Distinct trajectories of cortisol response to prolonged acute stress are linked to affective responses and hippocampal gray matter volume in healthy females

Roee Admon1, Michael T. Treadway2, Linda Valeri3, Malavika Mehta4, Samuel Douglas4 and Diego A. Pizzagalli1,5

1Department of Psychology, University of Haifa, Haifa, Israel, 3498838
2Department of Psychology, Emory University, Atlanta, GA, 30322
3Psychiatric Biostatistics Laboratory, McLean Hospital/Harvard Medical School, Belmont, MA, 02478
4Center for Depression, Anxiety and Stress Research, McLean Hospital/Harvard Medical School, Belmont, MA, 02478
5McLean Imaging Center, McLean Hospital/Harvard Medical School, Belmont, MA, 02478

DOI: 10.1523/JNEUROSCI.1175-17.2017

Received: 30 April 2017
Revised: 18 June 2017
Accepted: 5 July 2017
Published: 24 July 2017


Conflict of Interest: Over the past 3 years, Dr. Pizzagalli has received consulting fees from Akili Interactive Labs, BlackThorn Therapeutics, Boehringer Ingelheim, Pfizer and Posit Science for activities unrelated to the current research. Dr. Treadway has served as a paid consultant to Avanir Pharmaceuticals and the Boston Consulting Group. All other authors report no biomedical financial interests.

The authors would like to thank Dr. Nicolas Rohleder and Dr. Jutta Wolf, directors of the Laboratory for Biological Health Psychology at Brandeis University, for performing endocrinological assays.

Correspondence should be addressed to To whom scientific correspondence should be addressed: Roee Admon, PhD, Department of Psychology, University of Haifa, 199 Aba Khoushy Ave., Haifa, Israel, 3498838, radmon@psy.haifa.ac.il Or: Diego A. Pizzagalli, PhD, Center for Depression, Anxiety and Stress Research, McLean Hospital/Harvard Medical School, 115 Mill Street, McLean Hospital, Belmont, MA 02478, dap@mclean.harvard.edu

Cite as: J. Neurosci ; 10.1523/JNEUROSCI.1175-17.2017

Alerts: Sign up at www.jneurosci.org/cgi/alerts to receive customized email alerts when the fully formatted version of this article is published.

Accepted manuscripts are peer-reviewed but have not been through the copyediting, formatting, or proofreading process.

Copyright © 2017 the authors
Distinct trajectories of cortisol response to prolonged acute stress are linked to affective responses and hippocampal gray matter volume in healthy females

Roee Admon\textsuperscript{1*}, Michael T. Treadway\textsuperscript{2*}, Linda Valeri\textsuperscript{3}, Malavika Mehta\textsuperscript{4}, Samuel Douglas\textsuperscript{4}, Diego A. Pizzagalli\textsuperscript{4,5}

\textsuperscript{1}Department of Psychology, University of Haifa, Haifa, Israel, 3498838
\textsuperscript{2}Department of Psychology, Emory University, Atlanta, GA, 30322
\textsuperscript{3}Psychiatric Biostatistics Laboratory, McLean Hospital/Harvard Medical School, Belmont, MA, 02478
\textsuperscript{4}Center for Depression, Anxiety and Stress Research, McLean Hospital/Harvard Medical School, Belmont, MA, 02478
\textsuperscript{5}McLean Imaging Center, McLean Hospital/Harvard Medical School, Belmont, MA, 02478

\*These authors contributed equally to this work.

To whom scientific correspondence should be addressed:
Roee Admon, PhD
Department of Psychology, University of Haifa
199 Aba Khoushy Ave.
Haifa, Israel, 3498838
radmon@psy.haifa.ac.il

Or:
Diego A. Pizzagalli, PhD
Center for Depression, Anxiety and Stress Research
McLean Hospital/Harvard Medical School
115 Mill Street
McLean Hospital
Belmont, MA 02478
dap@mclean.harvard.edu

Abbreviated title: Trajectories of cortisol response to acute stress
Conflict of Interest: Over the past 3 years, Dr. Pizzagalli has received consulting fees from Akili Interactive Labs, BlackThorn Therapeutics, Boehringer Ingelheim, Pfizer and Posit Science for activities unrelated to the current research. Dr. Treadway has served as a paid consultant to Avanir Pharmaceuticals and the Boston Consulting Group. All other authors report no biomedical financial interests.

Acknowledgements: This work was support by the National Institutes of Mental Health (R01 and R37 MH068376, MH068376-09S1 to DAP). Dr. Admon was supported by the Israeli Council for Higher Education (Alon Scholarship). Drs. Treadway and Valeri were supported by K99 MH102355 and the Harvard Catalyst Biostatistics Consulting for Harvard Medical School affiliated researchers, respectively. The authors would like to thank Dr. Nicolas Rohleder and Dr. Jutta Wolf, directors of the Laboratory for Biological Health Psychology at Brandeis University, for performing endocrinological assays.
The development of robust laboratory procedures for acute stress induction over the last decades has greatly advanced our understanding of stress responses in humans and their underlying neurobiological mechanisms. Nevertheless, attempts to uncover linear relationships between endocrine, neural, and affective responses to stress have generally yielded inconsistent results. Here, 79 healthy females completed a well-established laboratory procedure of acute stress induction that was modified in order to prolong its effect. Endocrinological and subjective affect assessments revealed stress-induced increases in cortisol release and negative affect that persisted 65 and 100 minutes post stress onset, respectively, confirming a relatively prolonged acute stress induction. Applying latent class linear mixed modelling (LCMM) on individuals’ patterns of cortisol responses identified three distinct trajectories of cortisol response: hyper-response ($n=10$), moderate-response ($n=21$), and mild-response ($n=48$). Notably, while all three groups exhibited a significant stress-induced increase in cortisol release and negative affect, hyper-response and mild-response groups both reported more negative affect relative to the moderate-response group. Structural MRI revealed no group differences in hippocampal and amygdala volumes, yet a continuous measure of cortisol response (area under the curve) showed that high and low levels of stress-induced cortisol release were associated with less hippocampal gray matter volume compared to moderate cortisol release. Together, these results suggest that distinct trajectories of cortisol response to prolonged acute stress among healthy females may not be captured by conventional linear analyses; instead, quadratic relations may better...
Trajectories of cortisol response to acute stress describe links between cortisol response to stress and affective responses, as well as hippocampal structural variability.
Trajectories of cortisol response to acute stress  Admon et al.,

**Significance Statement**

Despite substantial research, it is unclear if and how individual neuroendocrine stress response patterns are linked to affective responses to stress and structural variability in neuroendocrine regulatory brain regions. By applying latent class linear mixed modelling on individuals’ patterns of cortisol responses to a prolonged acute stressor, we identified three distinct trajectories of cortisol response. Relative to the group showing moderate cortisol response, groups characterized by hyper and mild cortisol response were both associated with more negative affect. Moreover, a continuous measure of cortisol response showed that high and low levels of stress-induced cortisol release correlated with reduced hippocampal gray matter volume. Given that neuroendocrine stress responses are conceptualized as biomarkers of stress susceptibility, these insights may have clinical implications.
Introduction

Stress sensitivity is a key element in the etiology and pathophysiology of psychopathology (Harkness et al., 2015). Accordingly, extensive scientific effort has been devoted to the characterization of the neural, physiological, and affective responses to stress in humans, and their potential mutual interactions. This work established that the physiological response to acute stress involves the activation of endocrine stress-response systems, most prominently the hypothalamus-pituitary-adrenal (HPA) axis (Herman et al., 2016). The corresponding affective response to acute stress often include a temporary shift towards more negative affective states. As the end-point of HPA system, salivary cortisol is the most frequently used variable to evaluate endocrine stress response in laboratory settings, with a cortisol increase ≥2.5 nmol/l following stress induction typically taken as a threshold for “stress-response” (Foley and Kirschbaum, 2010). A wealth of laboratory acute stress procedures established that approximately 50-80% of individuals can be classified as “stress-responders” based on this cutoff (Dickerson and Kemeny, 2004). Interestingly, however, in most cases links between stress-induced cortisol and affective responses are not clear, with the majority of studies not reporting significant differences in affective responses to stress among “stress-responders” compared to “non-responders” (Campbell and Ehlert, 2012). Further, only about 25% of studies report a linear relation between cortisol and affective responses to stress (Campbell and Ehlert, 2012).

The endocrine stress-response systems have also been the focus of neuroimaging research, particularly targeting the hippocampus and amygdala structures owing to their pivotal roles in HPA regulation. Results have been mostly inconsistent,
with hippocampal volume being positively associated (Pruessner et al., 2007), negatively associated (Cho, 2001), or not associated (Liu et al., 2012) with the magnitude of cortisol response to stress. Similarly mixed results also emerged with regard to amygdala volume (Klimes-Dougan et al., 2014; Cacciaglia et al., 2017). Taken together, attempts to link patterns of stress-induced cortisol response to both affective responses and structural variability in HPA-regulating brain regions have yielded mixed results. One potential explanation for these inconsistencies may relate to the fact that studies typically report on mean cortisol response, or use predetermined values to classify individuals as “stress-responders” vs. “non-responders”, thus disregarding the important role of individual differences in determining stress sensitivity (Monroe and Simons, 1991; Liu, 2015). In addition, given that current laboratory protocols induce stress for a relatively short time period (typically < 20 minutes), it is possible that induced effects were too brief to allow for sufficient endocrine and emotional variability to evolve.

To address these limitations, the first aim of the current study was to effectively induce acute stress for a relatively prolonged time period among healthy females, while capturing individual endocrine and affective response patterns. To this end, healthy females completed the Maastricht Acute Stress Test (MAST), a robust laboratory acute stress procedure (Smeets et al., 2012), which was modified in order to prolong its effect by informing participants that, due to their poor performance, they would need to repeat the task later in the session. Our second aim was to identify distinct trajectories of cortisol response to such prolonged acute stress without a priori assumptions regarding the number, size, or pattern of change of these trajectories. This was accomplished by
applying latent class linear mixed modelling (LCMM) on individuals’ patterns of cortisol responses. Our final aim pertained to investigating in a subsample ($n=69$) with MRI data potential links between cortisol response to stress and structural variability, with a priori hypotheses relating to key regions implicated in HPA regulation, such as the amygdala and hippocampus. Overall, we hypothesized that applying a data-driven approach on cortisol patterns of response to stress may provide a more accurate account for individual variability, and that these insights may enable linking stress-induced cortisol responses with affective responses, as well as with structural variability in regions implicated in HPA regulation.

Materials & Methods

Participants: A total of 88 right-handed psychiatrically, medically and neurologically healthy female participants were included. Only females were investigated to avoid potential sex-dependent variability in HPA axis stress response (Kudielka and Kirschbaum, 2005). All participants were recruited using community advertisements. Exclusion criteria included any current or past psychiatric disorder as assessed by a Structured Clinical Interview for the DSM-IV [SCID; (First et al., 2005)]. Additionally, individuals were excluded for five or more lifetime exposures to any illegal substance, as well as due to recent use of illegal drugs, psychotropic medications or nicotine. For a complete summary of relevant demographic characteristics, please see Table 1.

Study procedure: Participants were tested in individual sessions between 11am and 4pm to minimize the effects of diurnal variation on endogenous cortisol levels (Blascovich et al., 2011). The stress procedure itself occurred between 1pm to 2pm.
Further, to allow for controlled saliva collection, participants were asked not to brush their teeth and to refrain from food, drinks, and intense physical exercise at least 1 hour prior to the test phase. None of the participants reported to have violated these directives. Upon arrival, participants received information about the study and the measurements that would be taken, and provided written informed consent to a protocol approved by the Partners IRB board. Next, participants completed two tasks (one reward task (PRT) and one reaction time task (RT); these data will be reported separately) and a clinical interview to determine eligibility (SCID). Following the interview participants completed the modified MAST stress task, which was then followed again by administration of the two tasks (Figure 1). Following this first laboratory session, participants were asked to return to the lab within approximately one month to complete an MRI scanning session (mean days = 25, SD = 21). The scanning session included an fMRI task, [described in (Treadway et al., in press)], as well as a high resolution anatomical scan.

**MAST stress task:** Stress was induced via a modified version of the Maastricht Acute Stress Test [MAST; (Smeets et al., 2012)], a laboratory acute stress procedure that was previously shown to yield robust endocrine and affective stress responses among healthy individuals (Smeets et al., 2012). The MAST consists of a 5 minute preparation phase and a 10 minute acute stress phase that combines the physical aspects of immersing one hand in ice-cold water from the Cold Pressor Test (CPT) with the unpredictability, uncontrollability, negative social-feedback, and mental arithmetic elements of the Trier Social Stress Test (TSST). See (Smeets et al., 2012) for additional details. Unlike the original task, immediately upon MAST completion participants were
told by a non-emphatic male study staff they had not yet met that their performance in
the math portion was not good enough and that they would need to repeat the task
following the administration of the remaining tasks and questionnaires. This
manipulation was intended to prolong the effect of the acute stressor. Later in the
session, participants were informed that repeating the task was not necessary since
their performance was “good enough” (i.e., relief was provided).

Stress measurements: In order to validate the effectiveness of the stress manipulation
endocrine and subjective indices of the stress response were assessed at multiple time
points throughout the session. Saliva samples were taken at six time points: upon
arrival (T -80 min), before the MAST (T 0 min), slightly following its completion (T+25 min), as
well as 50 (T+50 min), 65 (T+65 min), and 100 (T+100 min) minutes following stress onset.
These samples were used to track fluctuations in cortisol levels as well as in alpha-
amylase - an established measure of sympatho-adrenal-medullary (SAM) adrenergic
system (Nater and Rohleder, 2009). Saliva samples were obtained by placing a cotton
swab in participants’ mouth using salivette collection devices (Sarstedt, Germany) and
were stored at -20°C until analysis. Cortisol and alpha-amylase concentrations from
saliva samples were assayed at the Laboratory for Biological Health Psychology at
Brandeis University (Directors: Dr. Nicolas Rohleder and Dr. Jutta Wolf).

Affective responses to stress were assessed via a modified 100-point visual
analogue mood scale (VAMS) at the same six time points as the saliva collections, as
well as at two additional times: before the clinical interview (T -40 min) and immediately
following the relief (T+90 min). The VAMS consisted of five 100mm horizontal lines each
representing a bipolar dimensional mood state: Happy-Sad, Relaxed-Tense, Friendly-
Hostile, Sociable-Withdrawn, Quick Witted-Mentally Slow. Participants indicated their response by moving a computer cursor on each line to the point that best described their current mood state. Such continuous, on-line, assessment of mood was designed in order to avoid potential inaccuracies in retrospective post-stress affect reporting (Hellhammer and Schubert, 2012). Finally, self-report measures of positive and negative affect [PANAS; (Watson et al., 1988)] and state anxiety [STAI-S; (Spielberger et al., 1983)] were assessed at three time points: upon arrival (T_{-80\ min}), following the MAST (T_{+25\ min}), and 100 minutes following stress onset (T_{+100\ min}) (Figure 1).

MRI Acquisition and pre-processing: MRI data were acquired using a 3-Tesla Siemens Tim Trio scanner and a 32-channel head coil at the McLean Imaging Center. Scanning protocol included high-resolution T1-weighted MPRAGE images [TR = 2200ms; TE = 1.54ms; FOV = 230mm; matrix = 192x192; resolution = 1.22mm^3; 144 slices]. MRI data were analyzed using the voxel-based morphometry (VBM) module of the Computational Anatomy Toolbox (CAT12) (http://www.neuro.uni-jena.de/cat/) for SPM12 (Wellcome Department of Cognitive Neurology). VBM analysis incorporated the following preprocessing steps: (1) spatial registration to a reference brain, (2) tissue classification (segmentation) into gray and white matter and CSF, (3) bias correction of intensity non-uniformities, and (4) smoothing (8mm).

Statistical analysis: Out of the 88 participants who were recruited, saliva samples of nine participants were excluded from analyses due to missing data, leaving a total sample size of 79 participants for cortisol and alpha amylase analyses. Ten additional participants did not undergo structural MRI leaving a total sample size of 69 for MRI analyses. Cortisol responses were log transformed prior to statistical analysis to reduce
skewness. Main effects of time were tested using a repeated measures ANOVA with the six sampling time points as a within subject factor. A similar approach was implemented for alpha amylase. Regarding the VAMS, ratings were transformed so that higher scores indicate greater negative affect. Further, in order to probe parallel endocrine and affective patterns only the six VAMS ratings that were assessed alongside saliva samples were included in the analyses. VAMS ratings were analyzed using a repeated measures ANOVA with the six sampling time points and the five VAMS scales as within subject factors. Repeated measures ANOVAs were also separately implemented for self-report measures of state anxiety (STAI-S), positive affect (PANAS-PA), and negative affect (PANAS-NA) with the three time points as a within-subject factor. For all post-hoc tests alpha was set at 0.05 and adjusted (Bonferroni) for multiple comparisons.

Latent class linear mixed modelling (LCMM), also called growth mixture models (GMM) (Bauer and Curran, 2003), was used in order to identify distinct classes (i.e., groups) of participants featuring similar trajectories of cortisol response to stress. Specifically, we tested whether the model that best fits our data included 2, 3 or 4 classes of distinct trajectories of cortisol response. Within LCMM time was modeled as polynomials while allowing for linear, quadratic and cubic trajectories to be derived empirically based on trajectory subgroups. For continuous outcomes, such as cortisol values, the LCMM is an extension of the standard linear mixed model for handling various subpopulations of longitudinal trajectories (O'Brien and Fitzmaurice, 2005). Importantly, this approach captures all the heterogeneity in individual trajectories and identifies subgroups of participants with similar profiles of trajectories, independently of observed participant’s characteristics. In other words, LCMM estimates the number of
distinct trajectories of cortisol response that best capture variability in the data, without requiring a priori assumptions regarding the number, size, or pattern of change of these trajectories. Bayesian information criterion (BIC) was used to compare different models allowing for 2 vs 3 vs 4 classes and determine the optimal number based on variability in the data (Nylund et al., 2007). Low BIC values indicate a better fit of the model to the data. Analyses were performed using RStudio V0.99.879 [R foundation, Vienna, Austria] and the lcmm package (Proust-Lima et al., 2015). Notably, the first saliva sample, taken approximately 80 minutes prior to stress onset, was excluded from these analyses, leaving a total of five samples per participant. This was done in order to include in the models only stress-related cortisol responses while avoiding endogenous variation in cortisol levels.

Next, cortisol and affective responses to stress were examined with regard to the three classes identified using LCMM. Notably, despite the fact that the LCMM models were defined based on cortisol data, running an ANOVA on cortisol patterns with the resulted classes as a factor is not circular and is in fact regarded as the most valid post-hoc test (Jung and Wickrama, 2008). Accordingly, cortisol response to stress was examined using a mixed-effect ANOVA with the three classes as a between-subjects factor and the five sampling time points as a within-subjects factor (after omitting the first saliva sample, see above). For VAMS ratings, to compare only endocrine and affective data that were acquired concurrently, the first VAMS rating was used as an individual baseline score to which all other five VAMS samples were normalized. These data were then analyzed using a mixed-effect ANOVA with the three groups as a between-subjects factor and the five sampling time points and five VAMS scales as...
within-subjects factors. Significant interactions were pursued for each VAMS scale separately using a mixed-effect ANOVA with groups (3) and sampling time points (5) as between- and within-subjects factors, respectively. Similarly, STAI-S, PANAS-PA, and PANAS-NA scores were separately examined using a mixed-effect ANOVA with groups (3) and sampling time point (3) as between- and within-subjects factors, respectively. For all post-hoc tests alpha was set at 0.05 and adjusted (Bonferroni) for multiple comparisons.

In addition to LCMM, cortisol response to stress was examined by calculating the area under the curve (AUC), a continuous measure that captures the total hormone concentration versus time-dependent change (Pruessner et al., 2003). Both area under the curve with respect to ground (AUCg), and area under the curve with respect to increase (AUCi) were computed, as it was shown that these formulas may reveal different associations across variables (Pruessner et al., 2003). Finally, for descriptive purposes, a responder rate of participants showing a cortisol increase ≥2.5 nmol/l was also calculated.

For structural MRI, gray matter volumes were extracted for each participant from anatomical masks of the amygdala and hippocampus following a priori hypotheses relating to their key role in HPA regulation. Potential relations between amygdala and hippocampus gray matter volumes and cortisol response to stress were examined using a mixed-effect ANOVA with the three cortisol classes as a between-subjects factor and left and right amygdala and hippocampus gray matter volumes as a within-subjects factor (separately per region). In addition, exploratory analyses were also conducted to compare gray matter volumes of the hippocampus and amygdala with continuous
indices of cortisol release (individual AUCi and AUCg values) using hierarchical regression, with linear regression in the first step and quadratic regression in the second step, such that a significant $F$ change would indicate a quadratic effect. Given a total of eight regression analyses (2 regions, 2 sides, 2 AUC measures), $p$ value for significance was set at $0.05/8 = 0.00625$ (Bonferroni corrected).

**Results**

**Overall effect of stress:** Repeated measures ANOVA with cortisol responses to stress revealed a main effect of time ($F_{(5,390)} = 47.25$, $p < 0.001$), with a strong quadratic effect ($F_{(1,78)} = 40.70$, $p < 0.001$). Post-hoc analyses revealed significant stress-induced increase in mean cortisol levels from before the MAST ($T_{0 \text{ min}}$) to 25 ($T_{+25 \text{ min}}$), 50 ($T_{+50 \text{ min}}$) and 65 minutes ($T_{+65 \text{ min}}$) (all $p$’s < 0.001); but not 100 minutes following its onset ($T_{+100 \text{ min}}$; $p = 0.2$) (Figure 2A). For alpha amylase the main effect of time was also significant ($F_{(5,390)} = 9.29$, $p < 0.001$). Post hoc tests revealed some increase from before the MAST to slightly following its completion, though it did not reach significance level ($T_{+25 \text{ min}}$; $p = 0.151$). There was, however, a significant decrease in alpha amylase levels from 25 minutes following MAST onset to 40 minutes later ($T_{+25 \text{ min}} > T_{+65 \text{ min}}$; $p < 0.001$) (Figure 2B).

When considering VAMS ratings, repeated measures ANOVA also resulted in a highly significant main effect of time, indicating an overall increase in negative affect across all five VAMS scales ($F_{(5,400)} = 96.93$, $p < 0.001$), with the expected quadratic effect ($F_{(1,80)} = 124.40$, $p < 0.001$). Mirroring the cortisol results, negative mood across all scales was elevated 25 ($T_{+25 \text{ min}}$), 50 ($T_{+50 \text{ min}}$) and 65 minutes ($T_{+65 \text{ min}}$) following...
Admon et al.  

Trajectories of cortisol response to acute stress

stress onset relative to before stress ($T_{0\min}$; all $p$’s < 0.001). Furthermore, unlike cortisol, negative mood was still elevated at the final sampling time point, 100 minutes post stress onset, relative to before stress ($T_{100\min} > T_{0\min}$; $p < 0.001$), yet it was significantly less negative at that point relative to 65 minutes post stress onset (after additional 35 minutes that included the relief component; $T_{65\min} > T_{100\min}$; $p < 0.001$) (Figure 2C).

Repeated measures ANOVA on self-reported anxiety state (STAI-S) revealed a main effect of time ($F_{(2,162)} = 80.62$, $p < 0.001$), due to significantly higher anxiety levels after the MAST ($T_{25\min}$) compared to both arrival ($T_{-80\min}$) and session completions ($T_{100\min}$) ($p$’s < 0.001). Interestingly, anxiety levels were still significantly higher at session completion relative to arrival ($T_{100\min} > T_{-80\min}$; $p = 0.004$) (Figure 2D). A similar analysis for positive affect (PANAS-PA) also revealed a main effect of time ($F_{(2,162)} = 29.52$, $p < 0.001$) that was driven by significant reduction in positive affect after the MAST compared to arrival ($T_{25\min} < T_{-80\min}$; $p < 0.001$), a reduction that was still evident at session completion ($T_{100\min} < T_{-80\min}$; $p < 0.001$) (Figure 2E). Correspondingly, a main effect of time ($F_{(2,162)} = 38.88$, $p < 0.001$) for negative affect (PANAS-NA) was found to be driven by significantly higher negative affect after the MAST compared to both arrival and session completion ($T_{25\min} > T_{-80\min}$; $T_{25\min} > T_{100\min}$; $p$’s < 0.001) (Figure 2F). See (Treadway et al., in press) for additional analyses from this cohort.

Latent class linear mixed modelling (LCMM): Approximately 72% of participants from our sample (57/79) could be classified as cortisol “responders” based on a cortisol increase ≥2.5 nmol/l, suggesting that the laboratory procedure succeeded in activating HPA response. Despite these robust overall effects, patterns of cortisol response throughout the session greatly differed across participants, showing various patterns of
change over time (Figure 3A). Accordingly, individual cortisol response patterns were analyzed via LCMM aiming to estimate whether 2, 3, or 4 distinct trajectories of cortisol response best captured variability in the data without requiring a priori assumptions. BIC criteria comparing the different LCMM models yielded BIC$_2$ = 424, BIC$_3$ = 413, BIC$_4$ = 416, indicating that model allowing for 3 latent classes was optimal. These three classes were labeled based on their distinct trajectories of cortisol response to stress as hyper-response ($n = 10$), moderate-response ($n = 21$), and mild-response ($n = 48$). Figure 3B depicts the estimated mean trajectories of the three groups. Note that in the second-best model (4-class model) the 4th class was small (7.6% of the sample), thus contributing very little value beyond the 3-class model. The model allowing for 3 latent classes also had a good discrimination ability, with <15% of participants a posteriori classified in other classes than the one initially assigned (see Table 2 for full statistic description).

Mixed-effect ANOVA with the three groups from LCMM as a between-subjects factor and the five cortisol sampling time points as a within-subjects factor revealed, as expected, a significant main effect of time, which was not pursued by post-hoc tests. The ANOVA further revealed a main effect of groups ($F_{(2,76)} = 6.17, p = 0.003$) due to significantly higher overall cortisol release in the hyper-response class relative to the mild-response class ($p = 0.009$). Critically, a significant group by time interaction ($F_{(8,304)} = 36.92, p < 0.001$) also emerged, which was followed up by within- and between-group post-hoc comparisons. Within-group analyses raveled a significant stress-induced increase in mean cortisol release from before the MAST ($T_{0\text{ min}}$) to both 25 ($T_{+25\text{ min}}$) and 50 ($T_{+50\text{ min}}$) minutes following MAST onset (all $p$'s < 0.05) in all three classes (Figure
3B). Between group comparison, however, revealed that the moderate-response class exhibited significantly increased cortisol release 25 minutes following MAST onset (T +25 min) relative to both mild-response and hyper-response classes (p < 0.001, p = 0.008, respectively), while the hyper-response class exhibited significantly increased cortisol release 50 (T +50 min) and 65 (T +65 min) minutes following MAST onset relative to both mild-response and moderate-response classes (all p’s < 0.001) (Figure 3B).

Effects of stress by cortisol class: When considering the VAMS ratings, mixed-effect ANOVA revealed a main effect of time (F(4,304) = 23.96, p < 0.001), a group x scale interaction (F(8,1216) = 2.74, p = 0.006), and a group x time x scale interaction (F(32,1216) = 2.46, p < 0.001), which was pursued for each scale separately. This resulted in three out of the five scales showing a significant group x time interaction (Happy-Sad, F(8,304) = 2.06, p = 0.039; Relaxed-Tense, F(8,304) = 3.10, p = 0.002; Friendly-Hostile, F(8,304) = 2.15, p = 0.031). For the Happy-Sad scale post-hoc tests indicated that these results were driven by significantly higher sadness scores 50 minutes (T +50 min) following stress onset in the hyper-response class relative to both the mild-response and moderate-response classes (p = 0.006, p = 0.003, respectively) (Figure 4A). A similar pattern emerged with regard to the Relaxed-Tense scale, only that increased tension among hyper-response relative to the mild-response and moderate-response classes occurred 25 minutes following stress onset (T +25 min) (p = 0.05, p = 0.023, respectively) (Figure 4B). For the Friendly-Hostile scale, the analysis revealed that the mild-response class was driving the effect, exhibiting a trend towards more hostility 25 minutes following stress onset (T +25 min) compared to the moderate-response class (p = 0.080) (Figure 4C). For state anxiety (STAI-S) and positive affect (PANAS-PA) the mixed-effect
ANOVAs revealed no effect of groups (Figure 4D-E). For negative affect (PANAS-NA), however, there was a significant group x time interaction ($F_{(4,148)} = 3.17, p = 0.016$) driven by significantly more negative affect 25 minutes following stress onset in the mild-response class compared to the moderate-response class ($p = 0.037$) (Figure 4F).

**Structural variability and cortisol class**: Mixed-effect ANOVA with the three groups from LCMM as a between-subjects factor and left and right hippocampal gray matter volume as a within-subjects factor revealed only a significant main effect of side due to overall more gray matter volume on the right compared to the left side ($F_{(1,66)} = 33.52, p < 0.001$), with no group or group x side effects ($p = 0.99, p = 0.36$, respectively, data not shown). Analyses of amygdala gray matter volume yielded similar results, including a main effect of side (right > left; $F_{(1,66)} = 138.04, p < 0.001$), and no group or group x side effects ($p = 0.99, p = 0.16$, respectively, data not shown).

**Structural variability and cortisol AUC**: Hierarchical regressions with the continuous cortisol response measure AUCg separately for participants' gray matter volumes in the left and right amygdala and hippocampus revealed a significant relation only for the right hippocampus. Further, a significant $F$ change in the hierarchical regression model ($F_{(1,66)} = 12.56, p = 0.001$; significant after Bonferroni correction ($p < 0.00625$)) indicated that high and low AUCg values were both associated with less right hippocampal gray matter volume compared to moderate AUCg levels ($r = 0.421$) (Figure 5). Similar results appeared when using AUCi as the dependent measure in the regression, showing only a significant, quadratic, relation between AUCi magnitude and right hippocampal gray matter volume ($F_{(1,66)} = 4.86, p = 0.031; r = 0.262$). AUCi result, however, did not survive multiple comparison correction and should therefore considered with caution.
Discussion

The overarching goal of the current study was three-fold. First, we aimed to evaluate an acute stress laboratory procedure specifically designed to yield a prolonged effect. Towards this end, participants completed the MAST, a robust laboratory procedure for acute stress induction (Smeets et al., 2012), and were told immediately afterwards that they would soon need to repeat the task due to their poor performance. Results indicated that this revised version of the MAST yielded stress-induced increase in cortisol release 25, 50, and 65 minutes post stress onset, as well as a shift towards more negative affect in all of these time points and up to 100 minutes following stress onset. Taken together, this represents a relatively prolonged period of acute stress induction compared to previous reports, including studies using the original MAST procedure. Given the inherent delay between stress onset and increase in salivary cortisol, and uncertainties whether individuals differ in such latency, efficient manipulations that induce a sustained acute stress response could be useful in future research.

Trajectories of cortisol response to prolonged acute stress

By probing individual patterns of stress-induced cortisol release over this relatively prolonged time-period and analyzing these patterns using latent class linear mixed modelling (LCMM), our second aim was to investigate the number of distinct trajectories of cortisol response that best captured variability in our data without a priori assumptions. This analysis revealed three distinct trajectories of cortisol response, labeled as hyper-response, moderate-response, and mild-response, all exhibiting a
significant, quadratic increase in cortisol release. Generalization of this finding should proceed with caution. First, since only healthy females were included no predictions can be made regarding cortisol patterns that may emerge in response to stress in males, or among psychiatric samples. More critically, it is possible that specific sample characteristics influenced the final number of classes identified. In fact, we expect that a higher number of cortisol trajectories may emerge among larger as well as more environmentally and/or genetically diverse samples. While the exact number of classes may vary based on sample characteristics, our results point to the importance of adequately modeling individual differences in cortisol response to stress, as well as including multiple measures of the response trajectory when probing endocrine stress-response. In this regard it is important to note that the endocrine stress-response system, and most prominently HPA axis, acts via tightly regulated negative feedback loops that control the onset, magnitude, and duration of stress response activation (Joels and Baram, 2009). Accordingly, these different parameters of the stress response system may all contribute to determine its physiological and affective outcomes.

**Linking cortisol and affective responses to prolonged acute stress**

In parallel with variability in cortisol stress response patterns, individuals are also known to differ in their affective responses to stress, including in measures of response threshold, amplitude, and rise time to peak (Davidson, 2000). In the current study, we found that while all the three cortisol groups experienced a shift towards more negative affective state, individuals in the moderate response class exhibited less stress-induced
sadness and tension relative to individuals in the hyper-response class, as well as less
hostility (trend) and negative affect relative to individuals in the mild response class.
Such quadratic association between the magnitude of cortisol response to stress and
affective responses may account for the paucity of studies reporting a linear link
between the two (Campbell and Ehlert, 2012). This quadratic pattern also dovetails with
the well-established inverted U-shape relationship between basal levels of cortisol and
cognitive performance, with beneficial effects of moderately elevated cortisol levels on
cognition (de Kloet et al., 1999). Why “too little” and “too much” cortisol are associated
with reduced cognitive performance and/or heightened affective response to stress is
not clear. One potential explanation may relate to the notion that, at least for some
susceptible individuals, repeated hypersecretion of cortisol may have caused
desensitization of the HPA axis, eventually resulting in reduced HPA sensitivity (Heim et
al., 2000). This interpretation remains speculative in regard to our sample however, as
we did not assess participants’ life-time exposure to stress. In this regard, it has been
suggested that life-time exposure to stress itself may affect stress vulnerability via a
quadratic pattern, challenging the assumption that stress and negative outcomes show
a simple, linear relationship (Liu, 2015). Our findings suggest that cortisol and affective
response to stress may also exhibit a non-linear relation.

The third aim of the current study was to investigate potential relations between
cortisol response to stress and structural variability in the amygdala and hippocampus,
key HPA regulatory regions. Resembling the link between cortisol and affective responses, a quadratic link was also present between a continuous measure of cortisol response to stress (AUCg) and hippocampal gray matter volume. Specifically, both high and low levels of stress-induced cortisol release were found to be associated with less right hippocampal gray matter volume compared to moderate cortisol release. This finding may be discussed in light of extensive preclinical evidence showing that exposure to cumulative stress may translate to morphological damage, most pronouncedly in the hippocampus (Sapolsky et al., 1990; Joels et al., 2004). Increased susceptibility of the hippocampus to the effects of stress was attributed to its important role in stress regulation, as reflected by its substantial number of cortisol receptors (McEwen, 1999). In humans, reduced hippocampal volume has been the most commonly described neural structural abnormality in people who were exposed to traumatic stress and consequently develop an anxiety disorder such as posttraumatic stress disorder (PTSD) (Smith, 2005). Accordingly, reduced hippocampal volume among individuals exhibiting mild cortisol response may further link such response pattern to repeated hypersecretion of cortisol. This interpretation, again, could be tested in studies assessing participants’ life-time exposure to stress.

Limitations and conclusions

A few limitations of our study should be emphasized. First, it is not clear why alpha-amylase levels did not increase in response to stress in the current study, nor why only some affective scales showed a significant relation with cortisol trajectories while other did not. Second, our LCMM-based classes did not differ in their hippocampal
and amygdala gray matter volumes and the link between cortisol response and hippocampal volume only emerged when treating cortisol response as a continuous measure. This could relate to our modest sample size for these analyses, with only seven participants with MRI data in the hyper-response class (20 in the moderate-response class and 42 in the mild-response class). Whether the link between cortisol response to prolonged acute stress, affective responses, and hippocampal gray matter volume is categorical or continuous by nature, is the topic for future research. A third limitation relates to the fact that factors that were previously shown to impact emotional and cortisol responses to acute psychosocial stressor in healthy volunteers, including menstrual cycle phase (Duchesne and Pruessner, 2013), contraceptives usage (Roche et al., 2013), and personality traits (Childs et al., 2014), did not interact with cortisol classes in the current study [Chi-square tests revealed no effects of menstrual cycle phase or contraceptives use on cortisol class ($\chi^2 = 1.82$, $\chi^2 = 2.83$, respectively); mixed-effect ANOVA revealed no interaction of NEO five-factor personality scores with cortisol class ($p = 0.82$)]. These inconsistencies could stem from the fact that previous work did not apply LCMM to classify cortisol response but rather relied on mean group measures. Alternately, it may also be the case that additional factors we did not control for may have influenced current results, including age (Hostinar et al., 2014), fatigue (Bower et al., 2005), and body shape (Epel et al., 2000), to name a few. While controlling for all potential contributors to the stress response is practically impossible, future studies may choose to assess multiple factors and try to incorporate them into multi-dimensional models to account for additional variability in the data.
Taken together, our results suggest that investigating cortisol responses to stress without *a priori* assumptions regarding pattern of change can uncover distinct trajectories of cortisol response to prolonged acute stress among healthy females that would have been overlooked in conventional analyses parsing participants into cortisol responders and non-responders. Most critically, identifying such distinct cortisol trajectories enabled us to discover the often-hidden link between stress-induced cortisol release and patterns of affective responses to stress as well as hippocampal structural variability. Given that neuroendocrine stress responses are also conceptualized as biomarkers reflecting individual differences in stress resilience and susceptibility to psychopathology and disease (Feder et al., 2009), these insights regarding individual differences in trajectories of cortisol response to stress may have clinical implications.
References


Trajectories of cortisol response to acute stress

Admon et al.,


interplay between the limbic system and hypothalamic-pituitary-adrenal axis.

Development and psychopathology 26:1321-1335.


Trajectories of cortisol response to acute stress

Admon et al.


Trajectories of cortisol response to acute stress


Figure Legends

Figure 1. Timeline: Schematic diagram illustrating the design of the study session. Participants provided written consent before completing two tasks [one reward task (PRT) and one reaction time task (RT)], a clinical interview to determine eligibility (SCID), the MAST stress task, and the PRT and RT tasks again. To prolong the effect of the acute stressor, immediately upon MAST completion participants were told by a non-emphatic male study staff they had not yet met that their performance in the math portion was not good enough and that they would need to repeat the task following the administration of the remaining tasks and questionnaires. Later in the session participants were informed that repeating the task was not necessary since their performance was “good enough” (i.e., relief). Throughout the session participants provided six saliva samples, eight VAMS ratings, and three self-report measures of anxiety and affect. MAST - Maastricht Acute Stress Test; VAMS - Visual Analogue Mood Scale.

Figure 2. Overall effect of stress: Change in (A) cortisol, (B) alpha amylase, (C) VAMS rating, (D) state anxiety, (E) positive affect, and (F) negative affect throughout the session. Across all measures, there was a significant effect of time driven by stress induced increase in cortisol release and negative affect. Notably, cortisol levels and negative affect were still elevated 65 and 100 minutes post-stress onset, respectively, reflecting a relatively prolonged acute stress induction. Participants were fully debriefed and their mood had returned to baseline before they left the laboratory. * p < 0.05.
Figure 3. Latent class linear mixed modelling (LCMM): (A) Individual patterns of cortisol response throughout the session ($n=79$). (B) Applying LCMM on these data revealed that the model that best fit our data included three latent classes, labeled based on their distinct trajectories of cortisol response to stress as hyper-response ($n=10$), moderate-response ($n=21$), and mild-response ($n=48$). Note that all three groups exhibited a significant stress-induced increase in cortisol release 25 as well as 50 minutes following stress onset. * $p < 0.05$.

Figure 4. Effects of stress by cortisol class: Change in (A) Happy-Sad scale, (B) Relaxed-Tense scale, (C) Friendly-Hostile scale, (D) state anxiety, (E) positive affect, and (F) negative affect throughout the session as a function of cortisol class. Note that individuals in the moderate response class exhibited less stress-induced sadness and tension relative to individuals in the hyper-response class, as well as less hostility (trend) and negative affect relative to individuals in the mild response class. * $p < 0.05$.

Figure 5. Structural variability and cortisol AUCg: A hierarchical regression model revealed a quadratic link between the continuous cortisol measure AUCg and hippocampal gray matter volume ($r = 0.421, p = 0.001, n=79$), such that individuals with both high and low levels of stress-induced cortisol release had less right hippocampal gray matter volume compared to individuals with moderate cortisol release.
Consent

T_{80} → PRT1 → RT1 → SCID

0  20  40  60

Asked to repeat

T_0

T_{-25} → stress

80  100

T_{-50}

T_{-65} → relief

120  140  160

Session end

T_{100}

180 (min)

★ PANAS - Positive and Negative Affect
★ STAI-S - State Anxiety

★ VAMS - Visual Analogue Mood Scale
★ Saliva sampling - Cortisol & Alpha Amylase
Quadratic relationship between hippocampal gray matter volume and cortisol AUCg

$$r = 0.42$$
Table 1. Sample demographic information

<table>
<thead>
<tr>
<th></th>
<th>n (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Gender</strong></td>
<td>88 (100%)</td>
</tr>
<tr>
<td>Female</td>
<td>88 (100%)</td>
</tr>
<tr>
<td><strong>Handedness</strong></td>
<td>88 (100%)</td>
</tr>
<tr>
<td>Right</td>
<td>88 (100%)</td>
</tr>
<tr>
<td><strong>Race</strong></td>
<td></td>
</tr>
<tr>
<td>Caucasian</td>
<td>61 (69%)</td>
</tr>
<tr>
<td>Black</td>
<td>15 (17%)</td>
</tr>
<tr>
<td>Asian</td>
<td>10 (12%)</td>
</tr>
<tr>
<td>Unknown</td>
<td>2 (2%)</td>
</tr>
<tr>
<td><strong>Ethnicity</strong></td>
<td></td>
</tr>
<tr>
<td>Non-Hispanic</td>
<td>81 (92%)</td>
</tr>
<tr>
<td>Hispanic</td>
<td>5 (6%)</td>
</tr>
<tr>
<td>Unknown</td>
<td>2 (2%)</td>
</tr>
<tr>
<td><strong>Income</strong></td>
<td></td>
</tr>
<tr>
<td>&lt;$10,000</td>
<td>11 (13%)</td>
</tr>
<tr>
<td>$10,000–$25,000</td>
<td>10 (11%)</td>
</tr>
<tr>
<td>$25,000–$50,000</td>
<td>21 (24%)</td>
</tr>
<tr>
<td>$50,000–$75,000</td>
<td>18 (20%)</td>
</tr>
<tr>
<td>$75,000–$100,000</td>
<td>17 (19%)</td>
</tr>
<tr>
<td>&gt;$100,000</td>
<td>11 (13%)</td>
</tr>
<tr>
<td><strong>Marital Status</strong></td>
<td></td>
</tr>
<tr>
<td>Married</td>
<td>17 (19%)</td>
</tr>
<tr>
<td>Unmarried</td>
<td>71 (81%)</td>
</tr>
<tr>
<td><strong>Smoking Status</strong></td>
<td>88 (100%)</td>
</tr>
<tr>
<td>Non-Smoker</td>
<td>88 (100%)</td>
</tr>
<tr>
<td><strong>Menstrual cycle phase</strong></td>
<td></td>
</tr>
<tr>
<td>Follicular</td>
<td>62 (70%)</td>
</tr>
<tr>
<td>Luteal</td>
<td>26 (30%)</td>
</tr>
<tr>
<td><strong>Contraceptives usage</strong></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>44 (50%)</td>
</tr>
<tr>
<td><strong>Median (S.D.)</strong></td>
<td></td>
</tr>
<tr>
<td>Education (years)</td>
<td>16.5 (1.7)</td>
</tr>
<tr>
<td>BMI</td>
<td>22.6 (3.4)</td>
</tr>
</tbody>
</table>
Table 2. LCMM model statistics

<table>
<thead>
<tr>
<th>Between Model Comparison</th>
<th>Log likelihood</th>
<th>NPM&lt;sup&gt;a&lt;/sup&gt;</th>
<th>BIC&lt;sup&gt;b&lt;/sup&gt;</th>
<th>%class1&lt;sup&gt;c&lt;/sup&gt;</th>
<th>%class2&lt;sup&gt;c&lt;/sup&gt;</th>
<th>%class3&lt;sup&gt;c&lt;/sup&gt;</th>
<th>%class4&lt;sup&gt;c&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>2 Classes</td>
<td>-185.89</td>
<td>12</td>
<td>424.22</td>
<td>40.5</td>
<td>59.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3 Classes</td>
<td>-169.81</td>
<td>17</td>
<td>413.89</td>
<td>26.6</td>
<td>12.7</td>
<td>60.7</td>
<td></td>
</tr>
<tr>
<td>4 Classes</td>
<td>-159.67</td>
<td>22</td>
<td>415.47</td>
<td>7.6</td>
<td>26.6</td>
<td>11.4</td>
<td>54.4</td>
</tr>
</tbody>
</table>

Within the 3 Class Model - Fixed effects allowing for cubic time (t) trends

<table>
<thead>
<tr>
<th>n (%)</th>
<th>Coefficient</th>
<th>Se</th>
<th>Wald</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Class 1 21 (26)</td>
<td>t</td>
<td>8.47</td>
<td>3.06</td>
<td>2.78</td>
</tr>
<tr>
<td></td>
<td>t&lt;sup&gt;2&lt;/sup&gt;</td>
<td>32.88</td>
<td>5.51</td>
<td>5.97</td>
</tr>
<tr>
<td></td>
<td>t&lt;sup&gt;3&lt;/sup&gt;</td>
<td>0.13</td>
<td>1.84</td>
<td>0.07</td>
</tr>
<tr>
<td>Class 2 10 (13)</td>
<td>t</td>
<td>-40.52</td>
<td>3.46</td>
<td>-11.69</td>
</tr>
<tr>
<td></td>
<td>t&lt;sup&gt;2&lt;/sup&gt;</td>
<td>-34.27</td>
<td>3.95</td>
<td>-8.67</td>
</tr>
<tr>
<td></td>
<td>t&lt;sup&gt;3&lt;/sup&gt;</td>
<td>-12.14</td>
<td>1.62</td>
<td>-7.51</td>
</tr>
<tr>
<td>Class 3 48 (61)</td>
<td>t</td>
<td>4.29</td>
<td>2.52</td>
<td>1.71</td>
</tr>
<tr>
<td></td>
<td>t&lt;sup&gt;2&lt;/sup&gt;</td>
<td>-17.07</td>
<td>4.02</td>
<td>-4.25</td>
</tr>
<tr>
<td></td>
<td>t&lt;sup&gt;3&lt;/sup&gt;</td>
<td>-3.97</td>
<td>1.36</td>
<td>-2.92</td>
</tr>
</tbody>
</table>

Within the 3 Class Model - Mean of posterior probabilities (%) in each class

<table>
<thead>
<tr>
<th>n (%)</th>
<th>Class 1</th>
<th>Class 2</th>
<th>Class 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Class 1 (n=21)</td>
<td>91</td>
<td>4</td>
<td>5</td>
</tr>
<tr>
<td>Class 2 (n=10)</td>
<td>8</td>
<td>85</td>
<td>7</td>
</tr>
<tr>
<td>Class 3 (n=48)</td>
<td>4</td>
<td>1</td>
<td>95</td>
</tr>
</tbody>
</table>

<sup>a</sup>Number of Model Parameters

<sup>b</sup>Bayesian Information Criterion

<sup>c</sup>Posterior proportion for each class