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The Hypothalamic–Pituitary–Adrenal Axis and the Cognitive Functions: the Effect of Cortisol Level on Antisaccade Task Performance

POSTGRADUATE THESIS

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Abbreviations

Term	Abbreviation
ACC	Anterior Cingulate Cortex
ACTH	Adrenocorticotropic Hormone
ADHD	Attention Deficit/Hyperactivity Disorder
ANS	Antisaccade Task
ANOVA	Analysis of Variance
CAR	Cortisol Awakening Response
CLOCK	Circadian Locomotor Output Cycles Kaput
CRH	Corticotropin-Releasing Hormone
DCS	Diurnal Cortisol Slope
DLPFC	Dorsolateral Prefrontal Cortex
FEF	Frontal Eye Fields
EU	European Union
GDPR	General Data Protection Regulation
GRs	Glucocorticoid Receptors
HPA	Hypothalamic–Pituitary–Adrenal
IPS	Intraparietal Sulcus
LTD	Long-Term Depression
LTP	Long-Term Potentiation
M	Mean
MRs	Mineralocorticoid Receptors
NKUA	National Kapodistrian University of Athens
OCD	Obsessive-Compulsive Disorder
PEF	Parietal Eye Fields
pre-SMA	Pre-Supplementary Motor Area
PVN	Paraventricular Nucleus
RT	Reaction Time
SC	Superior Colliculus
SCN	Suprachiasmatic Nucleus
SD	Standard Deviation
SEF	Supplementary Eye Fields
TMT	Trail Making Test
UMHRI	University Mental Health Research Institute
WCST	Wisconsin Card Sorting Test

Abstract

The Hypothalamic-Pituitary-Adrenal (HPA) axis is a basic neuroendocrine system that regulates Circadian Cortisol Rhythm. Findings indicate that cortisol affects multiple aspects of cognitive functioning. In the present thesis we examined the relation between performance in the antisaccade task (ANS), an oculomotor task that is considered to measure executive functioning, and cortisol levels throughout the day, depicting the diurnal cortisol rhythm. A sample of 21 participants aged from 18 to 33 was used. To measure cortisol levels, saliva sampling was done five times a day, whereas participants had to execute the antisaccade task three times a day. Results showed that ANS Performance, as measured by Reaction time (RT), RT Variability and Percentage of Correct Responses, improves during the day. Surprisingly, this improvement was not correlated to the expected and observed Diurnal Cortisol Variation. Our half-split analysis is valid evidence against the hypothesis that the observed improvement is the result of a practice effect, but further research should be done since there are different opinions in bibliography.

Keywords: Antisaccade Task, Diurnal Cortisol Curve, Diurnal Antisaccade Performance Variation, Salivary Cortisol, Oculomotor tasks, Saccades.

Introduction

In the following thesis a study will be presented on the relation between cortisol levels and executive functions, and more specifically we will study if there is a connection between circadian cortisol rhythm and performance on a specific oculomotor task known as the Antisaccade Task (ANS). We will also study this task's diurnal variation.

Theoretical Background

Executive Functions. Executive functions are a set of top-down mental processes necessary for the cognitive control of behavior. As such they require effort, since they are often invoked when altering a current behavior or when resistance to an automatic response is needed. It is generally agreed that there are three core executive functions; inhibition or inhibitory control, working memory, and cognitive flexibility or set shifting. All other executive functions supposedly stem from these three (Diamond, 2013). Executive functions are considered to be "higher level" cognitive functions that serve the purpose of regulating other cognitive functions and, consequently, behavior.

As far as neurophysiology is concerned executive functions have always been linked to the prefrontal cortex (Robbins, 1998), with numerous examples of neuroimaging studies of healthy individuals (Yuan & Raz, 2014; Szameitat et al., 2002), lesion studies (Stuss, 2011), and animal studies with rodents (Dalley et al., 2004; Holmes & Wellman, 2009) and non-human primates (Petrides, 1998, 2005). However, other researches question the specificity and sensitivity of executive functioning related tasks to frontal lesions (Alvarez & Emory, 2006).

The executive function that will concern us in this study is inhibition. This function comprises cognitive inhibition, i.e. the ability to inhibit irrelevant information and selectively attend to goal-relevant information, and response inhibition, i.e. the ability to override or inhibit a prepotent response in order to engage in goal-directed rather than habitual, inappropriate and no longer required actions (Cutsuridis, 2017; Harnishfeger, 1995; Miyake et al., 2000; Shields, Bonner, & Moons, 2015; Shields, Sazma & Yonelinas, 2016), although factor analyses have suggested that these two types of inhibition constitute the same process in healthy young adults (Friedman & Miyake, 2004). Response inhibition deficits are linked to disorders such as ADHD, OCD, schizophrenia and substance abuse (Cutsuridis, 2017).

There are multiple tasks used to assess executive functions (Diamond, 2013), the most common of them being the Wisconsin Card Sorting Test (WCST), the Tower of Hanoi, and the Tower of London. Investigations of response inhibition employ a variety of behavioral paradigms as well, that share a mutual and closely related inhibitory mechanism. Task examples are the Stroop Task (Stroop, 1935), the Stop-Signal Task (Logan, 1994), and oculomotor tasks, including the antisaccade and countermanding tasks (Cutsuridis, 2017).

The literature on executive functions is presented with some issues. The use of different neuropsychological tests that measure more than one function makes comparisons impossible,

not to mention the differences in each function's definition across studies. Eye movement related tasks seem to overcome some of this ambiguity, them being very specific as to what they measure (Antoniades et al, 2013). This is why we chose to focus on this kind of tasks to measure executive functioning.

Cortisol and the HPA Axis. Cortisol is a steroid hormone, in the glucocorticoid class of hormones, which are synthesized and released by the adrenal cortex under the regulation of the HPA axis. Glucocorticoids affect profoundly synaptic physiology, circuit regulation of stress responsiveness and, ultimately, behavior (Myers, McKlveen, & Herman, 2014). The production of glucocorticoids by the HPA axis is pulsatile, demonstrating both circadian and ultradian rhythms, while it can also be induced by acute exposure to stress. The axis's function in summary is the following: Corticotropin-releasing hormone (CRH) and arginine vasopressin are secreted by the paraventricular nucleus (PVN) of the hypothalamus into the pituitary portal circulation, which stimulates adrenocorticotropic hormone (ACTH) release from the anterior pituitary gland. ACTH acts on the adrenal gland to induce steroidogenesis and the secretion of glucocorticoids such as cortisol in some species (e.g. humans) and corticosterone in others (e.g. rats, mice) from the zona fasciculate. This secretion creates negative endocrine feedback loops, as the glucocorticoids inhibit CRH expression and secretion, and ACTH output via two distinct mechanisms; a fast, non-genomic feedback system, sensitive to the rate of glucocorticoid secretion, and a delayed feedback that involves genomic, transcriptional alterations, regulated by the glucocorticoid receptors (GRs) (Evanson, Tasker, Hill, Hillard, & Herman, 2010; Smith & Vale, 2006; Taves, Gomez-Sanchez, & Soma, 2011).

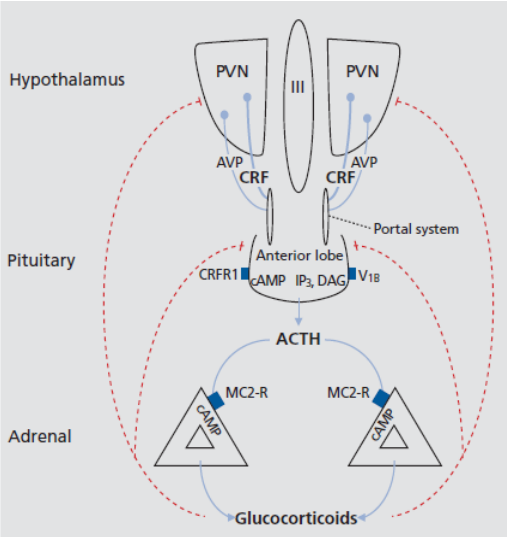

Figure 1. Schematic representation of the hypothalamic-pituitary-adrenal (HPA) axis (Smith & Vale, 2006).

Glucocorticoids secreted by the HPA axis act on the brain through two different types of receptors. The GRs mentioned above are widely distributed throughout the brain and peripheral tissue. They have a low affinity for glucocorticoids, a property suggesting that they mediate glucocorticoid negative feedback following stress, whereas they are largely unoccupied at basal levels. The other type, known as the mineralocorticoid receptors (MRs), bind glucocorticoids with an affinity 6 to 10 times higher, thus sensing their resting levels and promoting key functions associated with low glucocorticoid levels, including circadian drive of the HPA axis and mnemonic function, although recent data refers to some membrane-associated MRs that respond to stress-induced corticosteroid releases (Karst et al., 2005). GRs are abundantly expressed throughout the brain, in both subcortical and cortical structures including stress-regulatory sites, with preferential distribution in the prefrontal cortex, whereas MRs have a more restricted distribution, primarily in the limbic system (Myers, et al., 2006; Lupien, et al., 2005). With moderate cortisol increase, GRs occupation increases, supporting synaptic long-term potentiation (LTP), important for executive functioning, learning and memory, while increase of cortisol levels beyond that point, and therefore increasingly high GRs occupation is linked to synaptic long term depression (LTD) (Blair, Granger, & Razza, 2005).

As mentioned above, the HPA axis demonstrates circadian and ultradian rhythms. The very steady rhythmic secretion of glucocorticoids produced by diurnal changes in the HPA axis activity is critical to the maintenance of physiological homeostasis and metabolic balance in the organism, while dysregulation in this diurnal rhythm is associated with various pathologies, metabolic abnormalities, fatigue and poor quality of life (Debono et al., 2009). The circadian cortisol rhythm is regulated by the main circadian oscillator (pacemaker) in the suprachiasmatic nucleus (SCN) in the hypothalamus. In mammals SCN is light sensitive, as it is synaptically affected by afferents from the retina, in order to entrain the circadian clock to the environmental dark light cycle, or in other words, to the phase of the day-night cycle in which the individual is exposed. The main circadian oscillator's function is necessary to synchronize all other peripheral oscillators to each other and to the photoperiod, and glucocorticoids play a role in this temporal entrainment (Chan & Debono, 2010; Girotti, Weinberg & Spencer, 2009). The principal mediators of the internal rhythmicity of nearly all body functions are endocrine signals and the autonomic nervous system (Buijs & Kalsbeek, 2001).

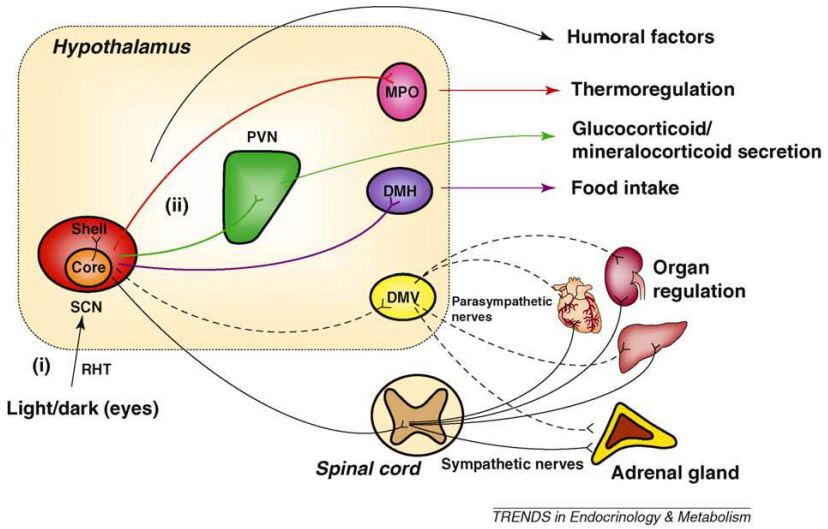

Figure 2. Central CLOCK synchronizes the peripheral CLOCKs and regulates peripheral organ activities via neural and humoral interactions (Nader, Chrousos & Kino, 2010).

The circadian cortisol rhythm is described as follows: At midnight healthy normal individuals have very low cortisol levels that proceed to build up overnight, starting to rise at around 02:00 to 03:00, to peak first thing in the morning, at around 08:30. Cortisol levels then decline slowly throughout the day to reach the nadir and complete the 24 hour cycle (Krieger, Allen, Rizzo & Krieger, 1971). During circadian trough glucocorticoids occupy more than 90% of MRs, but only 10% of GRs, while during circadian peak and/or stress MRs are saturated and approximately 67-74% of GRs are occupied (Reul & DeKloet, 1985). Another way to describe circadian cortisol cycle is according to the sleep cycle. Four unequal temporal phases can be described: a period of minimal secretory activity from 4-hours-prior to 2-hours-after sleep onset, a preliminary nocturnal secretory episode at the third-to-fifth hour of sleep, a main secretory phase during the sixth-to-eighth hour of sleep and continuing through the first hour of wakefulness, and an intermittent waking secretory activity in the rest of the waking period (Weitzman et al., 1971). These patterns of cortisol secretion result from changes in an underlying much faster ultradian rhythm: pulses of cortisol secretion that occur approximately once an hour. Differences in amplitude –and not frequency- of these pulses predominantly account for differing cortisol concentrations across the day/night cycle (Clow, Hucklebridge, Stadler, Evans & Thorn, 2010; Russel, Kalafatakis & Lightman, 2015).

It seems that elevation of cortisol secretion in the morning depends upon both sleep-wake and dark-light cycles (Clow, Thorn, Evans & Hucklebridge, 2004). Apart from these two, there is a number of other possible zeitgebers of the glucocorticoid diurnal rhythm. For example, it has been observed that in rats, when food availability is restricted to a few hours daily, time of meal presentation can be a powerful cue to glucocorticoid release, which peaks 1-2 hours before food availability (Krieger, Hauser, & Krey, 1977). Other factors might also affect circadian cortisol secretion. For instance, it has been observed that increasing age is linked with increased cortisol

secretion throughout the day (Deuschle et al., 1997). Another study found that the acrophase of the circadian rhythm of serum cortisol occurs earlier in morningness than eveningness types of healthy adults, with that preference being an innate phenomenon screened with the Horne and Östberg morningness-eveningness questionnaire (Bailey & Heitkemper, 2001).

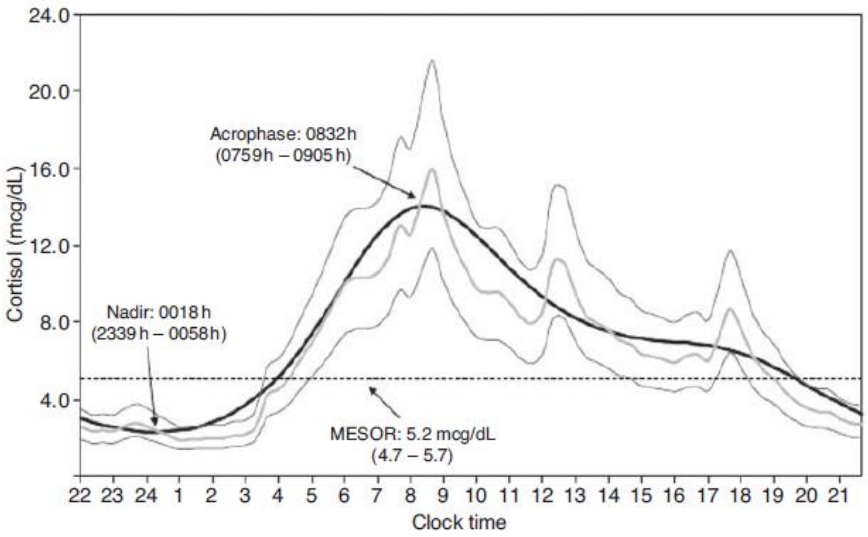

Figure 3. Physiological Cortisol Circadian Rhythm, derived from 20min sampling over a 24-h period in 33 healthy subjects The figure shows the geometrical mean -/+2 *SD* values of serum cortisol concentration (Debono et al., 2009).

A healthy HPA axis function is reflected by robust morning cortisol rise and afternoon and evening decline, also known as the Cortisol Awakening Response (CAR) and the Diurnal Cortisol Slope (DCS), while flattened profiles are considered unhealthy (Adam & Kumari, 2009). The CAR first described by Pruessner et al. (1997), is the dramatic cortisol level increase that happens 30 minutes post-awakening. It is considered as a distinctive part of the circadian cortisol cycle, in which salivary free cortisol concentrations increase between 50 and 160% in healthy adults. It appears that the CAR is independent from the rest of the cortisol diurnal cycle, and that it is genetically influenced unlike the remaining diurnal profile (Wüst, Federenko, Hellhammer, & Kirschbaum, 2000).

Similar to the general diurnal cortisol cycle, CAR has also been observed to be influenced by multiple factors, some of them being older age, smoking, awakening time, and day of the week (weekend vs. weekday), the later indicating that CAR is sensitive to the anticipation of a potentially stressful day. In contrast, it does not appear to be influenced by factors such as quality of sleep, body mass index, alcohol consumption, hormone replacement therapy, sleep duration, spontaneous vs. alarm waking, postural shift from supine to standing, blood glucose

levels and disrupted sleep (Clow et al., 2004). Finally, an important factor that enhances CAR is light exposure after awakening, contrarily to the cortisol secretion that happens the rest of the day, which doesn't seem to be affected. It is observed that under condition of total darkness after awakening the CAR is still apparent, but it can be enhanced by light exposure over the same post-awakening period (Sheer & Buijs, 1999). The same study demonstrates that evening cortisol levels remain unaffected by light exposure. Moreover, the effect of light on morning cortisol secretion can occur in absence of the sleep-wake transition, as exposure to light in sleep-deprived participants induced an immediate elevation of cortisol levels in the early morning but not in the afternoon (Leprout et al., 2001). However, later awakening, although associated with brighter natural light, is correlated with lower CAR (Edwards, Evans, Hucklebridge & Clow, 2001).

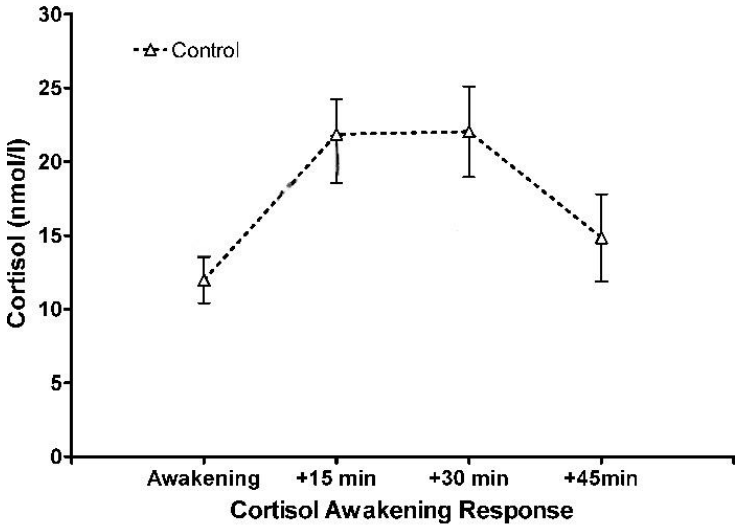

Figure 4. Cortisol Awakening Response of Healthy Adults (adapted from Roozendaal et al., 2010)

Cortisol & Executive Functions. There is extensive bibliography on the effect of glucocorticoids on the hippocampus and the mnemonic functions such as episodic memory. The most consistent finding in these studies is that high and augmenting cortisol levels are related with deterioration of said functions (Het, Ramlow, & Wolf, 2005; Lupien, et al., 2005). However, as mentioned above, cortisol acts on the prefrontal cortex as well, a region that has been associated with executive functions such as inhibition, through the GRs that exist there. Therefore, it could be hypothesized that cortisol might somehow affect executive functions.

The impact of cortisol on cognition can be best understood in terms of differential effects of MRs and GRs activation in the hippocampus, and our locus of interest, the frontal lobes. De Kloet, Oitzl and Joëls (1999), interpreting the inverted-U shape function between circulating levels of glucocorticoids and cognitive performance, stated that cognitive function is enhanced

when most of the MRs and only part of the GRs are activated. Lupien et al. (2005) suggest that working memory is more sensitive than declarative memory to an acute elevation of glucocorticoids, and that this is related to the fact that this acute elevation activates the GRs in the frontal regions. However, in line with recent data about membrane-associated MRs that respond to stress-induced corticosteroid releases (Karst et al., 2005), a study where healthy participants underwent a stressor after an MR antagonist or a placebo was administrated, indicated that stress facilitates inhibitory control and that these effects depend on MR functioning, as blocking these receptors blocks the stress effects (Schwabe, Höffken, Tegenthoff, & Wolf, 2013). The effect of cortisol in executive functions has been primarily studied through two different experimental paradigms; stress elicitation, and exogenous corticosteroid administration.

Stress Elicitation. As mentioned above, apart from circadian cortisol rhythm, glucocorticoids are also secreted as part of the stress response. Stress is defined as a state of real or perceived threat to homeostasis, which triggers the activation of a complex response that involves the endocrine, nervous, and immune systems. It is now suggested that stress response's primary role is to mobilize energy to promote context-specific behavior important for survival, and not necessarily sustain homeostatic systems at levels prior to a challenge (Nederhof & Schmidt, 2012). Stress can be divided into acute stress, which refers to a recent, transient occurrence of one or more stressors, and chronic stress, which refers to an ongoing difficulty facing a threat that may or may not be constantly present. It is well established that acute stress, which involves activation of the HPA axis and cortisol hyperproduction as part of the "fight-or-flight response", affects executive functions, although there does not seem to be a consensus in the literature about whether it enhances or impairs them (Leblanc, 2009; Shields, Sazma, & Yonelinas, 2016). One possible explanation of the effect of stress on cognition is found in cortisol. It is observed that blocking glucocorticoid receptors locally within the rat prefrontal cortex, and not the Ventral Tegmental Area, results in a reduction in stress-evoked dopamine efflux, and that it also attenuates stress-induced impairments in cognition, measured by a task sensitive to working memory impairment. It is therefore demonstrated that glucocorticoids in the prefrontal cortex modulate mesocortical dopamine efflux leading to cognitive impairments observed during acute stress (Butts, Weinberg, Young, & Phillips, 2011).

Stress might be impairing to executive control in order to force attention toward highly salient information (Vogel et al., 2016). This matches findings about stress impairing cognitive inhibition but enhancing response inhibition, producing in this way a cognitive phenotype to both approach and avoidance by impairing executive control over thoughts but improving it over motor actions (Shields et al., 2016). Moreover, findings suggest that changes in cortisol levels produced by acute stress impair executive functions as cognitive flexibility, not immediately after the stress experience, but gradually over time, paralleling the HPA-stress response time course (Plessow, Fischer, Kirschbaum & Goschke, 2011).

However, studies that examine stress-induced influence on executive functions might not be a reliable marker of cortisol effect on these functions. Shields et al. (2016) meta-analysis suggests

that, across all executive function tasks, stress effects significantly differ from corticosteroid administration effects. For example, stress enhances response inhibition and impairs cognitive inhibition whereas corticosteroid administration doesn't. As the authors point out, we cannot assume that these differences are due to the fact that stress is a more forceful manipulation of cortisol levels than exogenous administration, as the latter increases cortisol levels much more. The mechanisms behind stress response are far more complex than just cortisol elevation, and linking poor or high performance on neuropsychological tests designed to measure executive functions to high cortisol levels while inducing stress would be an unsafe deduction. For example Schwabe, Tegenthoff, Höffken and Wolf (2012) showed that the shift that stress promotes from goal-directed learning to habitual stimulus-response learning is mediated by the interaction of glucocorticoids and noradrenergic activity, and not by cortisol alone. This is a clear indicator that stress might influence executive functions through additional biological pathways rather than cortisol alone.

Exogenous Corticosteroid Administration. Experimentally manipulating cortisol through exogenous corticosteroid administration is another used method to determine if acute increases in cortisol actually influence cognition. It is safe to assume that, because endogenous cortisol is synthesized outside the brain and crosses the blood-brain barrier, exogenous cortisol will influence neural processes in the same way. As far as long-term memory retrieval is concerned, the impairing effects of cortisol through exogenous administration are clearly illustrated in Het, Ramlow and Wolf's meta-analysis (2005). However, regarding executive functions, less data is available. A meta-analysis suggests that, contrary to working memory, inhibitory control is enhanced from 15 to 135 minutes post cortisol administration, but starts getting impaired after that time frame. In other words, the rapid nongenomic effects of cortisol enhance inhibition, while impairing working memory, whereas the slow genomic effects of cortisol do the exact opposite to these two executive functions (Shields et al., 2015). However, studies with exogenous corticosteroid administration also have disadvantages, the main one of them being that they study an experimentally manipulated variable that cannot recur in everyday life, as cortisol levels are never that high as part of the natural cortisol cycle in healthy individuals.

Judging by the disadvantages of the two methods mentioned above, it could be suggested that cortisol effects on executive functions are studied by examining endogenous cortisol, and more specifically, by measuring the natural variance of cortisol during the day, or in other words the circadian cortisol rhythm.

Endogenous Cortisol and Executive Functions. Only a few studies examine how endogenous cortisol and its variance during the day affect cognition. Most of these studies are focused on episodic memory and the hippocampal function. Their main finding is that high morning cortisol levels are associated with episodic memory deterioration in the elderly (Lupien et al. 1994; Kuningas et al. 2007). There are very few studies examining the relation between executive functions and endogenous cortisol levels, and even less examining circadian cortisol rhythm. We present most of them below.

There are studies concerning both chronic and acute effects of endogenous cortisol on cognition. McLennan et al. (2016) found no relation between long term high cortisol exposure measured by hair cortisol, likely caused by chronic stress, and cognitive performance in various domains, such as executive functioning. Chronic effects should however be qualitatively different than effects of daily cortisol levels. Egeland et al. (2005) found that high morning salivary cortisol levels in subjects with major depression are related to executive dysfunction, assessed with the WCST, a widely used measure of executive functioning which is related to dorsolateral prefrontal cortex activation according to imaging studies, and the Stroop Color-Word Test. Nevertheless, only one saliva sample at 8 a.m. was collected for each subject. Lee et al. (2007) found that in healthy elderly elevated cortisol levels measured by taking saliva samples before, during and after cognitive assessment were related to poorer cognitive performance in seven cognitive domains, including executive functioning which was tested with the Purdue Pegboard Test, the Trail Making Test (TMT) - part B and the Stroop test. Another study that examines endogenous cortisol levels without exogenous manipulation in older aged individuals is that of Evans, Hucklebridge, Loveday and Clow (2012). In this study salivary cortisol samples were collected 0, 15, 30 and 45 minutes after awakening, in order to measure the CAR. Circadian cortisol rhythm was not examined. It has been found that the magnitude, but particularly the timing of the CAR is strongly related to executive functioning performance, measured by the TMT-A & B. This study suggests that the CAR could act as a biomarker and a regulator of cognitive functioning. Evidently, none of these studies examined diurnal cortisol.

There have also been studies where multiple cortisol samples were collected during the day. Li et al. (2006) also suggest that higher mean cortisol in non-demented elderly is associated with poorer performance on the TMT-A & B and the Stroop Test. In this study saliva samples were collected three times a day, at 8 a.m., 3 p.m., and 11 p.m., but cortisol measurement and neuropsychological assessment were executed on different days. Beluche, Carrière, Ritchie and Ancelin (2009) evaluated salivary cortisol secretion in healthy non-depressed elderly at a hospital examination and at home, at three time-points a day. They also evaluated cognitive performance on that day, at a two- and at a four-year follow-up. They found that high morning cortisol was associated with low verbal fluency and visuospatial performance in men, while moderately high morning cortisol and a moderately flat slope were associated with low verbal fluency performance -but not with visuospatial performance- in women. In the longitudinal analyses, high morning cortisol levels and a slow diurnal rhythm predicted a decline in non-verbal functioning in men and verbal functioning in women. It is worth mentioning that visuospatial performance was evaluated with the TMT-B task, which is also an executive functioning test. It's clearly demonstrated that most studies (Beluche et al., 2009; Evans et al., 2012; Karlamangla et al., 2005 Lee et al., 2007; Li et al., 2006; Segerstrom et al., 2016) examine healthy elderly, a population in which circadian cortisol rhythm and brain-neuroendocrine function relationship are notably different than in healthy young adults (Clow et al., 2004; Lupien, McEwen, Gunnar & Heim, 2009).

Some other studies with younger individuals exist as well. Stawski et al. (2011) observed that

higher cognitive functioning assessed by a phone-based battery was linked to healthier diurnal cortisol profiles, including higher morning cortisol levels, lower afternoon and evening cortisol levels and a steeper DCS in healthy middle-aged individuals. Moriarty et al. (2014) did a pilot study in healthy male adults, aged 30-60 years, and found a U-shaped relationship between endogenous cortisol and Spatial Working Memory. Nevertheless, the study, same as the one of Evans et al. (2012), concentrated on CAR and not the creation of a cortisol curve and the matching of each individual's performance to it. McCormick, Lewis, Somley and Kahan (2007) examined the effect of cortisol levels on performance on the WCST in young men and women, aged 17-22. They found that cortisol levels were associated to WCST performance, and not to performance on a test that measures mental rotation. As they stated, the arrival sample of cortisol probably best reflects cortisol action on performance due to the natural delay between cortisol secretion and cortisol action. It is important to mention that saliva samples were obtained upon arrival, before and after each test, four times in total, over a period of approximately 35 minutes, therefore neither here examining diurnal cortisol. Finally, in a study examining diurnal cortisol in a large sample of healthy adults (Singh-Manoux et al., 2014) there was no longitudinal association between any feature of the diurnal cortisol pattern and cognition. It can be stated that executive functions were part of the assessment, as a Verbal Fluency test was used.

From this brief bibliographical review it appears that existing studies on the matter have various deficits concerning the age and gender of the participants, the timing and frequency of cortisol measurements and the executive functioning tests used. To our knowledge there is no study of the effect of circadian cortisol rhythm on inhibition in young adults.

The Antisaccade Task. In the majority of the studies mentioned above, the tasks used to measure cognition all share a common problem: it is not clear which function is measured each time, as for most tests a number of functions must be used. This is a problem of specificity, quite common in the executive functions literature, as mentioned before. On the contrary, oculomotor tasks are considered very specific as to what they measure. They also have numerous other advantages that make them preferable when measuring cognitive functions. They are some of the few neuropsychological tasks that are easy to elicit, measure, and quantify, are not subjective and can be used in mathematical and computational approaches and comparisons between studies, just like biological markers. Moreover, they are non-invasive and they do not cause noticeable fatigue. Finally, another advantage is that the saccades, the particular type of eye movements measured in these tasks, are well understood and divided into a recognizable hierarchy with specific anatomical and psychological properties (Antoniades et al., 2013). For these reasons we have chosen to investigate the relation between circadian cortisol rhythm and inhibitory control, with the latter measured through the Antisaccade Task (ANS), an oculomotor tasks described below, based on the saccade.

A saccade is a quick, simultaneous movement of both eyes between two or more phases of fixation in the same direction, that shifts the eyes from one target to another (Leigh & Zee, 2015), bringing an object of interest into focus on the fovea where visual acuity is highest (Land,

1999). During the corresponding oculomotor task the subject initially fixates a centrally placed target that appears on an imaginary horizontal line. After a variable period of time a second target appears either to the left or to the right of the first target, which simultaneously disappears. The subject is instructed to follow the target as fast as possible (Constantinidis, et al., 2003). The word "reflexive" is commonly but misleadingly used to describe the saccade response, since the variability and duration of RT in the saccade task reflect the fact that its initiation is the culmination of a prolonged neural process of decision-making (Antoniades et al., 2013).). However, RT effects in this task are often small, proving the need of more complex tasks to study and compare brain functioning under different circumstances. One such task is the ANS, introduced by Hallet in 1978.

During the ANS the subject initially fixates a centrally placed target that appears on an imaginary horizontal line. After a variable period of time a second target appears either to the left or to the right of the first target, which simultaneously disappears. The subject is instructed to execute a saccade as quickly as possible in the opposite direction, suppressing the "reflexive" but erroneous saccade response (Evdokimidis, et al., 2002). This action requires executive control, especially the ability to inhibit a prepotent response, and secondarily, the ability to actively maintain the task goal in working memory (Meier, Smeekens, Silvia, Kwapil, & Kane, 2018; Unsworth, Schrock, & Engle, 2004). In general, the sudden appearance of a visual stimulus captures our attention automatically, causing our gaze to reflexively shift to the location of the stimulus. However, our gaze is not solely visually, but also voluntarily guided, and we can use internal motives to inhibit externally triggered behavior (Curtis & D'Esposito, 2003). The ANS makes use of this capability as it requires of the subject to inhibit an erroneous prosaccade towards the peripheral stimulus and to generate a volitional saccade to a position in the opposite direction (Cutsuridis, 2017). It is considered as a sensitive indicator of neurological dysfunction, and especially frontal lobe lesions, as such patients produce large numbers of errors, or can't perform the task at all (Guitton, Buchtel & Douglas, 1985).

In the ANS three possible oculomotor behaviors can be observed: the subject makes an antisaccade, the subject makes an erroneous prosaccade followed by a corrected antisaccade, or the subject makes only an erroneous prosaccade, a behavior which is very rarely observed. Errors are considered movements towards the peripheral stimulus. The erroneous prosaccade without correction is related to severe frontal lobe lesions or lack of understanding of the task instructions in normal subjects (Evdokimidis et al., 2002).

The ANS has four main measures; gain, RT, spatial error and error rate of "reflexive" prosaccades. Magnusdottir et al. (2019) reported that antisaccade RT, i.e. the time from target appearance to saccade initiation that reflects the speed of volitional response generation (Reuter & Kathmann, 2004), is significantly predicted by performance on TMT-B, while antisaccade error rate is predicted by Rapid Visual Information Processing (RVIP). Antisaccade gain and spatial error are measures of the ability to match saccade amplitude to target amplitude that depend on sensorimotor processes involved in transforming the covertly encoded visual target location into a motor output (Moon et al., 2007). In other studies the indices measured are

percentage of errors, and Mean and Standard Deviation (*SD)* of the correct antisaccade, the error prosaccade, and the corrections, if these should happen. Mean error rate in healthy population is 20% (Hutton & Ettinger, 2006). Optimal performance to the task appears to require both an intermediate mean RT and a small variability. RT and error rate on the ANS are not related to lateral preference as measured with the Porac-Coren questionnaire (Constantidis et al., 2003). It is observed that patients with frontal damage and patients suffering from schizophrenia make more antisaccade errors, and antisaccade latencies are more variable across these subjects (Cutsuridis, 2017). It seems that most of the ANS variables, and especially the ones that will concern us here, which are RT, RT Variability (or *SD*) and Error Rate, have very good Test-Retest reliability, in normal subjects (Ettinger et al, 2003; Klein & Berg, 2001; Klein & Fischer, 2004; Wöstmann et al., 2013), as well as in schizophrenia patients and their relatives (Calkins, Iacono & Curtis, 2003). Below we will briefly report findings regarding the ANS and brain activity.

Antisaccades and Brain Activity. Many different brain areas are involved in the generation of a saccadic movement, the most important being the Frontal Eye Field (FEF) related to the preparation and generation of all volatile saccadic movements, the Supplementary Eye Field (SEF) for the generation of multiple consecutive movements, and the SC of the brain stem, for the execution of the saccadic movement (Pierrot-Deseilligny, Milea, & Müri, 2004; Hall & Colby, 2016). First, visual information is processed via the retino-geniculo-cortical pathway to primary visual cortex and from there to the Parietal Eye Fields (PEF) that code for space and via the retinotectal pathway to the superficial layers of the SC. PEF then projects to both the intermediate layers of the SC (SCi) and frontal cortical oculomotor areas including FEF, SEF, Anterior Cingulate Cortex (ACC) and Dorsolateral Prefrontal Cortex (DLPFC) (Cutsuridis, 2019). The ANS can be divided into two phases, the inhibition phase and the generation phase. Regarding the inhibition of the prosaccade, the main regions that are most likely to be involved are the DLPFC that is considered to be responsible for the executive control of ocular movements and suppressing automated or reflexive responses, the Medial Prefrontal and ACC for mistake evaluation and conflict resolution, and the Right Supramarginal Gyrus, whose far greater activation during the inhibition phase suggests a role in saccade inhibition or stimulus detection. Regarding the generation phase, regions that are likely involved are the Intraparietal Sulcus (IPS) and the Supplementary Motor Cortex , especially the FEF and SEF, for visuo-motor aspects of the antisaccade programming and generation, and finally the Right Inferior Frontal Gyrus (Curtis, Cole, Rao, & D' Esposito, 2004; Scangos & Stuphorn, 2010; Emeric, Leslie, Pouget, & Schall, 2010; Stuphorn & Schall, 2006; Cameron, Riddle & D'Esposito, 2015; Ettinger et al., 2007; Aron, 2011). If activity in movement-related neurons can be kept below a critical threshold just long enough for the voluntary antisaccade to be programmed and initiated, then the decision to make a correct antisaccade is likely to be achieved. FEF and IPS activation during the stimulus-response phase of the task could be related to processes including covert orienting the visual stimulus, cancellation of the reflexive saccade or selection of the antisaccade location (Curtis & D'Esposito, 2003). In an fMRI study that involved the antisaccade task

(Raemaekers et al, 2007) it was found that although reproducibility of individual subject maps is highly variable across subjects, Test-Retest Reliability was high for group activation maps during the prosaccade and the antisaccade task.

A functional separation of SEF and pre-supplementary motor area (pre-SMA) is supported in some studies, with greater SEF activity during saccades and greater pre-SMA activity during successful antisaccades. More specifically, saccade-related neurons in the SC and FEF decrease their firing rate before antisaccades, compared with prosaccades (Everling & Munoz, 2000; Everling, Dorris, Klein, & Munoz, 1999; Everling, Dorris, & Munoz, 1998). On the contrary, event related fMRI showed greater prestimulus preparatory activity in the pre-supplementary motor area (pre-SMA) before voluntary antisaccades compared with prosaccades (Schlag-Rey, Amador, Sanchez, & Schlag, 1997) and this preparatory activity predicted whether a "reflexive" prosaccade was later successfully inhibited. These differences start to emerge only 300ms before the appearance of the saccade stimulus. The pre-SMA activation likely reflects a process related to supervisory control that facilitates appropriate motor behavior in cases where inhibition of a prepotent response is needed. However, the pre-SMA may not be necessarily responsible for actually inhibiting the reflexive response. Instead it might be preparing other oculomotor regions such as the SEF in some fashion such that reflexive responding is less likely, and SEF might be the primary oculomotor region that actually inhibits the reflexive prosaccade. All of these areas are abundant in GRs, which supports the hypothesis that cortisol might have on effect on oculomotor tasks such as the ANS (Curtis & D'Esposito, 2003).

Study Purposes – Hypotheses

Based on all of the above, we decided to study the relation between cortisol level and executive functioning. Primarily we wanted to examine whether ANS performance, as expressed by the measures of RT and Error Rate, would change during the day. Secondarily, we hypothesized that the diurnal cortisol curve would be different between subjects, reflecting different stress responsiveness, but that it would generally fit the expected cortisol curve usually observed in normal subjects. We finally hypothesized that this diurnal variation of cortisol would correlate with variation in the ANS performance, suggesting a role of normal cortisol variation in cognitive function.

Method

Study Design

We conducted an observational descriptive study with a repeated measures design. The sample consisted of one group measured three times on oculomotor performance. We also used three cortisol level measurements for each participant, estimated according to their Diurnal Cortisol Profile, which we acquired as explained in the respective section below.

Participants & Exclusion Criteria

For this study we recruited 21 healthy participants, nine men and twelve women, aged from 18 to 33 years old. Mean age was 24.33 and *SD* was 3.94. They all consisted of one group and comparisons were executed between and within subjects. Subject selection was done with non-randomized, opportunity sampling. Exclusion criteria that have been chosen based on previous studies because they could possibly affect ANS performance and/or diurnal cortisol levels were: neurological disorders, anxiety disorder or other psychopathology, disorders related to cortisol and the HPA axis, psychotropic drug use, use of benzodiazepine, contraceptives and antihistamines. Descriptive statistics regarding Age and Years of Education per Gender can be found in Table 1.

Table 1

Age and Years of Education per Gender

Sex	***N***	**Age**				**Years of Education**			
		M	***SD***	**Min**	**Max**	***M***	***SD***	**Min**	**Max**
Men	9	24.67	3.74	18	29	16.89	2.93	13	20
Women	12	24.08	4.23	18	33	15.83	1.99	13	18
Total	21	24.33	3.94	18	33	16.29	2.43	13	20

Ethics

The current study is in accord with the principles of the Helsinki Declaration of 1975, as revised in 2013 (World Medical Association Declaration of Helsinki, 2013). The procedures used in this study in no way affected the participants' health or caused nuisance. Participants were fully updated regarding the research purposes and the procedures that were followed, in written form. They were also informed that their participation is voluntary, that the use of their data is completely based on their consent, and that they have the right of quitting in any stage of the study without further consequences. They were encouraged to ask the researchers any

questions regarding the program. All of this information was part of the written consent that participants were asked to sign before entering the study. The University Mental Health Research Institute (UMHRI) did not bear any expenses from the execution of the present study

The study complies with the EU General Data Protection Regulation (GDPR*)*: Regulation (EU) 2016/679 of the European Parliament and of the Council of 27 April 2016 on the protection of natural persons with regard to the processing of personal data and on the free movement of such data, and repealing Directive 95/46/EC (GDPR), OJ 2016 L 119/1. Participants were informed in a written manner regarding which of their personal data will be collected, and were asked to declare their consent by signing a GDPR form. Protection of personal data and biological material was assured. All data was coded before being inserted in a data base, securing participants' anonymity. Researchers of the Institute are the only ones responsible of the data retention and processing. Any result publication will not contain reference to personal data.

The Study has been registered for approval at the UMHRI's Ethics Committee. The written consent forms are included in the Appendix B of this thesis.

Procedure – Measurements

Each subject's participation on the study lasted one day. In this day we collected saliva samples and asked participants to perform the oculomotor tasks as described in the respective sections below. The procedure followed a detailed experimental protocol. All procedures took place in a quiet and familiar environment for the participant, preferably their house. For optimal results, a rest day was selected, for example weekends or days-off, to ensure that participants felt relaxed and anxiety did not affect their cortisol levels. Apart from these measurements, demographic information was also collected, for potential use in the statistical analysis, or to ensure that no exclusion criteria were met. The information collected was: age, place of birth, residency, years of education, clinical, psychiatric and neurological history, smoking, and current use of medicine, alcohol, drugs and caffeine. None of this made it to the final analysis.

Apparatus

Two different measurements were conducted. We collected saliva samples to create a Diurnal Cortisol Profile for each participant and we also measured performance on four oculomotor tasks, of which only the ANS performance was used in the current study.

Saliva Sampling. Cortisol level estimation was done by taking saliva samples. Salivary cortisol is considered to be a reliable measure of HPA activity, highly correlated with free cortisol levels in plasma (Hellhammer, Wüst, & Kudielka, 2009). Sampling was done with the use of Salivettes® (Sarstedt, Rommelsdorf, Germany), that consist of a typical centrifuge tube that contains an absorptive cotton part. Participants have to chew on the cotton-made part of the salivette for approximately two minutes, stimulating saliva production. In this way sampling is conducted in a non-invasive manner. Next follows the centrifuging of the samples, to remove

solid materials, and are then deep frozen in a -80^{o} C freezer until the analysis. The analysis was done with the immunological method of Electrochemiluminescence, into a Cobas e411 analyser (Roche Diagnostics, Manheim, Germany), in the Choremeio Research Laboratory of the First Pediatric Clinic of the Children's Hospital "Agia Sofia", in association with Assistant Professor, Dr. P. Pervanidou.

To ensure correct sampling all the necessary rules were respected, such as no food consumption 30 minutes prior to sampling, no teeth brushing, no use of cosmetic's products on the lips, and mild mouth wash for the removal of any food residues that must be finished 10 minutes before sampling. Sampling was done five times a day, exactly after awakening, 30 minutes later and then 4, 8 and 12 hours after. All of the sampling was completed in one day. This sampling time and frequency allowed the optimum representation of the cortisol curve, according to what is known about circadian cortisol rhythm.

Oculomotor Tasks. Oculomotor tasks, and specifically the ANS that concerns us in this study, were performed using a device called Saccadometer Plus (Ober Consulting Sp. z o.o. Poznań, Poland). This device has been chosen because it is portable and can be easily used outside of laboratory environment. It detects horizontal eye movements, with sampling frequency 1 kHz, using the technology of Direct Infra-Red Oculography. The experiment took place in a uniformly illuminated environment. The infra-red censors and transmitters were placed on the head of the participant in a way that resembled that of eyeglasses, and each participant was seated facing a white wall, in a viewing distance of approximately 200 cm. The task target, a red dot, was projected by the same device via Light Amplification by Stimulated Emission of Radiation (LASER). For pictures of the device see Appendix B.

Every task was executed in 100-trial blocks, with small breaks between each block. Before each block there was a small training session for each task, to ensure that participants understood the task instructions and were able to execute it correctly. A calibration procedure was performed before starting the measurement, using a sequence of ten saccadic eye movements, five to the left and five to the right of the central fixation stimulus. The inter-trial interval was between 1000-2000 ms, chosen in a random manner by the device for each trial. For all the oculomotor tasks that were tested, we recorded the following: Error Rate, duration, movement magnitude (in degrees), maximum and mean speed, maximum and mean acceleration, maximum and mean deceleration, and RT. The order that the tasks were performed by was pre-fixed and different for each individual and for each session within the same individual's measurements, to ensure that there was no Order Effect

Specifically for the ANS the participant was given the instruction to focus his gaze on a central target. Then, after a small variable period of some ms, a peripheral target appeared, while simultaneously the central target disappeared. Participant was given the instruction to execute a saccade as quickly as possible, at exactly the opposite direction of that of the peripheral target, inhibiting the prepotent response towards the target. The subject was also given the instruction to try to blink only after completing the eye movement, for the device to correctly record the

movement.

Measurements were non-invasive and non-tiring for the participant. The whole procedure lasted approximately 40 min and was repeated three times a day, during one day for each participant, after the second, third and fifth saliva sampling.

Statistical Analysis

To analyze our data from the oculomotor measurements we first used the *Latency Meter* program to do the data preprocessing which included examining and discarding problematic trials. Then we used *Statistica 12*, a statistical analysis software, to perform all of our analyses, for both the oculomotor and cortisol measurements. For the sample demographic data analysis we used IBM SPSS Statistics 21.

We conducted all our analyses after first checking that all the necessary assumptions were met: our dependent variables were all measured at the continuous level and normally distributed, our independent variables consisted of at least two groups, and there were no significant outliers. Finally, we always tested Sphericity with Mauchley's Test of Sphericity and used the Greenhouse-Geisser correction wherever sphericity was violated.

We performed Repeated Measures Analyses of Variance (ANOVA) for all performance variables, as well as to create the cortisol curve. We then split our morning ANS session in half and performed a T-Test to compare the first and second half for potential practice effects. We also calculated differences between Morning and Evening Cortisol, as well as Morning and Evening Performance Variables, and conducted correlational analyses. Two other cortisol related variables that we calculated where CAR and Mean Cortisol. CAR was calculated by finding the difference between first and second cortisol measurement, while Mean Cortisol was calculated by adding all cortisol measurements of each participant and dividing them by the number of measurements, which was 5. Finally, we calculated the Performance Variables and Cortisol Slopes. In brief, we took the graphs that were created from each participant's cortisol, RT, RT's variability, and percentage of correct answer measurements, calculated each graph's slope and contrasted all of them with correlational analysis. Due to the fact that cortisol diurnal levels form a curve, we used a log transformation of the 30min, 4h, 8h and 12h cortisol measurements to get a more linear graph. The first sampling of cortisol corresponding to the awakening was excluded for the same reasons.

Results

Behavioral Analysis

1. ANOVA: RT of Correct Antisaccade Responses

First, we wanted to examine whether the RT of correct antisaccade responses changes during the day. We conducted an ANOVA on our data. There was a statistically significant effect of the

time of measurement on RT of correct responses, $F(2,40)= 4.88$, $p=.013$, $\eta^2_p= 0.196$. Statistical power was at 77% (Figure 5).

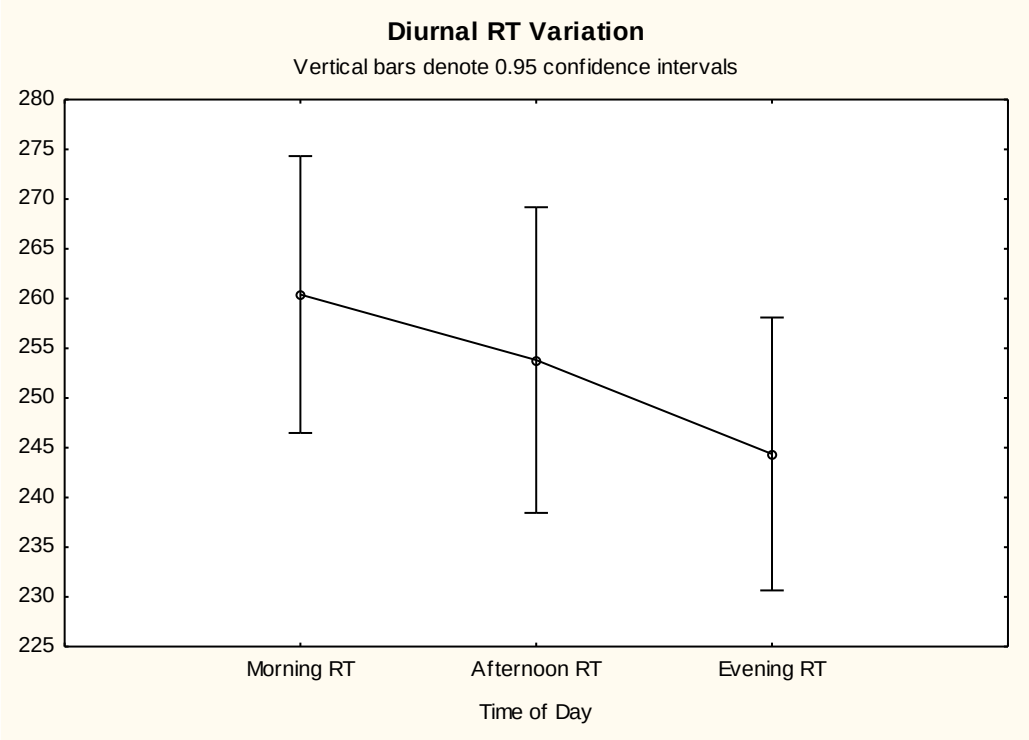

Figure 5. Diurnal Correct Antisaccade Responses' RT Variation

Since the ANOVA was significant, we proceeded to Post-Hoc Analysis, using the Bonferroni criterion, to examine which groups differed significantly. We found that the mean RT of the Morning Measurement's mean RT ($M= 260.40$) was significantly different ($p=.01$) from the Evening Measurement's ($M= 244.37$), with a mean difference of 16.03. We therefore observe that participants' correct antisaccade responses are faster as the day progresses, and they are significantly faster in the evening in contrast to the morning.

We continued by running a Repeated Measures ANOVA to also examine whether the RT's variability changes between measurements. To do this we used the RT's *SD* as a variability measure. Results showed a statistically significant effect of the time of measurement on RT variability, $F(2,40)= 5.68$, $p=.007$, $\eta^2_p= 0.221$. Statistical Power was at 84% (Figure 6). Post-Hoc Analysis with the Bonferroni Criterion showed that the morning RT's mean variability ($M=$ 64.62) was significantly different from the evening's ($M= 48.72$), with a mean difference of 15.9, $p=.005$. These results suggest that RT variability decreases during the day, meaning that participants' reaction times are more stable in the evening measurement.

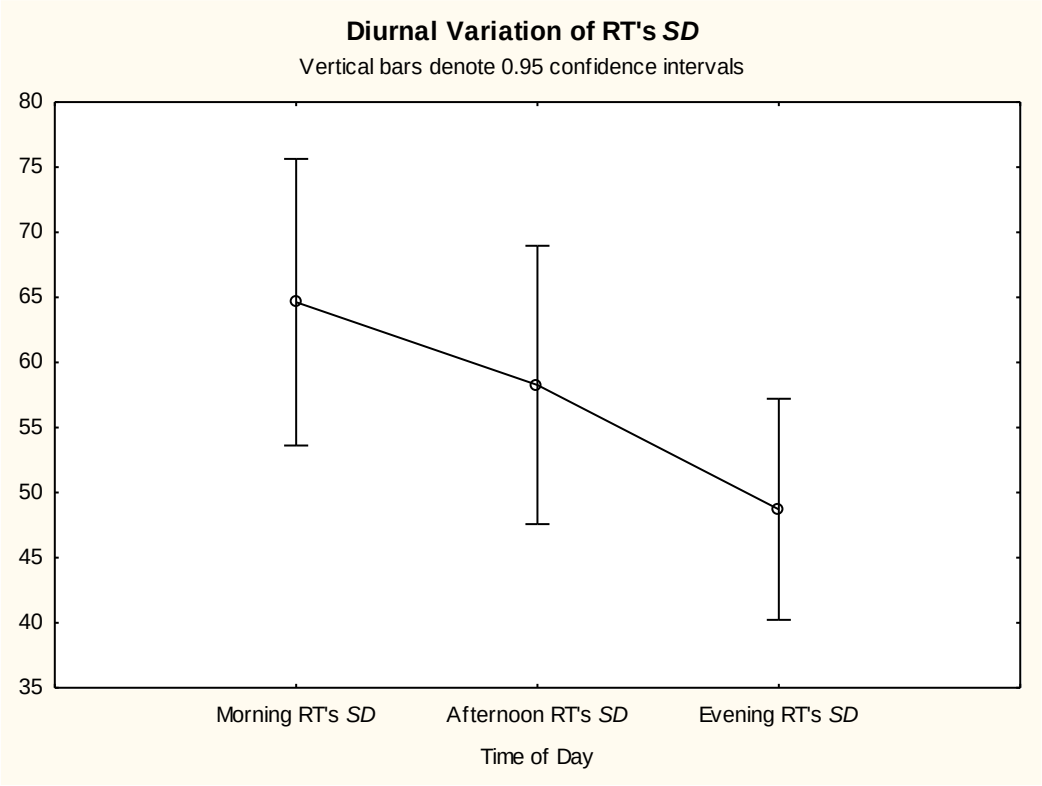

Figure 6. Diurnal Correct Antisaccade Responses' RT Variability Variation

2. ANOVA: RT of Incorrect Responses

We wanted to examine whether the time of the day that the measurement took place also affected the incorrect responses' RT. The ANOVA was not significant ($F(2, 36)= 2.59$, $p=.089$), although generally the same tendency of the RT mean dropping during the day was observed. Then we also run a Repeated Measures ANOVA on the RT's variability, using again the standard *SD* as its measure. Neither this analysis was significant ($F(2, 34)= 1.24$, $p=.302$) although it followed the same trend of the variability decreasing during the day.

3. ANOVA: Percentage of Correct Answers per Block

Next, we wanted to examine whether the percentage of correct antisaccade responses changed during the day. To do this we first transformed our categorical variable (correct, incorrect) into a continuous variable (0,1). Our Analysis after the Greenhouse-Geisser correction was statistically significant, $F(1.25, 25.01)= 7.88$, $p=.006$, $\eta^2_p= 0.283$. Statistical Power was at 94% (Figure 7). Since the analysis was significant, we proceeded to do Post Hoc tests with the Bonferroni criterion. We found that the percentage of correct antisaccade responses was significantly lower in the morning measurement (0.863=86.3%) than in the evening measurement (0.927=92.7%)

with p=.0009 and mean difference 0.064. In conclusion, what we observed was that participants' performance regarding percentage of correct answers is getting better during the day, and is significantly optimal in the evening.

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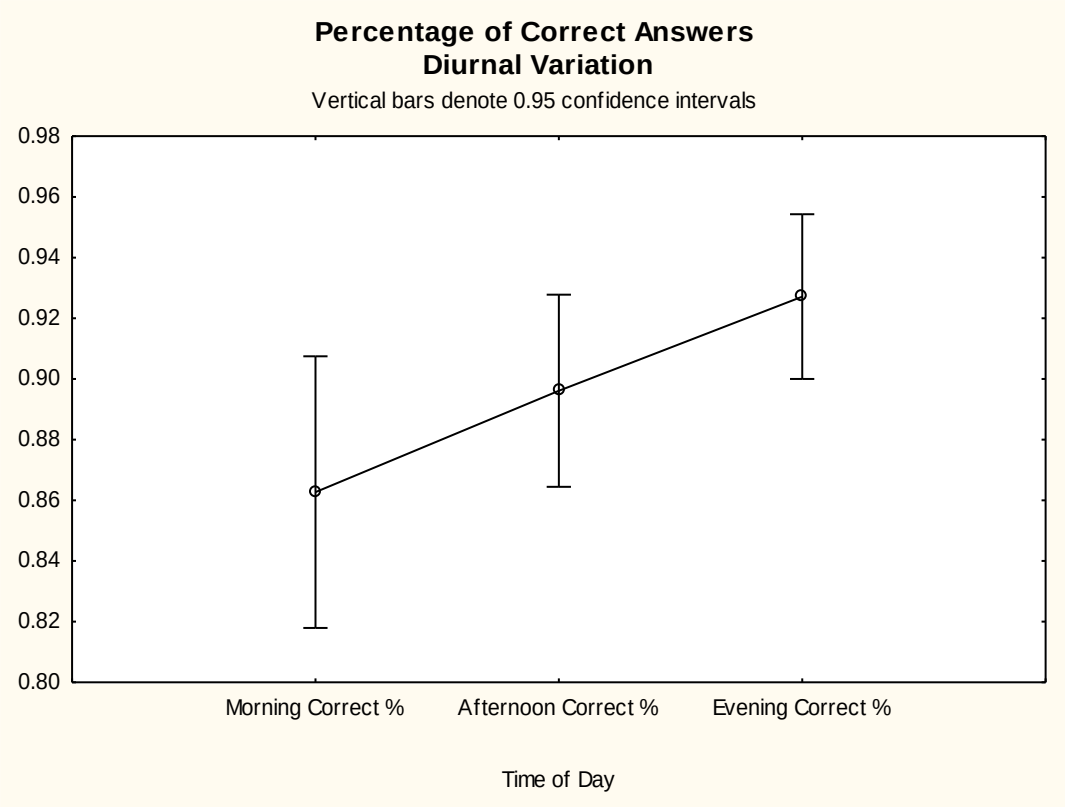

Figure 7. Diurnal Variation of Correct Antisaccade Responses' Percentage

Half Split Analysis

As established by the studies in test-retest reliability mentioned in the literature review of the introduction of the present thesis, there does not seem to be a learning/practice effect in the antisaccade task. However, we wanted to somehow test the possibility that performance gets better during the day because of repetition and learning. For this reason we split our Morning Session Data in two. Our hypothesis was that if there would be a learning effect, it would be most visible in the morning, when the participants encounter the task for the first time. Therefore, if there was a learning effect, participants would get better during the morning session, and this would be mirrored in their performance, which would be better in the second half of the session. To examine our hypothesis we did a T-test contrasting the percentage of correct answers and latency of the first and second half of the session.

As far as Percentage of Correct Answers was concerned, the t-test was statistically significant, $t(20)= 3.95$, $p=.0008$. More specifically participants answered correct more frequently in the first

half (0.886) than in the second half (0.833) of the morning sessions. As far as latency of correct answers is concerned, the t-test was not statistically significant ($t(20)= -1.179$, $p=.252$), but still the same trend was observed, meaning that participants were faster in the first half (256.21) than in the second half (317.81) of the morning session. Therefore, what we observe here is the opposite of what would be a learning effect. Participants don't get better in the second half of the session. In fact, they get worse, meaning that maybe there's an effect of tiredness by the end of each block, but certainly not a learning effect. It is safe to assume that our previously reported results are not due to learning.

Cortisol Analysis

Since we observed significant differences in antisaccade performance during the day and we assumed they were not a result of learning, we proceeded in testing whether cortisol levels during the day have something to do with these differences. We first took all five cortisol measurements and created the general cortisol curve. Our data seem to fit very well the expected and generally observed in healthy adults Cortisol Curve. Cortisol levels start at a medium point upon awakening, reaching their maximum 30 minutes later, and start dropping afterwards, until they reach a nadir 12 hours upon awakening.

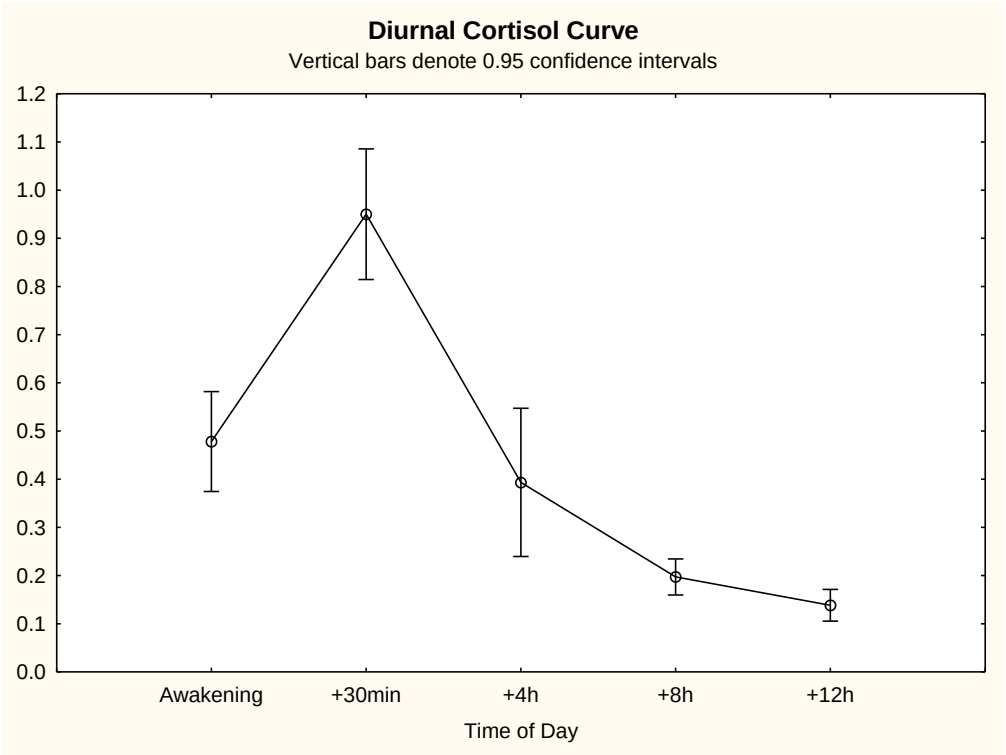

Figure 8: General Diurnal Cortisol Curve

Moreover, as it can be seen in Figure 8, *SD*s of each measurement are all very low, the highest of them being the ones of the second and third measurement, when variability is most expected. Therefore it is safe to assume that cortisol sampling was done correctly, and that no participant suffered any undiagnosed HPA-axis related or anxiety related disorder, since their data fit the expected cortisol curve with no exception.

Since we observed the same trend in cortisol and performance changing during the day, we then wanted to examine whether these two are correlated. To do this we first looked at our previously done Post Hoc tests to see which performance measurements differed significantly. These were the Morning and Evening Mean RT of correct responses, the Morning and Evening Variability of the correct responses' RT, and the Morning and Evening Percentage of Correct Responses. We then calculated each couple's difference for each participant, as well as the respective in time difference of cortisol level, which in this case was the difference of the second and the fifth cortisol measurement of each participant, since they were done exactly before the Morning and Evening Session of oculomotor tasks.

Surprisingly, no significant correlations between cortisol levels' difference (2-5) and any of the task variables' differences was observed. We also tried correlating the same three performance rates with two additional widely used cortisol markers, each participant's Mean Cortisol and CAR. Neither these correlations were high or significant. All the correlations and their significance level can be seen in Table 3. The Bayesian Factor that is also included in the table shows how possible it is to meet these results when the null hypothesis is valid. Numbers above 3 show moderate possibility.

Table 2

Correlations of Cortisol and Performance Differences

		RT Diff.	**RT *SD* Diff.**	**% of Correct Responses Diff.**
Cortisol Diff. (2-5)	*Pearson's r*	0.10	0.14	0.07
	p-value	.663	.546	.770
	BF_{01}	3.38	3.12	3.55
Mean Cortisol	*Pearson's r*	0.19	0.35	-0.02
	p-value	.403	.122	.935
	BF_{01}	2.66	1.21	3.69
CAR	*Pearson's r*	-0.04	0.15	-0.16
	p-value	.874	.508	.495
	BF_{01}	3.66	3.01	2.97

These results show no relation between cortisol level and performance. However, before completely excluding the possibility of these two being related, we wanted to somehow contrast

the entire Cortisol Variation with the entire Performance Variation of each participant, instead of just taking the levels that are significantly different and contrasting them. In other words, we wanted a marker that depicts how cortisol and performance change during the day, which we created by calculating the cortisol and the performance variables' slopes, as described in the Statistical Analysis section in *Method*. We then performed a correlation analysis. None of the correlations reached significance. More specifically there was no significant correlation between the Cortisol slope and the RT Slope (r= 0.09, p=.688), the RT Variability Slope (r= 0.02, p=.929) or the Percentage of Correct Answers Slope (r= 0.333, p=.141).

Discussion

In the present study we tested if there was any diurnal variation in the ANS performance, by contrasting the RT, RT *SD* and Percentage of Correct Answers that was measured for each participant in a three-point basis during the day, Morning, Afternoon and Evening. We also tested if the Diurnal Cortisol Curve created by the five saliva samples that we collected from each participant was the one we anticipated. Finally we tested whether these two diurnal variations were correlated. The results of our study confirmed some, but not all of our hypotheses. Our first hypothesis, suggesting that the ANS task performance would change during the day, was confirmed. It seems that the three task variables that we studied, those being RT, RT Variability and Percentage of Correct Answers, all changed during the day, and more specifically, overall performance improved. This is the only study to our knowledge examining diurnal variability of antisaccade performance, except for the Roy-Byrne et al. study (1995), which however only included Morning (8-10 am) and Afternoon (3-5 pm) measurements. In fact our results highly differ from those of said study, since Roy-Byrne and his colleagues showed that time of the day did not have a significant effect on antisaccade RT and error rate. Not only were results not significant, but also error rate and RT seemed to have the opposite trend than that of our results, as they both increased in the afternoon measurement. Finally, in said study, the morning measurement's RT *SD* was disproportionately high. These results are opposite to our observation of statistically significant improvement of ANS performance. It must be pointed out that said study only included eight subjects, of highly variable age.

As far as RT of Incorrect Responses is concerned, we did not get any significant results. However, this is not something we did not expect. It is possible that this lack of improvement is observed because of the process of making errors being involuntary and more "automatic". Also it is worth mentioning that RT values of said responses don't have high test-retest reliability (Klein & Fischer, 2004).

As far as our half-split analysis, we think that it serves as proof that our results are not caused by practice effect. However, there seems to be some ambiguity in the literature as to whether there is a practice effect in oculomotor tasks. As Lezak (1983) mentions, practice effect in

cognitive assessments is particularly observed in tests that "require an unfamiliar or infrequently practice mode of response" (p. 115), something that matches the antisaccade tasks description. Smyrnis et al. (2002) observed a practice effect in the ANS. More specifically they observed that within the same block the first 10 trials were worse regarding error rate, RT and amplitude. After these 10 first trials the performance was stabilized. However we could say that these first trials serve as a period of familiarization with the task, which in our case happens in the brief training before each session. This is highly supported by the fact that in said study the performance is then stabilized instead of continuing to improve within the same session. This matches our results of not observing an improvement in the first half of our blocks. Finally, the same study also showed a fatigue effect in error rate, which seems to increase again in the last 10 trials. This is in accord with our results that show a decrease in the percentage of correct answers in the second half of the block, probably as a result of fatigue.

Other studies have shown that ANS is susceptible to practice effects. Ettinger et al (2003) showed a between-sessions practice effect within a 2-month interval, expressed by reduced error rate and improved spatial accuracy at retest, but not significantly faster RT. A similar error rate reduction was observed by Green, King & Tremble (2000) within a 1-week interval. As Ettinger et al. (2003) mention, the stronger learning benefits seem to occur to participants with the worst performance, possibly due to the existence of a ceiling effect in the better-performing subjects. Another interesting study about practice effects in ANS is that of Dyckman and McDowell (2004). In this study 30 subjects were tested at a three-point basis over a 2-week period in the antisaccade task, while simultaneously they practiced four days per week their assigned task, which was one of the following three: a different antisaccade task, a prosaccade task or a fixation task. All of the participants' RTs of correct ANS responses decreased between sessions, regardless the task they had been practicing. However, only antisaccade practice led to improvement of the Percentage of Correct Responses. Authors suggest that the ANS practice leads to a kind of "automatization" of the antisaccade response, probably associated with decreased brain activity related to the task. On the other hand, the fixation practice had no effect on ANS performance, while the prosaccade practice led to more antisaccade errors. Authors hypothesized that prosaccade practice reinforces the prepotent response, making inhibition more difficult.

In our opinion this study does not pertain to our measurements, whose number we don't consider enough to be accounted as prior practice. In the absence of intervening eye-movement training, which is our case, ANS performance is typically interpreted as stable (Ettinger et al, 2003; Klein and Berg, 2001; Roy-Byrne et al., 1995). However this study is one of the few to mention the ocular system's plasticity, something that must be seriously taken into consideration. It also points out that there are conditions that might disrupt or improve the antisaccade task, as for example the previous task performed by the subject. In our study we chose to change the order of the different tasks performed in a fixed manner between sessions, within and between subjects. In this way, this potential order effect does not affect our results.

Our second hypothesis, that there would be some inter-subject variability in cortisol

measurements, but they would generally fit into the expected Diurnal Cortisol Curve, was also confirmed. As expected, *SDs* of cortisol measurements were considerably low, the highest of them being those of the second and third measurement, around when the cortisol peak is expected to take place. These high *SDs* are explained by the fact that the cortisol peak is neither always of the same size, nor does it happen at the same time for everyone, so it can happen anywhere between these two measurements.

Our final and most important hypothesis was that the Diurnal Variation of the ANS Performance would be correlated, or even predicted by the Diurnal Cortisol Curve. This hypothesis was not confirmed. This was rather surprising, considering the fact that both measurements followed the same trend during the day. More specifically, RT and RT variability seemed to gradually fall during the day in the same manner as cortisol levels, while Percentage of Correct Responses seemed to follow the exact opposite trend. The fact that none of the performance variables is correlated to cortisol offers a very interesting view regarding the interpretation of previous studies. Our study is the only one investigating the Diurnal Cortisol Curve and its relation with executive functioning, and specifically oculomotor tasks. Yet it failed to produce results that show any correlation between the two. We could go as far as to suggest that changes in cognitive functioning observed in studies involving stress induction or corticosteroid administration might have nothing to do with cortisol. As we mentioned before, there is no proof that when stress is elicited, cortisol is the one responsible for the observed changes. On the contrary, it is evident that during stress many more alterations happen neurophysiologically. Corticosteroid administration also causes stress as well as other physiological alterations that highly differ from those that happen due to normal diurnal cortisol variation. Therefore, the present study might serve as tinder for the research of other stress related mechanisms and alterations apart from cortisol that could be related to changes in cognitive functions such as inhibition.

This study has a few possible limitations. Because of the study's design we cannot ensure that the changes we observed in performance during the day aren't due to a learning effect. We could probably do this by having some participants perform the same task at the same time of the day in two consecutive days. This is something that we plan on doing in the near future to further support our claim that performance improvement is related to the time of day and not to learning. Moreover, due to the duration of measurements for each participant, which was one whole day, it was harder to gather a bigger sample, which would ensure the validity of our results.

In conclusion, our study, regardless its limitations, is the only one to have examined the Diurnal ANS Performance in a three-point basis. It is also one of the few studies that examined the relation of the diurnal cortisol variation with executive functioning, and more specifically inhibition, and the only one that tried correlating diurnal cortisol variation with an oculomotor task. The fact that no correlation between the two was found might serve as a refutation of the association of cortisol produced in the HPA axis stress response with changes in executive functioning.

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Appendix

Saccadometer Plus