sale et al., 2007). Testing sessions were never fewer than 3 days apart to minimize carry over effects from the rTMS protocols (Goldsworthy et al., 2012; Hamada et al., 2012). On each study day, a CAR was determined upon awakening and plasticity estimates were assessed on the same afternoon.

5.3.5 Estimation of the cortisol awakening response

Saliva samples were collected and assayed as per the protocol previously described in general methods sections 3.2.1 and 3.2.2; with samples immediately on awakening and at 15-, 30- and 45-min post-awakening (samples 1–4, respectively) on each study day. Electronic monitoring of awakening and sampling times as per the procedure previously described in general methods section 3.3.1, with wrist actigraphy further used to establish sleep efficiency. Adherence to the saliva sampling protocol was excellent, with no sampling time deviating >5min from the requested saliva collection times relative to verified awakening. It was established that 4 of the 144 saliva samples were below the limit of detection of the assay: three awakening samples, and one 30-min sample. Undetectable samples were treated as missing data, and all other cortisol measures were included in the final analysis. CAR magnitude was calculated as the MnInc from 0 to 45 min: sample 2 + sample 3 + sample 4)/3 sample 1, and overall cortisol secretion was calculated using the AUCg (also adapted to 4 sample form).

5.3.6 Transcranial Magnetic Stimulation and Electromyographic Responses

Muscle contractions in the hand in response to TMS stimulation of the motor cortex were recorded as EMG activity in the relaxed right FDI using surface electrodes placed in a belly-tendon configuration. The EMG signal was amplified (1000; CED 1902 amplifier, CED, UK), band pass filtered (20–1000 Hz) and digitized at a sampling rate of 2 kHz (CED 1401 interface, CED, UK). Single-pulse

stimuli were generated using the stimulator, delivered through a figure-of-eight coil (90 mm diameter) placed tangentially to the scalp with the handle pointing backward at a 45° angle away from the midline. Suprathreshold pulses were delivered over the left M1 at numerous sites in order to identify the optimal position for consistently evoking motor evoked potential (MEPs) in the relaxed right FDI and this site was marked on the scalp. The TMS intensity that elicited MEPs of 1 mV (SI1 mV) in the relaxed FDI was determined (for each testing session) at baseline and was used to examine changes in MEP amplitude after each protocol. Although individual neuronavigation was not employed in placing the coil, it is likely that there was little change in its position from day to day since the intensity employed for evoking baseline test MEPs, and the amplitude of these (1 mV) test MEPs was not significantly different between testing days. Any minor change in position or angle would have been random and could not contribute to the effects observed. Two blocks of 15 single-pulse TMS trials, with an inter-trial interval of 7 s (±10%), were delivered at baseline and one block of 15 single-pulse TMS trials was then delivered 0, 5, 10, 20 and 30 min after rTMS. Individual MEP data trials were excluded if EMG activity was present in the 100 ms immediately prior to TMS. The peak-to-peak MEP amplitude (in mV) was measured for each trial to give an index of the size of the muscle twitch and the mean amplitude at each post-rTMS time point was expressed as a ratio of the mean of the two baseline samples. This provided an index of the change in the size of the muscle response to the same brain stimulus after rTMS relative to baseline prior to rTMS: the MEP ratio or neuroplasticity index. The rTMS protocol adopted was continuous theta burst stimulation (cTBS) which was delivered using a figure of eight-shaped Double-Cooled-Coil-System coil (70 mm, Magstim, Whitland, UK). Bursts of three pulses were delivered at 50 Hz every 200 ms

continuously for 40 s (Huang et al., 2005). TBS intensity was set to 80% of active motor threshold (AMT); AMT was defined as the minimum intensity required to elicit a MEP in FDI of at least 200 mV in at least 5 out of 10 consecutive trials when performing a low-level voluntary contraction of FDI (10% of maximal voluntary contraction) and was determined for each testing session. This paradigm is known to induce long term depression (LTD)-like effects resulting in a smaller muscle response (hence a reduced MEP ratio) post-rTMS.

Bursts of three pulses were delivered at 50 Hz every 200 ms continuously for 40 s (Huang et al., 2005). TBS intensity was set to 80% of active motor threshold (AMT); AMT was defined as the minimum intensity required to elicit a MEP in FDI of at least 200 mV in at least 5 out of 10 consecutive trials when performing a low-level voluntary contraction of FDI (10% of maximal voluntary contraction) and was determined for each testing session. This paradigm is known to induce long term depression (LTD)-like effects resulting in a smaller muscle response (hence a reduced MEP ratio) post-rTMS, and is the same as that used in previous studies of cortisol secretion and motor cortex plasticity (Pitcher et al., 2012).

5.3.7 Treatment of data

Data were analysed using mixed regression modelling (Blackwell et al., 2006) of variation in the 180 MEP-ratio estimates, in line with the approach to statistical analysis as previously described in detail in section 3.7. CAR magnitude was included as the principal covariate, and was participant-centred. Participant centring expresses exclusively within-participant variation (i.e. participants'

deviations from their own study means) and is appropriate here since absolute differences in participants' average CAR magnitude was not the focus of the present study. Time-point of post-rTMS measurement within session (at 0, 5, 10, 20 and 30 min) and session number (1–4) were both modelled as fixed factors. Intercept effects were modelled as both fixed and random. Finally, further modelling was undertaken to check that any findings from initial modelling were not confounded by associations with awakening time, level of cortisol, or psychosocial variables recorded in the study diaries.

5.4 Results

Table 5.1 presents descriptive data for the study. As expected, following rTMS, the average MEP ratio was 51 (0.9 ± 0.29). In other words, the peak-to-peak MEP amplitude (in mV) post-rTMS was less than at base, indicating the induction of LTD-like synaptic plasticity. The average CAR across days showed a mean increase of 2.72 nmol/l cortisol in the 45 min after waking.

Table 5.1 Descriptives for sample (n=9) of TMS-ratios (mean of 0, 5, 10, 20, and 30 minutes after cTBS relative to base) cortisol concentrations (nmols/l) from 0-45 mins post-awakening, CAR mean increase (MnInc), and awakening time.

Measure	Mean	Sd
Average TMS ratio	0.90	0.29
Cortisol 1 (0 mins)	14.63	9.73

Cortisol 2 (15 mins)	16.37	7.06
Cortisol 3 (30 mins)	18.06	7.75
Cortisol 4 (45 mins)	17.14	7.43
CAR (MnInc)	2.72	9.29
Awakening time (hh:mm) am	7:49	1:25

5.4.1 Relationship between the CAR and motor cortex plasticity

Table 5.2 shows F-ratios and significances for the parameters in the modelled data. There was no significant difference between the MEP ratios measured at the five post-rTMS time points (sample numbers 1–5), allowing for summarisation the effect of rTMS as a single overall mean ratio (as shown in table 5.1). Similarly, there were no differences in the mean MEP ratios on each of the 4 days (session numbers 1–4), suggesting that there was no adaptation to the procedure over the period of testing. The magnitude of the CAR was significantly associated with participants' mean MEP ratios collected on the same day.

Table 5.2 F-ratios, df and significances associated with parameters in mixed regression modelling of TMS ratio data.

Model Parameter	**Df *(num,denom)***	**F**	**P**
Intercept	1, 49.43	55.76	<.001
Sample number (1-5)	1, 156.18	0.85	.359
Session number (1-4)	1, 158.06	3.51	.063
CAR (Between Participants)	1, 9.34	0.55	.479

CAR (Within Participants)	1, 156.18	9.58	.002

Calculation of the estimate coefficient (0.013) suggests that for each single nmol/l above their own average CAR on any testing day, the predicted MEP ratio would be 0.013 points (1.4%) lower than average. Since lower MEP ratios reflect a larger response to the rTMS protocol, the finding suggests that larger than average CARs in the morning predict greater neuroplasticity measured later in the afternoon. This relationship is presented in figure 5.1, with MEP ratios inverted for display purposes. The data show that if the CAR magnitude was larger than individual means, then there was a greater chance that the response to rTMS indicated greater neuroplasticity (i.e. an MEP ratio lower than the expected mean). Since lower MEP ratios reflect a larger response to the rTMS protocol, the finding suggests that, within-subjects, larger than average CARs in the morning predict greater neuroplasticity measured later in the afternoon.

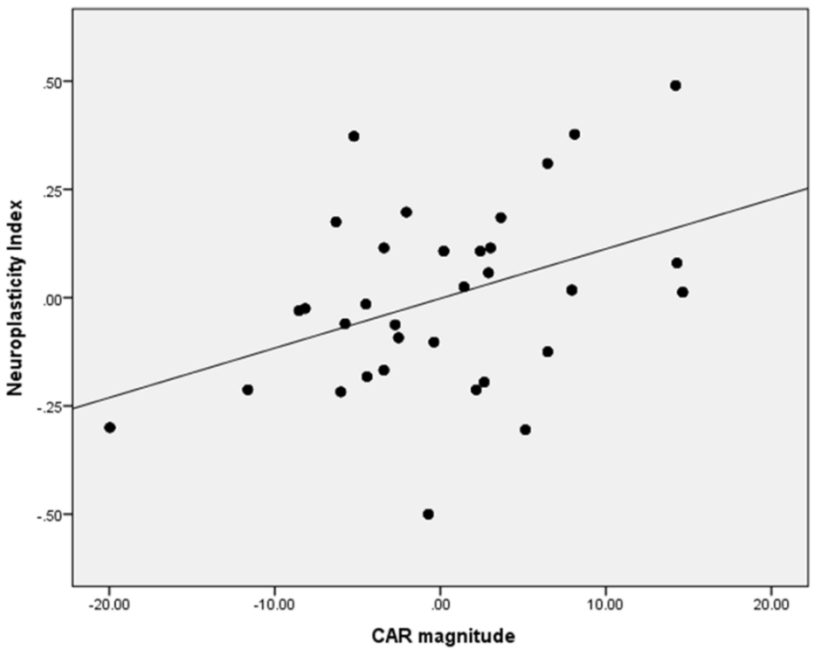


Figure 5.1 The relationship between morning CAR and afternoon rTMS response (MEP ratios inverted so that higher scores represent a larger response to the rTMS protocol). Data are expressed relative to mean responses for both measures over the 4 days.

5.4.2 Controlling for potential confounding variables

Further modelling examined whether CAR magnitude was confounded by the covariates of awakening time and/or awakening level of cortisol. The effect was robust to such statistical control, remaining independently significant with a similar effect size. There was no association between the total amount of cortisol secreted over the post-awakening period (the AUCg) and rTMS-induced cortical plasticity the same afternoon. Sleep efficiency, determined by wrist-worn actigraphy, was not associated with either the CAR or rTMS-induced cortical plasticity.

Finally, a series of additional models were produced to examine whether the observed effect was robust to inclusion of the simple, self-report psychosocial variables from the study diaries. These variables were separately added to the principal model to achieve a suitable ratio of variables to cases and conserve degrees of freedom, and the effect was robust to their inclusion in all cases, remaining independently significant with a similar effect size.

5.5 Discussion

This study of temporal variation with multiple sampling across days shows a highly significant relationship between variability in the cortisol awakening response and the capacity to induce synaptic plasticity in the motor cortex on that same day. Specifically, on days when individuals' CARs were bigger than their own individual averages there were greater changes in the size of the muscle response following the rTMS protocol, measured that afternoon, indicating greater neuroplasticity. Likewise, lower than average CARs predicted smaller changes in the size of the muscle response after rTMS. The rTMS protocol used here (continuous theta bust, cTBS) is thought to provide a measure of the responsiveness of early long-term depression (LTD)-like processes in the motor cortex (Huang et al., 2005, 2007). The measure varies considerably between individuals as it is affected by factors such as age and genetics (Hamada et al., 2012; Ridding & Ziemann, 2010). However, there are also large variations in an individual's response measured on different days (Sale et al., 2007). One cause of the within-subject variation in plasticity responses has been thought to be circadian changes in neuromodulators, such as cortisol (Sale et al., 2008). The present results suggest for the first time that the daily magnitude of the CAR may be responsible for some daily variation in synaptic plasticity. Furthermore, the observed effect proved robust to the effects of awakening time and awakening level of cortisol, suggesting that these potentially confounding factors in CAR research do not compromise the present model.

The TMS paradigm used in the present study has the clear advantage of being non-invasive and appropriate for use in healthy intact volunteers. Further,

although the present study was limited to exploration of this specific index of plasticity in the motor cortex, evidence suggests this measure serves as a representative marker of effects caused by any factor that affects the whole brain (e.g. secretion of glucocorticoids). For example, administration of an NMDA antagonist in humans can be detected by reduced rTMS-induced plasticity in the motor cortex (Stefan et al., 2002; Wolters et al., 2003). Similarly, the human Huntington's disease gene in mice reduces synaptic plasticity in many cortical regions; in humans, this is reflected in reduced plasticity in the motor cortex (Crupi et al., 2008). As such, the results of the present study might reasonably be hypothesised to reflect changes throughout brain regions with an affinity for cortisol, all of which could be affected by the CAR. The relationship between individual day to day changes in cortical plasticity to relevant day to day differences in behaviour have yet to be examined, so the functional implications of the changes in plasticity observed in such TMS protocols are not yet fully described. Nevertheless, some relationship might be expected as previous experiments have shown that when such TMS protocols are used to induce changes in plasticity in the motor cortex they simultaneously affect motor function (e.g. Jung & Ziemann, 2009).

As described in detail in chapters 1 and 2 of the present thesis, aberrant patterns of the CAR have been consistently linked with indices of impaired physical and mental health (Fries et al., 2009; Kudielka & Kirschbaum, 2003). In particular, attenuated CAR profiles are associated with lower hippocampal volume (Buchanan et al., 2004; Pruessner et al., 2007), amnesia (Wolf et al., 2005), deficits in verbal memory and processing speed (Aas et al., 2011; Evans et al., 2011), and worse executive function (Evans et al., 2012). Furthermore,

inhibition of the CAR using the cortisol synthesis inhibitor metyrapone impaired memory retrieval in healthy young participants (Rimmele et al., 2010). However, while the CAR has consistently been shown to be associated with cognitive function, the mechanism underlying this association has not previously been identified. Indeed, the precise purpose of the CAR in healthy function is unknown. However, as plasticity shows a circadian rhythm and variation between days (Sale et al., 2007) and is influenced by administration of exogenous glucocorticoids (Sale et al., 2008), and as the CAR is the most prominent aspect of the circadian pattern of cortisol secretion, this presents a possible mechanism for the previously observed relationship; one that is strongly supported by the present findings. Further, unlike previous studies of exogenous glucorticoids, the present study measured an aspect of the endogenous rhythm, and so has the advantage of demonstrating typical patterns of the circadian rhythm observed in the naturalistic setting.

Evidence suggests causal pathways linking circadian cortisol disruption to sub-optimal brain function and brain disorder (Jagannath et al., 2013; Wulff et al., 2010). One putative pathway involves dysregulation of the circadian CLOCK system (Menet & Roshbash, 2011). In this scheme, the SCN acts as the light-activated ‘‘master’’ CLOCK, which synchronizes the peripheral ‘‘slave’’ CLOCKs through neural and humoral pathways (Nader et al., 2010). It has been proposed in the present thesis that one of these pathways may involve the CAR, which is regulated by dual inputs from the SCN: via the hypothalamic pituitary adrenal axis as well as a direct neural pathway to the adrenal cortex (Clow et al., 2010b). These dual pathways allow the SCN to fine tune sensitivity of the adrenal cortex to adrenocorticotrophic hormone (the secretagogue for cortisol),

modulating the CAR, and making it ideally suited to relay messages to the periphery from the master CLOCK. Supporting this theory, glucocorticoids are known to affect peripheral CLOCKs in almost all organs and tissues by influencing the expression of several clock-related genes, which in turn have been shown to modify synaptic plasticity (Wang et al., 2009). It would be possible therefore for circadian fluctuations in glucocorticoid secretion (such as the CAR) to affect function in a sustained way over the day, not just by the influence of ambient levels (e.g. direct inhibition of NMDA receptor function). Certainly, this could provide a possible explanation for the current findings, as it would follow that a larger CAR may serve as a stronger cue to activate clock genes, and in turn activate and encourage enhanced neuroplasticity as an adaptive response to a challenging day. Although peripheral clock function was not directly examined in this study, circulating leukocytes provide an ideal tissue source for further work investigating the CAR and the circadian clock system in humans (see Kusanagi et al., 2008) and present an opportunity for further investigation. Day differences in mRNA expression of Per1, Per2, Per3 mRNA, in particular, at a set time in the afternoon relative to morning CAR would be of particular interest.

A further factor in this relationship, not measured here, may be the actions of brain-derived neurotropic factor (BDNF). BDNF is recognised as playing a role in determining the susceptibility of synapses to undergo the processes of long-term potentiation (LTP) and long-term depression (LTD) (Cheeran et al., 2008). BDNF is synchronised with, and thought to be physiologically co-regulated by, cortisol (Begliuomini et al., 2008), with cortisol modulating the BDNF producing gene and regulating the effects of BDNF on the brain (Suri and Vaidya, 2013).

As such, variation in BDNF levels in association with the CAR may be a candidate for further research to tease out the details of the CAR-plasticity relationship.

One limitation to the generalisability of the findings of the present study is the sample size employed here. However, the focus of the present study has been to demonstrate systematic covariation over time in two biological measures. The number of participants in this study was deliberately small and select for the purposes of ensuring adherence to the demanding study protocol and precision of recording (Smyth et al., 2013a; Stalder et al., 2016). The advantage of this emphasis on quality of measurement over quantity is perhaps reflected in the full adherence to the protocol in the present study – a rarity in CAR research, despite the many indications of its necessity for accurate and reliable CAR data (Smyth et al., 2016). However, the small number of participants is potentially relevant to issues of generalisability. While there is no theoretical reason to believe that such temporal co-variation would be restricted to the participants in this particular study, nevertheless the limited sample size affords the usual caution in matters of generalisation until the effect is replicated in other samples.

5.5.1 Conclusions

The study presented in this chapter is the first exploration of the relationship between the CAR and plasticity of the human brain, and demonstrates significant covariation between the cortisol awakening response and rTMS-induced synaptic plasticity of the motor cortex, measured 6–7 h later the same

day. These findings may indicate a pivotal role for the CAR in priming the brain for the day ahead (Clow et al., 2010a; Fries et al., 2009) possibly by entrainment of peripheral CLOCKs in the brain that can influence the sensitivity of synaptic plasticity. As well as shedding light on a possible role for the CAR and informing the marked state variation in this measure of neuroplasticity, these findings offer a plausible mechanism by which state factors which affect the CAR (such as stress) can affect brain plasticity.

6 Study III

The CAR and cognition in healthy young adults

6.1 Overview

The CAR has been proposed to play a role in regulating cognition by acting as a time-of-day signaller for peripheral clocks in the brain, under the influence of dual-pathway input by the SCN of the Hypothalamus (Clow et al., 2010 a & b). As discussed at length in the opening chapters of this thesis, studies have demonstrated associations between the CAR and various measures of cognition associated with structural and functional integrity of the HC and FC. For example, the CAR has been reported as positively associated with EF (Evans et al., 2011), and both positively and negatively related to measures of overall cognitive performance (e.g. Evans et al., 2011; Labad et al., 2016). However, the limited number of appropriately controlled studies, combined with the reliance on data from older adult and clinical samples, means that previous research offers limited insight into the CAR-cognition association, particularly that in healthy functioning young adults. The present study explored the association between the CAR and an index of EF in such a sample, using appropriate controls for monitoring participant adherence as recommended by Stalder et al. (2016). The results indicate a positive association between CAR magnitude and an index of EF in the afternoon of the same day, independent of known relevant CAR covariates, but only where CAR data was collected without delay exceeding 8-min post-awakening. These findings are discussed in terms of the potentially important implications for understanding the role of the CAR within the circadian rhythm of cortisol secretion, and in addition, in terms of the importance of sampling time accuracy for detecting associations with the CAR.

6.2 Introduction

As stated in chapter 1 (Intro I) there is accumulating evidence to suggest that the CAR is associated with various aspects of cognitive function, particularly for those areas of the brain with a high density of glucocorticoid receptors, such as the HC and FC. It has been speculated that the CAR is a product of the complex regulatory influences of these brain areas on ACTH and cortisol secretion around the time of awakening (Fries et al., 2009; Clow et al., 2010), and previous research has clearly established that CAR magnitude is positively related to HC integrity and associated memory functions (e.g. Baumler et al., 2014; Buchanan et al., 2004; Pruessner et al., 2007; Rimmele et al., 2010; Wolf et al., 2005). This includes both the demonstration that the CAR is attenuated in cases of bilateral HC lesions and severe global amnesia (Buchanan et al., 2004; Wolf et al. 2005). Such findings should be considered in the broader context that the circadian rhythm of glucocorticoids appears to support memory function by regulating plasticity in memory associated brain regions such as the HC (Oster et al., 2016). The CAR has been implicated as potentially influencing this association too, as it has been demonstrated that pharmacologic suppression of the CAR inhibits DM measured by free recall of previously learned texts and pictures, while item recognition (a memory process less dependent on the HC) is unimpaired (Rimmele et al., 2010). Further, it has also been demonstrated that CAR magnitude is positively associated with prospective memory in children (Baumler et al., 2014), indicating a potential role of the CAR in preparing the individual for the challenges of the coming day; as hypothesised to be a primary

function of the CAR in healthy function (Adam et al., 2006; Fries et al., 2009; Clow et al., 2010).

Despite the numerous explorations of CAR and HC associated functions, few studies have investigated the CAR in relation to pre-frontal (executive) functions. The FC plays an important role in regulating HPA-axis function (Diorio et al., 1993; Lupien et al., 2009), and while little may be known about CAR-EF associations, much research has explored the associations between EF and cortisol in a basal or acute response context (e.g. see Dierolf et al., 2016; Shields, Bonner, & Moons, 2015; Vaz et al., 2011). Such research has indicated that when controlling for basal cortisol levels, acute increases are positively associated with cognitive flexibility (EF) in the form of reaction time switch costs, particularly for negatively valent stimuli (Dierolf et al., 2016). However, results have been inconsistent, with some studies of exogenous cortisol administration failing to show any effects on cognitive flexibility measured over 1 hour later (Wingenfeld et al., 2011; Vaz et al., 2011). One limitation to the usefulness of this research for understanding CAR-EF associations is that EF can be divided into three separate functions; 'Shifting', 'Updating', and 'Inhibition' (Miyake et al., 2000). These three EFs are only moderately correlated (Miyake et al., 2000) and therefore it is unknown whether associations found between cortisol and 'shifting' (Dierolf et al., 2016) are reflective of a more general association with EF or rather specific to this discrete aspect.

A further limitation for understanding CAR-EF associations from studies of acute diurnal cortisol is that cognitive functions are subject to circadian and sleep factors (Schmidt et al., 2007), and this has been demonstrated in 'inhibition'

tasks as an index of EF (Manly et al., 2002). A relationship between the CAR and EF in the immediate post-awakening period has been investigated in study I of the present thesis, though associations between the CAR and EF at other points within the circadian and sleep-wake cycles have not previously been explored. It has been proposed in the present thesis and elsewhere (see Clow et al., 2010 a&b) that one function of the CAR may be to serve as a time-of-day marker, synchronising circadian rhythms in peripheral clocks within the body and brain under the regulatory influence of the hypothalamic SCN. If such a hypothesis were to hold true, then it may be expected that such associations would be observable, though subtly waning, across the waking day.

Of the few studies which have explored the CAR and FC-associated functions, there have been numerous methodological inconsistencies, the samples have exclusively been drawn from outside of the heathy functioning young adult population, and the findings have been contradictory. Evidence has been presented for a positive association between the CAR and both working memory and executive functioning in older adults (Almela et al., 2012; Evans et al., 2012), but negative associations with task updating, speed of memory, error monitoring, and serial sequence learning have been reported in other samples (Hodyl et al., *in press*, Maldonado et al., 2008; Oosterholt et al., 2016; Zhang et al., 2015). Most of this evidence comes from studies of abnormal function (e.g. Aas et al., 2011; Cullen et al., 2014; Hinklemann et al., 2013; Labad et al., 2016), or from samples of middle-aged or older adults (e.g. Almela et al., 2012; Evans et al., 2011; 2012). The inconsistency of results from the studies to date is likely due to variation in the nature and timing of the cognitive assessments, and irregularity with regards to monitoring of participant adherence to the CAR

protocol, which can lead to inaccurate assessment of the CAR (Stalder et al., 2016). Studies of diurnal cortisol secretion show reduced effect sizes when sampling accuracy is not monitored (Adam et al., 2017), and the potentially confounding effects are greater when measuring the CAR, due to the dynamic nature of this measure and the CAR increase starting around 8-min post awakening (Clow et al., 2004; Smyth et al., 2016). If selecting only the CAR-EF research conducted in accordance with the CAR expert consensus guidelines of Stalder et al. (2016), it is apparent that there is only one single study, conducted by Evans et al. (2012), which has investigated this area. The results of this study indicated positive associations between integrity of EF and both CAR magnitude and an earlier CAR peak in a sample of older adults (ages 60-91). This study measured EF using Form B of the TMT and, as discussed in chapter 4 (study I), this task measures task switching speed as an index of cognitive flexibility and as such the results are consistent with the within-subject findings of the first study of this thesis.

Finally, the possibility that the CAR-Cognition relationship seen in older adults (e.g. Evans et al., 2012) might be observed in the healthy young has only initially been explored using a single case study (in study I, Chapter 4, of the present thesis). One important contribution of the present study (study III) was to explore this relationship in a larger sample, and in a between-subjects context. From this it may be established whether the positive results of the initial study are generalisable beyond the single case, and at a trait (intraindividual) level. For the purposes of replication and comparison with any EF association, the association between the CAR and HC-dependant DM was also examined.

6.2.1 Aims

The aim of the present study is to investigate whether the CAR between–subjects predicts cognitive performance in the afternoon of the same day. The primary hypothesis is that the magnitude of the CAR will positively predict both EF and DM task performance. With regards to the prediction of the CAR itself, it is hypothesised that this will be influenced by both psychological anticipation of the day ahead and prior-day alcohol consumption, whilst both prior day exercise and stress will predict cortisol secretion in the later morning samples.

6.3 Method

6.3.1 Design

Ethical approval was provided by the Departmental Ethics Committee, and analysis of cortisol samples was conducted in accordance with the Human Tissues Act (UK). The study was designed to explore CAR-cognition associations in a between-subjects context, including the investigation of both EF and memory performance. The CAR sampling methodology complied with all

recommendations of the CAR expert consensus guidelines of Stalder et al. (2016) so as to maximise accuracy in estimation of the CAR and increase potential comparative value of the present data with that of other compliant CAR studies. Such controls included electronic monitoring of adherence to the sampling protocol using wrist-worn actigraphy and MEMS caps.

6.3.2 Participants

Participants were students recruited from the University of Westminster Psychology Department's Research Participation Scheme (RPS), in addition to volunteers from the academic community. Participants were recruited between September 2014 and February 2015, during normal study and outside of the examinations period. Interest was generated using a summary of the study advertised on the RPS internet webpage, in addition to announcements in undergraduate lectures. This description (and subsequent required fields on the volunteer form) indicated that participants must be in good health, non-smoking, and free from medication. Those recruited from the RPS scheme received research participation time. Participants received no other incentive to participate. Interested participants attended a briefing session which was conducted either on a one-to-one basis or in small groups (of no more than 3). This consisted of a detailed verbal description of the procedure, including emphasis on the importance of strict adherence to the CAR sampling protocol. Individuals were asked to only participate if they were confident that they would be able to fully adhere to the protocol, and were further advised that if any sampling errors be reported immediately, and that failure to do this (detected by electronic monitoring) would result in no research credits being awarded.

Participants were also given a demonstration of how to collect saliva using a salivette, and allowed to practice this prior to starting the study. Several participants subsequently withdrew when they understood the study requirements.

The initial sample population consisted of 55 participants, however complete data for one participant was excluded due to delays of >15min for all samples. The final sample therefore consisted of 54 healthy participants (44 females, 10 males), with a mean age of 20.2 (SD = 3.0) years. Of these, 48 were non-smokers, 5 were ex-smokers and 1 was an occasional smoker. 13 participants were using oral contraceptives at the time of participation, while no participants were taking any other medications known to effect cortisol secretion.

6.3.3 Materials

For the purposes of monitoring participant adherence to the CAR protocol, wrist actigraphy was used (Actiwatch, Phillips, UK), along with MEMS caps (The Aardex Group, Sion, Switzerland), as described in chapter 3. Participants were also required to complete a sample time self-report sheet on each morning of the study for verification of self-report with electronic measures. Study guidelines were also provided in the form of a participant checklist (see appendix 4) to ensure participants had a clear understanding of the study schedule, and to use as a first point of reference should they have trouble remembering any step of the procedure.

The computerised cognitive tests consisted of the CANTAB Attention Switching Task (AST) and the One-Touch Stockings of Cambridge task (OTS), as described in detail in chapter 3, section 3.5.1. The primary outcome measure of interest from the AST was switch cost; the same as in study I. The selected outcome measure of interest from the OTS was the mean latency to correct completion, measured from all trials (as described in section 3.5.3).

Participants were also required to complete the TMT as a separate index of EF (Tombaugh, 2004), specifically to assess set-shifting/cognitive flexibility (Arbuthnott & Frank, 2000), and since a positive association between performance on form B of this task and CAR magnitude has been previously demonstrated in older adults (Evans et al., 2012). The TMT consists of two forms: Form A, which assesses a simple motor task which is minimally dependent on EF (connecting numbered points on the sheet by pen, in ascending order), and Form B, a more complex version of the same task, requiring the participant to alternate between a series of letters as well as numbers. The EF measure is calculated by subtracting the time taken to complete Form A from the time taken to complete the more complex Form B, and as such producing a latency score which accounts for the time taken to switch between the task demands (EF) while controlling for simple differences in motor speed (Arbuthnott & Frank, 2000).

A word learning task was also devised, so as to further explore the CAR-memory associations found in previous studies and to control for memory in analyses of EF. This task involved viewing a total of 36 individual words selected from the MRC Psycholinguistic Database (Wilson, 1988). Similar to the methods of

Kuhlman et al. (2005), these items were selected in accordance with their emotional valence. Emotional valence was established using the Affective Norms for English Words guide (ANEW; Bradley & Lang, 1999) from which 3 categories of words were selected: Positive, Negative, and Neutral (12 words in each group). All of the words were selected on the basis of being no more than 3 syllables in length, and were matched for concreteness, and frequency of occurrence in everyday language using the frequency tables of Kucera & Francis (1967). As such, the words were (theoretically) of equal familiarity to all participants, so as to control for any confounding effects associated with the familiarity with, or memorability of, the words. The word learning task was also counterbalanced, such that for half of the participants the items would be presented in the reverse order, so as to control for any confounding effects of presentation order on learning. These words were presented on a computer screen for precisely 5 seconds per item, with participants instructed to learn the items (intentional learning). Recall on day 1 was assessed using a free recall task, while recall on day 2 was measured using both a free recall task and a recognition task. This involved presenting participants with a sheet of paper containing all 36 target words combined with 36 words which were not included in the original list. All words were selected using the same criteria as the original word list so as to prevent recognition task items being more or less memorable. The full list of words used in the learning task is presented in appendix 9, and the recognition task is presented in appendix 10.

Evening and morning diaries (described in detail in chapter 3; presented in appendix 8) were included to measure participants' self-reported psychosocial states, sleep-related variables, prior day alcohol consumption (self-reported, in

standard 10ml units of alcohol) and exercise (in approximated hours) throughout the study.

6.3.4 Procedure

All participants were required to complete two consecutive days of testing. Participants were briefed on the first day of the study and contacted by SMS message in the evening prior to data collection on both days of participation. This SMS message provided a reminder of the sampling procedure. Prior to going to sleep, participants filled in the evening diary and put on the Actiwatch, which was then worn throughout the night to assess both the timing of sleep and sleep quality.

Saliva samples were collected immediately upon awakening, and at 15, 30, & 45-min post-awakening using the standard procedure for salivary cortisol sampling, described in detail in chapter 3. Upon completion of the morning sampling, participants were required to fill out the morning diary. Participants then continued with their normal routine, before visiting the university in the afternoon of the same day for the cognitive testing session. Upon arrival for the afternoon session, participants were required to collect one further cortisol sample, using the same sampling procedure.

All cognitive tests were administered by the researcher in a controlled environment (a private, quiet lab cubicle), and each participant was tested individually. On the first day, the afternoon cognitive testing session involved both an executive function testing battery and a word-learning task. The

learning task was administered on a computer screen, while the computerised executive function tasks were completed using a specialised CANTAB computer and the TMT was completed using paper and pen. As the learning task required a distractor task (to control for the regency effect of short term memory) between learning and testing of learning on day 1, the TMT was completed at this stage. The testing schedule is presented in figure 6.1. Learning and recall were both time-restricted to 3-min. The complete cognitive testing session on day 1 took approximately 45 min to complete.

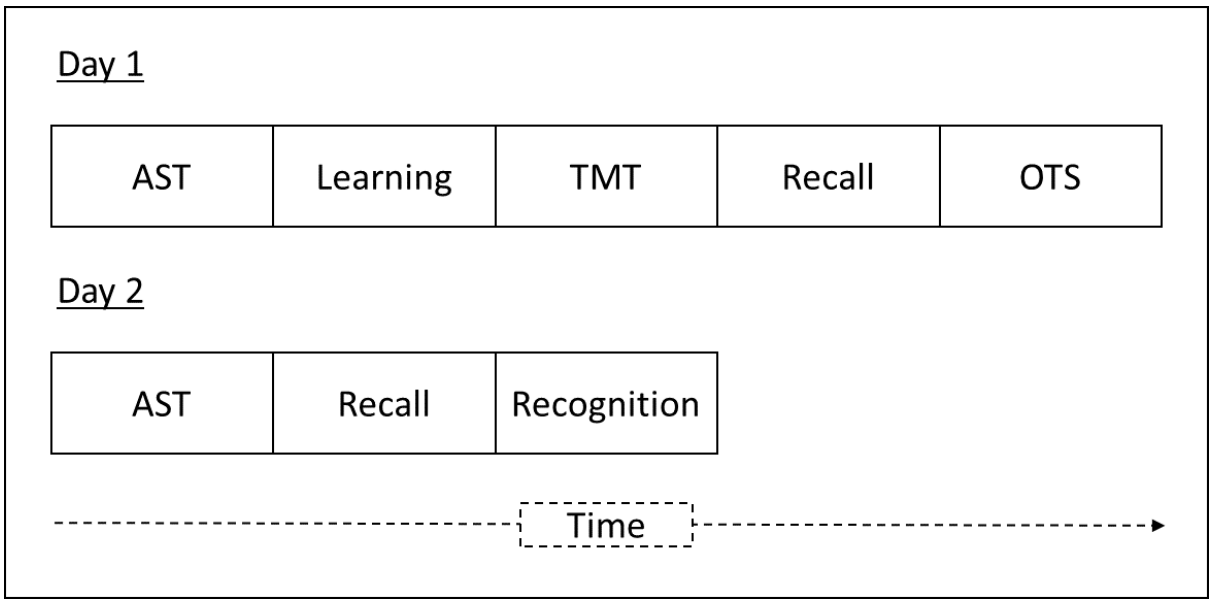


Figure 6.1 Cognitive testing protocol on days 1 and 2.

The testing procedure (including collection of timed saliva samples for determination of the CAR) was repeated on the second day but with the afternoon learning tasks replaced by timed recall and recognition tests of the learned items from the previous day (see figure 6.1). The recognition task was completed after the free recall task in this session, to control for any potential confounding effect of exposure to these cues from confounding the free recall

task. In line with the previous day's procedure, the recall and recognition tasks were both completed within a 3-min time limit. The complete cognitive testing session on day 2 took approximately 30 min to complete.

6.3.5 Treatment of data

In line with the analysis presented in the previous studies of this thesis (and as described in section 3.7), data were analysed using mixed regression modelling (Blackwell et al., 2006) of between-subject variation in EF performance. For the purposes of presentation and consistency with study I, the EF variable was produced by inverting the switch cost variable, such that high scores represent better EF performance. CAR Mninc was computed, and a fourth root transformation performed to counteract positive skewness. As some CAR values were negative, this transformation was performed on MnInc + 10 to ensure data could be transformed. The transformed MnInc variable on the 2 study days was used as the principal covariate. Both the EF and transformed MnInc variable were standardised for ease of comparison and interpretation of the results (represented as 'ZEF' and 'ZCAR', respectively), though for reference purposes descriptives, including means and standard deviations are presented for raw data only. Session (sampling day) was included as a main factor in the initial model. Sampling delay on each day of CAR assessment was computed using Actiwatch and MEMS cap recordings. Data were excluded if delay between electronically monitored awakening and collection of sample 1 exceeded 15 min, or if complete electronic monitoring data was not present, leaving a total *N* of 74 CARs from 41 participants, including 40 at session 1, and 34 at session 2. Delay data for the EM compliant sample was recoded into two categories; 0-8

(no delay/minor delay) and 9-15 min (moderate delay) between electronically monitored awakening and collection of sample 1 (inclusive of lower and upper scores), with CARs on 61 and 13 days in each group respectively (i.e. 82.9% meeting the criteria for ‘no delay/minor delay’, and 17.1% meeting the criteria for ‘moderate delay’). This delay variable was included as a main factor in a secondary model of the EF data, along with ZCAR (but without session). Intercept effects were modelled as both fixed and random. Since only two levels of the repeated-measures variable (session) existed, compound symmetry could be assumed and a random intercept only model was run. Associations with the alternative EF measures of OTS and TMT, were explored by Z-scoring and modelling of these as the dependent variable, replacing ZEF in the model. The relationship with memory performance was modelled using the same method, and then further investigated by way of memory performance being added to the principal model to check for a possible compromising effect on any initial findings. Finally, further modelling was undertaken to test for extraneous and potentially confounding variables, including cortisol levels upon awakening, afternoon cortisol levels, awakening time, gender, sleep duration and psychosocial factors.

6.4 Results

6.4.1 General descriptives

Table 6.1 presents the descriptive statistics on day 1 and day 2 for both cortisol sample and composite CAR measures, as well as the situational and cognitive variables measured on both days. Table 6.2 presents the descriptive statistics for variables measured on only one day (OTS, TMT, and recognition memory).

Though standardised in the data modelling, for clarity both EF and MnInc are presented in their raw form in table 6.1. The cortisol data showed a fairly typical pattern for the age and health status of the sample (Wüst et al., 2000b; Stalder et al., 2010 b). The average CAR across two days showed a mean increase of 8.59 nmol/l cortisol in the 45 min after waking, with the mean peak at 30 min post-awakening.

Table 6.1 Descriptive statistics on day 1 and day 2 for cortisol samples (0-45min), afternoon cortisol, sleep variables, AST

reaction time, EF, AST mean latency to correct response, word recall, prior day alcohol consumption, prior day exercise, and CAR measures.

	Day 1			Day 2		
	Mean	SD	N	Mean	SD	N
Cortisol S1 (0 min) (nmol/l)	8.31	5.35	40	7.55	3.73	34
Cortisol S2 (15 min) (nmol/l)	12.77	6.64	40	11.98	5.60	34
Cortisol S3 (30 min) (nmol/l)	16.71	7.70	40	16.89	8.15	34
Cortisol S4 (45 min) (nmol/l)	17.04	8.99	40	17.15	9.86.	34
Cortisol S5 (Afternoon) (nmol/l)	6.51	4.32	40	6.35	5.34	34
Time of awakening (hh:mm)	6:57	1:17	40	7:10	0:59	34
Sleep duration (hh:mm)	6:50	1:36	33	7:10	1:15	28
AST reaction latency (ms)	664.03	190.15	40	536.92	139.83	34
EF (AST switch cost inverted) (ms)	48.92	89.69	40	52.10	58.07	34
Word recall	12.20	5.14	40	10.08	4.74	36
Prior day alcohol (approx. units)	0.10	0.50	39	0.06	0.35	33
Prior day exercise (approx. hrs)	0.53	1.89	38	0.26	0.83	33
MnInc (nmol/l)	7.20	6.22	40	7.80	6.11	34
AUCg (nmol/l)	42.15	18.97	40	41.22	18.89	34

MnInc = Mean Increase (0-45 min post-awakening),

AUCg = Area under the curve with respect to ground (0-45 min post-awakening).

Table 6.2 Descriptive statistics for variables measured on only one day: OTS, TMT, and recognition memory.

	Mean	SD	N
OTS mean latency to correct (secs)	36.94	17.22	41
OTS mean choices to correct	1.37	0.22	41
Trail A (sec)	23.47	5.00	41
Trail B (sec)	51.18	18.88	41
TMT EF (B - A)	27.71	17.58	41
Word recognition	19.58	5.93	40

OTS, TMT = Measured at session 1,
Word recognition = Measured at session 2.

6.4.2 Modelling of data

6.4.2.1 The CAR and EF

Initial mixed modelling of ZEF and ZCAR with session indicated that the ZCAR was significantly positively associated with ZEF performance measured on the same day (F (65.237) = 4.280, p= .043). Both session (F (33.814) = 0.204, p= .654) and the session by ZCAR interaction (F (41.512) = 0.001, p= .979) were non-significant. The primary hypothesis, that CAR predicts same day EF, was therefore accepted. Table 6.3 shows F-ratios and significances for the parameters in this modelled data. In the second model, session was therefore excluded as a fixed effect, but this model investigated whether degree of delay in any way mediated or modulated the overall ZCAR-ZEF relationship. The proportion of data with delay of more than 8-min (but less than 15) was small

(17.1% of sample), so, unsurprisingly, no statistically significant effects of delay emerged, although post-hoc exploration of coefficients did indicate that where CAR data were measured accurately (i.e. first sample within 8-min of awakening) the association between ZCAR and ZEF remained significant and strong (coefficient (SE)= 0.28 (0.12) p= .025), while the effect was predictably smaller where CAR data was less accurately measured (9-15 min delay; coefficient (SE) = 0.09 (0.25), p= .721). Table 6.4 shows the coefficient estimates produced by the model. Figure 6.2 plots the line of fit equations for the predictive relationship between ZEF and ZCAR for accurately and inaccurately collected CAR data. Since EF and CAR were both entered into this model as *z*-scores, the slope coefficient of 0.28 for accurately measured data indicates a predicted improvement of 21 ms in attention switching performance on the AST for every 1 SD increase in magnitude of CAR MnInc.

Table 6.3 F-ratios, df and significances associated with parameters in mixed regression modelling of ZEF data.

Model Parameter	d*f* (num, denom)	*F*	*p*
Intercept	1, 40.17	0.016	.901
ZCAR	1, 65.24	4.280	.043
Session	1, 33.81	0.204	.654
ZCAR*Session	1, 41.51	0.001	.979

Table 6.4 Coefficient estimates for parameters in mixed regression modelling of ZEF data.

	Coefficent (SE)	*p*
Fixed effects		
Intercept	0.01 (0.15)	.970
ZCAR	0.28 (0.12)	.025
Accuracy 0-8 min	Red.	Red.
Accuracy 9-15 min	0.09 (0.25)	.721
ZCAR*Accuracy 0-8 min	Red.	Red.
ZCAR*Accuracy 9-15 min	-0.21 (0.22)	.342
	Variance (SE)	*p*
CS Diagonal		
CS diagonal offset	0.38 (0.09)	<.00

		1
CS covariance	0.64 (0.20)	.001

SE = Standard error, Red. = Redundant in model.

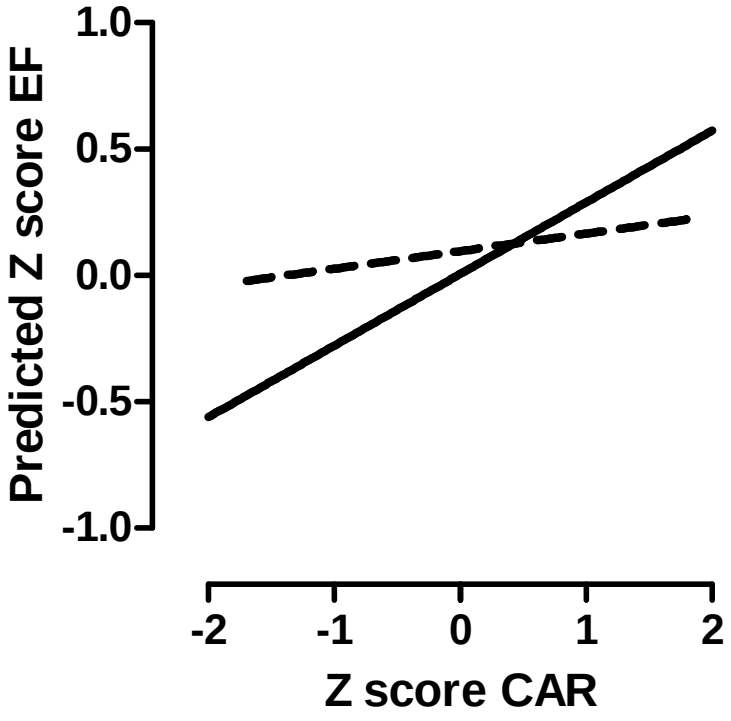


Figure 6.2 Line of fit equations from mixed modelling for predicted ZEF ('Fixed Predicted Values'; Y axis) and ZCAR (X

axis) by sampling accuracy. Solid line indicates accurately measured data; dashed line indicates inaccurately measured data.

6.4.2.2 The CAR and alternative EF measures

Further modelling explored associations with performance on the OTS. Noting that, as between-subject measure, OTS could only be included in the experimental procedure once (on day2), and with some data excluded due to failure to complete the task, a model was therefore produced with ZCAR as the dependent variable. Modelling of OTS with session indicated that there were no significant effects in this model and no interaction with session. Modelling of OTS latency to correct answer as a principal covariate and in terms of its interaction with accuracy indicated that this measure of OTS performance did not significantly predict ZCAR (F (67.049)= 0.369, p = .546), and this was also the case for both the main effect and interaction with accuracy. To explore TMT performance, a similar model was produced with ZCAR as the dependent variable. This model included session as a fixed factor and TMT as both as principal covariate and in terms of its interaction with session. Results of this model indicated that TMT was not associated with ZCAR (F (35.624) = 2.384, p = .131), and the interaction between session and TMT score was also non-significant (F (32.494) = 0.196, p = .661). Though it should be noted that the ZCAR-EF relationships falling short of significance in these models might be expected given the effect size of the association with EF in the principal model,

and the reduced statistical power when applying the sample size restriction in these subsidiary analyses.

6.4.2.3 The CAR and memory performance

Further modelling explored the relationship between ZCAR and memory performance measures. In the case of recall scores, as these were measured on both study days, this model followed the structure of the principal model but with recall employed as the DV. The results of this modelling indicated that session predicted free recall performance in the expected direction, such that recall scores were greater in the first session and reduced the following day (F (30.920) = 34.92, p <.001). Between-subjects ZCAR was not associated with same day free recall (F (42.337) = 1.03, p = .316), and neither was the ZCAR by session interaction (F (32.994) = 0.99, p = .327). To explore recognition scores, a model was produced with ZCAR as the dependent variable (much like the analysis of the TMT), given that recognition was measured only at session two. This model included session as a fixed factor while recognition was explored both as principal covariate and in terms of its interaction with session. Results of this model indicated that recognition was not associated with ZCAR (F (36.351) = 2.53, p = .121), and the interaction between session and recognition score was also non-significant (F (33.751) = 0.841, p = .366).

6.4.2.4 Exploration of moderating/mediating factors

Finally, a series of additional models were produced to examine whether the observed ZCAR-ZEF effect was robust to inclusion of potentially relevant

covariates (afternoon cortisol levels, awakening time, sleep duration, gender, reaction time scores, prior day exercise, prior day alcohol consumption, or psychosocial factors). These variables were separately added to the ZCAR-ZEF by accuracy model to achieve a suitable ratio of variables to cases and conserve degrees of freedom. The results of these analyses indicated that the association between ZCAR-ZEF in accurately measured data was robust to these effects in all but two cases. In all models, ZCAR-ZEF remained similarly significant, with a similar effect size, except for the modelling of the interaction between ZCAR and awakening time (where the coefficient, of .23 was similar to the earlier model, but fell short of significance at .082) and the model including S1 (in which the coefficient again remained similar, at .23, but fell short of significance at .084). In one other case the ZCAR-ZEF association fell short of significance, where sleep duration was included in the model, however this is likely due to the limited sample size (n = 33) as the effect size remained similar (.21). A further, non-hypothesised effect emerged from this modelling in that gender significantly predicted ZEF with females performing better than males (F (41.675) = 5.39, p = .025). However, as these subsidiary findings were non-hypothesised, the significance values should not be interpreted in the same way as a hypothesised finding, given the meaning of p. Aside from this unexpected finding, all of these control variables and their interactions with MnInc were non-significant.

6.5 Discussion

This study explored variation in the CAR and an index of executive functioning in healthy young adults. The results indicate that, in the case of accurately measured CAR data, the magnitude of the CAR positively predicts performance on this index of EF in the present sample, which is consistent with previous evidence of a CAR-EF association in older adults (Evans et al., 2012), and the within-subject study of morning EF presented in chapter 4 the present thesis. The hypothesis that the magnitude of the CAR positively predicts EF performance in the afternoon of the same day is therefore accepted. This is the first demonstration that between-subjects the CAR is related to same day measures of EF in the healthy young. However, this relationship was not significant in the inaccurately measured CAR data (where delay from awakening to first sample was >8min). With regard to DM, CAR magnitude did not significantly predict same-day DM measure used in this study, and the hypothesis that the CAR positively predicts DM in the afternoon of the same day is therefore rejected. The findings are discussed here in relation to previous literature, including the convergent findings of studies exploring the importance of sampling time for accurate assessment of the CAR and associated factors.

It is well established that cognitive functions fluctuate in relation to circadian and sleep factors (Schmidt et al., 2007), and this has been demonstrated in EF tasks such as those measuring inhibitory control (e.g. Manly et al., 2002; Schmidt et al., 2007). While specific set-shifting/cognitive flexibility tasks are less explored in this context, it is likely that such tasks are subject to circadian and sleep-wake influence given the correlations between the various EF measures (Schmidt et al., 2007). Importantly then, when compared with study I (chapter 4) of the present thesis, the results of this study indicate a relationship

with attention-switching performance in the afternoon, rather than only in the morning. These results therefore indicate that the CAR may be broadly associated with diurnal cognitive function, and not restricted to the immediate post-CAR period. A relationship with cognition throughout the waking day is also to be expected if the CAR does indeed play a role in synchronising peripheral clocks of the circadian system (Clow et al., 2010a), and as such the present data lend weight to this hypothesis.

Importantly, the results of this study clearly show that while the magnitude of the CAR MnInc does predict EF in accurately measured CAR data, and by comparison, in the cases where sampling was delayed beyond 8 min post-awakening this effect was no longer significant. This finding is in agreement with converging evidence from studies of CAR sample timing inaccuracy, showing that delay beyond 8-min post-awakening misses the start of the CAR and therefore will have a powerful impact on reliability of effects of this kind (Smyth et al., 2016). It is noteworthy that data presented here were gathered after a thorough briefing and also the application of strict monitoring of participant adherence that have not been present in previous studies of the CAR (Stalder et al., 2016) and these data are mostly non-delayed. In data sets with more substantial delay present (where delay is not appropriately controlled for, or where much of the data exceed 9 min in delay) it appears likely that a very serious confounding effect of delay could occur. This is a consistent issue in cortisol research, as even in studies of associations with the diurnal cortisol slope, appropriate adherence monitoring is associated with larger effect sizes (Adam et al., 2017). Noting that the CAR is a more dynamic effect and requires far greater precision of measurement than diurnal slope (Stalder et al. 2016;

Smyth et al., 2016), the importance of adherence controls and modelling of delay in CAR studies is apparent. This finding is of substantial importance, not only because it is indicative that the findings here are robust where sampling is accurate, but also because this suggests that studies without appropriate controls for participant adherence to the CAR protocol are likely to be unreliable in detecting CAR associations with other factors. This provides a potential explanation for the inconsistencies in previous CAR-cognition literature, as reviewed in chapter 1 of the present thesis, and marks an important requirement for future studies in this field.

The lack of an association between the CAR and the alternative EF measures of TMT and OTS may indicate that the association is restricted to the AST index alone, or may reflect the limited sensitivity of these alternative measures for use in the present study. Although moderately correlated, confirmatory factor analysis has shown the EFs of 'shifting', 'updating', and 'inhibition' to be clearly distinguished at separate processes (Miyake et al., 2000). The absence of an association between CAR and OTS performance then may be indicative of the specificity of the CAR-EF association to 'shifting' type tasks, as opposed to 'inhibition' and 'updating' as primarily measured by the OTS. This is convergent with evidence from studies of acute diurnal cortisol secretion and EF, which have demonstrated associations with cognitive flexibility ('shifting'), notably in a similar index of EF: switch cost on a reaction time task (Dierolf et al., 2016). In this study, Dierolf et al. used the mean CAR AUCg from three consecutive days prior to the cognitive testing as a measure of basal cortisol, and found participants with high basal cortisol showed worse cognitive flexibility than those with low basal cortisol. Importantly however, they also found that acute

cortisol administration in the ‘high basal’ group improved task switching performance, particularly for negatively valent stimuli, when compared to placebo. This suggests not only that cortisol secretion is associated with cognitive flexibility, but that the rapid effects of cortisol secretion are distinguishable from those of basal cortisol as measured by the AUCg (Dierolf et al., 2016). This may be particularly relevant for the CAR in the context of understanding it as a rapid, dynamic aspect of cortisol secretion. The association with the CAR may therefore be specific to ‘shifting’ or ‘task switching’ type processes, and unrelated to other aspects of EF.

Such an association between CAR and performance at shifting type EF tasks can quite conceivably be aligned with the ‘Boost’ hypothesis of the CAR (Adam et al., 2006; Clow et al., 2010a; Fries et al., 2009), in that task-switching and cognitive flexibility is required upon awakening, and the rapid re-attainment of such functions may be particularly important in the context of increased anticipation of challenge in the coming day. Such an association between the CAR and task-switching in the present study may therefore present a conceivable mechanism by which the CAR may play a homeostatic role in ‘boosting’ cognitive performance upon awakening. An alternative explanation here may be that the TMT and OTS measures are not sufficiently sensitive to detect such an effect in a young, healthy functioning population. Where variability in EF is more pronounced in older adults, a clearer effect is likely to be seen in tests such as the TMT as observed by Evans et al. (2011), but a more sensitive test such as the AST may be required to detect differences where variance is low, as in a healthy-functioning young sample.

Finally, the lack of a significant association between CAR and DM in the present study is at odds with previous research (e.g. Baumler et al., 2014; Buchanan et al., 2004; Pruessner et al., 2007; Oster et al., 2016; Rimmele et al., 2010; Wolf et al., 2005). The failure to replicate this finding may be indicative of 3 things; that the previous studies were not valid/reliable, that the measures used here were not valid/reliable, or that the study was underpowered to detect this effect. On balance, the first of these appears unlikely given the previous successful replications, and therefore one of the latter explanations is perhaps more likely to explain this inconsistency. As the measurement of the CAR in the present study was more carefully controlled than previous studies, any issue would likely come down to the measurement of DM in the present study. There exists the possibility that some unique factor to do with this test (for example, chance prior associations with learning targets, or implementation of rehearsal by some participants between testing sessions) might have prevented it from being a reliable index of DM. However, in the absence of any apparent reason to doubt the validity of the method, this should be registered as a failure to replicate this previously reported effect.

6.5.1 Conclusions

The results of the present study provide a first demonstration of a between-subject association between the CAR and an index of EF in healthy young adults. Taken with the earlier demonstration of a between-subjects CAR-EF relationship in older adults (Evans et al., 2012), in and the within-subject demonstration of a CAR-EF relationship in study II of the present thesis, these studies present a clear body of evidence for an association between the CAR

and this aspect of cognitive function in humans. Such an association has potentially important implications for understanding the function of the CAR as a time-of-day marker within the circadian rhythm of cortisol secretion, and thus as a potential regulator of pre-frontal cortex function. Further, that this effect was only observed in accurately measured CAR data further consolidates the evidence that adequate controls on accuracy of sample timing is of vital importance to studies of the CAR and the exploration of associated factors.

7

General discussion

7.1 Overview

The studies presented here expand upon previous published literature showing associations between the CAR and cognition in older adult and clinical populations by demonstrating that such a relationship exists in healthy young adults. Along with this, it is demonstrated that the CAR is associated with a TMS measure of neuroplasticity in the afternoon of the same day, and further these studies highlight methodological issues involved in measurement of the CAR. The practical implications of these findings are, first, that the association between the CAR and this same-day index of executive function, as well as TMS-induced neuroplasticity, in healthy young adults may present a role for the CAR in healthy function. Second, that this may present a mechanism by which the CAR can influence symptoms seen in clinical samples where diminished CAR profiles are observed. A third practical implication is that this presents a very clear case for strict monitoring of sampling accuracy in assessment of the CAR. Studies I and II are superior to many in that these both employed very well-briefed researcher-participants and had far greater adherence to the CAR sampling protocol than much of the published research in this field, while study III demonstrates that even in very well-briefed participants, the necessity of strict monitoring of adherence in CAR studies must not be understated. These studies should be viewed as components of an overall research programme, as the rationale for each study builds upon the findings of the previous. Accordingly, the intention behind this chapter is to integrate and discuss the findings of the research programme as a whole. To this purpose, methodological issues are presented first followed by discussion of the major findings: the CAR-

EF association, CAR-plasticity association, and implications for monitoring protocol adherence. The chapter ends with consideration of directions for future research.

7.2 Methodological issues

The same, well-established methodology was applied in all of the studies presented here as per the guidelines of Stalder et al. (2016). CARs were observed in each study, with minimal evidence of CAR non-responding. Free cortisol was measured in saliva in samples collected at 0, 15, 30 and 45 min post awakening at the least in all studies, providing detailed estimates of CAR profiles on each study day. Table 7.1 compares the mean cortisol concentrations at each sampling point for each study in this thesis, alongside normative data from previous research (table adapted from Thorn 2005; normative data from Clow et al., 2004). As can be seen from this table, the data presented here are almost all within 1 SD of the mean for the normative data and never outside of 2 SD, suggesting general validity of the CAR measurement.

Table 7.1 Cortisol data for the studies in this thesis and previously published normative data (adapted from Thorn 2007; data from Clow et al., 2004).

	Cortisol concentrations (nmol/l)				
	0 min PA	15 min PA	30 min PA	45 min PA	Increase in cortisol (nmol/l) from 0-30 min PA
Study I	10.2	13.9	17.9	17.3	7.7
Study II	14.6	16.4	18.0	17.1	3.4
Study III day 1	8.6	13.7	18.2	18.9	9.7
Study III day 2	8.7	14.8	20.3	19.5	11.6
Mean of the above studies	10.5	14.7	18.6	18.2	8.1
Mean of published studies (±SD between studies)	11.6 (4.6) n=12	16.3 (5.6) n=6	20.0 (5.9) n=12	18.9 (6.5) n=6	9.3 (3.1)

Key: PA = post awakening, n=number of studies used to calculate mean (see Clow et al., 2004).

As discussed in chapter 4, the timing of the cortisol peak was at 30 min post-awakening in study I, in line with previous research in males. There is evidence that males have an earlier and less-sustained CAR, with the typical peak at 30 min and reduced levels at 45 and 60 min post-awakening, compared to females (Wüst et al., 2000b; Oskis et al., 2009; Stalder et al., 2009). That these findings are convergent with previous studies supports the validity of the present thesis. In studies I and III however, the samples were predominantly female, and so the

timing of the average CAR peak at 30-min post-awakening in these studies is at odds with these previously published figures. However, recent research exploring the timing of the CAR in detail has shown that the typical peak is in fact between 30-40 min, regardless of sex (Smyth et al., 2013a). This raises some doubts over the previously published norms for the sexes, given that these studies examined cortisol levels only at 30 and 45 min post-awakening. Therefore, although the reason for the average peak falling at 30 min in the present studies is not entirely clear, and this could be considered a limitation to the generalisability of these studies, it is quite plausible that the previously published sex differences reflect an artefact of what are in fact more subtle sex differences in timing of the peak than is apparent when measured only at 15 min intervals.

A number of possible explanations for the discrepancies in the previous literature have been considered in the present thesis, and it is important to consider them in relation to the present studies. These include methodological inconsistencies of previous studies regarding adherence to the CAR sampling protocol, the validity of the cognitive assessments employed, and the absence of appropriate controls for other potentially confounding factors such as state factors or variance between samples. Certainly, some of the initial criticism of previous research in this field can be dismissed outright. For instance, in all the studies presented here the CAR was assessed on at least two days over a sampling period of 45 min following awakening, and careful electronic monitoring of adherence to the sampling protocol was ensured in each study. As such these studies are designed to ensure validity of the CAR measurement,

and the role of known potentially confounding situational and state influences were controlled for in the analyses.

A potential limitation to the implications of the findings of the studies presented here is that they are all based on young adults and may not generalise to other age groups. There is some evidence to suggest that CAR magnitude is negatively associated with age (Clow et al., 2010b), and as such, these findings can not necessarily be generalised to other age groups. However, with regard to age, the purpose of these studies was to explore the CAR only within this small and select sample and so such a limitation is integral to the design. Another factor to consider with regard to generalisability of data from any sample is the socioeconomic status (SES) of the sample population. However, despite early studies finding positive associations between CAR and SES (e.g. Wright & Steptoe, 2005), this has only previously been explored in cross-sectional studies and prior to the hypothesis that an increased CAR is a homeostatic response to challenge (Adam et al., 2006), and therefore did not consider within-subject. As such, there remains a possibility that SES might be associated with the CAR in a between-subjects context, but there is no reason to believe that CAR associations with EF or plasticity would be influenced by SES in a within-subjects context. As such, SES can be reasonably deemed irrelevant to within-subjects variation as explored in two of the three studies in the present thesis. With regards to study III, there were no hypotheses made regarding SES, and this variable was therefore not measured. However, based upon the effect sizes observed in studies of SES and the CAR, it would appear very unlikely that differences in SES could confound the association between CAR and EF observed in this study.

A further important consideration with regard to the samples of the present studies is motivation and the nature of their consent to participate. The samples were comprised primarily of undergraduate and postgraduate students, and while the participants in study II were not presented with any apparent incentive for participation beyond intrinsic motivation to support the research, the undergraduate participants in study III did receive study credits for their participation. This is unlikely to have influenced the results of the study in any major way; however it is possible that these participants may have been particularly motivated to be honest regarding their self-reported adherence to the sampling protocol compared to other studies. This is possible as the briefing included advice that informing the researcher about any errors with the sampling would still allow participants to claim study credits, but that failure to notify the researcher of non-adherence (as detected by electronic monitoring) would result in these being withheld. Nonetheless, any effect of such an incentive on the results appears unlikely as evidence from previous studies suggests participant adherence to the saliva sampling protocol is not increased by financial incentive (Halpern et al., 2012).

The results of Study III indicated very little consistency across days in suspected non-adherent individuals, suggesting that adherence is largely circumstantial rather than due to participant characteristics. Indeed, as with previous research, sampling accuracy was expressed as a day-level rather than person-level variable. From the present data, the same appears to be true for negative CAR profiles, as those individuals showing a negative response on one day were no more likely to show a negative response on the other day. Although such

findings taken at face value would appear to suggest that both non-adherence and negative CAR profiles are not trait characteristics, it is worth noting that no hypotheses were made regarding these variables and, in the absence of appropriate controls, such differences cannot be taken as evidence. This is especially true since assessments of extreme scores can often be explained by regression to the mean. Therefore, detailed assessment of the influence of participant characteristics on both protocol adherence and CAR non-responding would require further studies appropriately designed to test this, for example by inclusion of control groups.

Although predictive due to their temporal relationship, the findings presented here are nonetheless correlational in nature. Therefore, as with all correlational relationships, it is important to emphasise that causality cannot be assumed here. There exists the possibility that the correlations between the CAR and variables considered here could be explained by some unknown and unmeasured factor that influence both magnitude of CAR and the cognitive/physiological outcomes measured here. Regarding the CAR-EF association, for example, differences in general cognitive abilities of participants might provide an alternative account of the findings. By this account, participants with greater overall cognitive ability would be expected to do better at the cognitive tests, and perhaps have a larger CAR due to better general health, or perhaps simply a better regulated sleep-wake cycle. Indeed, EF is known to reflect separable but related functions (Miyake et al., 2000) and may therefore in some cases predict overall cognitive ability, perhaps giving some support to this alternative explanation for the between-subjects findings presented here. Further, as has been noted elsewhere (Stalder et al., 2016),

more competent participants might also be more likely to adhere closely to the sampling procedure, thereby influencing CAR estimates by non-random variation in delay (also increasing the risk of a self-selecting sample, as exclusion of data for non-adherent participants would be effectively non-random in such a case). However, though this alternative interpretation of the findings might seem potentially applicable to the between-subjects study (study III) presented here, it is perhaps less easily applicable to the within-subjects studies (studies I & II), suggesting that such an explanation is unlikely, but nonetheless possible. It must be noted that, beyond the present example of general cognitive ability, it remains a possibility that both EF and the CAR might be influenced by an unknown and unmeasured factor, and this could only be dismissed by further study including experimental manipulation of the variables. Nonetheless, there is no implication or apparent reason to believe that this is likely from the data presented here.

A similar limitation may be applied to the association with plasticity, as there exist other neuromodulators including BDNF and dopamine, which might influence this association. For instance, should temporal and circadian variation plasticity be the product of the BDNF rhythm and not related to cortisol, then this might result in the present finding of a same-day CAR-plasticity relationship, as diurnal BDNF and cortisol levels show synchrony and are thought to be physiologically co-regulated (Begliuomini et al., 2008). However, such an explanation would appear unlikely, especially since cortisol regulates BDNF effects on brain plasticity by modulation of the BDNF producing gene (Suri and Vaidya, 2013). It would be more plausible to expect that any association between diurnal plasticity variation and BDNF would be modulated by cortisol.

Further, the demonstration that administration of glucocorticoids inhibits plasticity in the same region of the brain measured here: M1 of the motor cortex (Sale et al., 2008) provides further strong support for the interpretation that the CAR influences plasticity as proposed in the present thesis.

7.3 The Cortisol Awakening Response and Executive Function

The findings of the studies presented here indicate that CAR magnitude is positively associated with EF, as well as TMS-induced neuroplasticity, in healthy young adults. These findings are convergent with studies of older adults (Evans et al., 2011) and afford the possibility that this may present a mechanism by which the CAR can influence mental health.

The findings of these studies contradict those of Ennis et al. (2016), who did not find an association between the CAR and processing speed or working memory in young, middle-aged, and older adults. A possible explanation for this may be the inconsistencies in study methodologies employed. Ennis et al. employed a simple delta 0-30 CAR measure and did not monitor participant adherence to the CAR protocol beyond self-report, which has been shown to be an inaccurate method (Stalder et al., 2016). However, the present studies employed the more detailed calculations of the CAR combined with strict controls on participant adherence, as recommended by the expert consensus guidelines (Stalder et al., 2016). Indeed, as the measurement of the dynamic CAR is very sensitive to even small inaccuracies, such as delays >5 min in sampling time (Smyth et al.,

2013a), there is good reason to consider rigorous study protocols as more reliable and to exercise caution in interpreting findings from studies where the established requirements for rigour are not met (Stalder et al., 2016; Smyth et al., 2016).

EF shows an inverted U-shaped relationship with age (Zelazo et al., 2004), such that performance improves from childhood until young adulthood and declines thereafter. Understanding what contributes to this relationship therefore has great implications for potential therapeutic interventions. In healthy individuals, cortisol secretion shows a marked circadian rhythm, peaking with the CAR and declining across the day, reaching nadir in the late evening and early part of sleep, before gradually increasing during late sleep prior to subsequent morning awakening (Weitzman, Fukushima, Nogeire et al., 1971; Linkowski, Mendlewicz, Leclercq, et al., 1985). Circadian rhythms, in addition to ultradian pulses, in cortisol secretion have been identified as a key factor in the homeostatic response to stress (McEwen et al., 2015). Chronic disruption to circadian rhythms is associated with increased risk for development of pathology (Yehuda et al., 1996; Linkowski, 2003; Menet & Rosbash, 2011; Cohen et al., 2012; McEwen et al., 2015), and it has been proposed that, rather than being just a symptom, abnormal circadian rhythms of glucocorticoids might be a contributing causal factor in development of pathology (e.g. Karatsoreos, 2014). The most prominent structure responsible for EF, the FC, has a high density of cortisol receptors, and the structure and function of the FC is also associated with circulating levels of glucocorticoids (e.g. Evans et al., 2011). Separate from the rest of the diurnal rhythm, the CAR has been specifically identified as associated with impaired executive functioning in patients with clinical disorders

as well as ‘at risk’ groups (e.g. Aas et al., 2011; Cullen et al., 2014). Further, it has been demonstrated that the CAR predicts preservation of EF in healthy older adults (Evans et al., 2012). The studies presented here provide support for the hypothesis that the CAR positively predicts EF in healthy functioning young adults, and therefore support the possibility that dysregulation of the CAR may contribute to the ageing process and the development of pathology. It is important to note that such a possibility is speculative at this point in time, as the mechanisms by which the CAR may interact with EF are not yet fully understood, and indeed, the results of the present studies are correlational in nature. However, this adds to a growing body of literature suggesting the need for future longitudinal and experimental research to test this hypothesis. Indeed, a plausible next step would be to explore the association between the CAR and CLOCK genes, as described in chapters 1, 2 and 5.

Rapidly accumulating evidence from studies of biological rhythms suggests that misalignment between central and peripheral clocks has widespread adverse effects on body and brain (Oster et al., 2016). The SCN acts as a master pacemaker in this system and uses diverse signals to synchronise peripheral clocks in different parts of the body, including direct innervation in some cases and also the coordination of hormonal circadian markers in others. The clinical significance of understanding the mechanisms by which the SCN communicates is therefore apparent. This may have important implications, not only for understanding and treatment in cases of pathology (Menet & Rosbash, 2011), but also for understanding healthy function and further elucidating the ageing process (Oster et al., 2016). However, the precise mechanisms of SCN entrainment of peripheral clocks have not been fully identified. The CAR is a

clear candidate as a mechanism in this process for many reasons: it is the most prominent and dynamic aspect of the circadian rhythm, a unique time-of-day marker distinct from baseline circadian secretion and signifying morning awakening (Dettenborn et al., 2007; Wilhelm et al., 2007), and is blunted in a range of clinical disorders in which circadian rhythms are implicated, such as depression (Fries et al., 2009; Clow et al., 2010a). Further, cortisol secretion is known to be modulated by the SCN, sensitivity to light in mediating cortisol secretion is greatest in the morning, and CAR magnitude is positively associated with levels of ambient light around the awakening period (Scheer and Buijs, 1999; Thorn et al., 2004). The finding of the present thesis, that the CAR positively predicts EF, provides support for the theory that the CAR plays a role in synchronising and optimising function to prepare the individual for challenges of the coming day. It has recently been demonstrated that human peripheral clocks are entrained by glucocorticoids (Cuesta, Cermakian, and Boivin, 2015), therefore implicating a role for the circadian cortisol rhythm, and perhaps the CAR, in entrainment of the peripheral circadian system in the body and brain. Although not directly studied in the present thesis, this presents a candidate mechanism to explain the present results.

7.4 The CAR and TMS-induced neuroplasticity

Further to the association with EF shown in studies I and III, the findings of study II indicate a within-individual positive association between CAR magnitude and

propensity for TMS-induced neuroplasticity in the afternoon of the same day. The hypothesis that the CAR may be associated with TMS-induced neuroplasticity is unique to the present PhD, and these two variables have never been explored together in any previously published study. Time of day effects in TMS indices of LTP-like neuroplasticity have been previously established, with cortisol proposed as a candidate modulator (Sale et al., 2010), due both to the impairing effect of elevated glucocorticoids on LTP (Dubrovsky et al., 1987) and the synchronisation of circadian rhythms of BDNF and cortisol (Yoo et al., 2007). The study presented here is the first demonstration of an association between such LTP-like neuroplasticity and the CAR and may have very important implications for research in both areas. Within the scope of understanding the CAR-cognition relationship, this finding offers a potential mechanism underlying the manifold CAR-cognition associations observed in previous research, including the cognitive deficits associated with abnormal CAR function (e.g. de Kloet et al., 2005; Wolf et al., 2005; Rimmele et al., 2010; Evans et al., 2011; Moriarty et al., 2014; Labad et al., 2016; Ennis et al., 2016).

As discussed at length in chapter 1, in recent years the role of circadian rhythms in the development of psychiatric conditions has become the focus of an increasing number of studies in psychoneuroendocrinology, psychology and medicine. It has recently been proposed that the disruption to sleep and circadian rhythms frequently observed in many mental health disorders (including depression, bipolar disorder, obsessive-compulsive disorder, and schizophrenia), rather than being merely a symptom, could be a contributing causal factor in their development (e.g. Menet and Rosbash, 2011; Jagannath et al., 2013; Karatsoreos, 2014). This hypothesis is supported by the growing

evidence for a role of the SCN and circadian rhythms in determining variations in mood and cognitive performance. This includes animal studies demonstrating SCN structural/functional integrity has downstream consequences on brain regions responsible for regulating mood and cognition, and associated behavioural outcomes (Stephan & Kovacevic, 1978; Devan et al., 2001; Ruby et al., 2008). It has been proposed that the CAR, under the influence of the SCN, may play a role in this relationship by serving as a hormonal time-of-day marker for regulation of peripheral clocks in the circadian system (Clow et al., 2010a), and this theory is supported by the data presented here. Further supporting this theory, and the present study, are the recent demonstrations of an effect of glucocorticoids in entraining peripheral clocks in humans (Pezük, Mohawk, Wang, & Menaker, 2012; Cuesta et al., 2015). Taken together, this presents a very strong case for a causal role for the CAR in entraining peripheral clocks of the circadian system and, in turn, influencing the diurnal rhythm of plasticity within the human brain.

The relationship between the CAR and synaptic plasticity demonstrated in this study might well provide an explanation for the processes underlying the consistently reported abnormal CAR profiles seen in cases of impaired structure and function of regions such as the HC and FC (e.g. de Kloet et al., 2005, Sullivan and Gratton, 2002, Buchanan et al., 2004, Wulff et al., 2010; Rimmele et al., 2010; Evans et al., 2012). Certainly, the results of Study II deserve further exploration, and a particularly important next step in this research would be to establish whether CAR magnitude is also associated with cognitive and behavioural outcomes of brain plasticity, such as learning and memory consolidation (both also known to be influenced by diurnal glucocorticoid

secretion; Roozendal, 2000; Monfils et al., 2007; Neves, Cooke, & Bliss, 2008; Born and Wagner, 2009).

A notable example of the implications of the present research is that of understanding motor, cognitive, and sensory impairments in individuals born preterm. As demonstrated in previous studies, when compared with term-born adolescents, both early and late pretermers show reduced LTD-like neuroplasticity in response to cTBS of the motor cortex (Pitcher et al., 2012), in the same region (M1) as studied here. Relevant to the present thesis, this reduced plasticity in preterm adolescents was also associated with lower levels of salivary cortisol. As there is some early evidence that early preterm birth is associated with long-term alterations in diurnal rhythms of cortisol secretion (Watterberg & Scott, 1995; Antonini, Jorge, & Moreira, 1999; Grunau et al., 2007; Sullivan et al., 2008), this provides some suggestion of a possible role for the CAR in determining these abnormalities. This is supported by the implication that the CAR may influence plasticity, as might be speculated from the present correlational study.

As has been noted elsewhere (Sale et al., 2010), mediation of the circadian modulation of plasticity by specific neuromodulators presents a novel opportunity for therapeutic intervention. Cortisol presents a candidate neuromodulator for such a relationship and intervention, as it has a circadian rhythm inversely matching that of plasticity (Sale et al., 2007; 2008), and as exogenous cortisol administration has been shown to be associated with reduced LTP in animal models and reduced TMS-induced LTP-like plasticity in humans (Sale et al., 2008). The findings presented here add to this literature by

demonstrating an association with the most prominent and dynamic aspect of the cortisol rhythm in humans, the CAR. It is well established that many psychiatric disorders are characterised by abnormalities in the CAR (Kudielka & Kirschbaum, 2003; Fries et al., 2009) and also in brain plasticity (Hallett, 2000), and the present research presents an association between these two factors for the first time. In turn, this might have implications for understanding neuroplasticity in the context of pathology and treatment. Indeed, if the predictive relationship between CAR and plasticity observed here is in fact causal, then the pharmacological alteration of the CAR presents a novel option for therapeutic intervention, with a significant benefit compared to other treatments in that it is would be a non-invasive method. This presents an opportunity for further research to explore this possibility, both by replication of these initial findings and by investigation of other co-related neuromodulators, such as BDNF and CLOCK genes, as described in chapter 5 of this thesis.

Cortical excitability, as measured by TMS and EEG, is affected by the duration of wakefulness and modulated by circadian phase (Ly et al., 2016). Time of day variation therefore presents a challenge for non-invasive stimulation therapy (Fratello et al., 2006; Sale et al., 2007) and for its usefulness for investigating pathophysiological changes in the brain (Pitcher et al., 2012). TMS treatment of neuropsychiatric disorders necessitates repeated sessions of rTMS across several days, and previous research has highlighted the importance of appropriate timing of sessions within the sleep-wake and circadian cycles to ensure efficacy of treatment (Cohen et al., 2010). The findings presented here add to a growing body of literature on associations between levels of cortisol in healthy intact participants and rTMS-induced LTD-like responses in the motor

cortex (Pitcher et al., 2012; Sale et al., 2008), implicating the diurnal rhythm of cortisol as a potential circadian system underlying this relationship. As such, these findings may too have important implications for understanding the potential for use of TMS in a therapeutic context.

In summary, better understanding of the cortisol-synaptic plasticity relationship may provide insight into the mechanisms underlying the relationship between circadian rhythms and cognitive function, and in turn, present new strategies for enhancing the efficacy of non-invasive stimulation in a therapeutic context.

7.5 Monitoring adherence to the CAR sampling protocol

A consistent issue within the field of CAR research has been the reliance on participant non-adherence to the sampling protocol within the domestic setting (Stalder et al., 2016). This remains a problem which limits the quality of published studies in this area, despite even widely-cited methodology reviews from over than 10 years ago, including statements such as: “it is not adequate to simply ask people whether they have accurately complied with the procedural protocol” (Clow et al., 2004). With only 6% of published CAR research between 2013-14 using electronic monitoring (Smyth et al., 2016), it is estimated that non-adherence in CAR research is widespread, and is likely to explain a large degree of the inconsistency in the literature (Stalder et al., 2016 Smyth et al., 2016). Although early examinations of the effect of sampling delays suggested that delays of up to 10 min allowed for inclusion of data in analyses (Griefahn and Robens, 2010), more detailed recent analysis of the CAR

has shown that delays of more than 5 min are enough to invalidate data (Smyth et al., 2013a). Applying such tight restrictions on accuracy may be perceived inconvenient by researchers due to cost, due to the need for electronic monitoring materials, increased demands placed upon participants, and the growing loss of data with tighter exclusion criteria. More so, the exclusion of such data may be non-random (some participants may be systematically more likely than others to fail to adhere to the protocol), resulting in self-selecting samples. As such, the recent CAR consensus guidelines review (Stalder et al., 2016) notes: “a fixed accuracy margin is necessarily a trade-off between scientific precision and practical feasibility”.

The challenge of the trade-off between scientific precision and practicality is minimised in well-briefed, highly adherent samples with careful electronic monitoring, as is the case in all of the studies presented here. Perhaps unsurprisingly therefore, these studies had far greater adherence to the CAR sampling protocol than much of the published research in this field. The employment of a researcher-participant case study design (study I) and a small but dedicated sample of research staff (study II) allowed for complete adherence to the CAR protocol – something very unusual in this field of research, and encouraging with regards to confidence in estimations of the CAR. Study III demonstrates that even in very well-briefed participants, the necessity of strict monitoring of adherence in CAR studies is paramount. In sum, the studies presented here present a very clear case for strict monitoring of sampling accuracy in CAR research. Indeed, from these data it is reasonable to hypothesise that the results of the many studies in this field which lack the appropriate monitoring of sampling adherence may often be confounded. The

implications of this may be far reaching, and certainly have the potential to explain the inconsistent findings of previous investigations of CAR-cognition associations as reviewed in chapter 1 of this thesis.

7.6 Future directions

The focus of this work has been upon exploring the association between the CAR and cognition and a possible mechanism underlying this association in healthy functioning young adults, so as to illuminate a potential role for the CAR in humans. The primary reason for seeking to understand this is to inform studies of ageing and abnormal function. In turn, it is hoped that this might bring about opportunities for preventative interventions. There is limited research for developing cortisol-targeting therapeutic methods for mental disorders, however, methods for administering exogenous cortisol by ingestion or corticosteroid nasal sprays have been established for other forms of therapy, and offer potential candidate means for such intervention. Further, as cortisol secretion might be manipulated non-invasively at a simple, behavioural level with no significant negative side-effects (e.g. through sleep-wake, light, or exercise-based interventions) this could offer an advantage for patients, for example as a potential non-invasive treatment method, perhaps suitable for use as a complementary therapy.

Future research in this area also needs to consider the methodological issues raised in this thesis and ensure that these standards of rigor are met. It was already firmly established, prior to the studies presented here, that careful

monitoring of participant adherence to the sampling protocol is vital for accurate estimation of the CAR (Clow et al., 2010a; Smyth et al., 2013a; Stalder et al., 2016). However, implementation of appropriate controls such as electronic monitoring of awakening and sampling times has only been ensured in a minority of studies, and this has likely contributed to the inconsistencies in the CAR literature (Clow et al., 2004; Clow et al., 2010a; Smyth et al., 2016). The findings of the present studies (in particular, those of study III) strongly emphasise this point. Not only was the significant association between CAR and EF only apparent in accurately measured data, but the studies in general showed a reduced frequency of negative CAR profiles compared to previous research (see Wüst et al., 2000; Stalder et al., 2009). The data presented here therefore strongly support both the CAR consensus guidelines of Stalder et al. (2016) and the statistical approach to managing sampling delay proposed by Smyth et al. (2016). Indeed, the significant effect found in study III would not have been apparent had this study employed only self-report measures of accuracy, rather than the detailed and careful electronic monitoring that was in place. This implies increased risk of type II error in studies which do not meet these recommended guidelines for CAR research (Stalder et al., 2016; Smyth et al., 2016). It is proposed therefore that implementation of these methodological standards will improve the accuracy and reliability of future CAR research, affording greater insight into associated factors, and therefore greater clarity with regards to a role for the CAR and any potential for therapeutic intervention.

A further method of ensuring greater adherence and therefore more accurate CAR estimates is the researcher-participant case study design. First employed as a method of investigation in this area by Stalder et al. (2009; 2010a), the

study presented here is only the second recorded example in CAR research. Such a study design is unconventional in psychology and physiology research primarily because of the severe limitations in terms of generalisability, however, this can be considered a trade-off in exchange for having a very well-informed and highly motivated participant. When used in an appropriate context, such as study I of the present thesis which aimed to explore solely within-subject variation, this approach can offer a clear advantage in eliminating the reliance on participants to complete the protocol independently within the domestic setting. As CAR sampling requires strict adherence at the nadir of the circadian rhythm of cognitive performance; a period in which there is apparent risk of sleep inertia (Tassi & Muzet, 2000), reliance on a researcher-participant is a convenient method of alleviating the risks this presents for valid CAR measurement. The studied case in this thesis was a young adult male, as was the only other recorded researcher-participant case study of the CAR (Stalder et al., 2009; 2010a), however, such a methodological approach could be very useful if applied to a female case as this might illuminate state variation in females. For example, such a methodology could provide further insight into timing of the CAR peak in females, and variation in the CAR across the menstrual cycle; which has so far only been explored in a between-subjects context (Kudielka & Kirschbaum, 2003; Wolfram et al., 2011).

Further, the work presented here explores a novel hypothesis: that the circadian rhythm of cortisol modulates TMS-induced neuroplasticity. The results support this hypothesis, in so much as demonstrating that a relationship exists between intra-individual variation in features of the cortisol circadian rhythm and same-day propensity for TMS-induced neuroplasticity. Evidence suggesting causal

pathways link circadian cortisol disruption to sub-optimal brain function and brain disorder (Jagannath et al., 2013; Wulff et al., 2010) and it is thought that one of these pathways involves dysregulation of the circadian CLOCK system (Menet & Roshbash, 2011). As the SCN ‘‘master’’ CLOCK synchronizes the peripheral ‘‘slave’’ CLOCKs through both neural and humoral pathways (Nader et al., 2010) and the extent and nature of these pathways have not been illuminated, it has been proposed in the present thesis that this may involve the CAR, which is regulated by dual inputs from the SCN: via the HPA axis as well as a direct neural input to the adrenal cortex (Clow et al., 2010b). Fine-tuning the sensitivity of the adrenal cortex to ACTH via these dual pathways allows the SCN to modulate the CAR, and makes it an ideal candidate for a time-of-day marker from the master CLOCK to the periphery. As discussed in chapter 5, this could provide a possible explanation for the current findings, as it would follow that a larger CAR may serve as a stronger cue to peripheral CLOCKS to encourage plasticity as an adaptive homeostatic response to challenge. Strong support for this theory is provided by the very recent demonstrations that glucocorticoids affect expression of clock-related genes throughout almost all organs and tissues, entrain peripheral clocks, and in turn modify synaptic plasticity (Wang et al., 2009; Cuesta et al., 2015). Future research should therefore seek to examine the relationship between the CAR and peripheral clock function in humans, perhaps via circulating leukocytes (see Kusanagi et al., 2008). In particular, day differences in mRNA expression of Per1, Per2, Per3 mRNA, at a set time in the afternoon relative to morning CAR would be of particular interest for investigating this possible mechanism of action.

As asserted throughout this chapter, future research will need to determine if manipulation of the CAR has beneficial effects on brain plasticity and associated

cognitive performance. Early signs are promising, for example the CAR has been shown to predict behavioural outcomes associated with enhanced neuroplasticity of the motor cortex region M1 (Hodyl et al., 2016), which is the physiological function measured in the present thesis. However, correlational and observational studies are useful only in identifying associations, and are only a first step towards understanding the relationship between the CAR and brain function. Longitudinal and experimental studies are required to clearly determine any cause and effect associations. There is a detailed literature describing the physiological pathways mediating brain function and bodily health across the life span, but examination of the CAR within this context is needed. The present thesis emphasises the possibilities within this field of research, offering promise for the understanding of connections between mind and body, and for the future development of therapeutic intervention in cases of abnormal function.

7.7 Conclusions

In summary, the studies in this thesis have sought to go some way towards elucidating a role for the CAR in healthy function, such that interpretation of abnormal function might be better understood. The findings of these studies provide novel insight with regard to a positive predictive relationship between the CAR and EF and an index of brain plasticity, challenging previous inconsistent findings from CAR-cognition studies, and informing best practice in CAR research methodology. It is hoped that the present research may mark opportunities for further work in this field, with the ultimate aims of better

understanding the psychological and physiological functioning of the human body and potentially developing interventions in cases of ill-health associated with abnormal cortisol function.

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Appendices

Appendix 1

Information sheet and consent form for Study I (Case study):

UNIVERSITY OF WESTMINSTER

PARTICIPATION INFORMATION SHEET

Patterns of stress/arousal hormones in human adults.

Researcher: Robin Law

Staff Supervisor: Angela Clow

You are being invited to take part in a case study project on cortisol, a hormone which is associated with stress and arousal. The Cortisol Awakening Response (CAR) is considered to be an important link between mind and body in psychosomatic medicine, and cross-sectional studies have shown that dysregulation of the CAR occurs in psychological and somatic illness including depression, anxiety, cancer and diabetes. The aim of this research is to explore the role and function of the CAR in healthy participants, in particular its relationship with cognitive function and psychological arousal upon awakening.

The study will involve you:

1) Collecting saliva samples for 50 days. This is done by chewing on a cotton swab and storing it in an eppendorf tube (a small, plastic test tube). You will need to do this **five times a day for fifty days**. These will be the times for each sample:

1st – as soon as you wake up (e.g. around 7:00am)
2nd – 15 minutes after you wake up (e.g. around 7:15am)
3rd – 30 minutes after you wake up (e.g. around 7:30am)
4th – 45 minutes after you wake up (e.g. around 7:45 am)
5th – 1 hour after you wake up (e.g. around 8:00am)

Also for at least 30 minutes before collecting each saliva sample you: cannot eat or drink anything (except water), smoke, clean your teeth, or do any rigorous physical exercise.

2) Taking a short battery of cognitive tests on a Cantab™ computer, measuring your critical flicker perception, and taking a personal diary for 50 study days.

3) Wearing a light measuring device on a lanyard around the neck for 50 study days and mornings (kept beside your bed during sleep).

Please note:

- Participation is entirely voluntary.
- You have the right to withdraw at any time without giving a reason.
- You have the right to ask for your data to be withdrawn as long as this is practical, and for personal information to be destroyed.
- You do not have to answer particular questions either on questionnaires or in interviews if you do not wish to.

- Your responses will be confidential. No individuals will be identifiable from any collated data, written report of the research, or any publications arising from it.
- All personal data will be kept in a locked cupboard on University premises.
- Please notify us if any adverse symptoms arise during or after the research.
- If you wish you can receive information on the results of the research.
- The researcher can be contacted during or after participation by email (Robin.law@my.westminster.ac.uk) or by telephone (0207 911 5000 ext 2170).

----------*please separate*

CONSENT FORM

Title of Study: Patterns of stress/arousal hormones in human adults.

Lead researcher: Robin Law

I have read the information in the Participation Information Sheet, and I am willing to act as a participant in the above research study.

Name: _____

Signature: _____ Date: _____

This consent form will be stored separately from any data you provide so that your responses remain anonymous.

I have provided an appropriate explanation of the study to the participant

Researcher Signature _____

Appendix 2

Information sheet and consent form for Study II:

THE UNIVERSITY OF ADELAIDE
AUSTRALIA

The Relationship Between the Cortisol Awakening Response and Motor Function

Study Overview

This study involves collecting morning saliva samples, participating in Repetitive Transcranial Magnetic Stimulation (RTMS) and completing questionnaires on four weekdays, at 3 to 9 day intervals.

We will be using the saliva to measure levels of a natural hormone called cortisol. Known as a stress hormone as it is produced in response to psychological stress, cortisol is also essential for normal physiological functioning. It has a marked 24hour rhythm, with peak levels about 45 minutes following awakening and low levels in the early part of sleep.

Changes in this rhythm between days can be an important factor in physical and psychological health. The study will investigate variations in cortisol levels upon awakening and throughout the day in relation to variations in motor cortex function (measured by RTMS) later in the same day.

You are required to take 1 saliva sample immediately upon awakening and then once every 15 minutes for the first 45mins after awakening. One further sample will be required immediately after completing the RTMS session.

You are also required to complete an RTMS session at approximately 3pm on each testing day, and complete a couple of brief questionnaires, which assess things like happiness, workload and stress.

You will be given a sampling and equipment pack to take away with you. This includes all of the necessary equipment for saliva sampling and sleep/wake assessment. You will be expected to return this equipment pack upon completion of each testing session.

Please complete the first one of the questionnaires, entitled ‘evening diary, when you are in bed, prior to sleep. Please also remember to put on the actiwatch, on your non-dominant hand, prior to going to sleep. You should wear this throughout the night, and remove it upon awakening. Please be careful not to get the actiwatch wet (e.g. by leaving it on when you wash your hands or go in the shower etc.)

The other questionnaire (entitled ‘morning diary’) is to be completed as soon as you have taken the final morning saliva sample (at 45 minutes post-awakening).

To get accurate results, in the morning during the sampling period you cannot eat, drink anything (apart from water), brush your teeth, or smoke until you have taken the last morning saliva sample (at 45 minutes).

It’s really important for the research that you adhere to the sampling protocol, especially for the samples you take in the first 45minutes following awakening, when cortisol levels are rapidly increasing. You will find a saliva sampling time record sheet in the pack with your samples, please fill this in as you complete the morning saliva sampling.

If you have any questions or concerns please contact Robin Law by email or phone:
Email: robin.law@my.westminster.ac.uk Phone: 0458549652

The Relationship Between the Cortisol Awakening Response and Motor Function

Consent Form

Please read the information given below and sign the consent form. This form will be stored separately from any data you provide so that your responses remain anonymous.

1. I, ……. *(please print name)*

   consent to take part in the research project entitled: **Relationship between the cortisol awakening response and motor function.**

2. I acknowledge that I have read the attached Information Sheet entitled:

   Relationship between the cortisol awakening response and motor function.

3. I have had the project, so far as it affects me, fully explained to my satisfaction by the research worker. My consent is given freely.

4. Although I understand that the purpose of this research project is to improve the quality of medical care, it has also been explained that my involvement may not be of any benefit to me.

5. I have been given the opportunity to have a member of my family or a friend present while the project was explained to me.

6. I have been informed that, while information gained during the study may be published, I will not be identified and my personal results will not be divulged.

7. I understand that I am free to withdraw from the project at any time and that this will not affect medical advice in the management of my health, now or in the future.

8. I am aware that I should retain a copy of this Consent Form, when completed, and the attached Information Sheet.

……...
(signature) *(date)*

WITNESS

I have described to …….. *(name of subject)*

the nature of the procedures to be carried out. In my opinion she/he understood the explanation.

Status in Project**:** ……

Name: ……

……...
(signature) *(date)*

Transcranial Magnetic Stimulation† (TMS) Adult Safety Screen

Name:
Date:
Age:

Please answer the following:

Do you have epilepsy or have you ever had a convulsion or a seizure?	Yes	No
Have you ever had a fainting spell or syncope? If *yes*, please describe in which occasions in the space provided below.	Yes	No
Have you ever had severe (i.e., followed by loss of consciousness) head trauma?	Yes	No
Do you have any hearing problems or ringing in your ears?	Yes	No
Are you pregnant or is there a chance you might be?	Yes	No
Do you have cochlear implants?	Yes	No
Do you have an implanted neurostimulator? (e.g., DBS, epidural/subdural, VNS)	Yes	No
Do you have a cardiac pacemaker or intracardiac lines or metal in your body	Yes	No
Do you have a medication infusion device?	Yes	No
Are you taking any medications? *(Please list)*	Yes	No
Have you had a surgical procedure to your spinal cord?	Yes	No
Do you have spinal or ventricular derivations?	Yes	No
Did you ever undergo TMS in the past?	Yes	No
Did you ever undergo MRI in the past?	Yes	No

Subject signature:

Experimenter name: **Signature:**

*If you answered **yes** to any of the above, please provide details (use reverse if necessary):*

_____

_____

_____

_____

_____

_____

Appendix 3

Information sheet and consent form for Study III:

UNIVERSITY OF WESTMINSTER

PARTICIPATION INFORMATION SHEET

Patterns of stress hormones and cognitive performance

Researcher: Robin Law
Staff Supervisor: Professor Angela Clow

You are being invited to take part in a study on cortisol, a hormone which is essential for life and associated with stress and arousal. The Cortisol Awakening Response (CAR) is considered to be an important link between mind and body and has been shown to be related to various aspects of cognitive functioning. The aim of this research is to explore the role and function of the CAR in healthy participants, in particular its relationship with psychological arousal upon awakening and cognitive performance later in the same day.

The study will involve you:

1) Collecting saliva samples on 2 days. This is done by chewing on a cotton swab and storing it in a salivette (a small, plastic tube). You will need to do this **five times a day, for two days**. These will be the times for each sample:

1st – as soon as you wake up in the morning (e.g. around 7:00am)
2nd – 15 minutes after you wake up (e.g. around 7:15am)
3rd – 30 minutes after you wake up (e.g. around 7:30am)
4th – 45 minutes after you wake up (e.g. around 7:45 am)
5th – In the afternoon during the cognitive testing session (see point 2)

N.b. During the 1 hour post awakening that you are collecting saliva samples you cannot eat or drink anything (except water), smoke, clean your teeth, or do any rigorous physical exercise. This also applies for 30 min prior to taking the afternoon cortisol sample.

2) Visiting the university at a set time in the afternoon of the two study days to take part in a cognitive testing session. These tests will be administered on a computer screen and will take approximately 30 min.

3) Taking a brief personal diary in the evening before bed, and in the morning on both of the study days. This will be completed at home (approx. 3 min to complete) and assesses mood and anticipations of the day ahead, as well as estimates of the amount of exercise you have done in the day, and the amount of alcohol (if any) consumed in the evening prior to the study.

Please note:

- Participation is entirely voluntary.
- You have the right to withdraw at any time without giving a reason.
- You have the right to ask for your data to be withdrawn as long as this is practical, and for personal information to be destroyed.

- You do not have to answer particular questions either on questionnaires or in interviews if you do not wish to.
- Your responses will be confidential. No individuals will be identifiable from any collated data, written report of the research, or any publications arising from it.
- All personal data will be kept in a locked cupboard on University premises.
- Please notify us if any adverse symptoms arise during or after the research.
- If you wish you can receive information on the results of the research.
- The researcher can be contacted during or after participation by email (Robin.law@my.westminster.ac.uk) or by telephone (0207 911 5000 ext 2170).

----------*please separate*

CONSENT FORM

Title of Study: Patterns of stress hormones and cognitive performance

Lead researcher: Robin Law

I have read the information in the Participation Information Sheet, and I am willing to act as a participant in the above research study.

Name: _____

Signature: _____ Date: _____

This consent form will be stored separately from any data you provide so that your responses remain anonymous.

I have provided an appropriate explanation of the study to the participant

Researcher's Signature _____

Appendix 4

Study guidelines for Study III:

'Wake-Up Study' - Participant Check-list

PRE-STUDY - Before bed

Put on actiwatch & make sure salivettes are by your bed ready for the morning

Complete *evening diary*

DAY 1

Morning

Immediately on awakening – Take sample 1

Start filling in sample time record sheet

Take remaining samples at scheduled times

Complete *morning diary* at 45 min

Take off actiwatch

Afternoon

Visit the University for cog test session (remember to bring day 1 saliva samples with you)

Evening

Put on actiwatch & make sure salivettes are by your bed ready for the morning

Complete *evening diary*

DAY 2

Morning

Immediately on awakening – Take sample 1

Start filling in sample time record sheet

Take remaining samples at scheduled times

Complete *morning diary* at 45 min

Take off actiwatch

Afternoon - Visit the University for cognitive testing session

Remember to bring back:

- Saliva samples
- Morning & evening diaries
- Saliva sample time record sheet
- Actiwatch
- MEMS

Appendix 5

Sample time self-report sheets:

Date…… ID:

DAY 1

Please be as honest as you can about the actual times you took the saliva samples

Please calculate the times to take your samples (1st column) and the exact times you actually took your samples (2nd column)

Tube 1: Wake time:_____ taken at: _____

Tube 2: 15 mins: _____ taken at: _____

Tube 3: 30 mins: _____ taken at: _____

Tube 4: 45 mins: _____ taken at: _____

N.B. Please do not forget to bring the samples with you to the afternoon cognitive testing session

Please make a note of any problems with sampling……

……...

……

……

……

Date…… ID:

DAY 2

Please be as honest as you can about the actual times you took the saliva samples

Please calculate the times to take your samples (1st column) and the exact times you actually took your samples (2nd column)

Tube 1: Wake time:_____ taken at: _____

Tube 2: 15 mins: _____ taken at: _____

Tube 3: 30 mins: _____ taken at: _____

Tube 4: 45 mins: _____ taken at: _____

N.B. Please do not forget to bring the samples with you to the afternoon cognitive testing session

Please make a note of any problems with sampling……

……..

……

……

……

Appendix 6

Example diaries for Study I:

EVENING DIARY (Fill this out before going to bed)

Day: …….. Date: …. / …. / ….

This evening I have had ……. alcoholic drinks

I have had my last meal ……. hours ago

I have done ……. hours of exercise today

SLEEP IN THE COMING NIGHT

I am going to bed at ……... I expect that I can sleep for at least ……. hours

How well do you expect to sleep this night? (place a mark along the line)

very bad _____ very good

TODAY

How happy have you felt over today?

not at all happy _____ very happy

How stressed have you felt over today?

not at all stressed _____ very stressed

How much of today have you spent fulfilling obligations (work or other)?

nothing at all _____ very much

How much of today have you spent doing leisure activities?

nothing at all _____ very much

NOW

How happy do you feel now?

not at all happy _____ very happy

How stressed do you feel now?

not at all stressed _____ very stressed

TOMORROW

How happy do you feel as you face tomorrow?

not at all happy _____ very happy

How stressed do you feel as you face tomorrow?

not at all stressed _____ very stressed

MORNING DIARY (fill this out after taking the 15 minutes saliva sample)

Day: …….. Date: …. / …. / ….

SLEEP

Went to bed last night at…..... awakened by: (check one)

Went to sleep last night at ……..... alarm clock / radio ……...

someone whom I asked to wake me ……...

finally woke up …… noises ……...

just woke ……...

after falling asleep, woke up this many times during the night (circle)

0 1 2 3 4 5 or more

RATINGS (place a mark somewhere along the line)

SLEEP QUALITY:

very bad _____ very good

MOOD ON FINAL WAKENING:

very tense _____ very calm

ALERTNESS ON FINAL AWAKENING

very sleepy _____ very alert

COMING DAY

How happy do you feel as you face today?

not at all happy _____ very happy

How stressed do you feel as you face today?

not at all stressed _____ very stressed

How much of today do you expect to spend fulfilling obligations (work or other)?

nothing at all _____ very much

How much of today do you expect to spend doing leisure activities?

nothing at all _____ very much

CHECKLIST – Morning 15 min

Please read the following adjectives and **circle** the most appropriate response for each one based on how you feel **now**.

If a word definitely describes how you feel, circle “DEF”
If a word slightly describes how you feel, circle “SL”
If you do not understand or cannot decide, circle “UN”
If a word does not describe how you feel, circle “NOT”

Do make sure you circle one of the responses for each word.
Please complete these questions quickly; your first reaction is best.

Worried	DEF	SL	UN	NOT	Pleasant	DEF	SL	UN	NOT
Peaceful	DEF	SL	UN	NOT	Comfortable	DEF	SL	UN	NOT
Active	DEF	SL	UN	NOT	Lively	DEF	SL	UN	NOT
Drowsy	DEF	SL	UN	NOT	Restful	DEF	SL	UN	NOT
Fearful	DEF	SL	UN	NOT	Tired	DEF	SL	UN	NOT
Calm	DEF	SL	UN	NOT	Sleepy	DEF	SL	UN	NOT
Activated	DEF	SL	UN	NOT	Bothered	DEF	SL	UN	NOT
Stimulate	DEF	SL	UN	NOT	Apprehensive	DEF	SL	UN	NOT
Contented	DEF	SL	UN	NOT	Tense	DEF	SL	UN	NOT
Dejected	DEF	SL	UN	NOT	Nervous	DEF	SL	UN	NOT
Up-tight	DEF	SL	UN	NOT	Sluggish	DEF	SL	UN	NOT
Vigorous	DEF	SL	UN	NOT	Aroused	DEF	SL	UN	NOT
Alert	DEF	SL	UN	NOT	Distressed	DEF	SL	UN	NOT
Idle	DEF	SL	UN	NOT	Relaxed	DEF	SL	UN	NOT
Passive	DEF	SL	UN	NOT	Somnolent	DEF	SL	UN	NOT
Cheerful	DEF	SL	UN	NOT	Energetic	DEF	SL	UN	NOT
Uneasy	DEF	SL	UN	NOT	Jittery	DEF	SL	UN	NOT

Appendix 7

Example diaries for Study II:

EVENING DIARY (Fill this out before going to bed)

Day: (Circle) MON / TUES / WEDS / THURS / FRI Date: …. / …. / …. ID: ……

This evening I have had ……. units of alcohol

I have had my last meal ……. hours ago

I have done ……. hours of exercise today

SLEEP IN THE COMING NIGHT

I am going to bed at ……... I expect that I can sleep for at least ……. hours

How well do you expect to sleep this night? (place a mark along the line)

very bad _____ very good

TODAY

How happy have you felt over today?

not at all happy _____ very happy

How stressed have you felt over today?

not at all stressed _____ very stressed

How much of today have you spent fulfilling obligations (work or other)?

nothing at all _____ very much

How much of today have you spent doing leisure activities?

nothing at all _____ very much

NOW

How happy do you feel now?

not at all happy _____ very happy

How stressed do you feel now?

not at all stressed _____ very stressed

TOMORROW

How happy do you feel as you face tomorrow?

not at all happy _____ very happy

How stressed do you feel as you face tomorrow?

not at all stressed _____ very stressed

MORNING DIARY (fill this out after taking the 45 min saliva sample)

Day: …….. Date: …. / …. / …. Study ID: ……

SLEEP

Went to bed last night at…..... awakened by: (check one)

Went to sleep last night at ……..... alarm clock / radio ……...

someone whom I asked to wake me ……...

finally woke up …… noises ……...

just woke ……...

after falling asleep, woke up this many times during the night (circle)

0 1 2 3 4 5 or more

RATINGS (place a mark somewhere along the line)

SLEEP QUALITY:

very bad _____ very good

MOOD ON FINAL WAKENING:

very tense _____ very calm

ALERTNESS ON FINAL AWAKENING

very sleepy _____ very alert

COMING DAY

How happy do you feel as you face today?

not at all happy _____ very happy

How stressed do you feel as you face today?

not at all stressed _____ very stressed

How much of today do you expect to spend fulfilling obligations (work or other)?

nothing at all _____ very much

How much of today do you expect to spend doing leisure activities?

nothing at all _____ very much

CHECKLIST

Please read the following adjectives and **circle** the most appropriate response for each one based on how you feel **now**.

If a word definitely describes how you feel, circle “DEF”
If a word slightly describes how you feel, circle “SL”
If you do not understand or cannot decide, circle “UN”
If a word does not describe how you feel, circle “NOT”

Do make sure you circle one of the responses for each word.
Please complete these questions quickly; your first reaction is best.

Worried	DEF SL UN NOT	Pleasant	DEF SL UN NOT
Peaceful	DEF SL UN NOT	Comfortable	DEF SL UN NOT
Active	DEF SL UN NOT	Lively	DEF SL UN NOT
Drowsy	DEF SL UN NOT	Restful	DEF SL UN NOT
Fearful	DEF SL UN NOT	Tired	DEF SL UN NOT
Calm	DEF SL UN NOT	Sleepy	DEF SL UN NOT
Activated	DEF SL UN NOT	Bothered	DEF SL UN NOT
Stimulate	DEF SL UN NOT	Apprehensive	DEF SL UN NOT
Contented	DEF SL UN NOT	Tense	DEF SL UN NOT
Dejected	DEF SL UN NOT	Nervous	DEF SL UN NOT
Up-tight	DEF SL UN NOT	Sluggish	DEF SL UN NOT
Vigorous	DEF SL UN NOT	Aroused	DEF SL UN NOT
Alert	DEF SL UN NOT	Distressed	DEF SL UN NOT
Idle	DEF SL UN NOT	Relaxed	DEF SL UN NOT
Passive	DEF SL UN NOT	Somnolent	DEF SL UN NOT
Cheerful	DEF SL UN NOT	Energetic	DEF SL UN NOT
Uneasy	DEF SL UN NOT	Jittery	DEF SL UN NOT

Appendix 8

Example Diaries for Study III:

EVENING DIARY - PRIOR TO DAY 1 (Fill this out before going to bed)

Day: (Circle) MON / TUES / WEDS / THURS / FRI Date: …. / …. / …. ID: ……

This evening I have had ……. units of alcohol

I have had my last meal ……. hours ago

I have done ……. hours of exercise today

SLEEP IN THE COMING NIGHT

I am going to bed at ……... I expect that I can sleep for at least ……. hours

How well do you expect to sleep this night? (place a mark along the line)

very bad _____ very good

TODAY

How happy have you felt over today?

not at all happy _____ very happy

How stressed have you felt over today?

not at all stressed _____ very stressed

How much of today have you spent fulfilling obligations (work or other)?

nothing at all _____ very much

How much of today have you spent doing leisure activities?

nothing at all _____ very much

NOW

How happy do you feel now?

not at all happy _____ very happy

How stressed do you feel now?

not at all stressed _____ very stressed

TOMORROW

How happy do you feel as you face tomorrow?

not at all happy _____ very happy

How stressed do you feel as you face tomorrow?

not at all stressed _____ very stressed

CHECKLIST – EVENING PRIOR TO DAY 1

Please read the following adjectives and **circle** the most appropriate response for each one based on how you feel **now**.

If a word definitely describes how you feel, circle “DEF”
If a word slightly describes how you feel, circle “SL”
If you do not understand or cannot decide, circle “UN”
If a word does not describe how you feel, circle “NOT”

Do make sure you circle one of the responses for each word.
Please complete these questions quickly; your first reaction is best.

Worried	DEF SL UN NOT	Pleasant	DEF SL UN NOT
Peaceful	DEF SL UN NOT	Comfortable	DEF SL UN NOT
Active	DEF SL UN NOT	Lively	DEF SL UN NOT
Drowsy	DEF SL UN NOT	Restful	DEF SL UN NOT
Fearful	DEF SL UN NOT	Tired	DEF SL UN NOT
Calm	DEF SL UN NOT	Sleepy	DEF SL UN NOT
Activated	DEF SL UN NOT	Bothered	DEF SL UN NOT
Cheerful	DEF SL UN NOT	Apprehensive	DEF SL UN NOT
Tense	DEF SL UN NOT	Contented	DEF SL UN NOT
Dejected	DEF SL UN NOT	Nervous	DEF SL UN NOT
Up-tight	DEF SL UN NOT	Sluggish	DEF SL UN NOT
Vigorous	DEF SL UN NOT	Energetic	DEF SL UN NOT
Alert	DEF SL UN NOT	Distressed	DEF SL UN NOT
Relaxed	DEF SL UN NOT	Stimulated	DEF SL UN NOT
Uneasy	DEF SL UN NOT	Jittery	DEF SL UN NOT

Exercise scale

Please only include exercise at *level 15* or above when calculating your daily exercise in the diary.

Level	Example
6	Reading a book, watching television
7 - Very, very light	Tying shoes
8	
9 - Very light	Gentle walking
10	
11 - Fairly light	Walking through the supermarket
12	
13 - Moderately hard	Brisk walking – but not out of breath
14	
15 – Hard	Bicycling, swimming, running
16	
17 - Very hard	The highest level you can sustain
18 -	
19 - Very, very hard	
20 – Exhaustion	The very hardest exercise you can do

Units of alcohol guide (approximate)

MORNING DIARY – DAY 1 (fill this out after taking the 45 minutes saliva sample)

Day: …….. Date: …. / …. / …. Study ID: ……

SLEEP

Went to bed last night at…..... awakened by: (check one)

Went to sleep last night at ……..... alarm clock / radio ……...

someone whom I asked to wake me ……...

finally woke up …… noises ……...

just woke ……...

after falling asleep, woke up this many times during the night (circle)

0 1 2 3 4 5 or more

RATINGS (place a mark somewhere along the line)

SLEEP QUALITY:

very bad _____ very good

MOOD ON FINAL WAKENING:

very tense _____ very calm

ALERTNESS ON FINAL AWAKENING

very sleepy _____ very alert

COMING DAY

How happy do you feel as you face today?

not at all happy _____ very happy

How stressed do you feel as you face today?

not at all stressed _____ very stressed

How much of today do you expect to spend fulfilling obligations (work or other)?

nothing at all _____ very much

How much of today do you expect to spend doing leisure activities?

nothing at all _____ very much

CHECKLIST – DAY 1

Please read the following adjectives and **circle** the most appropriate response for each one based on how you feel **now**.

If a word definitely describes how you feel, circle “DEF”
If a word slightly describes how you feel, circle “SL”
If you do not understand or cannot decide, circle “UN”
If a word does not describe how you feel, circle “NOT”

Do make sure you circle one of the responses for each word.
Please complete these questions quickly; your first reaction is best.

Worried	DEF SL UN NOT	Pleasant	DEF SL UN NOT
Peaceful	DEF SL UN NOT	Comfortable	DEF SL UN NOT
Active	DEF SL UN NOT	Lively	DEF SL UN NOT
Drowsy	DEF SL UN NOT	Restful	DEF SL UN NOT
Fearful	DEF SL UN NOT	Tired	DEF SL UN NOT
Calm	DEF SL UN NOT	Sleepy	DEF SL UN NOT
Activated	DEF SL UN NOT	Bothered	DEF SL UN NOT
Cheerful	DEF SL UN NOT	Apprehensive	DEF SL UN NOT
Tense	DEF SL UN NOT	Contented	DEF SL UN NOT
Dejected	DEF SL UN NOT	Nervous	DEF SL UN NOT
Up-tight	DEF SL UN NOT	Sluggish	DEF SL UN NOT
Vigorous	DEF SL UN NOT	Energetic	DEF SL UN NOT
Alert	DEF SL UN NOT	Distressed	DEF SL UN NOT
Relaxed	DEF SL UN NOT	Stimulated	DEF SL UN NOT
Uneasy	DEF SL UN NOT	Jittery	DEF SL UN NOT

Appendix 9

List of word learning test items:

Real items:

Positive	Neutral	Negative
FRIEND	CIRCLE	DEATH
GIFT	PLAIN	FIRE
GLORY	CHAIR	BULLET
ANGEL	COLUMN	DAMAGE
KISS	EVENT	PRESSURE
BLOSSOM	CANE	ANGER
HEAVEN	CHIN	CANCER
BEACH	CABINET	ASSAULT
BABY	CELLAR	PRISON
LEADER	CLIFF	DEBT
REWARD	GLASS	FEVER
TREAT	HOTEL	GLOOM

False (matched) items:

Positive	Neutral	Negative
JUSTICE	TOOL	INSULT
CASH	VEST	FRAUD
CHAMPION	INSECT	GRIEF
THRILL	IRON	JAIL
PUPPY	ITEM	PAIN
DINNER	JOURNAL	SLAVE
EARTH	KETTLE	WASTE
HOLIDAY	LAMP	ULCER
HEART	MARKET	PANIC
LIBERTY	PATIENT	FEAR
LUXURY	RAIN	DISASTER
NATURE	SEAT	ACCIDENT

Appendix 10

Word recognition task:

P: D:

Word Recognition

HEAVEN	GLASS	HEART
VEST	GLOOM	GLORY
INSULT	JOURNAL	DAMAGE
ANGEL	FEAR	SEAT
TOOL	CHAIR	RAIN
PRESSURE	PUPPY	CABINET
NATURE	ITEM	LIBERTY
CLIFF	CANCER	DEBT
ASSAULT	DISASTER	ULCER
THRILL	LAMP	LUXURY
FIRE	CELLAR	CIRCLE
CASH	DINNER	DEATH
PLAIN	PANIC	PATIENT
BABY	CHIN	LEADER
EVENT	PRISON	BULLET
INSECT	EARTH	PAIN
FRAUD	TREAT	FRIEND
IRON	FEVER	CHAMPION
ANGER	HOLIDAY	MARKET
JUSTICE	KISS	HOTEL
GRIEF	ACCIDENT	REWARD
CANE	BLOSSOM	SLAVE
GIFT	KETTLE	WASTE
JAIL	BEACH	COLUMN