Cortisol levels in survivors of the Utøya shootings two to three years after the event

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Master's program in psychology

Specialization:Behavioural Neuroscience

THE UNIVERSITY OF BERGEN

FACULTY OF PSYCHOLOGY

SPRING 2016

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Sammendrag

Overlevende etter Utøyamassakren i 2011 står i fare for å utvikle symptomer på PTDD/PTSS. Tidligere forskning har befattet seg med de biologiske markørene som assosieres med utvikling av PTSD/PTSS, og med særskilt oppmerksomhet rettet mot HPA-aksen og hormonene som utskilles.

I den foreliggende studien forsøker vi å identifisere noen av de biologiske markørene (kortisol) for så å drøfte mulige forklaringer på de forandringene som er blitt observert.

Overlevende fra Utøya ble ytterlige delt inn i undergrupper: De som utviste PTSD/PTSS-symptomer, og de som ikke gjorde det, sammenlignet med en ikke utsatt kontrollgruppe. Kortisol fra spytt ble samlet inn ved spytteprøver hjemme, og før og etter en fMRI-scanning. Prøvetakingen foregikk over en tredagers periode for å kunne vurdere en normal døgnrytme med henhold til kortisol, og av særskilt interesse var da kortisolets oppvåkningsrespons. Dette inntreffer like etter oppvåkning. Omtr. 15 til 30 minutter etter oppvåkning øker kortisolnivåene. Utøyaoverlevende med PTSD/PTSS viste en lavere gjennomsnitt kortisolproduksjon enn de to andre gruppene, særlig med hensyn til kortisolstigning (AUCi).

Resultatene som foreligger viser tegn på endringer i HPA-aksen hos personer som utviklet PTSD/PTSS etter Utøya-skytingen og bringer økt forståelse av noen av de biologiske markørene vedrørende PTSD.

Nøkkelbegreper: Kortisol, HPA-aksen, kortisolets oppvåkningsrespons, posttraumatisk stresslidelse, traume.

Abstract

Survivors of the Utøya shootings 2011 are at risk at developing PTSD/PTSS symptoms. Previous research has addressed the biological markers associated with the development of PTSD/PTSS, with special attention to the HPA axis, and the hormones secreted.

In the present study, we try to identify some of these biological markers (cortisol) and discuss possible explanations to the observed changes.

The survivors of the Utøya shootings were further subdivided into those with PTSD/PTSS symptoms and those who did not compared to a non-exposed control group. Salivary cortisol was collected by home assessments, and before and after a fMRI scanning sequence The sampling occurred over a three day period to assess a normal circadian cortisol rhythm, and particularly the cortisol awakening response was of interest. This phenomenon occurs right after awakening. Approximately 15 to 30 minutes after awakening the cortisol levels increase.

The Utøya subjects with PTSD/PTSS showed a lower overall cortisol output than the two other groups, especially with respect to the cortisol increase (AUCi).
The results provide evidence of alterations in the HPA axis in the subjects who developed PTSD/PTSS after the Utøya shootings, and provide increased understanding of some of the biological markers of PTSD.

Key words: Cortisol, HPA-axis, cortisol awakening response, Posttraumatic stress disorder, trauma

Acknowledgements

I would like to thank my main supervisor, Robert Murison. The door to your office was always open whenever I ran into problems. With many cups of coffee, you took the time to give me feedback and discuss questions. Your useful comments, remarks, ideas and engagement inspired me. You have supported and challenged me through the learning process of this master thesis, and I want to thank you for giving me more knowledge on this topic.

To my co-supervisor Anne Marita Milde, thank you for the opportunity to be a part of this research, and for the feedback on my thesis and steering me in the right direction I have learned allot.

Finally, I want to thank my family and my boyfriend Christ-Ove for the tremendous support and believe in me, and continuous encouragement throughout my years of study and through the process of writing this thesis. This accomplishment would not have been possible without them. Thank you.

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Introduction

General introduction

The exposure to extreme traumatic stress could have devastating consequences for the individuals exposed, and some may develop posttraumatic stress disorder (PTSD). Although the majority of people will experience a traumatic episode in their life, only a minority of those will develop PTSD or other mental health problems like depressive or anxiety disorders. This indicates that there is great individual variation in the emotional and neurobiological components in response to extreme traumatic stress. This also indicates that the risk of developing PTSD cannot be identified solely by the stressor, but entails a specific phenotype unable to recover in the aftermath of an extreme traumatic stress experience. The exposure of acute traumatic stress and the consequences this might have should focus on pre and posttraumatic risk factors (Olff, Langeland & Gersons, 2005; Yehuda & LeDoux, 2007).

Posttraumatic stress disorder

People undergoing intense trauma resulting from life threatening situations or physical injuries may respond to the traumatic event with intensive fear, horror or helplessness, and as a result, the development of a psychiatric condition called Post-traumatic stress disorder (PTSD). The distress in this condition is maintained by the constant reliving of the traumatic experience by invasive flashback memories (Yehuda, McFarlane & Shalev, 1998). Often these invasive memories are triggered by cues in the environment that resemble the traumatic event. Eventually the individual may develop a pattern of avoidance of situations that trigger these invasive memories (Zoladz & Diamond, 2013).

The abnormal behavioral and physiological symptoms which often occur with PTSD are: a higher autonomic arousal, exaggerated startle response, lower baseline levels of cortisol, and cognitive impairments. The diagnosis is based on three clusters: (1) re-experience, including nightmares, (2) avoidance including a numbing of general responsiveness, and (3) increased

arousal, which may include disturbances of sleep and concentration. These symptoms need to be present for one month after the traumatic event and are associated with distress and impairment of daily life functioning. If not all these criteria are met, but still some clusters are filled the description of posttraumatic stress symptoms (PTSS) is used (American Psychiatric Association, 2013; Miller, Chen, & Zhou, 2007; Yehuda et al., 1998)

The Neuroendocrinology of Stress

*HPA axis.*The HPA axis is an important hormonal response system which is present in a wide variety of organisms and is activated by internal and external signals, like mental and physical stressors. The hypothalamus controls the secretion of hypophysiotrophic neurons where the neurons synthesize and secrete corticotrophin releasing hormone (CRH), and arginine-vasopressin (AVP). CRH proceeds through the hypophyseal portal circulation to the anterior pituitary gland. The result is the secretion of adrenocorticotropic hormone (ACTH), which travels through the systemic circulation until binding in the adrenal cortex, where glucocorticoids (GCs) are released (mostly cortisol in humans) (Herman, Ostrander, Mueller & Figueiredo, 2005). Cortisol is a glucocorticoid hormone released by the adrenal glands, and is the end product of the HPA axis.

Cortisol. Cortisol plays an important role in physiological functions of the human body, such as in the central nervous system, processes involving learning, memory and emotions, as well as the metabolic system, influencing glucose storage and usage, and mediating the rapid mobilization of amino acids and fat from cells, making them accessible for the use of energy, This function enables the body's managing ability to respond to different stressors both physiological and psychological (Herman et al., 2005).

Cortisol also has an important effect on the immune system, and is the most potent anti-inflammatory hormone in the body. Glucocorticoids control and act on the immune system by both suppressing and stimulating the inflammatory responses and the growth of lymphocytes.

This happens during all forms of stress like infections, physical trauma, and psychological stress, which results in the activation of the immune system. Cortisol has profound inhibitory effects on the components of the immune/ inflammatory system by restraining the inflammatory reaction and preventing tissue damage. The development of stress related alternations of the HPA axis such as in PTSD may have adverse health outcomes, which could result in increased inflammatory responses, because PTSD individuals are thought to have lower cortisol levels. Evidence to this is supported by a study that measured elevated levels of pro-inflammatory cytokine in PTSD patients. Over a longer time this could have adverse health effects (Fries, Hesse, Hellhammer & Hellhammer, 2005; Levine, Zagoory-Sharon, Feldman, Lewis & Weller, 2007; Rohleder, Joksimovic, Wolf, & Kirschbaum, 2004; Tsigos & Chrousos, 2002).

There is a significant circadian rhythm in glucocorticoid secretion in response to pulsatile trophic hormone stimulation, with higher peaks during the active phase of the diurnal rhythm, which usually means higher levels of cortisol in the morning 15-30 min after awakening, and lower in the evening. The pulses of CHR and ACTH vary in magnitude during the day. There are indications that the pulsatile and circadian secretory mechanisms can be regulated independently. Stress and the circadian rhythm are closely connected with HPA axis activity. There can be some variations based on changes in light, feeding patterns, and activity. It has long been understood that different cortisol levels can have adverse medical outcomes. There are however different results and inconsistencies in the literature (Clow, Hucklebridge, Stalder, Evans & Thorn, 2010; Miller et al., 2007; Levine et al., 2007; Tsigos & Chrousos, 2002).

*Glucocorticoid negative feedback.*To stop the glucocorticoid release there are mechanisms in place which reduce the degree and the length of the glucocorticoid release. This is called the glucocorticoid negative feedback where the released glucocorticoids inhibit the further release

of ACTH. There seem to be at least two ways to regulate the negative feedback mechanisms: fast feedback and delayed feedback. The fast feedback mechanism seems to be sensitive to the amount of glucocorticoids, and is most likely non-genomic, which means that it does not directly influence gene expression. The other method is the delayed feedback mechanism which is sensitive to the levels of glucocorticoids, and appears to be genomic. At present there are two known glucocorticoid receptors in the brain; the glucocorticoid receptor (GR) and the mineralocorticoid receptor (MR). The GR is only bound during high glucocorticoid secretion like during the peak of the circadian rhythm and during stress. The MR has a higher binding rate than the GR also during the basal glucocorticoid secretions. An important brain area located within the temporal lobe is the hippocampus. Because of its plentitude of GR and MR it seems to play a crucial role in the negative feedback mechanism of the HPA axis (Herman et al., 2005; McEwen et al., 1992).

Biological markers in a highly traumatized group

During the aftermath of a traumatic experience, some individuals may develop psychological disorders like PTSD, major depression or anxiety. However not all trauma exposed individuals will develop disorders. The question remains why some trauma-exposed individuals are resilient and some are vulnerable when the same amount of trauma is received. Therefore researchers are trying to identify the individual basal biological markers and the difference between those who develop disorders and those who do not (Yehuda & LeDoux, 2007).

One of those biological markers that has received great attention is cortisol. During the mid and late nineties, the focus shifted from the pathological consequences that high cortisol levels have, to the pathological effects low levels of cortisol may have. This change of focus was a result of studies with individuals suffering from PTSD, which showed lower basal

cortisol levels compared to healthy controls, or individuals who did not develop psychopathological disorders (Doom & Gunnar, 2013; Miller et al., 2007; Yehuda, 2005).

There are however some controversies, and some PTSD studies fail to show lower cortisol levels. A study conducted by Lemieux and Coe (1995) found elevated cortisol levels in women with PTSD who had a history of childhood sexual abuse. A more recent systematic review and meta-analysis studied the basal cortisol levels among adults with PTSD and a control group. Thirty-seven studies were included. Combining all the available data, they found no difference in basal cortisol levels between the PTSD group and the controls. However, subgroup analyses showed that studies using plasma or serum showed significantly lower cortisol levels in those with PTSD, specifically in female only studies (Meewisse, Reitsma, de Vries, Gersons, & Olff, 2007). These different findings can be the result of different methodological designs, developmental factors, timing, difference in type of trauma and gender.

An interesting longitudinal study tried to address the developmental course of basal cortisol levels, at six time points: during childhood, through adolescence and young adulthood. This was a female only study where 84 subjects who had experienced substantiated familial sexual abuse were included, and 102 women served as a healthy control group. They found on average the normative developmental course of the HPA axis, where there is a steady increase from middle childhood into early adulthood for non-stress cortisol levels, followed by a leveling off. The females with a history of sexual abuse showed higher levels of cortisol in childhood and lower cortisol levels by early adulthood. These results may indicate that early and traumatic stress leads to a higher stress response in the beginning, which is attenuated as time goes by (Trickett, Noll, Susman, Shenk & Putnam, 2010)

In neurobiological stress research, the low dose dexamethasone suppression test (DST) is often used. This means that the subject takes a sample of 0.5 mg dexamethasone at 11 p.m. on

the evening before the test day. This causes a downregulation of the HPA axis because of feedback inhibition, which causes a moderate suppression of the HPA axis. By using this method researchers are able to look at the different results of normal or heightened suppression or non-suppression. This means that the HPA axis undergoes a pharmacological challenge (Klaassens, Giltay, Cuijpers, van Veen & Zitman, 2012; Meewisse et al., 2007).

An interesting meta-analysis looked at the HPA axis functioning in adults who experienced trauma in healthy subjects and PTSD patients. They assessed two meta-analyses that compared mentally healthy trauma exposed individuals to individuals who were not exposed to trauma, plus PTSD patients. The results showed that there was not a significant difference in cortisol values between the trauma-exposed versus the non-exposed individuals, neither in the PTSD versus the non-exposed. Although subgroup analysis showed that the trauma-exposed showed an increased cortisol suppression after the DST compared to the non-exposed, the authors concluded that PTSD and/or trauma exposure on the whole were not related to HPA axis alterations (Klaassens et al., 2012).

The cortisol awakening response (CAR)

In addition to the circadian rhythm of cortisol secretion, there is a rapid increase of cortisol within proximally 20 to 30 min after awakening in the morning hours. This phenomenon is called the cortisol awakening response (CAR). This is different from the diurnal HPA axis activity, but can be seen as a supplementary process linked to awakening, unrelated to the further cortisol release during the rest of the day (Wilhelm et al., 2007). One of the important structures regulating the circadian rhythms of physiological body systems is the suprachiasmatic nucleus (SCN) also known as the endogenous biological clock. The SCN is also sensitive to light signals by the retinal ganglion cells. In addition, the SCN also regulates HPA axis via input to the hypothalamus. The change in cortisol results from a difference in the fast ultradian pattern: pulses that occur around once every hour. The difference of the

amount of cortisol but not the frequency, by which the pulses occur, makes the difference of the day/night cycle of cortisol concentrations (Clow et al., 2010).

Another important brain area that seems to have an influence on the CAR is the hippocampus. It not only plays an inhibiting role on the HPA axis, but also an activating one. This has been shown in clinical studies with bilateral and unilateral hippocampus damage which showed an absence of CAR (Buchanan, Kern, Allen, Tranel & Kirschbaum, 2004), and that a larger hippocampal volume is associated with a greater CAR (Pruessner, Pruessner, Hellhammer, Pike & Lupien, 2007). These results indicate that the hippocampus has an influence on the regulation of the CAR (Clow et al., 2010).

The CAR is a phenomenon that has been extensively studied in recent decades, since it is a stable biological marker of the acute activity of the HPA axis both in the healthy population but also in those who have physical or psychological disorders. The exact function of the CAR is still not entirely certain but Fries, Dettenborn and Kirschbaum (2009) speculate that the CAR is associated with anticipation of the upcoming day.

Altering factors of the CAR

The HPA axis is an eminently flexible system with great variability. Several factors are known to influence the CAR. Depending on the research question these altering factors are confounders or of interest. Some of those are constant factors: like age, gender, genetic factors, and female reproductive factors like menstrual cycle, or oral contraceptive usage, health conditions both physical and psychological. Variable factors include: mood, food intake, smoking, sleep related factors, and stress exposure.

With regard to gender, some studies have found that the menstrual cycle in pre, peri and postmenopausal woman shows different CAR levels compared to men (Pruessner et al., 1997; Wright & Steptoe, 2005).There is a peak in cortisol levels in both male and females, but females show a slower decrease of cortisol levels compared to men (Kudielka, Gierens,

Hellhammer, Wüst & Schlotz, 2012). However, other studies have not replicated the sex difference of the CAR. A large prospective population study conducted by Bouma, Riese, Ormel, Verhulst and Oldehinkel, (2009) used adolescents in the age of 15-17 years examine CAR. They did not found a significant effect of gender and menstrual phase but they did however find that girls who were using oral contraceptives (OC) showed a slightly blunted response. On the other hand, the studies that did report a difference in the CAR had quite small effect sizes (Pruessner et al., 1997). It can also be a matter of different study designs, when an applied stressor/challenge is used. For example The Trier Social Stress Test (TSST) is a standardized laboratory stress comprising a preparation period (3 minutes), a free speech task (5 minutes), and a mental arithmetic task (5 minutes) in front of an audience. This is a different method in regard to the CAR and "resting" cortisol levels, as it is thought that a stress challenge will increase cortisol levels. This task also contains factors that include uncontrollable and social-evaluative aspects (Kudielka, Hellhammer & Wüst, 2009). The study of Bouma et al. (2009) thus also contained an applied stressor in addition to the CAR measurements. They found that boys and girls who were not using any OC showed a different result. Whereas boys showed a strong cortisol response, the girls using OC showed no response at all to the challenge. It must be said, however, that when reporting sex differences, the differences that were found are quite small.

With regard to age, the reported effects have been inconclusive. One study did not find any age effects concerning the CAR (Pruessner et al., 1997). An interesting longitudinal study with elderly healthy subjects showed that there was a great variability in the subgroups, with increasing levels of cortisol in one group, decreasing levels in another group, and stable levels in another group. Age however was not related to the cortisol levels or the pattern of change over the years (Lupien et al., 1996). One study showed a lower CAR with increasing age

(Kudielka & Kirschbaum, 2003). These results show clearly little consensus, which could be due to the sample characteristics and the method of analysis.

It is interesting to question whether the CAR is also dependent on the time of awaking, but studies have shown inconsistent results. Some studies did not find an association between awaking time and the CAR (Pruessner et al., 1997), whereas others did find an association between the time of awakening and the CAR levels, where earlier awakening was associated with a higher CAR (Federenko et al., 2004). They investigated shift-working nurses and students. The early shift showed a profoundly higher CAR compared to the other shifts and the students. In addition, students were studied after taking a short nap (one to two hours) during two weekdays. The results showed no CAR after the short nap, implying that a longer period of night-time sleep is needed to induce CAR. Similar results were found by Kudielka and Kirschbaum, (2003) with higher CAR in subjects waking up early compared to subjects waking up later. The majority of studies report that early awakening is associated with a higher CAR, but with some contrary results.

Not many studies have explored the body posture in relation to the CAR or "resting" cortisol states. There are however two studies with contradicting results regarding different body postures. The study that did find an influence used healthy volunteers and subjected them to three different body positions (sitting, lying, and an upright position) for a 20 minute time interval in each condition (Hennig et al., 2000). The results showed a decrease in cortisol concentrations for the sitting and lying conditions, and an increase for the upright position. In this study they used resting state cortisol values and not the CAR. Another study (Hucklebridge, Mellins, Evans & Clow, 2002) did not find the same results. In addition to the resting state cortisol values, they included the CAR in their study, and how this could be influenced by standing shortly after awakening or remaining supine during the response study period. They did not find a difference in cortisol values in accordance to postural change in

both conditions. This is an interesting topic since different body postures in accordance to HPA axis activity could be an unexpected factor.

Other factors know to influence the CAR are socioeconomic status, where low status seemingly is associated with a higher CAR, the use of medications, food consumption, normal meals but also distinct foods types like licorice , caffeine and alcohol although the influences on CAR seem low. Also extensive physical exercise (especially at high intensity), sleep wake factors (in particular shift work), and smoking although it goes in both directions, since acute nicotine consumption is associated with HPA axis stimulation. However, basal HPA axis activity is only marginally changed in habitual smoking (Kudielka et al., 2012; Kudielka et al., 2009; Pruessner et al., 1997).

Hypocortisolism

Studies not only of PTSD but also of other stress-related disorders have reported low cortisol levels, for instance chronic fatigue syndrome, fibromyalgia, rheumatoid, arthritis, atypical depression (Fries et al., 2005; Gold & Chrousos, 2002; Heim, Ehlert & Hellhammer, 2000). There are several factors that could cause hypocortisolism; (1) lower release at different levels of the HPA axis: CRF from the hypothalamus, ACTH from the pituitary, and cortisol from the adrenal glands. This could also be just temporarily during the circadian cycle, (2) a lower adrenocortical reactivity from cortisol to the target cells, (3) a heightened negative feedback of the HPA axis, or (4) morphological changes (Heim et al., 2000).

With regard to reduced release at different levels of the HPA axis, one possible explanation might be reduced hormone availability from the HPA axis. If there is a reduction of cortisol, it could be due to a decreased biosynthesis of the hormone, or a dysfunctional adrenal gland a decrease in the reactivity of the glands. The insufficiency could be due to failures of the adrenals or because of higher order HPA axis dysfunctions, but the exact evidence for this is still uncertain (Fries et al., 2005; Heim et al., 2000).

With regard to increased negative feedback, hypocortisolism and increased negative feedback mechanisms could be associated with one another. Animal studies have shown that monkeys with repeated shock avoidance sessions did not develop hypocortisolism because of an adrenal depletion, but because of suppressive mechanisms. The same findings have been found in PTSD patients, including the findings of increased GR binding in lymphocytes making the assumption of an increased negative feedback. This model however, also needs more research since the findings are not consistent (Heim et al., 2000).

Hypocortisolism may well be the result of morphological changes of the HPA axis. This may be caused either by a predisposition or because of the corticosteroids, but information about the adrenal glands is quite sparse in clinical studies. However, there is evidence from animal studies that the adrenal glands increase in size with chronic stimulation, suggesting that hypocortisolism can be associated with smaller adrenal glands (Heim et al., 2000). In PTSD patients there is additional evidence of hippocampal changes, where the PTSD patients show smaller hippocampal volumes as compared to healthy controls. The hippocampus is as mentioned earlier an important brain area in relation to the negative feedback mechanisms of the HPA axis. This is due to the large amount of GR and MR receptors, and the evidence that glucocorticoids cause alterations in the hippocampus comes from many animal studies where the glucocorticoids cause damage to neurons in the hippocampus (McEwen et al., 1992). In addition, several magnetic resonance imaging (MRI) studies in patients with PTSD have shown this effect, where the PTSD patients show smaller hippocampi than healthy individuals. These results are however not a consistent finding and some studies fail to see a difference in hippocampus volumes (Bremner, 1999; Doom & Gunnar,2013; Frodl & O'Keane, 2013; McNally, 2003). The question remains however, whether glucocorticoids causes hippocampal changes in PTSD individuals, or if a smaller hippocampus is predisposing, making the individuals more vulnerable when experiencing a traumatic event.

The groundbreaking study done by Gilbertson et al. (2002) has shown some evidence for a predisposing role of hippocampal volume. They measured the hippocampal volume in a series of monozygotic twin pairs differently exposed to trauma. The trauma exposure was combat in the Vietnam War. The individuals who developed PTSD showed smaller hippocampal volume than the combat exposed group who did not develop PTSD. In addition, the non-exposed brothers of those who developed PTSD had also smaller hippocampal volume than combat-exposed who did not develop PTSD. These findings provides evidence that the predisposition of having smaller hippocampal volume could predict a greater vulnerability to develop PTSD when exposed to trauma (Gilbertson et al., 2002).

Sampling methods

To get a valid measure of the CAR one needs accurate saliva samples from throughout the post-awakening period. The assessment of cortisol data from saliva requires that the participants take saliva samples at distinct time points, starting immediately after the awakening, followed by repeated measures with a 10 or 15 minute intervals for up to 30 to 60 minutes (Stalder et al., 2016).

Inaccuracies in Sampling. Missing data points are a great problem in assessment protocols especially when subjects are collecting saliva samples at home. Inaccurate sampling times could result in biased cortisol data. The cortisol increase could be missed in the collected samples if a subject does not start sampling immediately after awakening, perhaps because of sleepiness or the inability to follow instructions, with the consequence that the other samples also become postponed or too early. The timing of the first awakening sample is critically important for the first hour (Kudielka et al., 2012). A failure to correctly report the first awakening sample or the delay can have a great impact on the CAR data. This has been shown in a study by Griefahn and Robens (2011) using a sample of 510 CARs. The time of awakening and of the saliva collection were verified by the use of polysomnography or

actimetry. Delays of 15 minutes or more showed a significantly aberrant CAR as compared to the subjects with correct measurements. Smaller sampling delays (<15 minutes) are thought to be acceptable but a study conducted by Smyth, Clow, Thorn, Hucklebridge and Evans (2013) showed that moderate delays between 5 and 15 minutes result in an over-estimation of CAR magnitude and earlier CAR peaks. Thus, an absent CAR or even a negative CAR with the first awakening sample being extremely high as compared to the rest of the CAR could be due to sampling errors as opposed to HPA axis dysfunction.

The repeated salivary cortisol assessments can be accumulated to a summary index to give an estimate of the subjects' ultradian and circadian changes of cortisol secretion, and to determine the overall cortisol output over a certain time period. An often used method is the area under the curve (AUC). There are two formulas presented by Pruessner, Kirschbaum, Meinlschmid, and Hellhammer (2003) that can be used to calculate AUC: the area under the curve with respect to increase (AUCi) and area under the curve with respect to ground (AUCg). In CAR research there is often a large amount of data because of the repeated measurements, and the use of these two measures of AUC decreases the large amount of data, which makes it easier to handle. In addition, it limits the number of statistical comparisons between groups. The AUCi gives more of a reflection about the sensitivity to the HPA axis and the cortisol changes over time, whereas the AUCg gives the reflection of the total cortisol secretion (Pruessner et al., 2003).

Comorbid disorders

A high percentage of individuals with PTSD have comorbid disorders. These include major depressive disorder (MDD), substance abuse, and anxiety which are significant confounding influences in studies, especially in relation to neuroendocrine abnormalities (Breslau, Davis, Peterson & Schultz, 2000). Within the PTSD paradigm, having a comorbid diagnosis of MDD may have profound effects on the circadian cortisol levels and the CAR in particular. This is

because one of the most robust findings in MDD is a hyperactivity of the HPA axis and increased levels of cortisol (Pariante & Lightman, 2008). A reduced negative feedback sensitivity to glucocorticoids seems to be at least partly the cause of increased cortisol levels This may cause difficulties in analyzing the results in PTSD subjects with MDD comorbidity, because of the complete opposite findings in PTSD.

In addition to MDD, anxiety disorders are also a common comorbidity. The HPA axis in current anxiety with agoraphobia also shows a higher CAR. Other anxiety phobias on the other hand, like panic disorder without agoraphobia or social phobia do not show different cortisol levels compared to healthy control subjects (Vreeburg et al., 2013). Although the neuroendocrine profile is different in MDD compared to in PTSD, there have been found lower cortisol levels in MDD as well, showing the complexity of this system (Dedovic & Ngiam, 2015). A meta-analysis has addressed this problem by looking at PTSD patients with and without MDD. The results showed that the daily cortisol output was lower in PTSD and PTSD + MDD subjects, as well as in the CAR and after the post-dexamethasone test. In the afternoon cortisol levels, PTSD + MDD patients showed higher cortisol levels compared to only PTSD patients and healthy controls. This meta-analysis suggests that MDD could influence HPA axis functioning in PTSD patients distinctly in the evening hours. In addition to this finding, they revealed that trauma exposure alone was linked to lower afternoon cortisol and post-dexamethasone levels. This would suggest that exposure to trauma alone also could alter HPA axis functioning and is not a specific PTSD marker (Morris, Compas & Garber, 2012).

Cortisol awakening response in PTSD/PTSS

Alteration of the HPA axis is seen in a number of physical and psychological disorders, and the reason may be that there is a bi-directional causal relationship between these disorders and HPA axis function (Kudielka et al., 2012).

Studies that have investigated the CAR in relation to PTSD/PTSS are sparse, but the few studies that have been conducted found a reduced CAR, but with some inconsistencies regarding the results. A study by Wessa, Rohleder, Kirschbaum and Flor (2006) examined the CAR in PTSD subjects, trauma-exposed subjects, and non-exposed subjects. They measured the saliva cortisol levels at eight different time points until 8 p.m. In addition, to self-reports of awakening they also used an electronic monitoring device (MEMS Track Cap) to control for accurate sampling. The results showed that there was no significant difference between the three groups on the first awakening sample, but the cortisol increase after awakening 30 - 60 minutes later was significantly blunted in PTSD patients compared to the other 2 groups. A negative correlation between overall cortisol secretion (AUCg) and PTSD symptoms was found (Wessa et al., 2006). In addition to these findings, another study also investigated whether hypocortisolism was shown among Bosnian war refugees with PTSD compared to healthy controls. Similar to the study by Wessa et al. (2006), PTSD patients showed significantly blunted cortisol levels after awakening as compared to controls (Rohleder et al., 2004).

The CAR has also been investigated in relation to PTSD severity in intimate partner violence in sheltered battered woman. Interestingly, the results showed that the intimate partner violence PTSD and abuse chronicity have contrary effects on the CAR. Whereas PTSD severity was correlated with a significant greater cortisol output during the first hour after awakening, abuse chronicity on the other hand was correlated with a lower cortisol output

during the first hour after awakening. This indicates that longer period of abuse may cause a more flattened CAR compared to more recent abuse PTSD symptoms. These findings provide some evidence that there is a difference between HPA axis functioning in chronic stress and the onset of stress in addition to PTSD symptoms (Johnson, Delahanty & Pinna, 2008; Miller et al., 2008).

The study of police officers gives a unique opportunity in PTSD/CAR research, since it is a homogeneous group with continual risk factors like being injured, witnessing others getting injured or, even more drastically, witnessing death. They thus experience a great deal of traumatic stress exposure, making them an interesting research target group in relation to basal cortisol levels and PTSD symptoms, since they experience acute and chronic stress on a daily basis. Neylan and colleagues, (2005) used police officers in their study, with the use of a pre- and post-dexamethasone challenge, and measurements of peritraumatic emotional distress (negative emotions and arousal when the trauma took place, including panic attacks), peritraumatic dissociation, duty related trauma and PTSD symptoms. They found that higher PTSD symptoms, peritraumatic distress, and peritraumatic dissociation were correlated with lower CAR levels. Older age was also correlated with lower CAR levels, although there were no significant differences in the post-dexamethasone challenge (Neylan et al., 2005).

Although there have been findings of a flattened CAR in PTSD subjects, not all studies report this. A study that did not find any differences in CAR and PTSD symptoms used a group of highly traumatized males from Rwanda with and without PTSD. The subjects were refugees who fled at the time of the Rwandan genocide in 1994 to a refugee camp Nakivale in southwestern Uganda. They were unable to find a significant result in the CAR compared with males with and without PTSD. One explanation could be the disadvantaged living conditions in the refugee camp in this study, with poorer hygienic, nourishment and water resources. The subjects in the studies that did report lower CAR levels were living under quite

stable conditions (Johnson et al.,2008; Neylan et al., 2005; Rohleder et al., 2004) as compared to the males in the present study. The fact that it was an only male study gives more emphasis to gender differences in CAR and PTSD research (Eckart et al., 2009).

Utøya

The 22nd of July 2011, Norway was the victim of two terrorist attacks, committed by one and the same perpetrator. The first terrorist attack was a bombing in central Oslo at the government building, and the second one the shooting on Utøya (an Island outside Oslo), a summer camp for the Norwegian Labor Party`s youth organization. During the shooting, 564 people were located on Utøya, ages ranging from 13 to 57. Sixty-nine people were killed by the terrorist, and several were seriously injured. Some of the factors make the attack on the Labor Party's youth organization even more devastating. Firstly, the terrorist, in his inhumane intention to kill them all, shot the victims multiple times. Secondly, he chased the young people around on Utøya for over 90 minutes, with limited possibilities for the victims to escape or hide. The only escape route was swimming across the cold lake to the other side, at the risk of drowning, making Utøya into a virtual trap. Some sought cover inside the buildings, or behind trees and rocks. Many of the adolescents knew each other, and thus a great many of the survivors lost friends or loved ones, as well as witnessing traumatic sights, sounds and experiencing life threatening situations. Adding to it all was the fact that the terrorist was dressed in a police uniform, leaving the survivors uncertain of whom to trust when the real police officers arrived on Utøya. The survivors were first taken to a hotel on the mainland for medical examination and crisis intervention before they returned to their homes, and an intervention system for the affected families was carried out by the healthcare services (Dyb et al., 2014; Hafstad, Dyb, Jensen, Steinberg & Pynoos, 2014).

The survivors of the Utøya shootings had approximately the same amount of exposure to this life threatening situation, which makes it a relevant source of study seeking to explore the

developing of or the resilience to psychological disorders, specifically post-traumatic stress disorder (PTSD), since the group is so homogenous (Dyb et al., 2014).

There have been a few studies that have examined mental health problems in victims after terrorist attacks. In connection with a terrorist attack in Beslan in Russia in 2004, 58 school children were examined 3 years after the attack. Fifty percent of the children who were inside the school during the terrorist attack met criteria for PTSD (Scrimin et al., 2011). At a high school shooting in Finland where a student shot 8 people and himself, a 4 month follow up study revealed that in almost half of the exposed students posttraumatic distress was observed. Especially gender and the amount of exposure were factors that predicted PTSD; 27% in females and 7 % in males (Suomalainen, Haravuori, Berg, Kiviruusa, & Marttunen, 2011). These studies concentrated mainly on the psychological consequences and did not include cortisol measurements of the survivors. Measuring cortisol levels of the victims might have provided more information about the biological markers associated with the different psychopathological consequences of a terrorist attack.

There is however a study of cortisol after the 9/11 terror attacks, not of survivors but of bereaved children. They used a salivary baseline cortisol measurement and a salivary dexamethasone suppression test for HPA axis function during a time frame of 2 years. The results showed that morning and afternoon baseline cortisol levels were significantly higher for the bereaved compared to the non-bereaved children. Compared with bereaved children without psychopathological disorders, those with PTSD had significantly lower afternoon baseline cortisol levels (Pfeffer, Altemus, Heo & Jiang, 2007).

The present project

In the following project, cortisol is measured by collecting saliva. This collection procedure provides biologically active cortisol, which means that it is unbound to carrier proteins (Yehuda, 2005). The aim was to collect a circadian cortisol cycle in the highly traumatized youth and adolescents who had survived the Utøya shootings, compared to a non-exposed control group matched on age, gender and political engagement. The cortisol measurements were done at home at 5 different time periods on a regular day, before they went to bed on previous day, at awakening, 15 min after the previous test, and 15 minutes after that again. The last sample was collected before they went to bed. They also participated in a test day which included neuropsychological testing and fMRI scanning. Thus in addition to the regular cortisol testing at home, on the test day they delivered saliva samples just prior to the fMRI scanning, and before the neuropsychological testing right after the fMRI scan. The intention was to asses a stress reactivity response as a result of being in the scanner for approximately 1 hour. The first aim is to see whether there is a difference in cortisol levels between Utøya victims and the control group. Later on we examine whether the symptoms of PTSD/ and post-traumatic stress syndromes (PTSS) can be identified in the Utøya group and if there is a significant difference in cortisol levels compared to Utøya subjects without PTSD/PTSS. In addition to the regular CAR profiles, we also calculated the AUCg and AUCi by the formulas of Pruessner et al., (2003) to get an estimate over the subjects overall cortisol secretion in relation to increase and ground, to see if there is a difference between the Utøya group and the control group, in addition to between Utøya survivors with and without PTSD/PTSS.

There are several theories that PTSD is associated with biological vulnerability factors, already present prior to the onset of symptoms rather than consequences of trauma exposure (Heim & Nemeroff, 2009). Since not everybody exposed to trauma will develop PTSD, there

needs to be some form of vulnerability factors such as; certain genetic variabilities, and the insufficient glucocorticoid-signaling pathway (van Zuiden et al., 2013). Low circulating cortisol levels measured shortly after the traumatic event have been found (Delahanty, Raimonde & Spoonster, 2000; Delahanty, Raimonde, Spoonster & Cullado, 2003; Schelling et al., 2006; Yehuda et al., 1998). However, opposite results are found in children they show elevated cortisol levels shortly after the traumatic experience (Carrion et al., 2002; Delahanty, Nugent, Christopher & Walsh, 2005). In addition, alterations in the number of GR and the strength of sensitivity have been reported. Whether this is a counterbalance in response to low cortisol levels, or some other alterations is not clear. However, the observation of a higher number of GR prior to military deployment provides evidence for an elevated negative feedback mechanism (van Zuiden et al.,2011).

Several studies have compared cortisol levels between subjects with and without PTSD. The presence of PTSD is associated with lower cortisol levels during the entire circadian rhythm shown in several meta-analyses (Meewisse et al., 2007; Morris et al., 2012). There are findings that exposure to trauma alone can cause lower cortisol levels in subjects without PTSD (de Kloet et al., 2007; Klaassens et al., 2012). However, a reduced CAR is particularly associated with the presence of PTSD (de Kloet et al., 2007; Morris et al., 2012)

Measurements of the CAR prior to PTSD symptoms before military deployment did not predict PTSD symptoms after return. Similar result were found in a prospective study of police officers, where a higher CAR was associated with higher peri-traumatic distress and symptoms of acute stress disorder post trauma exposure (Inslicht et al., 2011).

Several animal studies have further provided evidence that low (corticosterone) levels are associated with the development of PTSD related symptoms in rats (Cohen et al., 2006; Milde, Sundberg, Røseth & Murison, 2003), including alterations of the GRs (Whitaker, Farooq, Edwards & Gilpin, 2016). This further supports the increased sensitivity of the HPA

axis in PTSD. The developmental path of the stress system could provide further clarifications in the conflicting literature of biological markers in PTSD (Gunnar & Quevedo, 2007; Lupien, McEwen, Gunnar & Heim, 2009; Heim & Nemeroff, 2001).

Methods

The present study is part of a larger investigation evaluating the long-term effects of the 2011 Utøya attacks on the survivors' cognition and neural networks, in collaboration with the Resource Center of Violence, Traumatic stress and Suicide Prevention Western region (RVTS Vest). The overarching protocol includes the collection of cortisol data, brain-imaging (MRI and fMRI), and neuropsychological assessments from survivors of the 2011 Utøya attacks in the age range from 16 to 25. A total of 28 Utøya survivors were recruited in the study, and a control group of 35 subjects matched on age and gender were additionally recruited. Participants were recruited by RVTS Vest by sending written invitations through the post in February 2013. RVTS Vest holds a confidential register with the names and addresses of Utøya survivors in Rogaland and Hordaland. The recruitment of the control group was made by making contact with the head of different youth political parties in Hordaland in order to get permission for handing out letters to the members in the political youth parties. The intention here was to have a control group comprised of subjects with a similar level of political commitment and activity as the Utøya survivors, but from a different political party.

Inclusion/ Exclusion criteria

Inclusion criteria for the Utøya group (U) criteria comprised survivors of the Utøya massacre, both males and females, in the age range 17 to 24. Criteria for the Control group (C) comprised the same age range, males and females, and active involvement in a political party. Exclusion criteria for both groups included neurological illness or previous head trauma leading to more than 10 minutes of unconsciousness, endocrinological illness, metal implants

not compatible with MRI scanning, and pregnancy. Additional exclusion criteria for the control subjects (C) were current or previous history of psychiatric illness as determined by a short interview, substance abuse, personal involvement in the events at Utøya and knowing someone involved in those events.

Ethical approval

This project has been approved by the Regional Committee for Medical Research Ethics (Ref: 2012/1464) in accordance with Norwegian laws and regulations. During the collection of brain images the subjects were offered professional assistance if needed, providing an physician or clinical psychologist during participation and available at all times to the subjects. All data were anonymized and could not be traced back to individual participants.

Psychiatric Measures

All Utøya subjects completed the M.I.N.I. which is a commonly used psychiatric diagnostic interview instrument to assess psychiatric evaluation, and has established reliability and validity.

Saliva sampling

Subjects were instructed not to brush their teeth or to use toothpicks and to abstain from breakfast (at least 30 minutes) or a night snack (1 hour) before providing samples. High physical activity and smoking needed to be avoided before the sampling period. This instruction was given through detailed written information, which the subjects had with them at home. The Salivette tubes with the saliva samples were stored and numbered for the different time points (S1 to S11; see below). The date and time data were written on the tubes when the subjects were taking saliva samples. After the sampling at home the tubes were stored in a refrigerator for a maximum of 5 days before being delivered as soon as possible to the laboratory. After centrifugation, samples were analyzed for free cortisol levels using

enzyme-linked immunosorbent assay (ELISA) methodology. Cortisol levels are reported as nanomoles per liter (nmol/L).

Saliva samples were collected in Salivette tubes at home, and at the hospital before and after the fMRI session on day 3. Sampling took place over three days. On the first day, subjects were instructed to provide a sample just before bed (S1; between 9 p.m. and midnight). On the second day, subjects were instructed to collect saliva samples immediately (0 min) after awakening (S2) , 15 min after awakening (S3) and 15 min after the previous collecting (S4) (between approximately 7 to 9 am). These three morning samples were used to calculate the Cortisol Awakening Response (CAR) and Areas Under the Curve (AUCi and AUCg). A further evening sample was collected (S5). On the third day, the three morning samples (S6 to S8) and an evening sample (S11) were repeated. On this third day, subjects were also tested in the MR scanner. Here, the subjects underwent fMRI scanning and a neuropsychological test battery, and saliva samples were collected before and after the fMRI scanning (immediately before the neuropsychological testing).

fMRI protocol (data not included)

The MRI sequence consisted of two fMRI experiments, where the first one was a working memory protocol, using a modified version of 2-back working memory (WM) which consists of two conditions in a block design. Emotional and neutral images were used. The participants completed 5 blocks of emotional WM task, and each block consisted of 10 emotional trials. Between these blocks there was a neutral WM task consisting of 5 blocks of 10 trials. The trial was shown for 2 seconds with 500 ms inter-stimulus interval, the entire block was 24.5 seconds. The blocks were separated with 15 seconds of rest, where the subjects were shown a fixation cross on a black background on the screen. During the block designs, the subjects were instructed to press a key with their right index finger if the stimulus that was shown was

identical to the stimulus shown two trials before. Only one stimulus was shown at a time, in a sequential order. This MRI session lasted approximately 12 min.

The second fMRI experiment consisted of a resting state protocol with the same conditions as for the above protocol, the only difference being that there were not shown any stimuli. This protocol lasted about 5.5 minutes.

Statistical analyses

The experimental protocol consisted of a mixed design with independent factors (groups) and repeated measures (sample). The design covered 3 days of saliva sampling at home with 5 different sample points (S1 to S5), followed by a test day with 4 regular saliva samples (S6 to S8, and S11). These are designated as "resting samples" since they were unrelated to the fMRI session. In addition, two samples were taken in connection with the fMRI session, one sample before (S9) and one sample after (S10).

Statistical analyses were conducted using IBM SPSS statistics 23.

Groups were stratified according to Utøya victims (U), controls (C), and further to Utøya victims with and without PTSD/PTSS (U-1 and U-0, respectively). Data from 1 participant were not used in analyses as a result of difficulties identifying cortisol samples, making the total ($N = 62$). In addition, extreme exclusion criterion was set to ± 3 standard deviations from the mean. This led to the rejection of 5 samples. Cortisol levels were first analyzed for S1 to S8, plus S11 (i.e. excluding the samples immediately prior to and following the scanner session on day 3). Complete sample profiles were obtained from 11 Utøya survivors and 19 Controls. Cortisol "resting levels" were first analyzed at the nine different time points using a repeated measure analysis of variance (ANOVA), performed in IBM SPSS Statistics 23, comparing differences between the Utøya and Control groups. The second repeated measures ANOVA examined cortisol levels over the nine sample points, but with the Utøya group divided into those with and without PTSD/PTSS (U-1 and U-0, respectively).

Repeated measures ANOVA was also used to compare the Cortisol Awakening Response (CAR) on both days, the regular day (day 2), and the test day (day 3) between Utøya and Controls. CAR is defined here as the difference between the third and first samples on each of the two days (S4 minus S2 and S8 minus S6 respectively). The further analysis is of the CAR response on both days (2 and 3) comparing Controls with Utøya survivors with PTSD/PTSS (U-1) and Utøya survivors without symptoms (U-0). Repeated measures ANOVAs were also performed on the area under the curve (AUCg; Pruessner et al., 2003) calculated by multiplying the single cortisol sample levels by the time interval between the sampling points (S2, 3, 4) for day two and, (S6, 7, 8) for day three. This was done because AUCg gives a reflection of total cortisol output or the overall cortisol intensity. In addition to AUCg we also calculated the AUCi, which is more sensitive with respect to changes over time, between Controls and Utøya survivors, and between Controls and the two Utøya sub-groups (U-0 and U-1). The possible effect of gender is included as a covariate in the ANOVAs.

Finally, repeated measures ANOVA were used to analyze cortisol levels associated with the fMRI session (S9 and S10), with Utøya and Controls, and with Controls vs Utøya with and without symptoms.

Results

Comparison of Utøya survivors and Controls

Cortisol levels. Of the in total 30 participants used in analyses before averaging the samples, (19 controls, 11 Utøya) 6 met criteria for PTSD/PTSS in the Utøya group. A repeated measures ANOVA was conducted to assess the impact of being a survivor of the Utøya shootings (U) compared to a matched control group (C) on participants' levels of saliva cortisol across the 9 "resting state" samples. There was no significant group effect between U and C, $F(1, 28) = 0.070$, $p = .793$, partial eta squared = .002 (see Table 1 and Figure I). There was a main effect for samples, $F(8, 224) = 51.72$, $p = <.001$, partial eta squared = .649. There

was no significant group × sample interaction, F (8, 224) = 0 .36, p = .943, partial eta squared = .013. Levene's test showed no significant heterogeneity of variance.

SEE FIGURE 1 & TABLE I

Cortisol Awakening Reponses. Repeated measures ANOVA across the two days showed no main effect of either group, F(1, 39) = 0.205, p=.653, partial eta squared = .005, (see Table II) nor day, F(1, 39) = 2.490, p=.122, partial eta squared = .060. The group by day interaction was not significant, F(1, 39) = 1.525, p=.224, partial eta squared = .038 Levene's test showed no significant heterogeneity.

SEE TABLE II

AUCg. Analysis of AUCg across the two days (days 2 and 3) for the U and C groups yielded no significant group effect, F(1, 40) = 0.002, p=.964, partial eta squared = .000, (see Table III) no day effect, F(1, 40) = 0.000, p=.995, partial eta squared = .000, and no group by day interaction, F(1, 40) = 0.017, p=.896, partial eta squared = .000. Levene's test showed no significant heterogeneity.

SEE TABLE III

AUCi. Analysis of AUCi across the two days for the U and C groups yielded no group effect, F(1, 41) = 1.936, p=.172, partial eta squared = .045(see Table IV)
, no day effect, F(1, 41)=0.439, p=.511, partial eta squared = .010, and no group by day interaction, F(1, 41) = 1.444, p=.236, partial eta squared = .034. Levene's test showed no significant heterogeneity.

SEE TABLE IV

Pre and post fMRI cortisol levels. Analysis of cortisol levels prior to and following the fMRI session showed no group effect, F(1, 55) = 0.465, p=.498, partial eta squared = .008, but a significant sample effect, F(1, 55) = 23.751, p=.000, partial eta squared = .302, the interaction group by samples was not significant, F(1, 55) = 2.958, p=.091, partial eta squared =.051.(see

figure 2 and table V) Levene's test showed no significant heterogeneity. Both groups showed a fall in cortisol from before until after the fMRI session.

SEE FIGURE 2 & TABLE V

Comparison of Controls with Utøya survivors with (U-1) and without symptoms (U-0) of PTSD/PTSS,

Cortisol levels. Controls were compared with Utøya survivors without symptoms (U-0) and those with symptoms (U-1). This led to N of 19 for Controls, N of 5 for U-0 and N of 6 for U-1. Repeated measures ANOVA (Sample by Group) showed that the main group effect was not significant, F (2, 27) = 2.88, p =.073, partial eta squared = .176 (see figure 3 and table VI). There was a substantial main effect for samples, F (8, 216) = 46.51, p = < .001, partial eta squared = .63. There was a group by sample interaction, F (16, 216) = 2.47, p = .002, partial eta squared = .16. Levene's test showed no significant heterogeneity of variance.

SEE FIGURE 3 & Table VI

Cortisol Awakening Response. Repeated measures ANOVA on the two CAR's across the 3 groups showed no significant group effect, F (2, 38) = 1.60, p = .22, partial eta squared = .078 (see table VII), no sample effect, F (1, 38) = 2.93, p = 0.071, partial eta squared = .095, and no group by sample interaction, F (2, 38) = 1.25, p = .29, partial eta squared = .061. Levene's test showed no significant heterogeneity of variance.

SEE TABLE VII

AUCg: Repeated measures analysis of AUCg across the two days showed no significant group effect, F(2, 39) =1.945, p=.156, partial eta squared = .091,(see table VIII) no day effect, F(2, 39) = 0.001, p=.974, partial eta squared = .000, and no group by day interaction, F(1, 39) = 0.021, p=.979, partial eta squared = .001. Levene's test showed no significant heterogeneity of variance.

SEE TABLE VIII

AUCi. Repeated measures ANOVA of AUCi over the two days revealed a main group effect, F (2, 40) = 3.67, p = .035, partial eta squared = .155 (see table IX). There was no effect of day, F (1, 40) = .97, p = .33, partial eta squared =.024 and nVo significant group by day interaction, F (2, 40) = 2.12, p = .13, partial eta squared = .096. Post hoc Fishers LSD test show that Utøya survivors with PTSD/PTSS (U-1) had lower levels of AUCi than both Controls (p=.015) and survivors without symptoms (p=.028). Levene's test showed no significant heterogeneity of variance.

SEE TABLE IX

Pre and post fMRI cortisol levels. Repeated measure ANOVA of cortisol levels prior to and after the scanner session yielded no significant group effect, F(2, 54) = 0.266, p=.767 partial eta squared = .010 (see figure 4 and table X). The repeated measures term was significant, F(1, 54) = 15.076, p=.000, , partial eta squared = .218. The group by sample interaction was not significant, F(2, 54) = 1.739, p=.185, partial eta squared = .061. Levene's test showed no significant heterogeneity of variance. All three groups showed lower cortisol following the fMRI session than prior to the session.

SEE FIGURE 4 & Table X

To see if sex was a possible confounding factor the analyses were repeated with sex as a covariate. Sex had no significant impact on the reported results (data not shown).

Discussion

The aims of this study were to investigate the cortisol awakening response (CAR) and the cortisol circadian rhythm in a severely traumatized group of adolescents who survived the Utøya shootings in Norway the 22 July 2011. Of special interest was the development of PTSD/PTSS in this group, and whether they showed a different cortisol circadian rhythm and CAR, in comparison with a healthy control group, in addition to the individuals who did not develop PTSD/PTSS after the Utøya shootings. This study is the first one to record the CAR and cortisol circadian rhythm in a group that is so homogeneous. The results will be discussed in relation to previous studies.

When combining all the available cortisol data there was no difference in the overall cortisol levels between the Utøya group and the control group,in addition to, the CAR, AUCg, AUCi and before and after the fMRI session. The overall cortisol output represents the diurnal cortisol secretion with higher levels at around awakening and decreasing cortisol levels throughout the day to reach low cortisol levels in the evening. This basal HPA axis activity occurs through several secretory episodes of CHR and ACTH with short duration and high amplitude. Under non-stress situations this phenomenon occurs in a circadian fashion which

means approximately 24- hours in a pulsatile fashion. Under stressful situations and acute stress the secretory episodes increase both in amplitude and synchronization which result in increases of ACTH and cortisol secretion (Fries et al., 2009; Levine et al., 2007; Tsigos & Chrousos, 2002). Although the comparison between Utøya and the control group did not revealed any differences in the overall cortisol output, a subdivision of the Utøya group into Utøya with PTSD/PTSS (U-1), Utøya without PTSD/PTSS (U-0), and the control group revealed that PTSD/PTSS subjects showed a lower overall cortisol output compared to the control group, and Utøya without symptoms, especially 15- 30 minutes after awakening samples (S3, S4). Thus, a comparison between Utøya survivors and the control group did not reveal any changes to the overall cortisol output and circadian rhythmicity during the three days of sampling. Subgroup analyses showed however significantly lower levels of cortisol in the PTSD/PTSS subjects compared to controls and Utøya survivors without symptoms. This indicates that the trauma-exposure of being at Utøya is not responsible in itself for a dysregulation of cortisol levels but rather the development of PTSD/PTSS in the aftermath.

This is inconsistent with other findings where trauma exposure rather than PTSD produced differences in cortisol levels (Klaassens et al., 2012; Meewisse et al., 2007). In the meta-analysis conducted by Klaassens (2012), they only found significant results after subgroup analyses between PTSD, trauma- exposed, and non-exposed individuals in studies that used the low dose dexamethasone suppression test (DST). The dexamethasone strongly inhibits the cortisol awakening response because the synthetic glucocorticoids imitates negative feedback signals to the ACTH secreting cells of the pituitary. The main finding was that trauma-exposed individuals showed no difference in basal cortisol levels compared to non-exposed individuals, nor did PTSD patients compared to trauma- exposed individuals. They found an increased cortisol suppression after the DST test in the trauma-exposed subjects compared to

the non-exposed subjects, but they did not find a difference between the PTSD subjects and the trauma-exposed subjects.

One of the studies mentioned in Klaassens et al. (2012) showed that both trauma-exposed and PTSD patients revealed significantly more salivary cortisol suppression after the DST test compared to a healthy control group (the salivary cortisol sampling occurred at 1600h), using combat veterans with and without PTSD. In addition to salivary assessments at six time points, including the CAR, before and after the (dexamethasone suppression test 2300h), they also assessed plasma cortisol, ACTH and corticotrophin binding globulin (CBG) in response to dexamethasone in PTSD patients and trauma controls. The CAR was significantly different between PTSD patients compared to healthy control subjects where PTSD patients showed lower cortisol levels. Comparing trauma-exposed subjects to healthy controls, the trauma-exposed subjects revealed lower cortisol levels. There was no difference between PTSD patients and trauma-exposed controls. Plasma cortisol, ACTH and CBG did not show any significant differences between PTSD patients and trauma-exposed controls (de Kloet et al., 2007). The different results could be due to the use of dexamethasone, which disrupts the natural HPA axis responses In addition there was greater trauma variance in the meta-analyses; war veterans, sexual/physical abuse, refugees and others (Klaassens et al., 2012; Meewisse et al., 2007), while our group is highly homogenous in both age, and trauma type, something that might explain the divergent results.

The Cortisol awakening response in Utøya subjects

The cortisol awakening response (CAR) superimposed on the circadian cortisol pattern are secretory episodes. CAR is the change in cortisol levels from the time of awakening to approximately 30 minutes after awakening. First described by (Pruessner et al., 1997), it appears to be a reliable and easy measure for the acute sensitivity of the HPA axis. After awakening the free cortisol levels increase by approximately 50-75 % reaching a maximum

30 minutes after awakening. The process of awakening involves the activation of multiple cortical and subcortical brain regions, and it is thought that the CAR is actively involved with the process from sleep to consciousness (Clow et al., 2010; Fries et al., 2009).

A highly controlled laboratory study measured both plasma ACHT and serum/salivary cortisol levels pre and post awakening, during the 30 minutes post awakening plasma. ACTH and serum cortisol levels increased significantly steeper than two to five hours pre awakening. Participants were instructed to lie supine and in dark conditions for the first hour after awakening to control for the possible effect light can have on the CAR. The increasing levels 30 minutes post awakening indicates that the CAR is a phenomenon in part caused due to the processes of awakening (Wilhelm et al., 2007). The underlying mechanisms that regulate the CAR as a distinct phenomenon of the HPA axis is not entirely clear, as is whether the same known general HPA axis activity is the same or whether other regions play an important part activating the CAR. The suprachiasmatic nucleus, a structure in the hypothalamus (and known as the biological clock), is a possible structure that might act as an additional important input for the CAR. Another important brain structure is the hippocampus known for its inhibitory role of the HPA axis due to the large amount of glucocorticoid and mineralocorticoid receptors (Clow et al., 2010; Fries et al., 2009). However, there is evidence for a stimulatory effect on the CAR. The evidence comes from clinical studies with bilateral and unilateral damage to the hippocampus. The patients lacked the CAR, but the normal circadian rhythm was unaffected (Buchanan et al., 2004). In addition, a larger hippocampal volume was associated with a greater CAR (Pruessner et al., 2007), which demonstrates the importance of the hippocampus in relation to the CAR.

In order to understand alterations of the CAR a general understanding of the normal functioning is needed. Dysfunctions in the HPA axis activity are known for a wide variety of physical and mental health disorders (de Kloet, Joels & Holsboer, 2005; Heim et al., 2000).

Subtle changes of the CAR can indicate HPA axis alterations and the causal relationship with physical and mental health disorders. In the present study we were mainly interested on whether the Utøya subjects with PTSD/PTSS showed a different CAR compared to the Utøya subjects without PTSD/PTSS and a control group. The results showed that the CAR was not significantly different between the Utøya group and the control group, in addition to the subdivision of the three groups. This was in contrast to the overall cortisol output, especially on day two where sample 3 and 4 were significantly lower in the PTSD/PTSS group (samples 3 and 4 were the samples taken 15 and 30 minutes after awakening). Why the CAR did not show any significant results could be due to sampling errors. The saliva samples were collected at home which confers ecological validity, but lacks the control of the researchers. This could be problematic because the validity of CAR critically relies on participant's ability to correctly follow a planned sampling procedure.

Different results to ours were found in the study conducted by Wessa et al., (2006) where the PTSD subjects showed significantly lower CARs as compared to both healthy-controls and trauma-exposed controls. However, if we look at the overall cortisol output in our study we can see that PTSD/PTSS subjects showed a significant lower cortisol output 15 to 30 minutes after awakening. Taking in consideration that it was difficult to calculate the CAR in our study due to incomplete cortisol profiles, it might have had similar results to the study conducted by Wessa et al., (2006) if the cortisol profiles had been complete in all subjects.

The CAR is unrelated to cortisol secretory activity throughout the day (Clow et al., 2010; Fries et al., 2009). An altered CAR profile has been associated with a series of stress related psychopathology and general health conditions. Burnout and multiple bodily complaints in a group of nurses was associated with decreased basal salivary cortisol in the morning and relatively high cortisol levels in the afternoon and evening (Heim et al., 2000), something that not only indicates a disturbance of the CAR, but also of the circadian rhythm of cortisol

release . Like other studies (Johnson et al., 2008; Rohleder et al., 2004; Neylan., 2005; Wessa et al., 2006), a flattened overall cortisol output 15 and 30 minutes after awakening was observed in the PTSD/PTSS subjects.

Area under the curve with respect to ground (AUCg)

The area under the curve (AUC) is a method to quantify the repeated measurements of the cortisol. It is a method used to compute changes of the circadian and diurnal cortisol rhythm, and to determine the overall cortisol secretion over a specific time period, also called area under the curve with respect to ground (AUCg) computed by the trapezoid formula described by Pruessner et al., (2003). The AUCg is more related to the total hormonal output. In the present study; we were particularly interested in the overall cortisol secretion over the post-awakening period, in part due to the complexity identifying and calculating the evening samples. Our results therefore represent the total post awakening cortisol concentrations. The AUCg in both the Utøya and the control group, and the subgroup analysis showed no significant results. This is inconsistent with prior research with both higher total cortisol output (Johnsen et al., 2008) and lower total cortisol output (Neylan et al., 2005; Wessa et al., 2006) in PTSD patients. This could be due to our low number of participants in the PTSD/PTSS group, and different methodological procedures, and the use of post-dexamethasone measurements in other studies (Neylan et al., 2005).

Area under the curve with respect to increase (AUCi)

In addition to AUCg we also computed the area under the curve with respect to increase, different to AUCg that reflects the total cortisol amount. The AUCi measures the change of cortisol levels, and is able to capture the diversity of the post-awakening cortisol changes. Therefor the AUCi is a more preferred summary indicator when being referred to the CAR (Stalder et al., 2016).

Between the Utøya and the control group, the AUCi did not yielded any significant result, but the subgroup divisions showed a significant group effect. The Utøya group with PTSD/PTSS showed significant lower AUCi post- awakening than both the control group, and the Utøya group without symptoms. These results indicate that the overall concentration as measured by AUCg does not differ in the three groups, but the expected change (increase) post awakening (AUCi) is significantly lower in the PTSD/PTSS group.

The indication of a reduced AUCi in PTSD/PTSS subjects indicates some interesting findings on the development of PTSD/PTSS and the neurobiological changes for this condition.

Cortisol levels before and after the fMRI sequence

Most of the subjects also delivered two cortisol samples pre and post fMRI scanning on day three. fMRI was used to investigate the effects of acute traumatic stress, and the impact this might have on cognitive brain networks. Both on resting- state cortical network activity, this means that the subjects were not shown any stimuli in the scanner, and a working and emotional working memory task, with emotional and neutral stimuli. The results from before to after were significant, all subjects showed lowered cortisol levels post- fMRI scanning. This is surprising because we might have thought that the novelty, noise, and the limited amount of space would result in some form of HPA axis activation, and thus higher levels of cortisol post-fMRI scanning. The effects of supine position on the HPA axis activity seems to be limited, although Hennig et al. (2000) found that the circadian decrease of cortisol levels was associated with sitting and lying positions, and an increase to a upright position. The participants were exposed for 20 minutes in each condition in the different body positions. The time of salivary cortisol measurements was not mentioned but the subjects were at the institute when the salivary cortisol measurements were taken before, during and after each body condition, so the CAR can be ruled out. However, another study did not find any influences of body postures on cortisol levels with respect to the CAR. They investigated

whether the CAR could be influenced by remaining supine or standing upright in the post-awakening period. In addition, the subjects were requested to attend to the laboratory during the afternoon 14.00-16.00 hours. This was to determine whether the change from supine to standing influenced the salivary cortisol levels. The subjects lay supine for 15 minutes, and after 10 minutes a saliva sample was collected. After the supine condition, the subject was requested to stand in an upright position for a further 15 minutes. They found no influence on the CAR by postural change, nor did they detect a cortisol response in the afternoon due to the orthostatic challenge (Hucklebridge et al., 2002).

The results from our study could be the normal diurnal cortisol decline; the samples were collected in the morning and afternoon approximately between 08.55 and 16.00 with the exception of one subject (18.24 pre scanner and 19.40 post scanner). So rather the normal declining diurnal cortisol cycle as opposed to a supine posture in the scanner would give a possible explanation to the lower cortisol levels post-fMRI, in addition to the fact that that being in the scanner did not pose any threat to the subjects, and thus no higher activation of the HPA axis.

Hypocortisolism in PTSD/PTSS

There are several theories assuming that hypocortisolism is associated with a chronic stress period were there in the first case is a hyperactivity of the HPA axis (this could also explain the changes of the hippocampus). After a prolonged period with high glucocorticoids, this could cause alterations in the HPA axis going from hyperactivation to hypoactivation. A possible explanation for this phenomenon is the self-adjusting ability of the body. By the constant increased levels of glucocorticoids the body needs to adapt a sort of counterbalance to prevent damage from these effects. It may be the case in hypocortisolism that this counterbalance is too drastic, an overshoot. Possible counterbalance mechanisms are the down regulation of the receptor cells at different levels of the HPA axis, a reduction or deficiency of

the biosynthesis at the HPA axis, or higher negative feedback mechanisms (Fries et al., 2005; Miller et al., 2007).

These higher negative feedback mechanisms in the form of glucocorticoid receptors could be a preexisting vulnerability factor, prior to the development of PTSD after an acute traumatic stress experience. The receptor system consists of two related receptors molecules - the mineralocorticoid receptor (MR) and the glucocorticoid receptor (GR) which binds to circulating cortisol in the brain. The difference between the receptors is the affinity; MRs are higher in affinity than GRs and are saturated at low basal cortisol levels. The GRs respond in accordance with the ultradian rhythm, and during stress and the circadian rhythm, with secretory cortisol bursts increasing in both amplitude and frequency (Herman et al, 2005). GRs are found in the ACHT producing cells of the pituitary, in addition to CRH producing neurons of the hypothalamus, and hippocampus. Low circulation cortisol levels can lead to an increase of the GR, to improve the response ability and to receive a homeostatic state. The number and sensitivity of the MRs and GRs can cause influences on HPA axis activity in particular the cortisol levels by the strength of negative feedback. A prospective cohort study investigated the GR numbers prior to employment in military personnel. The subjects were assessed from 455 men, 6 months after deployment the subjects were assigned to a PTSD group that resulted in 34 subjects. They analyzed the GR binding by investigating them in peripheral blood mononuclear cells (PBMCs) measured with a whole cell dexamethasone binding assay, in addition to mRNA expression for GR subtypes and GR target genes. The results showed that the mean number of GR before deployment gave a high level of PTSD symptoms after a 4-month deployment to Afghanistan, compared to matched subjects without PTSD symptoms In addition, the number of GR in PBMCs predicted the development of a higher level of PTSD symptoms (van Zuiden et al., 2011). Some factors could be due to previous deployments, smoking or alcohol use, or traumatic childhood experiences, but none

of these variables were associated with the GR numbers. (van Zuiden et al., 2011), which would indicate that higher GR numbers in individuals could be a vulnerability factor for the development of PTSD/PTSS after a traumatic stress episode.

Early life stress and the development of the HPA axis

What causes the development of a higher number of GR could be discussed, the number of traumatic events in childhood could be a possible factor. When the brain is still under development, the effects of persistent sensitization of the HPA axis could cause major risk factors for the development and persistence of mental disorders especially under rapid brain development, which could cause an increased vulnerability to stress throughout the lifespan. Brain structures go through major growth and decline periods at different time points of development, the exposure to early life stress have development-dependent effects on brain structures, something that could lead to increased reactivity to stress and cognitive deficits in adulthood. There is evidence in children that there is a hyporesponsive period to glucocorticoids, Over the course of the first year of age, infants show no sign to elevated cortisol levels to stressors that typically would provoke HPA axis activity. The exact period of this hyposensitivity is not entirely clear, but adolescents show significant elevated levels to psychosocial stressors. The function of this hyposensitive period could be to protect the brain from glucocorticoids, if the infants are provided with a supportive and caring environment to buffer the HPA axis activity. However, if there is not a safe and stable environment, this could cause a stress response in children, and the capacity to produce elevated cortisol levels (Bouma et al., 2009; Gunnar & Quevedo, 2007; Lupien et al., 2009;).

A longitudinal study followed sexually abused women over a time period of childhood- to early adolescence (6-13), mid- to late adolescence (14-19) and early adulthood (20-32) compared to a control group, to determine whether childhood abuse results in disrupted cortisol activity. They measured diurnal cortisol levels between 09.00 and 10.00 a.m. after a

30 min resting state. They found that children who had experienced sexual abuse had higher levels of cortisol, but those levels became significantly lower in early adulthood, suggesting that sexual abused females showed a significant slower rate of growth in cortisol activity (Trickett et al., 2010)

Another study investigated the diurnal salivary cortisol level in children (mean age 10.7 years) with a history of exposure to trauma and exhibiting PTSD symptoms. The salivary cortisol levels were compared to a previous control group. They found that the children with PTSD symptoms had higher cortisol levels, the levels of cortisol falling during the day in both groups, but the children with PTSD symptoms had significantly higher cortisol levels than the controls. There was also a difference in cortisol levels between girls and boys in the traumatized group; girls had higher levels of cortisol than boys did and this difference was not observed within the control group (Carrion et al., 2002).

The cortisol levels assessed shortly after trauma in children aged 8-18, were contacted 6 weeks after the trauma to see if the children had developed acute PTSD, based on the urinary cortisol levels right after the traumatic event. They found that for boys, the elevated cortisol levels after traumatic experience were significantly correlated with their acute PTSD symptoms (Delahanty et al., 2005). These findings suggest that the exposure to early and severe stress leads to a heightened stress response, but that those effects becomes blunted over time showing a gradual attenuation of cortisol activity. Giving support for the attenuation hypothesis, this gradual suppression may be an adaptive response to the chronic exposure of glucocorticoids. Although this could be an adaptive response, low cortisol levels are not the optimal condition, as they are associated with several psychological and bodily disorders including PTSD (Heim et al., 2000; Miller et al., 2007).

In the present study we did not check for possible traumatic experiences in childhood, as a possible vulnerability factor to develop PTSD/PTSS in the aftermath of the terror attack. The

victims in our sample were adolescents and young adults in the age from 17 to 24. During adolescence, there are several psychosocial and biological changes associated with transition. The period is associated with increased basal and stress activated cortisol levels after a relative hyporesponsive period in childhood (Gunnar & Quevedo, 2007). The findings of a relative decreased overall cortisol level in PTSD/PTSS subjects suggest some form of alteration in this sensitive system.

Pre trauma cortisol levels

There have been several studies that compared cortisol levels before trauma-exposure and cortisol levels right after. Because not everybody will develop PTSD after trauma-exposure it has been hypothesized that there needs to be some biological vulnerability factors already present before trauma-exposure. However, the exposure to traumatic events are not that predictable in the normal population. These biological vulnerability factors could be retrieved from populations with greater risk for trauma exposure, e.g. police and military personnel, or data collections relatively fast after the traumatic event, e.g. at trauma centers, or hospitals after motor vehicle accidents, and major surgery. Soldiers who are deployed to combat regions may experience severe psychological distress, and be exposed to potentially traumatic stressors. The deployment to conflict areas is further related to a chronic stress state, and soldiers are at greater risk to develop PTSD. The investigation of pre-trauma salivary cortisol levels before deployment to an UN-mission in Bosnia and the association with post-trauma changes in cortisol, psychological distress and general health has been conducted by a study of Aardal-Eriksson et al. (Aardal-Eriksson, Eriksson & Thorell, 2002). They found that lower cortisol levels pre trauma significantly predicted self-rated psychological distress. The development of post-traumatic distress was further predicted by low cortisol levels pre-deployment in the morning and evening. The cortisol levels shortly after deployment in the

individuals with higher post traumatic distress showed low cortisol levels in the morning (8 a.m.) and higher cortisol levels in the evening (10 p.m.).

The CAR pre trauma in police recruits has been studied during academy training before the exposure to critical accidents, and 12, 24 and 36 months following the start of an active police service. The results showed that a higher CAR was associated with higher peri-traumatic dissociating (meaning they felt higher distress at the time the critical accidents occurred e.g., emotional numbing, reduced awareness, depersonalization, derealization) and acute stress disorder during an active police service. The CAR did not predict PTSD in the aftermath, which could be due to limited PTSD symptoms in the subjects (Inslicht et al., 2011).

The cortisol levels shortly after a traumatic incident have also been conducted in several studies consisting of samples from normal populations, and not those specifically at risk to experience traumatic events. Samples were assessed from motor vehicle accident victims. Fifteen-hour urinary cortisol samples were collected from the victims who were admitted to an ER, in addition, to a one month follow up on the presence of acute PTSD. The victims who met criteria for PTSD showed lower urinary cortisol levels, indicating that the initial hormone levels shortly in the aftermath of a traumatic exposure can be a vulnerability factor to develop PTSD (Delahanty et al., 2000). In addition, that prior trauma history, and the injury severity scores served as predictive factors to the development of PTSD where the urinary cortisol levels served as a mediator (Delahanty et al., 2003).

Patients who have been treated in an intensive care unit (ICU) for example could develop PTSD from extreme traumatic memories at the ICU. A stress dose of hydrocortisone given during ICU therapy of critically ill patients has shown to reduce the development of PTSD in long-term survivors, suggesting that low cortisol levels pose a vulnerability factor for PTSD, and the administration of glucocorticoids (hydrocortisone) before or right after a traumatic event could prevent the development of PTSD (Schelling et al., 2006).

Coping factors

Individual differences and mental health outcomes after the exposure to psychosocial stressors are tremendous. Why some develop PTSD while others do not remains one of the intriguing questions in mentalhealth science. Cognitive factors play an important role on the trauma response with respect to PTSD like negative beliefs and appraisals of ongoing threat. The perception of the stressful event as a threat is as relevant as the trauma exposure, as was seen in the injury severity scores in the motor vehicle accident patients. The victims who met criteria for PTSD received significantly lower injury severity scores than victims who did not develop PTSD (Delahanty et al., 2003), which shows the individual perception of the trauma exposure to be important.

The appraisal of the acute symptoms and an association to PTSD has been proposed, primarily because of the effect of creating a sense of serious and current threat (Ehlers & Clark, 2000). There are different coping strategies which are related to the distinct types of stress. The perceived controllability and predictability seems to be associated to active coping styles, which includes confrontation, fight or escape. Thus if the stressor is controllable and escapable the reaction of active coping could occur. If however the stressor is uncontrollable and inescapable (as with the Utøya terror attack), there could be evoked passive coping strategies with immobility and disengagement (Koolhaas et al., 2011; Olff et al., 2005) After a traumatic stress experience active coping strategies are related to beneficial outcomes. Examples of active coping are actively dealing with problems, and positive thinking. Passive coping is associated with dysfunctional outcomes and the development of PTSD. Social isolation and withdrawal, alcohol consumption, rumination and denial are examples of passive coping strategies. The importance of social support in the aftermath of a traumatic experience is also considered as active an coping strategy and could dampen and even protect against the development of PTSD (Olff et al., 2005)

Strengths and limitations

A major strength of the present study is that we were able to identify a change in cortisol levels in the PTSD/PTSS group even though our number of subjects was quite low, including PTSD/PTSS symptoms in the Utøya group. Another striking factor is the homogeneity of the Utøya group as everybody experienced the same type of trauma, and the subjects were approximately of the same age range, although the perceived experience is individually diverse. The use of fMRI examinations in addition to cortisol data is likely to increase our understanding on the effects of traumatic stress, which ultimately will guide current and future treatment to survivors of traumatic stressful experiences.

One of the major limitations of the present study is incomplete and potentially inaccurate sampling. Incomplete sampling and inaccurate marking of tubes led to a low number of subjects with full cortisol profiles, and because of that, we may have lacked statistical power to detect important associations. There are several methods to avoid inaccurate sampling; the first method is to identify noncompliance. This can be done by the use of a compliance check in the sampling procedure. A rather accurate electronic monitoring system is quite effective, employing track caps, which administer automatically date and time stamps when the container to take the saliva sample is opened. There are however some issues to this system. As it does not confirm the actual saliva sampling, subjects may open the device without taking a saliva sample, making the time and date inaccurate in relation to the CAR. Another devise is polysomnography (PSG) a well-used instrument in sleep research, and could be used to verify waking times in CAR assessments. The issue with using this devise is however that it takes time, is expensive, and it could be disruptive to subjects. A more appropriate method could be wrist actigraphy, which can be used to monitor sleep and authenticate the waking time. It is little disturbing as compared to the PSG, as well as rather inexpensive if PSG should be the other alternative (Kudielka et al., 2012; Smyth et al., 2013; Stalder et al., 2016).

A chest-worn motility monitor is an additional actigraphy based method. In addition to sleep patterns this devise also measures the heart inter-beat interval, because waking up is linked to a higher heartbeat. These devices provide the researcher with correct awakening times which helps profoundly in providing a valid and correct CAR assessment (Hucklebridge et al., 2002; Stalder et al., 2016). If these devices for some reason should not be available due to cost and time, there are other methods to increase the sampling accuracy and the subject's compliance. This can be done be simply informing the subjects about the procedure, and how important it is to be accurate about time.

Other possible strategies come from Stalder and colleagues, (2016) without having formally published the evidence for these strategies. The way sampling accuracy could be conveyed, and heighten the subjects compliance is face-to-face meetings, which could give the subjects the extra motivation to be precise and correct with their sampling procedure, plus the importance of correct sampling for the research. Precise instructions from the researcher are important such that the subjects understand the saliva sampling protocol, and no mistakes are made because of uncertainty or unawareness. It could be advisable that the researchers practice the procedure beforehand. The moment of awakening should be made clear to the subjects. Stalder and colleagues, (2016) recommend that the moment of awakening needs to be clarified in the moment they get conscious from sleep (*e.g., "When you are awake, i.e., you are conscious: you know who and where you are ; you are in a state that is clearly different from when you were sleeping even though you may still feel tired.")*. It should be made understood that nightly awakenings are not the right moment to start the sampling procedure. Additionally to personal contact, instructions in written form that can be taken home are proven to be a useful tool to increase the subject's compliance. Other methods that have had positive effects are text messages, phone calls, or emailing before the sampling day. These

methods also give a positive image to the researchers, which emphasize how important it is for the subjects to be compliant and precise with the sampling procedure (Stalder et al., 2016).

Conclusion

The results of the present study show that Utøya survivors and healthy controls did not differ in cortisol levels. However, the support of low overall cortisol levels in the Utøya survivors was obtained after a subdivision in the Utøya group, Utøya survivors with PTSD/PTSS symptoms and Utøya survivors without symptoms. Significantly lower overall cortisol levels were found in Utøya survivors with PTSD/PTSS symptoms compared with controls, and Utøya survivors without symptoms, especially 15-30 minutes after awakening. The expected change in cortisol levels right after awakening (increase) calculated by the AUCi showed that Utøya survivors with PTSD/PTSS had significantly lower cortisol levels than the other two groups. These results indicate that the development of PTSD/PTSS causes changes in cortisol levels, and induces circadian disturbances. This study is in line with other studies which have found some of the biological markers of PTSD/PTSS. However, it is difficult to identify whether low cortisol levels were present before, or were due to the development of PTSD/PTSS. It is clear that PTSD/PTSS is triggered by trauma, with a complex interplay of developmental, cognitive, genetic, endocrine, and neurobiological abnormalities, although this study has provided some clarity in the myriad factors associated with PTSD/PTSS.

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Figures

Figure 1

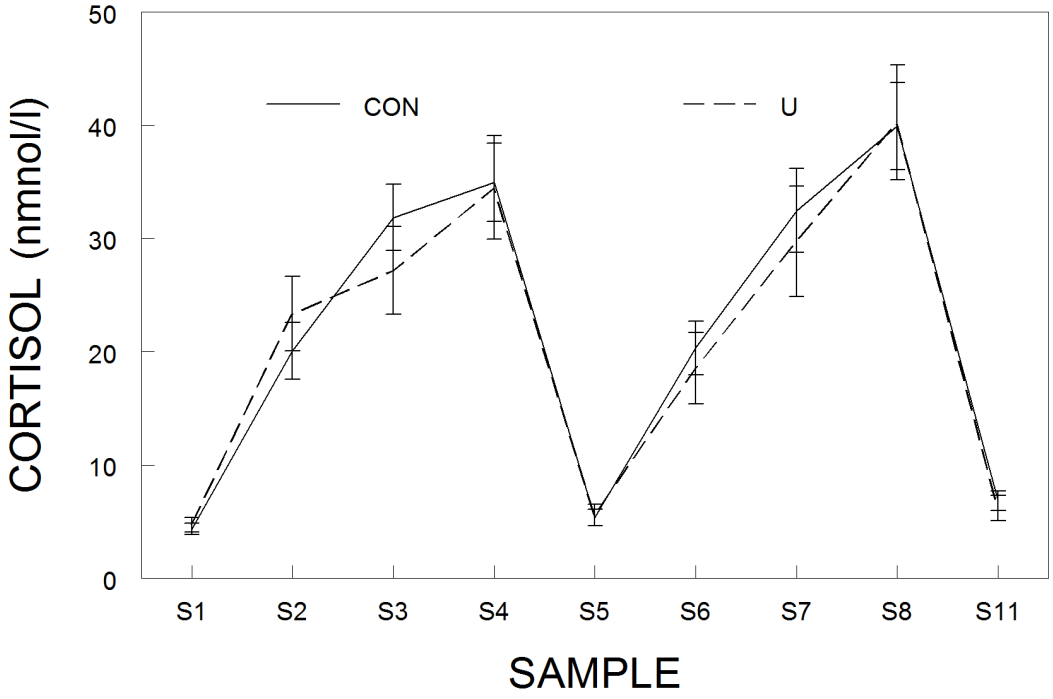

Figure 1. Mean salivary cortisol levels over a three- day sampling period between Controls (CON) and Utøya survivors (U). Note: Bars represent standard errors of the mean(S.E.M.).

Figure 2

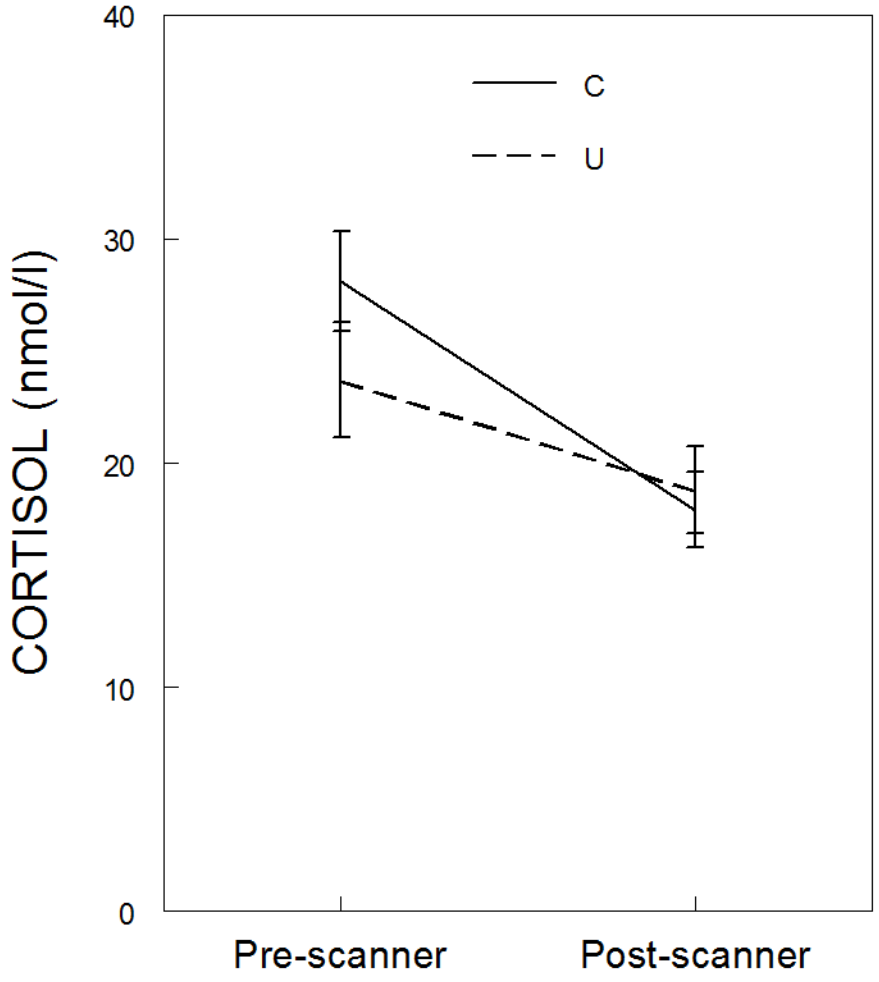

Figure 2. Mean salivary cortisol levels pre and post fMRI between Controls (C) and Utøya (U)survivors.

Figure 3

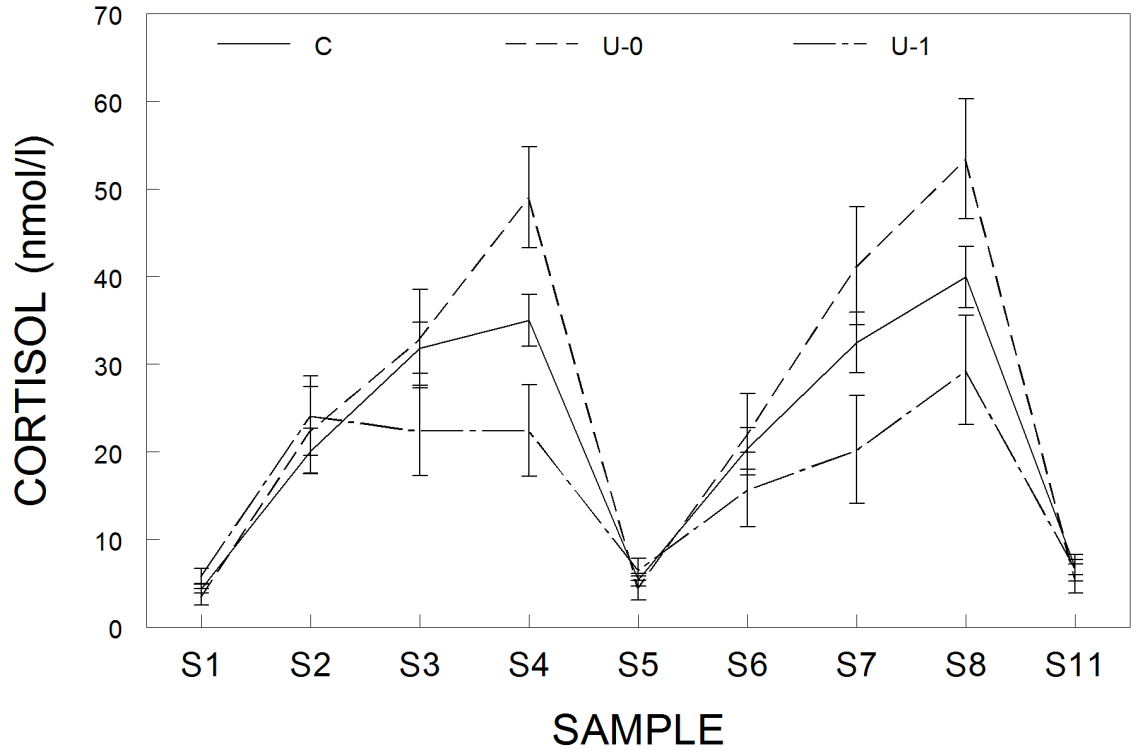

Figure 3. Mean salivary cortisol levels over a three- day sampling period between Controls (C), Utøya survivors without (U-0), and with PTSD/PTSS (U-1). Note: Bars represent standard errors of the mean (S.E.M.).

Figure 4

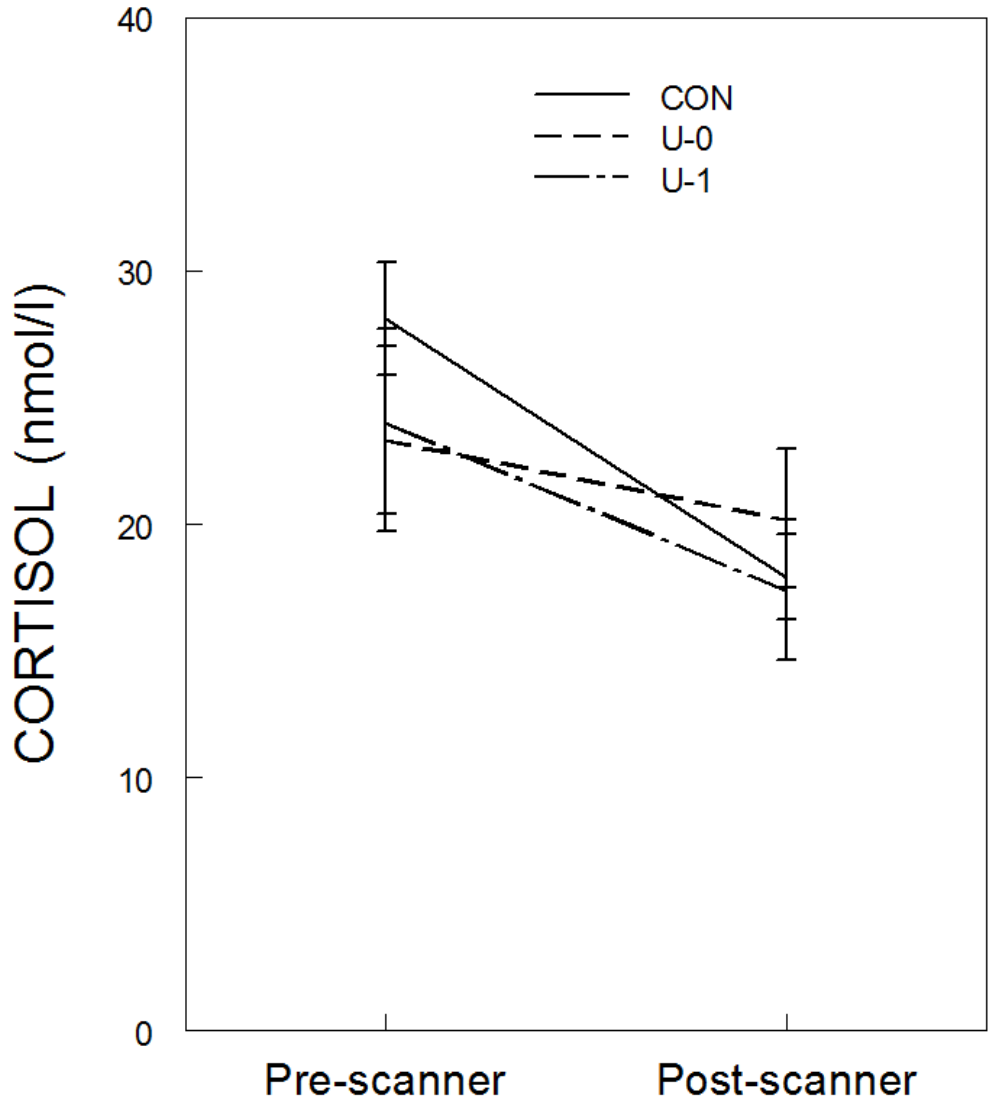

Figure 4. Mean salivary cortisol levels pre and post fMRI between Controls (CON) and Utøya survivors without (U-0), and with PTSD/PTSS (U-1).

Tables

Table I Mean salivary cortisol samples over a three-day period

	Group	M	SEM	N
Sample 1	C	4.38	0.47	19
	U	4.73	0.73	11
Sample 2	C	20.09	2.61	19
	U	23.37	3.07	11
Sample 3	C	31.86	3.20	19
	U	27.20	3.13	11
Sample 4	C	34.98	2.99	19
	U	34.52	5.49	11
Sample 5	C	5.37	0.54	19
	U	5.60	1.24	11
Sample 6	C	20.34	2.69	19
	U	18.55	2.25	11
Sample 7	C	32.48	3.59	19
	U	29.78	5.17	11
Sample 8	C	39.94	3.52	19
	U	40.28	5.78	11
Sample 11	C	6.85	0.79	19
	U	6.21	1.21	11

C control group
U Utøya group
SEM standard error of the mean

Table II CAR day two and three

	Group	M	SEM	N
CAR DAY 2	C	15.04	2.80	22
	U	11.92	4.26	18
CAR DAY 3	C	19.45	3.52	22
	U	22.09	3.52	18

C control group
U Utøya group
SEM standard error of the mean

Table III AUCg day two and three

	Group	M	SEM	N
AUCg DAY 2	C	59.52	5.21	23
	U	61.31	5.34	18
AUCg DAY3	C	60.50	5.52	23
	U	61.93	5.50	18

C control group
U Utøya group
SEM standard error of the mean

Table IV AUCi day two and three

	Group	M	SEM	N
AUCi DAY 2	C	21.25	3.24	24
	U	12.41	5.07	18
AUCi DAY 3	C	21.21	4.03	24
	U	19.97	4.49	18

C control group
U Utøya group
SEM standard error of the mean

Table V Pre and post fMRI mean salivary cortisol samples

	Group	M	SEM	N
Pre	C	28.12	2.25	33
	U	23.71	2.46	24
Post	C	17.91	1.46	33
	U	18.83	2.22	24

C control group
U Utøya group
SEM standard error of the mean

TableVI Mean salivary cortisol samples over a three-day period

	GROUP	M	SEM	N
Sample 1	C-0	4.38	0.46	19
	U-0	3.45	0.86	5
	U-1	5.79	0.98	6
Sample 2	C-0	20.09	2.60	19
	U-0	22.48	2.23	5
	U-1	24.11	5.57	6
Sample 3	C-0	31.86	3.19	19
	U-0	32.92	4.12	5
	U-1	22.42	3.80	6
Sample 4	C-0	34.98	2.99	19
	U-0	49.01	7.26	5
	U-1	22.43	3.34	6
Sample 5	C-0	5.37	0.54	19
	U-0	4.47	1.33	5
	U-1	6.55	2.02	6
Sample 6	C-0	20.34	2.68	19
	U-0	21.98	1.51	5
	U-1	15.69	3.67	6
Sample 7	C-0	32.48	3.58	19
	U-0	41.21	7.98	5
	U-1	20.25	3.93	6
Sample 8	C-0	39.94	3.51	19
	U-0	53.41	7.92	5
	U-1	29.34	5.27	6
Sample 11	C-0	6.85	0.79	19
	U-0	5.56	1.38	5
	U-1	6.74	1.99	6

C control group

U-0 Utøya without PTSD/PTSS

U-1 Utøya with PTSD/PTSS

SEM standard error of the mean

Table VII Car day two and three

	Group	M	SEM	N
CAR DAY 2	C-0	15.04	2.80	22
	U-0	20.95	4.96	8
	U-1	4.70	5.77	10
CAR DAY 3	C-0	19.45	3.52	22
	U-0	25.11	6.64	8
	U-1	19.67	4.38	10

C control group

U-0 Utøya without PTSD/PTSS

U-1 Utøya with PTSD/PTSS

SEM standard error of the mean

Table VIII AUCg day two and three

	Group	M	SEM	N
AUCg DAY2	C-0	59.52	5.21	23
	U-0	69.63	4.53	9
	U-1	52.99	9.14	9
AUCg DAY3	C-0	60.50	5.52	23
	U-0	71.47	7.44	9
	U-1	52.40	7.10	9

C control group.

U-0 Utøya without PTSD/PTSS

U-1 Utøya with PTSD/PTSS

SEM standard error of the mean

Table IX AUCi day two and three

	Group	M	SEM	N
AUCi DAY 2	C-0	21.25	3.24	24
	U-0	25.09	6.35	9
	U-1	-0.27	5.37	9
AUCi DAY 3	C-0	21.21	4.03	24
	U-0	22.62	8.36	9
	U-1	17.32	3.76	9

C control group.

U-0 Utøya without PTSD/PTSS

U-1 Utøya with PTSD/PTSS

SEM standard error of the mean

Table X Pre and post fMRI mean salivary cortisol samples

	Group	M	SEM	N
Pre	C-0	28.12	2.25	33
	U-0	23.38	2.92	12
	U-1	24.05	4.11	12
Post	C-0	17.91	1.46	33
	U-0	20.24	3.72	12
	U-1	17.42	2.53	12

C control group.

U-0 Utøya without PTSD/PTSS

U-1 Utøya with PTSD/PTSS

SEM standard error of the mean

Appendix

General guidelines for the saliva sampling that the participants received.

SAMLING AV SPYTTPRØVER Samling av spyttprøver gjøres med tildelt beholder Salivette®, den skal oppbevares tørt ved romtemperatur. Hormonet kortisol måles i spyttet, men da det er stor døgnvariasjon av dette hormon må spyttsamling skje om **kvelden mellom kl 21-24** og **morgenen mellom kl. 07-09** (straks man våkner). Skjer det ved andre tidspunkt, blir det vanskelig å fortolke analyse resultatet.

Følgende må unngås før prøvesamling:

Stor fysisk aktivitet eller røyking, dessuten tannpuss, tannpirker, mat og drikke, eller annet som kan lage rifter eller sår i tannkjøttet. Dette fordi lekkasje fra blod til spytt kan gi feil måleresultat. Ved kveldsprøve bør det ha gått ca 1 time etter kveldsmaten før prøven tas, og den må tas før tannpuss. I tilfelle noe av dette glemmes, vent da 1 time, skyll så munnen i kaldt vann 5 minutter før prøven tas.

Prøvetakning:

Fjern korken på røret, ta ut tampongen, putt tampongen i munnen, tygg gjerne på tampongen for å få i gang spyttproduksjon, sørg for at tampongen er gjennomtrukket av spytt før den tas ut. Legg tampongen tilbake i røret (der den var før røret ble åpnet) Røret har en indre hylse som tampongen ligger i, tampongen skal legges tilbake i denne hylsen, og hylsen skal ligge i røret, og korken på røret skal settes på. Slik leveres den til laboratoriet.

Oppbevaring og forsendelse:

Hjemme oppbevares prøven helst i kjøleskap og leveres ved første anledning til personen som utleverte Salivette®. Prøven er holdbar i 5 dager i kjøleskap, men kortere i romtemperatur.

Husk å notere tidspunkt for prøvetakning på røret! Husk å legge kveldsprøvene i kjøleskap før du legger deg! 2

Du skal ta prøvene til fastsatte tider på en helt vanlig dag:

1. Kveld, det vil si før du legger deg (om du må spise, drikke, røyke, pusse tenner så gjør dette 30 min før du tar prøven).
2. Med en gang du våkner på morgenen (selv om du våkner tidligere enn normalt).
3. 15 minutter etter forrige prøve (du må da ikke ha sovnet igjen).
4. 15 minutter etter forrige prøve (det vil si ca 30 minutter etter oppvåkning).
5. Kveld, før du legger deg

På testdagen:

1. Med en gang du våkner om morgenen (selv om du våkner tidligere enn normalt)
2. 15 minutter etter forrige prøve
3. 15 minutter etter forrige prøve (det vil si 30 minutter etter oppvåkning
4. like før fMRI scanning
5. like før nevropsykologisk testing, etter fMRI scanning
6. Kveld, før du legger deg

Glassene er nummerert til de ulike tidspunktene. Skriv dato og klokkeslettet på glasset når du tar prøven. Skulle du glemme å ta prøven til oppsatt tid er dette spesielt viktig! For at prøvene skal kunne brukes må du føre opp korrekt tidspunkt, selv om tidspunktet for prøven avviker fra instruksjonen.

OBS! Dersom du må drikke eller ta medisiner før du har tatt alle de tre morgenprøvene, så kan du drikke litt vann (ikke kaffe/te/sukkerholdige drikker), men du må da vente 30 min før du tar neste spytteprøve. Dersom du må røyke før alle prøvene er tatt, gjør du det på samme

måte. Husk å skriv ned om du har drukket, røkt (eller ved en forglemmelse spist) samt tidspunkt.