Locus Coeruleus Drive in Relation to Trait Anxiety and Behavioral Performance during Stress

Master thesis

Mirthe Regelink

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Supervisors:

Dr. Nils Kohn

Anne-Kathrin Brehl MSc.

Second reader

Dr. Erno Hermans

Radboud University Radboudumc

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M. Regelink

Abstract

The noradrenergic drive from the locus coeruleus (LC) plays an essential role in the neural stress response, as it induces a neural network shift to enable an adaptive behavioral response. In addition, LC drive influences the risk of developing an anxiety disorder, as it regulates brain areas involved in anxiety. The relationship between LC drive and anxiety has not yet been studied in healthy individuals. We therefore investigated the relationship of pupil dilation differences between a threatening and safe environment as a proxy for LC drive, with varying levels of preclinical anxiety and behavioral performance. Participants were selected with a pre-screening for elevated trait anxiety using the Hospitalized Anxiety and Depression Scale (n = 202). We employed an oddball paradigm with a threat-of-shock manipulation to engage the LC. Our results point towards an inverted-U-shaped relationship between anxiety and the pupil dilation differences between the threatening and safe blocks. This indicates that individuals with moderate anxiety levels had a larger dilation difference between the threatening and safe blocks as compared to individuals with lower and higher anxiety. A larger dilation difference indicates that the LC distinguished more between threat and safe blocks. In addition, a moderated mediation model shows that LC drive mediated the relationship between anxiety and behavioral performance (reaction times) and that the relationship between anxiety and LC drive in this mediation was moderated by the level of threat. This model aims to explain individual differences in behavior during stress and points to the existence of non-linear moderation processes.

Introduction

In a stressful encounter, the human physiological response typically involves a heightened heart rate, rapid breathing and perspiration. This results from upregulation of the sympathetic nervous system, which leads to a release of catecholamines (Jansen *et al.,* 1995). This response allows the individual to actively cope with the stressor and is characterized as the fight or flight response (Jansen *et al.,* 1995). On a neural level, it is accompanied by the release of noradrenaline, which induces a network shift in the brain allowing a rapid, comprehensive and coordinated stress response (Bouret and Sara, 2005; Hermans *et al.,* 2011). This noradrenaline induced neural shift results for example in decreased executive functioning as well as increased vigilance and negative affect (Hermans *et al.*, 2014). The combination of physiological, cognitive and (noradrenaline induced) neural responses ensures that humans can cope adaptively with stressors.

Neural noradrenaline (NA) is synthesized in the locus coeruleus (LC), a group of noradrenergic nuclei located in the dorsorostral pons (Aston-Jones and Cohen, 2005). The LC is the main source of NA in the forebrain and the sole NA source in the cortex (Valentino and Van Bockstaele, 2008; Sara, 2009). These widespread projections allow it to exert influence over the activity throughout the entire brain. As a result, the LC promotes optimal processing of sensory information with the NA induced neural network shift (Hermans *et al.*, 2011; Sara and Bouret, 2012). The LC achieves this by maintaining the necessary arousal level, which is supported by rapidly switching between its two firing modes, phasic and tonic (Aston-Jones and Cohen, 2005). Phasic firing is initiated by discrete sensory stimuli, and is sensitive to novel, unexpected and other salient stimuli (Krebs *et al.,* 2018). A phasic response is often described as a temporal attentional filter, allowing processing of the relevant stimulus (Aston-Jones and Cohen, 2005). Tonic firing on the other hand, is positively correlated to the state of arousal and plays a role in vigilance, influencing whether the individual is task-engaged or more broadly oriented. Tonic LC drive has been linked to the Yerkes-Dodson curve, which states an inverted-U relationship between arousal and performance (Aston-Jones and Cohen, 2005). During low and high tonic firing, performance is relatively poor because of an inattentive or distractible state, respectively. In contrast, during moderate LC tonic activity there is a task-engaged state. The combination of peripheral arousal with these firing modes allows the LC to mediate cognition through a reorientation of cortical networks and enable an adaptive behavioral response.

As the LC is located in the brainstem and only contains between 22.000 and 51.000 neurons in humans, detecting its activity with fMRI is challenging (Mouton *et al.*, 1994). One fMRI study in humans used a structural scan sensitive to neuromelanin, a byproduct of NA synthesis, to localize the LC. They reported a relationship between pupil diameter and BOLD LC activity using individual neuromelanin based masks for their fMRI analysis (Murphy *et al.,* 2014). As this method requires considerable resources and effort, a more economic proxy might be sensible. Therefore, studies have investigated pupil dilation as a proxy for LC drive (Bradley *et al.*, 2008; Joshi *et al.*, 2016; Reimer *et al.*, 2016). For example, Joshi and colleagues (2016) used single-cell recordings to record LC drive and pupil dilation simultaneously in monkeys. They concluded that pupil activity is related to LC drive, as they are driven by the same mechanism. Another animal study concluded that pupillometry can be used to track the activity of multiple neuromodulators, including NA (Reimer *et al.,* 2016). Furthermore, Bradley and colleagues (2008) showed that, just as LC drive, pupil dilation is sensitive to emotional arousal and not the valence of a stimulus. Taken together, animal and human studies point towards a relationship between pupil diameter and LC drive, indicating that pupil dilation can be used as a valid proxy for LC drive.

LC drive plays an essential role in the neural stress response. Hendrickson and Raskind (2016) for example state that the LC system is a central, modifiable link within a network of stress-response systems. Brehl and colleagues (2020) propose a similar model stating that increased amygdala responsiveness, a characteristic of anxiety, can result from multiple regulatory networks, including the LC. The LC regulates activity in different brain areas by different levels of NA. Moderate NA levels engage high affinity α2 adrenergic receptors, strengthening prefrontal cortex (PFC) activity and supporting higher cognitive functions whilst weakening amygdala activation. In contrast, during stress higher NA levels engage lower affinity α1 and β adrenergic receptors leading to increased amygdala function and lower PFC firing rates which reduces cognitive control (Hermans *et al.*, 2014; Arnsten *et al.*, 2015; Giustino and Maren, 2018). In addition, higher NA levels impede GABA transmission in the amygdala, thereby also increasing its excitability (Tanaka *et al.,* 2000; Tully *et al.,* 2007). Based on these mechanisms, the LC accurately modulates cognition; phasic activity for example influences attention processes and tonic drive affects vigilance. The LC thus plays a crucial role in the mammalian stress response as different arousal states promote different cognitive functions.

An excessive stress response is characteristic of anxiety (Anon, DSM-5, 2013), indicating that the LC also plays a role in the risk for developing anxiety disorders. Studies have for example observed increased NA levels or altered NA reactivity in posttraumatic stress disorders (PTSD) (Geracioti *et al.*, 2001; Hendrickson and Raskind, 2016). As mentioned above, the LC also regulates the amygdala, which is hypersensitive in anxiety disorders (Brehl *et al.*, 2020). Because of this relationship between LC drive and anxiety disorders, studies have investigated the specific role of the LC in anxiety. Animal studies using photostimulation or optogenetics to stimulate the LC have observed an increase in anxiety-like behaviors with increased LC firing rates (McCall *et al.,* 2015; McCall *et al.,* 2017). In addition, NA receptor antagonists have anxiolytic effects and have been used for treatment of PTSD (Singh *et al.,* 2016; Strawn and Geracioti, 2008). However, there are only a few human studies investigating LC activity or pupil dilation in relation to anxiety. Naegeli and colleagues (2018) found evidence of LC hyperactivity in PTSD. After exposing patients to sound bursts, they exhibited increased autonomic arousal such as increased skin conductance and increased pupil dilation as well as increased neural activation in a brainstem cluster identified as the LC. Another study observed larger pupil dilation in a high-PTSD-symptom group compared to a low-symptom group (Kimble *et al.,* 2010). Finally, Cascardi and colleagues (2015) showed that PTSD patients exhibited significantly more pupil dilation to threat-related stimuli compared to trauma-exposed controls, whilst their responses to neutral elements were similar. The present literature shows that increased LC firing promotes anxiety-like behavior in animals and has observed increased pupil responses in PTSD patients, particularly in response to trauma-related stimuli.

Taken together, individual differences in LC drive might indicate the risk for developing anxiety disorders. However, in healthy populations LC drive or pupil dilation in relation to varying levels of anxiety has received little attention. Therefore, we will investigate the relationship between pupil dilation differences between a threatening and safe environment, as a proxy for LC drive, and varying levels of preclinical trait anxiety. We expect an inverted-U-shaped relationship, in accordance with the Yerkes-Dodson curve. In addition, we are interested in the effects of LC drive and anxiety on behavior. Therefore, we will investigate the effects of both pupil dilation differences (threat minus safe) and anxiety on accuracy and reaction times. We expect a decrease in reaction times as pupil dilation differences (threat minus safe) increase, in accordance with Murphy and colleagues (2014) who found decreased reaction times with increasing LC BOLD activity in an oddball paradigm. For pupil dilation in relation to accuracy, we have not defined a specific direction for the expectancy. For the effect of anxiety on reaction times and accuracy, we expect inverted-U-shaped curves in accordance with the Yerkes-Dodson curve. Finally, we aim to explore the relationship between pupil dilation differences (threat minus safe) as a proxy for LC drive, the level of anxiety, the threat induction in the task and our behavioral measures. To address these questions, we will study pupil dilation and behavioral performance in a population of healthy individuals with elevated anxiety traits (HADS-A > 7) while performing an oddball paradigm with a threat-of-shock manipulation to manipulate LC firing rates.

Methods

Pre-screening

Participants were recruited with advertisements on university websites, announcements during lectures and posters and flyers that were spread on campus. These advertisements contained a link to the online screening (www.soscisurvey.de), which consisted of the Dutch version of the Hospitalized Anxiety and Depression Scale (HADS, supplemental material) (Zigmond and Snaith, 1983), demographical questions and an MRI screening. To oversample for high trait anxiety, all individuals with elevated scores (8 or higher) on the HADS anxiety subscale (HADS-A) received an invite to participate in the fMRI study. In addition, individuals with normal scores on the HADS-A (7 or lower) had a 25% chance to get an invite. All participants that filled in the questionnaire, regardless of whether or not they participated in the fMRI study, took part in a lottery to win an iPad Mini.

Participants

A total of 2206 participants finished the online questionnaire and 485 of them received an invite to participate in the fMRI study. Of those, 250 were interested and scheduled an appointment with the researchers. Five participants eventually did not participate due to various reasons, and six participants did not perform the LC paradigm, mainly due to technical or time issues. Of the remaining 239, eight participants were excluded because of additional reasons, such as incidental findings or clinical diagnosis. When evaluating the data quality, eleven participants were excluded because of problems during the paradigm such as missing shocks or incorrect responding, and seventeen participants were excluded because their eye tracking data was either not available or of bad quality. The total number of participants included in the analysis was 202, 54 of which were male and the mean age was 22.74 (*SD* = 4.88, *min* = 18, *max* = 47). After participation in all tasks and paradigms, they received 35 euros as a financial compensation for their time. The Commissie Mensgebonden Onderzoek (CMO) of the Radboud UMC in Nijmegen in the Netherlands approved the study protocol.

Procedure

This study was part of a larger study into biomarkers of anxiety. For this thesis, only the relevant part of the procedure, design and data will be discussed in detail. The other parts, including the fMRI data for the LC task, will be discussed elsewhere. After arrival, the participants received information about the study protocol and gave written informed consent. Next, they filled out an MRI screening form along with some demographics. After preparing the electrodes for the physiological measurements, the participant went into the scanner room. There, we adjusted the shocker intensity based on a stepwise protocol, to ensure the shock was uncomfortable but not painful (Gladwin *et al.*, 2016). Then, the participant went into the MRI scanner for a session of roughly one hour, which consisted of three paradigms. The first was one was the Hariri task, which required the participant to match either emotions or shapes and this lasted roughly 10 minutes (Hariri *et al.,* 2002). The second task was an emotion regulation paradigm, in which participants had to indicate how they felt after viewing a neutral or a negative picture. For a part of the negative pictures, the participants were asked to regulate their emotions (Kanske *et al.,* 2015). This paradigm lasted roughly 20 minutes. The final paradigm was the LC task, which was an oddball paradigm with a threat-of-shock manipulation and lasted roughly 16 minutes (Yoshiura *et al.,* 1999). This paradigm will be discussed in detail below. The order of the emotion regulation and LC task alternated between participants. In addition to these tasks, a structural T1-weighted image and a resting-state scan were collected. Throughout the scan, we recorded four physiological measures: pupil dilation, heart rate, skin conductance and an electromyography recording of the trapeze muscle. After the scan, the participants completed several questionnaires applied in Dutch. These were the HADS, State-Trait Anxiety Inventory, Inventory of Depressive Symptomatology, Depression Anxiety Stress Scale, Coping Inventory for Stressful Situations, FEEL-E (Emotion Regulation in Adults), NEO Personality Inventory and Youth Trauma Questionnaire. In addition, if the participant had a HADS-A score above 14, they also completed a structured interview with a psychologist (Mini International Neuropsychiatric Interview, version 5, based on DSM-IV). If a psychiatric diagnosis was confirmed based on this interview, the participant was excluded. The appointment lasted approximately three hours in total.

Paradigm

To engage the LC, we employed an oddball paradigm with a threat-of-shock manipulation (Figure 1). Participants were instructed to attend the fixation cross in the middle of the screen in front of them. In this location, the train of stimuli consisting of ‘O’s and ‘X’s, was presented on a grey background. Participants were instructed to ignore the non-target (‘O’), and respond to the target, the oddball ‘X’, with a button press with their right index finger. In total, there were 32 blocks (16 threat-of-shock and 16 safe blocks). Each block lasted 30 seconds and consisted of 15 stimuli. The order of the stimuli was semi randomized. The stimuli were presented for 100ms and in between the stimuli the fixation cross was shown. The inter-trial interval (ITI) was jittered pseudo-randomly, and varied between 1400ms and 2400ms. Each block contained between one to three oddballs, with an average of two oddballs per block. Following each oddball, the ITI was 2400ms.

In addition to the oddball paradigm, there was a threat-of-shock manipulation. All stimuli were surrounded by green or red frames. The participants were informed that if a red frame was present, a shock might be applied during this block (threat block). The green frame on the other hand indicated a safe block in which no shock would be administered. We did not inform the participants about the total number of shocks they would receive. In reality, each participant received two shocks throughout the task. The first shock was administered in the fourth threat block (block number seven or eight in total), the specific trial number varied between participants. The second shock was administered at the same time point for each participant; in the eighth threat block (block number fifteen or sixteen in total) following the tenth stimulus. Half of the participants started with a threat block, the other half

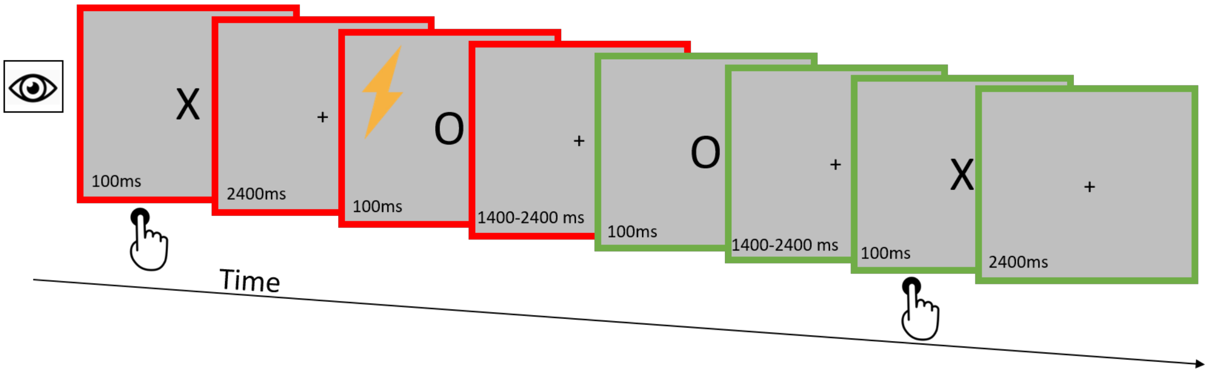


Figure 1: The oddball task with a threat-of-shock manipulation. Participants had to respond to the target (the oddball 'X') with a button press and had to ignore the non-targets ('O'). Following a target, the fixation cross was presented for 2400ms. Following a non-target, the fixation cross was presented in between 1400ms and 2400ms. During the threat-of-shock blocks (red frame), there was a chance that the participant would receive a shock. During the safe blocks (green frame), there was no such threat. In total there were 32 alternating blocks, 16 threat-of-shock and 16 safe blocks, all containing 15 stimuli and on average 2 oddballs. The participants received two shocks throughout the task. We recorded pupil dilation throughout the entire paradigm.

started with a safe block. The threat-of-shock blocks and safe blocks alternated for each participant. The task was presented using the Presentation software, version 20.2. (Neurobehavioral Systems, Inc., 2012).

Eye tracking and processing

Throughout the task, we recorded pupil dilation of the left eye with an SMI Eyetracker camera, using the iView X software (SensoMotoric Instruments). This camera had a sampling rate of 50Hz. The preprocessing of the data was partly based on Kret and Sjak-Shie (2019). Following each stimulus, we extracted the pupil dilation in pixels in a time window of 800ms, which included 40 samples. Outliers were removed if they were more than three standard deviations away from the mean. In addition, trials in which the shocks were administered and the four trials following these were excluded. To correct for eye blinks, two samples preceding and two succeeding a sample with a dilation of fewer than 10 pixels were removed. Next, the remaining data was z-standardized based on the individual mean and standard deviation. For each trial, we calculated the average dilation. If 30 or more samples within the trial were not useable due to reasons mentioned above, such as blinks, the trial was excluded. Finally, for each individual we calculated four means: the mean pupil dilation during the threat blocks for the targets (1) and the non-targets (2) and the mean pupil dilation during the safe blocks for the targets (3) and the non-targets (4).

Peripheral stimulation

The electric shocks were delivered with two Ag/AgCl electrodes attached to the middle phalanges of the fourth and fifth fingers of the left hand. The shock was delivered with an Innostim tens 2K pulse generator, shock duration was 250µs and the intensity varied between 0–80mA. The intensity was adjusted for each participant with a stepwise protocol (Supplemental Material). The pulse generator was placed in an MRI compatible box to prevent interference.

Behavioral data

The behavioral data was recorded by the Presentation Software. A hit was defined as a button press within 2400ms following a target. False alarms were defined as a button press following a non-target and preceding the subsequent stimulus. Multiple button presses within these timeframes were considered as one hit or one false alarm. Using the number of hits and false alarms, we calculated the sensitivity index (d'), by subtracting the normalized false alarm rate from the normalized hit rate, as a measure of accuracy. Next, we extracted the time between the target presentations and the button presses. For this reaction time (RT), outliers were removed if they were more than three standard deviations away from the group mean. Finally, the RT was standardized using the group mean. The mean RT was then calculated for all targets together, and separately for the threat and safe blocks.

Statistical analysis

Statistical analysis were conducted with R-Studio version 1.2.5019 as IDE for R for Windows version 3.6.1 (R-studio Inc.) and SPSS version 25 (IBM Corporation). To start, we checked if the task manipulation worked in two steps. Firstly, we tested if the threat induction and oddball influenced pupil dilation as expected. To test this, we used a two-way ANOVA with pupil dilation as the dependent variable (DV) and block type and stimulus type as predictors. Because we observed inhomogeneity of variance, we conducted an ANOVA with a robust test criterion. In addition, post-hoc tests were performed for a pair-wise comparison. Secondly, we tested if the threat induction influenced behavioral performance, in particular the reaction times (RT) and the accuracy of responses (sensitivity index d'). To this end, we used paired samples t-tests to compare reaction times, number of hits and false alarms and the sensitivity index between the threat and safe blocks.

For our further statistical analysis, we included three parameters of interest: pupil dilation, trait anxiety scores and behavior (RT and d'). Our aim was to investigate how these parameters related and order to test this, we investigated the bivariate associations between the parameters. This consisted of six linear regression models, all of which were tested for the threat and safe blocks separately, and combined. The first model tested the relationship between our behavioral measures reaction time (predictor) and the sensitivity index (DV). The second model tested the effect of the HADS-A scores (predictor) on the sensitivity index (DV). The third model tested the relationship between pupil dilation (predictor) and the sensitivity index (DV). The fourth model tested the effect of the HADS-A scores and squared HADS-A scores (predictors) on pupil dilation (DV). We performed this regression not only for the threat and safe blocks separately, but also for the difference in pupil dilation between the threat and safe blocks. Because we observed an opposite effect for the threat and safe blocks, we were interested in the interaction between anxiety and the threat induction. Therefore, we performed a moderation analysis with pupil dilation as a DV and HADS-A scores, block type and their interaction as predictors. The fifth model tested the effect of pupil dilation (predictor) on reaction times (DV). As we observed a negative relationship in the threat blocks and a positive relationship in the safe blocks, we were interested in the interaction between the LC drive and the threat induction. To this end, we performed a moderation analysis, which tested the effect of the pupil dilation, block type and their interaction (predictors) on reaction time (DV). The sixth and final model tested the effect of the HADS-A scores and squared HADS-A scores (predictors) on the reaction times (DV).

Finally, to integrate all bivariate models, we built and tested a model to explore the causal relations between the parameters of interest. Therefore, we designed a moderated mediation model (Figure 2). This tested if LC drive explained the association between anxiety and reaction times and if this mediation of LC drive depended on the level of threat in the environment. The model was tested using the PROCESS macro for SPSS Model, template number 7. The dependent variable in the model was reaction time, as a behavioral measure. The predictor was a corrected HADS-A score, which was first demeaned and then squared (dsHADS-A), to account for the quadratic relationships of the HADS-A scores with pupil dilation and RT. This anxiety score was demeaned again in the statistical analysis to facilitate interpretation of the results. A positive relationship between the dsHADS-A scores and RT indicated a U-shaped relationship between the HADS-A scores and pupil dilation, and a negative relationship indicated an inverted-U-shaped relationship. The model mediator was pupil dilation and the moderator was block type. For statistical inference, 5000 bootstrapping samples were calculated and the results were presented in a 95% confidence interval.

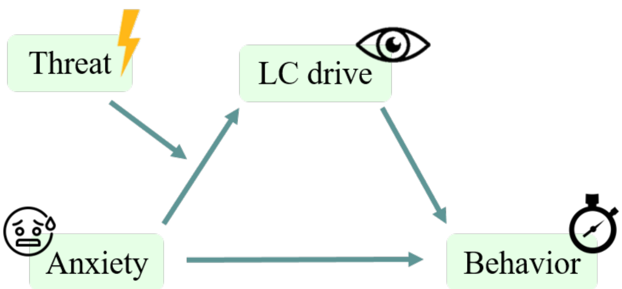


Figure 2: The moderated mediation model. It models the relationship between anxiety and reaction times. We propose that this relationship is mediated by pupil dilation as a proxy for LC drive. In addition, we expect a moderation of the threat induction (block type) on the relationship between anxiety and LC drive.

Results

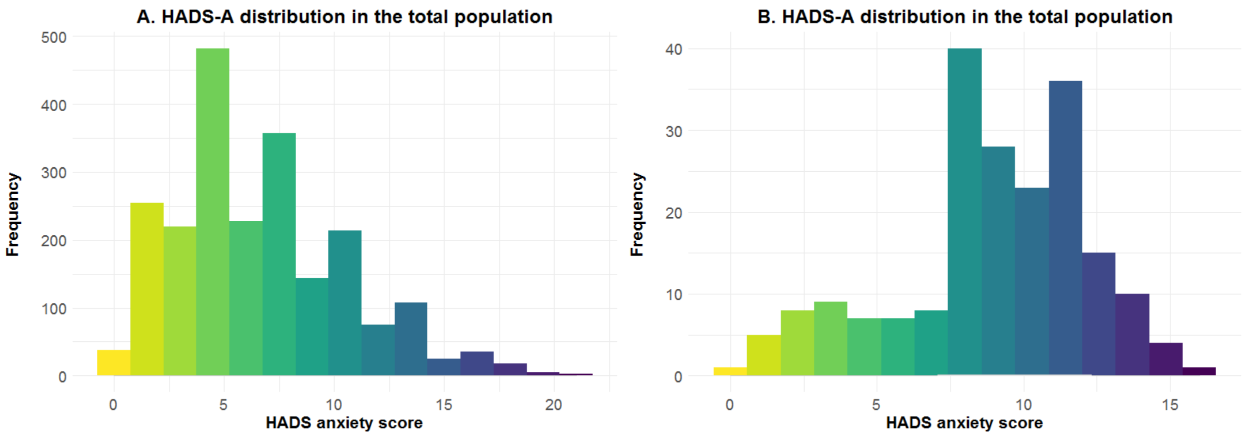


Figure 3: HADS anxiety score distribution. a) HADS-A distribution of all individuals that filled in the online screening questionnaires (*mean* = 6.62, *SD* = 3.90, *n* = 2206). b) HADS-A distribution of all individuals that participated in the study and were included in the data analysis (*mean* = 8.97, *SD* = 3.33, *n* = 202).

The distribution of the HADS-A scores in our population is displayed in Figure 3. Our study population contained 157 individuals with elevated anxiety (HADS-A > 7) and 45 individuals with normal anxiety levels (HADS-A <= 7). The mean HADS-A score for the study population was 8.97 (SD = 3.33, n = 202). There was no significant difference in the mean HADS-A score for females (*mean* = 9.14) and males (*mean* = 8.50) ($p > 0.05$).

<u>Pupil dilation: task manipulation</u>

To test whether the task manipulation was effective, we compared the mean pupil dilation between the four conditions (Figure 4). As pupil dilation was standardized per subject, its values represent the difference from the mean pupil dilation across all blocks. A two-way ANOVA revealed a significant effect of the threat induction on pupil dilation ($F(1, 804) = 1159.29$, $p < 0.001$). In addition, the effect of stimulus type on pupil dilation was not significant ($p > 0.05$) and there was no significant interaction between the threat induction and stimulus type ($p > 0.05$).

When performing post-hoc tests, a pairwise comparison revealed that within both the non-targets ($p < 0.001$) and within the targets ($p < 0.001$), the difference in mean pupil dilation between safe and threat blocks was statistically significant. When comparing the non-targets and targets, there was only a significant difference within the threat blocks (threat: $p = 0.018$; safe: $p > 0.05$). These results show that the threat induction was effective, but the effect of the stimulus type on pupil dilation was less evident, and only appeared in the threat blocks. A detailed record of the post-hoc tests can be found in the supplemental materials, table A.

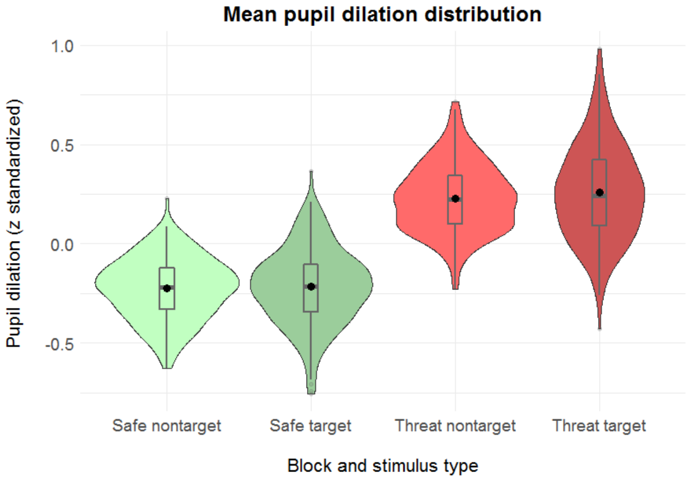


Figure 4: Plot showing the mean pupil dilation for the four conditions, safe non-target, safe target, threat non-target and threat target. The black dots represent the mean group dilation, the grey frame shows the boxplot including the group median.

<u>Threat induction influence on behavior</u>

Next, we evaluated the effect of the threat induction on task performance based on RT and accuracy (d'). Firstly, the mean RT during the threat blocks was lower than during the safe blocks ($t(201) = -7.805$, $p < 0.001$), indicating that people responded faster during threat blocks. Secondly, there was no significant difference between d' during the threat and safe blocks ($p > 0.05$). These results show that the threat induction did influence RT, but did not influence d'. A detailed record of the behavioral data can be found in the supplemental materials, table B.

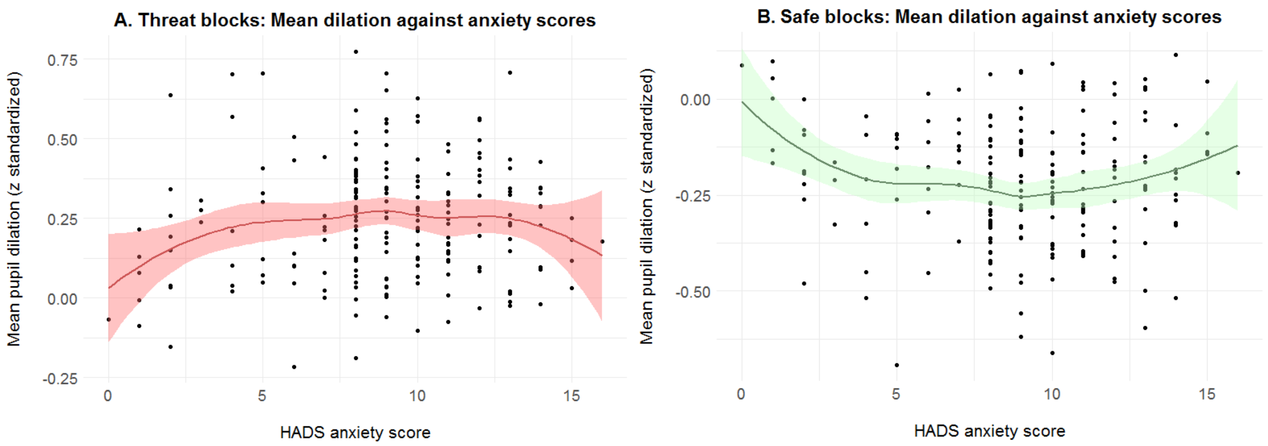
A linear regression model was conducted to test the relationship of d' with RT, d' with anxiety and d' with pupil dilation. The relationships of RT with anxiety and RT with pupil dilation will be discussed below. Firstly, in the safe blocks there was no significant relationship between d' and RT ($p > 0.05$). However, within both the threat blocks ($\beta = -0.205$, $t(200) = -2.031$, $p = 0.044$) and across all blocks ($\beta = -0.202$, $t(200) = -2.318$, $p = 0.022$) there was a negative relationship between d' and RT. This shows that as the reaction time decreased (faster responding), the accuracy increased. Secondly, there was no significant relationship between d' and the HADS-A scores ($p > 0.05$). Finally, there was only a significant positive relationship between d' and pupil dilation, when including the means across all blocks ($\beta = 5.070$, $t(200) = 3.809$, $p < 0.001$). This shows that as pupil dilation increased, the accuracy of responses also increased.

<u>Pupil dilation and anxiety</u>

For our first research question, we related the HADS-A scores to mean pupil dilation in the two block types. Figure 5 shows the relationships between the HADS-A scores and pupil dilation (a) in the threat blocks, (b) in the safe blocks and (c) difference between threat and safe blocks. A regression analysis showed a significant combined effect of the HADS-A scores (threat: $\beta = 0.039$, $t(199) = 2.618$, $p = 0.010$; safe: $\beta = -0.041$, $t(199) = -0.041$, $p = 0.001$) and the squared HADS-A scores (threat: $\beta = -0.002$, $t(199) = -2.356$, $p = 0.019$; safe: $\beta = 0.002$, $t(199) = 3.186$, $p = 0.002$) on pupil dilation during both the threat and safe blocks. The relationships for the threat and safe blocks were opposite in shape, with an inverted-U-shaped relationship during the threat blocks, and a U-shaped relationship during the safe blocks. To test this apparent opposite effect of threat on the relationship, we conducted a moderation analysis. This revealed a significant interaction of block type with both the HADS-A scores ($t(398) = -4.15$, $p < 0.001$) and the squared HADS-A scores ($t(398) = 3.889$, $p < 0.001$). Finally, there was a significant inverted-U-shaped relationship between anxiety and the difference in pupil dilation between the threat and safe blocks, when combining the HADS-A scores ($\beta = 0.080$, $t(199) = 3.126$, $p = 0.002$) and the squared HADS-A scores ($\beta = -0.004$, $t(199) = 2.900$, $p = 0.004$). Individuals in the middle range of the HADS-A score had the largest dilation difference between the threat and safe blocks, whereas individuals with a lower or higher HADS-A score had a smaller difference.

<u>Pupil dilation, anxiety and task performance</u>

For the next two research questions, we were interested in the effect of both pupil dilation and anxiety on RT. Firstly, we observed a significant negative relationship between RT and pupil dilation across all blocks ($\beta = -2.505$, $t(200) = -2.301$, $p = 0.022$) and in the threat blocks only ($\beta = -1.175$, $t(200)= -3.267$, $p = 0.001$). In contrast, in the safe blocks there was a marginally significant positive relationship ($\beta = 0.831$, $t(200) = 1.908$, $p = 0.058$). A moderation analysis revealed a significant interaction between pupil dilation and block type ($t(400) = 3.655$, $p < 0.001$). These results show that



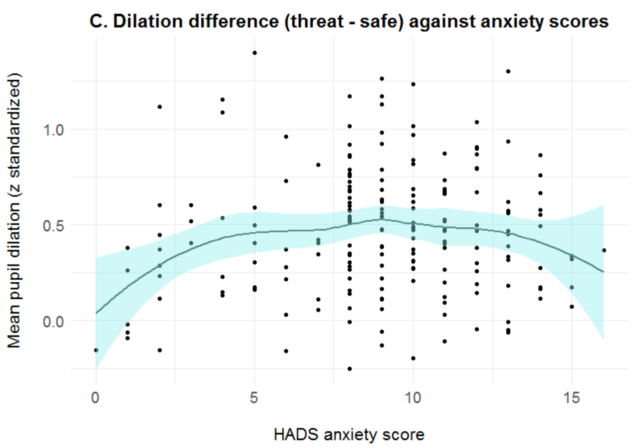


Figure 5: Scatterplots presenting the relationships between pupil dilation and the trait anxiety scores, including the LOESS lines and a 95% confidence interval. a. The HADS anxiety scores plotted against the mean pupil dilation for the threat blocks. b. The HADS anxiety scores plotted against the mean pupil dilation for the safe blocks. Note the y-axis in comparison to figure 5a. c. The HADS anxiety scores plotted against the mean pupil dilation difference between the threat and the safe blocks.

across all blocks and in the threat blocks only, participants responded faster with increasing pupil dilation, whereas during the safe blocks they responded more slowly with increasing pupil dilation.

Secondly, we investigated the effect of anxiety on RT. Within both the threat and safe blocks, there was a marginally significant relationship between RT and anxiety. The effect of the HADS-A scores (threat: $\beta = -0.147$, $t(199) = -1.865$, $p = 0.063$; safe: $\beta = -0.132$, $t(199) = -1.685$, $p = 0.094$) and the squared HADS-A scores (threat: $\beta = 0.009$, $t(199) = 1.938$, $p = 0.054$; safe: $\beta = 0.009$, $t(199) = 1.817$, $p = 0.070$) together yielded U-shaped relationships. Across all blocks, there was a marginally significant effect of the HADS-A scores ($\beta = -0.139$, $t(199) = -1.894$, $p = 0.060$) and a significant effect of the squared HADS-A scores ($\beta = 0.009$, $t(199) = 2.003$, $p = 0.047$) on RT. These results point to a U-shaped relationship between RT and anxiety, indicating that people in the middle range of the HADS-A scores responded faster compared to those with lower or higher HADS-A scores.

Moderated mediation model

Finally, we combined these bivariate associations between pupil dilation, anxiety and behavior (RT) into a moderated mediation model (Figure 2). This tested if the relationship between anxiety and RT was influenced by pupil dilation, and whether this mediation was dependent on the level of threat. Specifically, we used the anxiety scores as a predictor of reaction times, pupil dilation as a mediator in this relationship, and block type as a moderator of the relationship between anxiety and pupil dilation. For the anxiety scores, we used the demeaned, squared HADS-A score (dsHADS-A) to account for the quadratic relationship of the HADS-A scores, with pupil dilation and RT.

There was a significant direct positive effect of the dsHADS-A score on RT ($\beta = 0.0067$, $t(396) = 2.194$, $p = 0.029$). This indicates a U-shaped relationship between the HADS-A scores and RT, in accordance with previous analysis. In addition, the indirect effect of the dsHADS-A scores through pupil dilation on RT was tested. For the threat blocks, this was a positive significant relationship ($\beta = 0.0014$, *lowerCI* = 0.0003, *upperCI* = 0.0027), indicating a U-shaped mediated relationship between the HADS-A scores and RT. In contrast, for the safe blocks there was a negative significant effect ($\beta = -0.0015$, *lowerCI* = -0.0026, *upperCI* = -0.0004), indicating an inverted-U-shaped mediated relationship between the HADS-A scores and RT. Finally, there was a significant moderated mediation effect (*lowerCI* = -0.005, *upperCI* = -0.001). These results show that the relationship between anxiety and reaction time is mediated by pupil dilation, while the specific mediation depends on whether the individual is in a threatening or a safe environment.

Discussion

Regulatory LC drive and NA levels play an important role in the risk of developing anxiety disorders. However in healthy populations, LC drive or pupil dilation in relation to varying levels of non-clinical anxiety has received little attention. We therefore investigated the relationship between pupil dilation differences between a threatening and a safe environment as a proxy for LC drive, and varying levels of preclinical anxiety in an oddball paradigm with a threat-of-shock. Our results show an inverted-U-shaped relationship between anxiety and the pupil dilation difference scores. In addition, we explored the relationship between LC drive, anxiety, behavior and the threat induction. The moderated mediation model showed a mediation of LC drive on the relationship between anxiety and reaction times and a moderation by block type on the relationship between LC drive and anxiety. Different anxiety levels are associated to different behavioral performances and according to our model, this is brought about by varying levels of LC drive and dependent on the level of threat in the context.

To study these relationships, we employed an oddball paradigm with a threat-of-shock. The threat manipulation had a significant effect on reaction time but not on accuracy, possibly because the task was fairly easy. In addition, the threat manipulation had a significant effect on pupil dilation, with a larger dilation during the threat blocks. As pupil dilation was used as a proxy for LC drive and the LC is sensitive to arousal, this finding is in line with the current literature (Valentino and Van Bockstaele, 2008; Joshi *et al.*, 2016). The oddball in our task on the other hand, only elicited a significant pupil response in the threat blocks and not in the safe blocks, even though the LC is sensitive to all task relevant stimuli (Krebs *et al.,* 2018). As during safe blocks, the state of arousal is generally lower, there is less tonic LC firing and individual is less task-engaged (Aston-Jones and Cohen, 2005). Therefore, the effect of the phasic LC drive in response to the target may be less pronounced in safe blocks and the dilation difference between the targets and non-targets may be too small for us to detect.

With this paradigm, our first main finding was the inverted-U-shaped relationship between anxiety and the dilation differences (threat minus safe), indicating that individuals with moderate anxiety levels show a larger dilation difference as compared to individuals with lower or higher anxiety levels. Pupil dilation was interpreted as a proxy for LC drive (Joshi *et al.*, 2016). Throughout the task, tonic LC drive must discriminate between the two block types to cope adaptively with the threatening situation. Therefore, the large dilation difference in individuals with moderate anxiety levels reflects an adaptive response and optimal performance in dealing with stress. For individuals who were less or more anxious the dilation difference was smaller, thus reflecting suboptimal differentiation in performance. This is also reflected in the U-shaped relationship between reaction times and anxiety, indicating that individuals with moderate anxiety levels responded faster than those with lower or higher anxiety levels. Possibly, trait anxiety influenced the state of arousal, resulting in an inattentive state for participants with low anxiety levels and a distractible state for highly anxious individuals, explaining their suboptimal performance. Importantly, this study extends the current knowledge on the Yerkes-Dodson relationship between arousal and performance, by regarding the role of trait anxiety and its influence on LC drive and behavior. Both LC drive and behavioral performance are thus not only dependent on the current state of arousal, but are also influenced by trait anxiety.

Our second main finding was the moderated mediation model, showing that the relationship between anxiety and behavior is mediated by pupil dilation, as a proxy for LC drive, and that the specific mediation depends on the level of threat in the context. During the threat blocks, the mediated relationship between anxiety and reaction times was U-shaped whereas in safe blocks, it was inverted-U-shaped. The combination of these relationships shows that individuals with moderate anxiety levels have a larger speed difference between threat and safe blocks compared to individuals with lower or higher anxiety levels who show a smaller speed difference. This difference might again result from the effect of trait anxiety on the state of arousal. Our model shows that LC drive does not only influence behavioral performance directly, but also mediates the relationship between trait anxiety and behavior. The influence of anxiety on behavior is thus explained by LC drive, while the relationship between anxiety and LC drive is moderated by the threat level in the context. The model therefore provides a non-linear explanation of individual differences in behavioral performance during stress.

To summarize the behavioral results, we observed faster responses in the threat compared to the safe blocks and a positive relation of accuracy with speed and pupil dilation. In addition, between pupil dilation and reaction times there was a negative relationship in the threat and a positive relationship in the safe blocks. These results are partially in line with Murphy and colleagues (2014), who found faster responses with more LC drive in their oddball paradigm. Our results are however not in line with Hu and colleagues (2012), who reported increased accuracy and slower reaction times during stress in a stroop task, another vigilance paradigm. Interestingly, they propose that reaction time and accuracy present two parallel processes mediating performance. Kohn, Hermans and Fernandez (2017) support this in their study investigating the underlying neuronal patterns for these dissociative cognitive effects. As stress is associated with an NA-induced neural network shift (Hermans *et al.*, 2011), they investigated if cognitive performance was modulated by different neural connectivity profiles in a stroop paradigm. They studied four prototypic profiles, comparing up- and downregulation of two neural networks, the salience network (SN) and the executive control network (ECN) and observed differences in cognitive performance between these profiles. Specifically, ECN upregulation was associated with an increase in accuracy, whereas SN upregulation was associated with an increase in speed. Both our moderated mediation model and the neural connectivity profiles from Kohn and colleagues (2017) thus aim to explain individual differences in behavioral or cognitive performance during stress and hint at the existence of non-linear moderation processes.

In the stress induced neural network shift, the LC plays an important role as NA is one of the neuromodulators inducing it (Hermans *et al.*, 2011). The LC projections to different brain areas such as the amygdala or PFC influence neural connectivity, up- or downregulating different neural networks (Hermans *et al.*, 2014; Arnsten *et al.*, 2015). One could reason that differential levels of noradrenergic LC drive might result in different neural connectivity profiles. As Kohn and colleagues (2017) showed that these neural profiles were associated to different cognitive performances, an interesting next step is to relate LC drive to the four connectivity profiles. For each profile linked to LC drive, we can investigate its associated cognitive performance during stress in the oddball paradigm. By combining the LC drive to these brain connectivity profiles, this approach can build a bridge between trait anxiety, noradrenergic LC drive, neural network connectivity and behavior.

<u>Limitations and future suggestions</u>

There were a few limitations to our study. Firstly, we did not record LC activity with fMRI and used pupil dilation as a proxy. Even though the pupil dilation results show a pattern that is typical for the LC, we must be cautious when interpreting the results. For future studies, neuromelanin sensitive imaging can be used to create an LC mask and extract its BOLD time course (Keren *et al.*, 2015). Secondly, our task aimed to arouse the participants with a threat of an electrical shock however, we noticed that the task was rather tiresome. Luckily, we did observe behavioral and dilation differences between the block types so our threat induction was effective. However, for future studies investigating the effect of arousal on LC drive, it might be better to use a vigilance task that is slightly more engaging. One could for example use a more difficult task, or switch between multiple tasks. The former could also lead to differences in accuracy between the threat and safe conditions. The latter can be interesting as Aston-Jones and Cohen (2005) state that tonic LC activity reflects a balance between exploiting the task at hand, and exploring other options in the environment. Finally, our study did not include a subjective measure of arousal. We linked performance to trait anxiety rather than a state of arousal. However, trait anxiety may influence the state of arousal and it can therefore be interesting to subjectively measure the arousal state to control for the effect of anxiety on arousal.

<u>Conclusion</u>

We found an inverted-U-shaped relationship of trait anxiety with both LC drive and behavioral performance. In addition, the relationship between anxiety and behavior is mediated by LC drive, and is particularly dependent on the level of threat in the environment. Our model aims to explain why individuals respond differently during stress and hints at the existence of non-linear moderation processes. Varying anxiety levels are associated with different behavioral performances, and the LC drive to several brain areas and networks moderates this relationship differently depending on the threat level in the environment. By linking this model of LC drive, anxiety and behavior to different neural connectivity profiles, we can take the next step in explaining individual differences in cognitive performance during stress.

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Supplemental Materials

Tables

Table A

Pairwise comparison of the mean pupil dilation data. The table shows four pairwise comparisons, comparing the targets and non-targets in the safe (1) and the threat (2) blocks and comparing the safe and threat blcoks within the targets (3) and the non-targets (4). The means represent the standardized mean pupil dilation, which was standardized across all blocks using the individual mean and standard deviation.

Pair	Group 1			Group 2			Pairwise comparison		
	Group	Mean	SD	Group	Mean	SD	Statistic	df	p
Safe	Target	-0.216	0.195	Non-target	-0.226	0.157	-0.921	201	0.358
Threat	Target	0.261	0.243	Non-target	0.229	0.172	-2.37	201	0.018*
Target	Safe	-0.216	0.195	Threat	0.261	0.243	-20.2	201	<0.001**
Non-target	Safe	-0.226	0.157	Threat	0.229	0.172	-18.3	201	<0.001**

* Significant at α=0.05

** Significant at α=0.001

Table B

Behavioral data from the oddball paradigm with a threat-of-shock manipulation. The table shows the means and standard deviation for the reaction times, number of hits, number of false alarms and the sensitivity index (d') separately for the threat and safe blocks. The reaction times were standardized using the group mean and standard deviation. The table also shows the results of the paired-samples-t-tests between the threat and safe blocks.

	Threat		Safe		Paired samples t-test		
	Mean	SD	Mean	SD	t	df	p
Reaction time	-0.187	0.985	0.187	0.982	-7.805	201	<0.001**
Hits	31.4	1.00	31.2	1.19	2.92	201	0.004*
False alarms	17.8	3.70	11.9	2.09	-5.341	201	<0.001**
d'	0.012	1.40	0.03	1.38	-0.163	201	0.871

* Significant at α=0.005

** Significant at α=0.001

Hospitalized Anxiety and Depression Scale (HADS)

Tick the box beside the reply that is closest to how you have been feeling in the past week.
Don't think too long about your replies: your immediate is best.

D	A		D	A	
		I feel tense or 'wound up':			**I feel as if I am slowed down:**
	3	Most of the time	3		Nearly all the time
	2	A lot of the time	2		Very often
	1	From time to time, occasionally	1		Sometimes
	0	Not at all	0		Not at all
		I still enjoy the things I used to enjoy:			**I get a sort of frightened feeling like 'butterflies' in the stomach:**
0		Definitely as much		0	Not at all
1		Not quite so much		1	Occasionally
2		Only a little		2	Quite Often
3		Hardly at all		3	Very Often
		I get a sort of frightened feeling as if something awful is about to happen:			**I have lost interest in my appearance:**
	3	Very definitely and quite badly	3		Definitely
	2	Yes, but not too badly	2		I don't take as much care as I should
	1	A little, but it doesn't worry me	1		I may not take quite as much care
	0	Not at all	0		I take just as much care as ever
		I can laugh and see the funny side of things:			**I feel restless as I have to be on the move:**
0		As much as I always could		3	Very much indeed
1		Not quite so much now		2	Quite a lot
2		Definitely not so much now		1	Not very much
3		Not at all		0	Not at all
		Worrying thoughts go through my mind:			**I look forward with enjoyment to things:**
	3	A great deal of the time	0		As much as I ever did
	2	A lot of the time	1		Rather less than I used to
	1	From time to time, but not too often	2		Definitely less than I used to
	0	Only occasionally	3		Hardly at all
		I feel cheerful:			**I get sudden feelings of panic:**
3		Not at all		3	Very often indeed
2		Not often		2	Quite often
1		Sometimes		1	Not very often
0		Most of the time		0	Not at all
		I can sit at ease and feel relaxed:			**I can enjoy a good book or radio or TV program:**
	0	Definitely	0		Often
	1	Usually	1		Sometimes
	2	Not Often	2		Not often
	3	Not at all	3		Very seldom

Scoring:

0-7 = Normal
8-10 = Borderline abnormal (borderline case)
11-21 = Abnormal (case)

Shocker calibration

Instruction: "You will get some electrical stimulation now and you will have to rate every electrical stimulation from 1 to 5 on the aversiveness of the stimulation. A rating of 1 means the electrical stimulation is not aversive at all, a rating of 5 means it is strongly aversive and painful."

Check if everything is clear and if the participant is ready.

Start with a test shock (bar 1) and ask whether the subject felt something, then proceed to bar 2 and start calibration

** Never use the term shock! Ask the subject after every choice whether he/ she confirms the rating and adjust the bars of the shocker. If the number of bars does not change, pretend to adjust the number of bars anyway. Take time between shocks to prevent habituation effects and always be predictive about the next stimulation.

If subjects informs about the shock level beforehand, respond with this: "The electrical stimulations are by most considered to be unpleasant but not painful. We have tested many subjects before with this procedure and have never experienced any lasting side effects. The study was also approved by the medical ethical committee as having negligible risk."

<u>Shocker workup</u>

shocker level (bars):	subjects rating (tick one) (change for next level)					comments
2 (start level)	1 (+2)	2 (+1)	3 (=)	4 (=)	5 (-1)	
	1 (+2)	2 (+1)	3 (+1)	4 (=)	5 (-1)	
	1 (+3)	2 (+2)	3 (+1)	4 (=)	5 (-1)	
	1 (+3)	2 (+2)	3 (+1)	4 (=)	5 (-1)	
	1 (+3)	2 (+2)	3 (+1)	4 (=)	5 (-1)	
Final intensity shock:	Total:					

Maximum level possible: 10 bars. The final level should be found in exactly 5 exposures.