Donders Graduate School for Cognitive Neuroscience
Master of Science Programme
MSc Thesis

Can stress induced Amygdala – Frontal connectivity changes predict perceived stress?

by

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Date of final oral examination: 22 August 2016

Abstract

Stress-related disorders are one of the most common mental disorders and clarifying the influence stress exerts over our physiological systems could help screen vulnerable individuals and aid in early detection of disorders. To elucidate how stress affects our brain we explicate an amygdala – frontal cortex connectivity model, distinguishing between their sub regions. Participants (N=71, age=24.5, 19 females) underwent two resting-state scans with a well-established stress induction protocol in between to be able to detect changes in connectivity due to stress. These connectivity changes were then related to perceived stress later in life. Connectivity between the basolateral amygdala (BLA) and the dorsal and ventral frontal networks increased significantly due to stress induction. Stress induced connectivity increases of the BLA and centromedial amygdala (CMA) with the dorsal network were marginally predictive of perceived stress, however only for a subsample of our participant pool (p=.073 and p=.06 respectively). Indicating that stress induced amygdala – frontal connectivity changes could be predictive of later perceived stress. Future research could focus on increasing sample size and predictive validity of amygdala – frontal connectivity in relation to other stress-related symptomatology.

An introduction to stress responsivity

Approximately 20-25% of adolescents will meet criteria for a mental disorder once in their lifetime, with stress-related disorders (anxiety/mood disorders) contributing to over 40 % of the cases (Merikangas et al., 2010). Therefore it is important to clarify the influence stress exerts on our systems. Broadly, stress can be defined as 'the state of threatened balance, equilibrium or harmony', and can be separated into physical and psychological stress (Chrousos, Loriau and Gold, 2013, p4). Psychological stress, rather than physical stress, has the most effect on chronic stress disorders (e.g. anxiety disorders, depression and post-traumatic stress disorder (PTSD)) (Sapolsky in Gross, 2007) through its influence on biological as well as behavioral systems (Cohen, Janicki-Deverts and Miller, 2007). Psychological stress occurs when someone perceives the demands of the situation to exceed his or her coping capacities (Cohen, Janicki-Deverts and Miller, 2007).

One physiological system involved in stress that has been widely studied is the Hypothalamic-Pituitary-Adrenocortical axis (HPA axis). The major function of the HPA axis is to control the hormonal reaction to stress (i.e., regulate the release of cortisol) through an activation cycle incorporating the hypothalamus, pituitary and adrenal glands (Smith and Vale, 2006). A rise of the cortisol level in response to a situation is often used as an indicator of experienced stress (for an extensive review on cortisol reactivity see Dickerson and Kemeny, 2004). To investigate the influence of stress on the brain, researchers have developed stress induction protocols and used them in both animal and human studies, where cortisol increase is often used as an objective measure of stress reactivity (see for example Foley and Kirschbaum, 2010; Giles, Mahoney, Brunyé, Taylor and Kanarek, 2014; Koolhaas et al., 2011; Hostinar, Sullivan and Gunnar, 2014; Holmes and Welman, 2009).

Additional objective stress measures include autonomic nervous system reactivity, which is characterized by increased heartrate after the stressor (Dickerson & Kemeny, 2004; Kudielka, Buske-Kirschbaum, Hellhammer & Kirschbaum, 2004). In relation to acute stress induction protocols there have been mixed findings, some protocols seem to be more capable of eliciting an autonomic response than others (Giles et al., 2014). Research groups using a combined protocol with a physical as well as an elaborate social evaluative component have reported increased heart rate after stress induction (for example Vogel et al. 2015, however see Schwabe, Haddad and Schachinger, 2008). Additionally, subjective report after stress induction has also been widely used, where increased negative affect, but not decreased positive affect, is associated with increased stress levels (Watson, Clark & Tellegen, 1988).

Neuroimaging research into acute stress and related constructs implicated in stress-related disorders, for example abnormal emotion regulation (Tull, Barrett, McMilland and Roemer, 2007) have yielded a wide variety of insights. Two brain areas in particular are frequently shown to be involved in stress-related disorders. Firstly, the amygdala plays an important role (Hostinar, Sulivan

and Gunnar, 2014); in general it is found to be hyperactive when emotion is abnormally regulated (Shin et al., 2005). Apart from the amygdala the frontal regions, Anterior Cingulate Cortex (ACC) and medial Prefrontal Cortex (mPFC), are also involved in stress-related disorders. However, with regards to the frontal regions neuroimaging findings are mixed. Some studies reported decreased activity in orbitofrontal regions and increased activity in the ACC after stress (Dedovic, D'Aguiar & Pruessner, 2009), while others reported the opposite pattern of decreased frontal activation, in particular in the ACC (Pruessner et al. 2008). Differences in activity patterns between studies may result from the particular nature of the stressor, sex of the subject and early life experiences (Dedovic, Duchesne, Andrews, Engert and Pruessner, 2009). Importantly, some discrepant results may also stem from a lack of anatomical specificity in labeling stress-induced changes in the brain. Research into sub-regions of the frontal area revealed a more consistent pattern. When emotion was abnormally regulated the rostral ACC (rACC) and the ventral mPFC (vmPFC) were hypofunctioning, while the dorsal ACC (dACC) and the dorsal mPFC (dmPFC) were hyperfunctioning (Shin and Liberzon, 2010; Phan, Fitzgerald, Nathan, Moore, Uhde and Tancer, 2005; Etkin, Buchel and Gross, 2015).

In addition to abnormal activity patterns, abnormal connectivity patterns are also reported. The amygdala showed disrupted connectivity with frontal regions with regards to abnormal emotion regulation, among which are pathological anxiety and post-traumatic stress disorder (PTSD) (Banks, Eddy, Angstadt, Nathan, and Phan, (2007); Pezawas et al., 2005; Williams et al., 2006). It is postulated that the inhibitory connection between the ACC/mPFC and the amygdala is reduced by the encounter of a (life threatening) stressor based on task-based (Williams et al., 2006) and resting-state functional magnetic resonance imaging (fMRI) (Sripada et al., 2012). As the brain's response to stress is intricate, we will start with a delineation of the neurocircuitry of the amygdala and frontal regions with respect to stress processing. Thereafter we continue with the convergence between animal and human literature into one neurocognitive amygdala-frontal connectivity model and its response to stress.

Neurocircuitry of stress: the amygdala

The amygdala consists of several sub-regions, a three-way division is the most widely accepted in humans: the basolateral amygdala (BLA), centromedial amygdala (CMA) and Superficial Amygdala (SFA) (Amunts et al., 2005). It is postulated that the BLA is the main input node for information coming from the neocortex as well as the cingulate gyri and the hippocampal complex. This subnucleus then sends its efferent connections to the CMA, the main output node, which projects to the hypothalamus and periaqueductal grey (PAG) leading to anxiety-related behaviour (Bear, Connors and Paradiso, 2007). Therefore the focus of this project is on the BLA and CMA, as is increasingly common in other stress-related research (for example Vogel et al., 2015).

As mentioned previously, research suggests that an inhibitory link exists between the amygdala and the mPFC, where the amygdala was taken as one single functional unit (Williams et al., 2006; Sripada et al., 2012). However, in animal studies, Lihtik et al., (2005) revealed an excitatory link between the mPFC and the BLA, the main input node of the amygdala. Researchers from the same lab postulated that the frequently found inhibitory connection between the mPFC and the amygdala could be due to an inhibitory cluster, the Intercalcated (ITC) neurons, in between the BLA and the CMA (Lihtik, Popa, Apergis-Schoute, Fidacaro and Paré, 2008). The mPFC seems to activate the BLA, which in turn activates the ITC, thereby the ITC inhibits output from the CMA. Suggesting that excitatory projections from the mPFC can lead to decreased amygdala output. In addition, Jovanovic and Ressler (2010) suggested that the vmPFC can activate the ITC directly, instead of through excitation of the BLA. In recent years, there have been advances in human functional imaging techniques that hint towards a similar division of the subnuclei in humans (Etkin, Prater, Schatzberg, Menon and Greicius, 2009; Roy et al., 2009). These studies highlight the necessity to distinguish between amygdala subnuclei in stress-related research.

Neurocircuitry of stress: the medial Prefrontal Cortex

Koenings and Grafman (2009) showed that when the amygdala is lesioned, there was a decreased chance of developing PTSD. Contrary to their expectations, a vmPFC lesion also led to a decreased chance of PTSD, the inhibitory hypothesis of mPFC amygdala connectivity would predict an increased chance of PTSD due to less inhibition of the amygdala. This contradictory finding, together with mixed findings on medial prefrontal connectivity to the amygdala in relation to stress processing (Dedovic, D'Aguiar and Pruessner, 2009; Pruessner et al., 2008) may warrant a similar segmentation approach for the medial PFC as for the amygdala subnuclei.

When functional partitioning of the medial prefrontal region (ACC and mPFC) is considered, the region can be divided into a dorsal and a ventral network (Margulies, Kelly, Uddin, Biswal, Castellanos and Milham, 2007; Quirk and Beer, 2006; Philips, Drevets, Rauch and Lane, 2003). According to the literature: the ventral network comprises of the perigenual and subgenual portion of the ACC (pgACC and sgACC) and the ventral mPFC (vmPFC). The dorsal network comprises of an anterior and posterior portion of the ACC (adACC and pdACC) and the dorsal mPFC (dmPFC)(Etkin, Egner and Kalisch, 2011).

Animal research suggested that the ventral network and dorsal network have differential effects on neuroendocrine and autonomic responses to stress (Radley, Arias & Sawchenko, 2006). More specifically, it is postulated that the ventral network plays an inhibitory role and the dorsal network an excitatory role (Quirk & Beer, 2006). Excitation from the infralimbic cortex (ventral network) may inhibit CMA output activity through activation of the BLA and the ITC, the inhibitory

cluster between the BLA and CMA (Lihtik, et al., 2008; Jovanovic and Ressler, 2010). However, opposite findings have also been reported indicating that the infralimbic cortex was associated with increased stress responsivity and the prelimbic cortex (more dorsally located) with a decreased stress response (Radley et al., 2006). Similar findings have been reported by Ulrich-Lai and Hermans (2009), they suggested the prelimbic cortex is responsible for inhibition and the infralimbic cortex for stimulation of the HPA axis. However, it is worth noting that although the prelimbic cortex is located more dorsally, it is generally regarded as part of the homologue of the human vmPFC (Quirk & Beer, 2006).

Numerous studies in humans suggest a similar division between the dorsal and ventral networks in the PFC: the dorsal network (in particular the dmPFC) is more involved in emotion expression/appraisal and the ventral network in inhibition/regulation. (Etkin, Buchel and Gross, 2015; Etkin, Egner and Kalisch, 2011; Milad, Rauch, Pitman and Quirk, 2006; Quirk, Garcia, Gonzalez-Lima, 2006; Yamasaki, LaBar and McCarthy, 2002). Attempts to translate animal amygdala connectivity studies to human research have been made utilizing resting-state functional connectivity paradigms. Roy et al. (2009) found that the BLA had a positive association with regions in the ventral network and a negative association with regions in the dorsal network, in addition, the CMA had a positive association with the regions in the dorsal network. These results converged with animal literature suggesting that the ITC may act as an inhibitory cluster between the BLA and the CMA (Lihtik et al., 2005; 2008; Jovanovich and Ressler, 2010). Similarly, Brown et al. (2014) found that the BLA and CMA display opposite resting state functional connectivity patterns with the pgACC (ventral network)/dmPFC (dorsal network) in their combined sample (PTSD plus trauma-exposed controls). Unfortunately, in Brown et al.'s (2014) study the pgACC/dmPFC was taken as one single region, no distinction was made between the possible differential contribution of the ventral and dorsal networks. In addition, task-based research reported stronger BLA-ventral and CMA-dorsal network functional connectivity during an emotion regulation task, after stress induction (Vogel et al., 2014). These studies suggest an opposite pattern of BLA/CMA connectivity with both the ventral and dorsal network, and that their connectivity could be altered after stress.

Integrating the Amygdala and mPFC in one connectivity model

The goal of the current paper is three-fold: explicate a stress processing model based on previous work in animal and human research, empirically test the connectivity changes after acute stress induction and the model's predictive value for perceived stress at a later time point. Firstly, we expected to replicate the results from Roy et al. (2009) using resting-state fMRI, for we made use of the same anatomical atlas to define the amygdala subnuclei seeds (see Methods). Specifically, we expected to find a negative association between the BLA and the dorsal network, a positive

association between the BLA and the ventral network and a positive association between the CMA and the dorsal network (Roy et al., 2009). In addition, we expected a negative association between the CMA and the ventral network, based on animal literature (Lihtik et al., 2005; 2008; Jovanovich & Ressler, 2010) (see figure 1A).

Secondly, to assess how the network connectivity changes in response to acute stress, we will compare resting-state fMRI scans before and after a stress induction protocol. Few studies have utilized a similar combination of resting-state fMRI following a stress induction protocol. Among these studies, findings include enhanced functional connectivity of the amygdala with the dACC (Van Marle, Hermans, Qin and Fernandez, 2010) and enhanced amygdala-vmPFC resting state functional connectivity up to an hour after psychosocial stress induction (Veer et al., 2011). However, both studies did not distinguish between the amygdala subnuclei. Furthermore, from a functional perspective, the dorsal network is involved in explicit expression/appraisal and the ventral network in implicit regulation (Etkin, Buchel and Gross, 2015). It is expected that induced stress leads to enhanced expression/appraisal (enhanced BLA-dorsal connectivity) of stress-related behaviour and thus more regulation (enhanced BLA-ventral connectivity) is needed to bring the system back to homeostasis. In addition, the basolateral nucleus is the largest subnucleus. Therefore we expected to find similar increased connectivity between the BLA-dorsal and BLA-ventral network as in Van Marle, Hermans, Qin and Fernandez (2010) and Veer et al. (2011). As a stress induction check, we will correlate the connectivity change with known physiological and subjective measures indicating increased stress.

Finally, predictive validity was assessed by correlating connectivity changes due to stress induction to perceived stress at a later moment in life. A recent study by Brown et al. (2014), comparing PTSD patients and trauma exposed controls, found that the connectivity between the right BLA and part of the ventral network (sgACC) and dorsal network (dmPFC), and the connectivity between the left BLA and dorsal network (dACC) were enhanced in PTSD patients in comparison to the controls. Indicating that people vulnerable to developing PTSD may show enhanced connectivity between the BLA and ventral and dorsal network after a stressful experience. However, in the same study, Brown et al (2014) did not find connectivity differences of the CMA between PTSD patients and trauma-exposed controls. In addition, Qin et al. (2014) found that BLA connectivity patterns, but not CMA connectivity patterns, were predictive of (subclinical) childhood anxiety, indicating that BLA connectivity is important in emotion regulation. Based on these findings, we expected that the stress-induced connectivity increase between the BLA and dorsal network, and between the BLA and ventral network, would be predictive of perceived stress. We did not expect any differences in CMA connectivity to be correlated to perceived stress (see figure 1B).

To test these hypotheses, we used data from an ongoing study consisting of two groups of participants, police recruits and a control group. During the four months following the stress induction procedure the police recruits experienced a potentially stressful time performing emergency aid as part of their training. Controls did not experience a similar training.

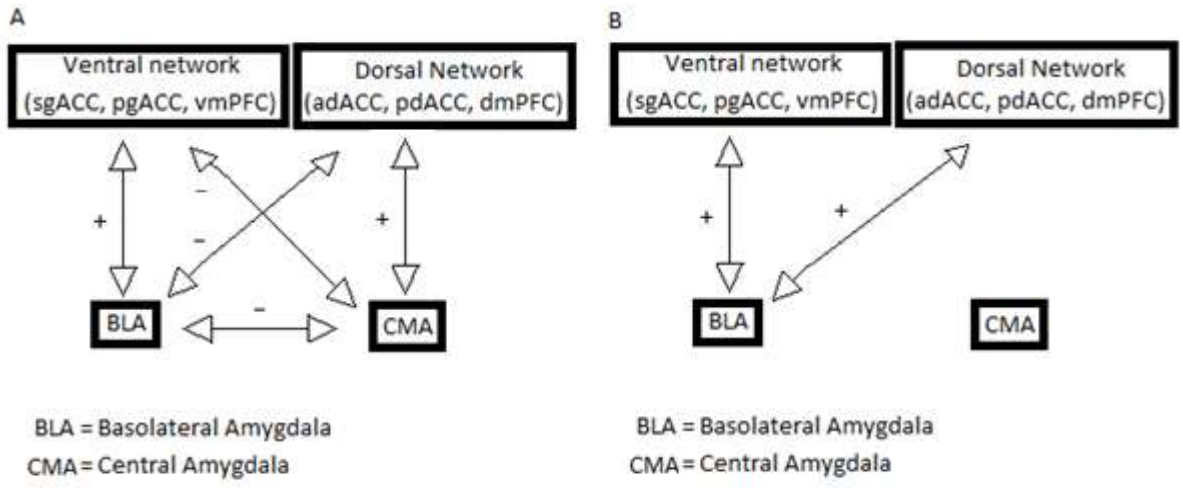


Figure 1: Amygdala – frontal connectivity model. A) before stress induction we expect that the BLA has a stronger association with the ventral than with the dorsal network, the CMA has a stronger association with the dorsal network than with the ventral network. B) After stress induction we expect that the BLA shows enhanced connectivity with the ventral and dorsal network. Vulnerable individuals will obtain a higher difference score on the Perceived Stress Scale (PSS), this will be correlated with BLA-Dorsal and/or BLA-Ventral increase after stress induction.

Methods

Participants

Our sample included 55 participants (of which 10 were female) recruited from the police academy in the Netherlands, ranging between the ages of 20 and 43 (*M*= 27.20 *sd*=5.94), as well as 51 age- and education-matched controls (of which 15 were female) between the ages of 18 and 35 (*M*= 22.20 *sd*=2.84) as part of a larger prospective study on predictive biomarkers of trauma-related symptomatology. The study was approved by the Independent Review Board, Nijmegen (IRB Nijmegen). Written consent was obtained prior to testing. Out of 106 available subjects' datasets three subjects were excluded due to technical error, two due to bad quality of the functional data, in particular ghosting artifacts. Finally, as we were particularly interested in two adjacent networks in the dorsal and ventral prefrontal cortex, due to limited time, a conservative approach was taken to ensure the proper registration of functional imaging data particularly in those regions. Thirty-one

subjects were excluded due to normalization abnormalities caused by bias fields, after extensive checks by two raters. The final subject sample consisted of 35 police recruits (age: *M*= 27.2, *sd*= 6.7, 8 females), and 36 control participants (age: *M*= 22.0 *sd*= 2.9, 11 females). Police recruits were recruited through presentations on their academies, control participants were recruited mainly through advertisements online and flyers at a nearby college. Participants were then screened by phone for inclusion. Standard exclusion criteria for MRI experiments applied, most importantly absence of metal in the upper body, >18 years of age, absence of pregnancy. Participants with a metal wire and tattoos were included. Study-specific exclusion criteria included: history of neurological, psychiatric or hormonal disease, abnormal vision or hearing, use of medication (especially corticosteroids) and excessive use of drugs/alcohol. Perceived stress was assessed on the test day and again after four months, during which police recruits performed emergency aid as part of their training.

Procedure

In short, the design of the entire project included two testing days (six-and-a-half hours each) which were approximately one year apart, with two intermediate questionnaires that were filled in online, once after four months and another after eight months from the first visit. In the year between testing days the participants recruited from the police academy performed two four-month periods of emergency aid. This involved being the first responder to a variety of situations including but not limited to traffic accidents, suicides and medical emergencies (e.g. performing CPR). The control participants did not experience similar training. The current paper will focus on a subset of the data, particularly on the stress induction task on the first day and the first intermediate questionnaire after four months, only relevant procedures will be discussed in detail below.

To measure stress processing/reactivity in the brain, two sessions of resting-state functional MRI scans were acquired with a stress induction procedure employed in between. The two sessions of resting state scans were each followed by an extra scan, not analyzed here. Each session of the resting state scan lasted approximately 8 minutes (see below for further scanning parameters). The stress induction protocol consisted of two tasks. The first task of the stress induction protocol was the Socially Evaluated Cold Pressor Test (SECPT, Schwabe, Haddad and Schachinger, 2008), where participants were instructed to submerge their foot into cold water for three minutes. The second part of the stress induction protocol consisted of a mental arithmetic task, lasting for three minutes. The stress induction protocol was supervised by an experimenter new to the participant, acting as neutral as possible. To ensure that the hypothesized connectivity differences between resting-state scans can be attributed to the stress induction procedure rather than irrelevant factors, negative

affect, heart rate and cortisol reactivity were used as a stress induction check. The entire stress induction protocol was performed inside the room where the MRI scanner was located.

Materials

Stress Induction Protocol

The stress induction protocol consisted of two tasks. Firstly, we conducted the Socially Evaluated Cold Pressor Test (SECPT, Schwabe et al.,2008), with minor adaptations as to ensure the entire procedure could be performed inside the MRI scanner. There were always two experimenters present (instead of one in the original experiment, (Schwabe et al, 2008; Vogel et al., 2015)), one of which was always of the opposite sex as the participant to induce an extra social evaluative component. The primary experimenter leading the SECPT was new to the participant, and acted as neutral as possible while wearing a white lab coat. We opted for a new experimenter to ensure that the participants were feeling stressed without experiencing, for example confusion, by a sudden behavior change of the main experimenter, who was present throughout the day (i.e. not as friendly as they were during the day).

The participants were instructed to submerge their foot (instead of their hand as in the original experiment by Schwabe et al., 2008) into cold water of approximately 1 degree Celsius. They were required to keep their foot in as long as possible until the primary experimenter asked them to stop and helped to take it out (limit was set to 3 minutes). The main experimenter of the day timed the procedure with a stopwatch. The participants were instructed not to move nor speak and look upwards and were told they were videotaped as to inspect their facial expressions. If they did not comply, the primary experimenter firmly repeated the instructions (e.g. Please, do not move). When participants took their foot out themselves before the limit of 3 minutes had passed, the primary experimenter asked participants to put their foot back in the water and continue the task, unless the participants insisted not to. 11 out of 71 participants did not complete the SECPT protocol by removing their foot from the water before the full three minutes had passed (approximately 15 %). To enhance and prolong stress responsivity we added a mental arithmetic task (Vogel et al., 2015). After the SECPT, the participants continued directly with the mental arithmetic task, that required them to count backwards in steps of 17 from 2059 for three minutes as fast and accurate as possible, under direct scrutiny of the primary experimenter. When the participant made a mistake the primary experimenter instructed them to start over from the same or a different number.

Stress induction check

As a stress induction check we employed two physiological (cortisol reactivity and heart rate increase) and one subjective (increased negative affect on the Positive and Negative Affect Scale

(PANAS; Watson, Clark & Tellegen, 1988)) measure. Increases in these measures are in general related to increased stress (Dickerson & Kemeny, 2004; Schwabe et al., 2008; Vogel et al., 2015) and were selected to ensure that any connectivity changes found between resting state scan one and two were due to the stress induction protocol.

Cortisol reactivity

Cortisol was measured at five timepoints. Unfortunately, the collection of the sampling differed slightly for a subset of our participant sample (18 police recruits). Therefore we will only use the four samples all participants had in common. Time of sampling in the afternoon (between 16.30h and 18.30h) was kept consistent across participants as cortisol levels fluctuate in a circadian rhythm, they increase on awakening and decrease gradually throughout the day (Dickerson & Kemeny, 2004).

The first sample was taken approximately twenty minutes after the subject entered the fMRI scanner, during that time participants performed an approach/avoidance task (AAT) which will not be discussed in the current paper. The second sample was taken right after the first resting state scan, the third sample after the stress induction protocol and the final sample was taken right after the second resting state scan (figure 2). To capture cortisol reactivity in one scalar value we employed a delta peak approach, Area Under the Curve with respect to increase (AUCi) is also a frequently employed measure for cortisol reactivity (Pruessner et al., 2003) however it uses the first measured value as an implicit baseline, which was not optimal in our case. The sample taken before the first resting state-scan could be regarded as a suboptimal baseline due to mild stress induced by scanner naivety in some subjects leading to a neuroendocrine and subjective stress response (Muehlhan, Lueken, Wittchen & Kirschbaum, 2011). Cortisol reactivity to stress will be defined as peak (sample after resting-state scan two) minus baseline (sample after resting-state scan 1). The sample taken directly after the stress induction protocol may not yet show cortisol reactivity because the effect size of peak cortisol response is largest 20-40 minutes after the task (Dickerson & Kemeny, 2004).

Cortisol data were cleaned by removing outliers 3 standard deviations above the mean per sample (Chiu et al., 2003, Adam & Kumari, 2009). In our sample 14 out of 280 samples in total had to be removed/were missing (approximately 5%), this was divided fairly evenly over our 4 samples (4, 4, 2 and 4 missing data points respectively). Inspection of the data revealed that the cortisol sample was not normally distributed. We employed a natural logarithm transformation to correct for the positive skew (Adam & Kumari, 2009). Due to missing values/removal of outliers the final sample for cortisol reactivity included 62 subjects (30 recruits, 32 controls).

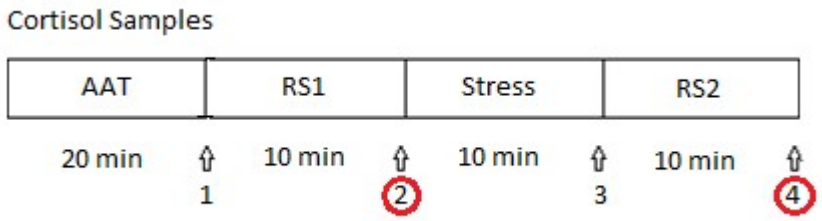


Figure 2: In total five cortisol samples were taken. However for a subset of participants the timing of cortisol samples was slightly different. The four common cortisol samples are shown here. The encircled samples represent the peak (4) and baseline (2).

Heartrate reactivity

Heartrate was measured throughout the entire procedure with an MRI compatible device. Due to time constraints, only data from 27 participants (14 recruits, 13 controls) was analyzed. Average beats per minute (BPM) was calculated for resting-state scan one and two separately using in house software (Hera). We expected the average heartrate (BPM) to increase from resting-state scan one to resting-state scan two (Vogel et al., 2015).

Negative Affect

Finally we included a subjective measure, the Positive And Negative Affect Scale (PANAS, Watson et al., 1988). The questionnaire consists of 20 affective states on which participants can rate their current feelings from 1 (not at all) to 5 (very strong). The 20 affective states can be divided into 10 statements reflecting negative affect and 10 statements reflecting positive affect. Sumscores were calculated for both positive and negative affect before and after stress induction. The difference score of negative affect was used in subsequent (correlational) analyses. We expect an increase in negative affect due to stress induction and no change in positive affect based on previous literature employing the same stress induction (Vogel et al., 2015; Bos, Schuijer, Lodestijn, Beckers & Kindt, 2014; Watson et al., 1988). In addition to negative affect we also asked the participants to rate the unpleasantness of the cold water on a scale from 1 to 9. Unfortunately, data on unpleasantness for eighteen police recruits is not available.

Perceived Stress Scale

The PSS is a self-report 14-item questionnaire which measures perceived stress (Cohen et al., 1983). Participants completed the questionnaire on the first day (PSS W1) they were tested and four months later (PSS IQ) when they participated in their first emergency aid training. A minor revision was made to the PSS IQ. Instead of asking about perceived stress the previous year we asked about

the previous *four months*, as our sole purpose of the second measurement was to measure perceived stress differences related to the emergency aid performed over those four months. We utilized the difference score (PSS IQ minus PSS W1) as the dependent variable in a regression analysis with connectivity coefficients, group, gender and the Dutch version of the Youth Trauma Questionnaire – short list (JTV; Bernstein et al, 2003) as predictors. Group was incorporated as a predictor because the police recruits and controls reacted differently to the stress induction, as measured by differences in negative affect and cortisol responsivity (see Results). As gender and previous traumatic experiences are among the main predictors of post-traumatic stress disorder (see for example Tolin and Foa, 2006), and women and men do not experience (nor cope with) stress in the same manner (see for example Frankenhaeuser, 1996), we decided to incorporate them into the initial regression analysis to control for possible confounding effects. Furthermore we repeated the regression analysis without controlling for the JTV, because youth trauma can influence amygdala connectivity patterns (see for example Qin, Young, Duan, Chen, Supekar and Menon, 2014) this may introduce interesting variation in the connectivity coefficients. To control for collinearity problems arising due to high correlations between BLA-Dorsal and BLA-Ventral, and between CMA-Dorsal and CMA-Ventral (both r>.7, p<.001), we performed the regression analysis separately for the dorsal and ventral network. Standardized betas are reported as none of the predictors is on the same scale and standardization thus ensures predictor contributions can be compared. Internal consistency coefficients were high for both the PSS W1 and PSS IQ with Lambda 2 = .870 and coefficient Alpha = .862 for PSS W1 and Lambda 2 = .855 and coefficient Alpha = .847. Although Lambda 2 is generally a better lower boundary for internal consistency than Alpha (Ellis, 2013), Alpha was also reported for comparing results between papers.

FMRI data

Acquisition parameters

The resting-state functional MRI data were acquired using a Siemens Prismafit 3 Tesla. A total number of 500 volumes were acquired with a multi-band EPI sequence for each session (slices= 64, TR= 735ms, TEs= 39ms, flip angle= 52°, voxel size = 2.4x2.4x2.4mm^3, slice gap= 0 mm, FOV= 210x210mm^2).

Preprocessing

Image preprocessing was conducted using SPM12 (Wellcome Trust Center for Neuroimaging, London, UK) as implemented in Matlab2015b (The Mathworks, Natick MA). The first five volumes were discarded to allow for T1 equilibrium to set. As slice timing correction was deemed unnecessary as the TR is short (0.735 seconds) and slice timing correction can result in loss of high frequency

signal (Smith et al., 2013), we did not implement slice timing correction during data preprocessing. Following steps were taken for the preprocessing: to remove subject motion artifacts we realigned all images with the first image using a least squares approach and rigid body transformation (6 parameters). The images were then resliced to match the first image voxel-by-voxel, registered and normalized to MNI space. Afterwards, the images were spatially smoothed using a 6 mm full width at half maximum (FWHM) Gaussian kernel and were temporally filtered using a high pass filter with >0.01 Hz. Subsequently, linear regression was used to regress out influence of head motion by adding 24 motion parameters (six realignment parameters, their derivatives and squared values).

FMRI statistics

To replicate Roy et al.'s (2009) findings we employed a seed-based two-step procedure where we first looked at whole-brain connectivity of the amygdala subnuclei and afterwards utilized an ROI-based approach looking into the BLA and CMA connectivity with the dorsal and ventral networks respectively. The BLA and CMA masks were defined using the Juelich atlas (Amunts et al., 2005) for each hemisphere separately and all were thresholded using a 50% probability. Then the masks for the left and right hemisphere were combined and finally binarized. The mean time series of the two subnuclei (BLA and CMA) were extracted using the MarsBar toolbox in Matlab (Brett et al., 2002). In total we ran four first level models: first we ran two models with the BLA and CMA extracted time series as a regressor. Thereafter, we ran two models with BLA and CMA extracted time series as regressor with the residual images of the other subnucleus as input. The latter two models could be regarded as partial correlation models, where we try to elucidate the functional connectivity pattern of one amygdala subnucleus while controlling for the influence of the other subnucleus on the connectivity. Unless otherwise reported, the results from the partial correlation model are used as input for subsequent analyses.

Furthermore, to distinguish connectivity changes due to our stress induction protocol in amygdala-frontal projections, we employed a second level model in which we performed a paired samples t-test. Resting-state scan one and two were treated as within-subject variables, to investigate which regions show stronger or weaker connectivity with the BLA and CMA due to stress induction. Finally, as we were especially interested in the dorsal and ventral mPFC networks, we performed region-of-interest analyses by applying predefined PFC ROI masks (see below)

Frontal ROIs construction

Firstly, we selected ROIs from Van Oorts Instantaneous Connectivity Parcellation atlas (van Oort, 2014) to match the definition of ventral and dorsal networks proposed by Etkin et al. (2011)(figure 3B). Van Oorts atlas was used because other atlases did not sufficiently distinguish

between the dorsal and ventral network. The functional parcels defined in Van Oort's atlas however were not specific to the medial PFC and some of them also incorporated part of the lateral prefrontal cortex. Therefore we masked the ROI with a medial PFC mask that was constructed by combining and binarizing the masks of anterior cingulate gyrus, frontal medial cortex, paracingulate gyrus and subcallosal cortex from the Harvard-Oxford cortical structural atlas (Jenkinson and Smith, 2001). Figure 3A displays the final ROIs used for ROI-based analyses.

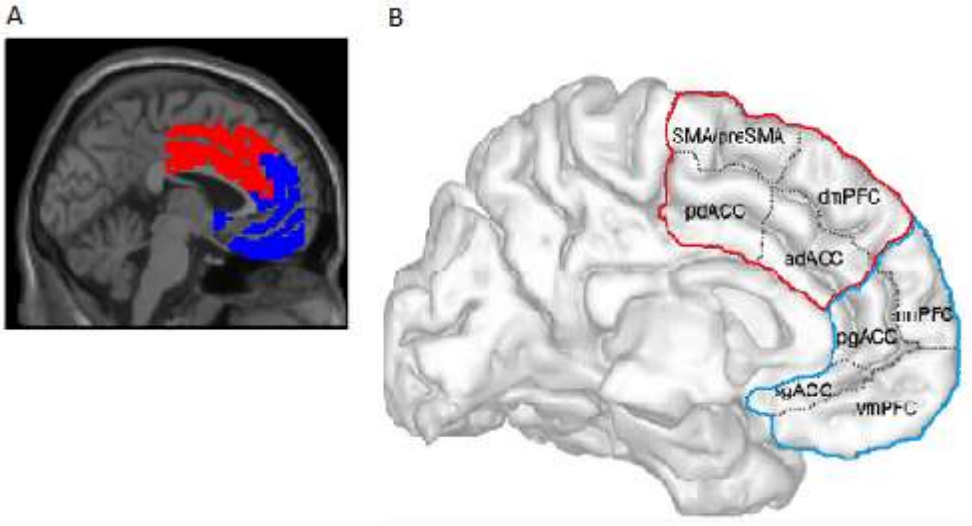


Figure 3: A) the ROIs as defined for this study, the dorsal network is specified in red and the ventral network is specified in blue. B) the anatomical definition of the brain regions our ROIs are based on, adapted from Etkin et al. (2011).

Results

Stress Induction check

Before we carried out any analyses we checked for motion differences between scans and groups. In analyses where sphericity is violated we report Greenhouse-Geisser values. Finally, when the assumptions of normality were violated nonparameteric correlations/tests are reported.

Motion differences

We calculated mean framewise displacement values (FD) for each subject (Power, Barnes, Schnyder, Schlagar & Petersen, 2012). Two participants were identified as outliers with FD values three standard deviations above the mean during resting-state scan two, these participants were excluded from further analyses. Wilcoxon rank test showed there was no difference in motion between resting-state scan one and two (z = -1.626, *p*=.104). Mann Whitney U tests showed controls moved significantly more than recruits during resting-state scan 1 (z=-2.241, *p*=.025 and *d*= -.622) and

resting-state scan 2 (z=-2.573, *p*=.010 *d*= -.649). The groups were similar in motion difference between scans. In all connectivity-related analyses we incorporated FD as covariate.

Cortisol Reactivity

Repeated measures Analysis of Variance (RM ANOVA), with time and group as within- and between-subject variable respectively, yielded a main effect of time ($F_{(1.35,81)}$=25.543, *p*<.001 and η_p^2=.299), further planned pairwise comparisons revealed all samples to differ significantly from each other (p<.05) except for 2 and 3 (*p*=.107). A significant main effect of group revealed that police recruits and controls differ in their cortisol response ($F_{(1,57)}$=8.692, *p*=.005 and η_p^2=.127) with controls having higher cortisol values than police recruits (see figure 4). There was no significant interaction effect. These results indicate that the stress induction protocol increased cortisol levels and both groups responded similarly.

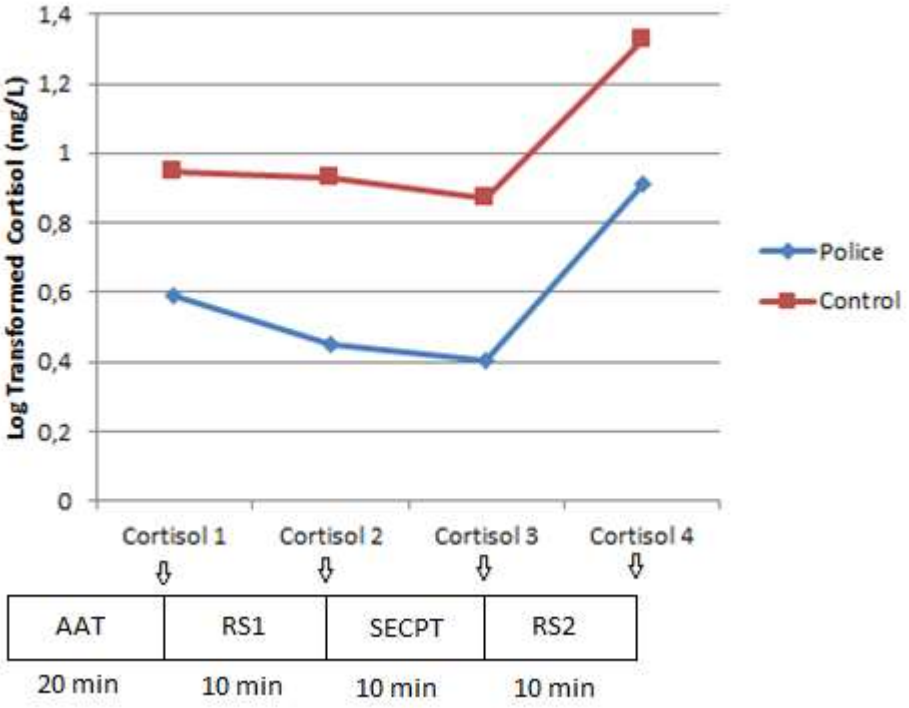


Figure 4: Cortisol response over time for police and control participants, there was approximately ten minutes between each cortisol measure. The first cortisol sample was taken approximately twenty minutes after fMRI scanning, the second cortisol sample was taken after the first resting-state scan, the third immediately after stress induction, the final one after the second resting-state scan.

Heartrate Reactivity

For heartrate reactivity an RM ANOVA with BPM during resting-state scan (one/two) as within-subject variable and group as between-subject variable was conducted. The analysis yielded no significant main effects of time ($F_{(1,25)}$=.086, *p*=.772, M_{rs1}=62.4 and M_{rs2} = 62.6) or group ($F_{(1,25)}$=2.516,

p=.125, M_{police}=60.3 and $M_{recruits}$ = 64.8), nor a significant interaction effect. These results indicate that the stress induction protocol did not increase heartrate.

Negative Affect

Wilcoxon rank test showed a significant increase of negative affect after the stress induction protocol (z=-3.448 p = .001, d=-.482). Mann Whitney U test indicated that controls show a stronger increase in negative affect than police recruits (z=-2.725, p=.006, d=-.634). The increase in negative affect was significant for controls (z=-3.288, p<.001, d=-.679), however not for police recruits (z=-.518, p=.605). Surprisingly, there was no significant difference in unpleasantness with respect to the stress induction between groups as indicated by Mann Whitney U test (z=-.283, p=.777). The mean scores for both groups were 5.1 on a scale from 1 to 9. This suggests police recruits experienced the water to be as unpleasant as controls did, which however seemed to have different impact on their mood.

Conclusion

Even though heart rate did not yield the expected effect, cortisol and the negative affect rating (however only for controls) did show an increase following stress induction. Therefore we conclude that the stress induction was successful.

Stress processing in the brain

Amygdala – frontal connectivity: before stress induction

BLA-based whole-brain connectivity analysis revealed significant connectivity widely distributed throughout most of the cortex, except the dorsal medial frontal region. CMA-based whole-brain connectivity analysis revealed similarly widely distributed significant connectivity in, most notably the putamen, (middle) temporal and frontal gyri, (figure 5A). For the ROI analysis the cross correlation connectivity parameters were used instead of the partial correlation connectivity parameters. We conducted an RM ANOVA with seed region (BLA/CMA) and ROI (dorsal/ventral) as within-subject parameters to verify the predicted amygdala-frontal connectivity model before stress induction. The BLA showed stronger connectivity overall with both frontal networks than the CMA ($F_{(1,68)}$=7.668, p=.007 and η_p^2=.101). A significant interaction effect of seed*ROI ($F_{(1,68)}$=18.991, p<.001 and η_p^2=.218) was further elucidated by separate paired samples t-test. The BLA and CMA did not differ in their connectivity with the dorsal network (p=.166), however the BLA did show stronger connectivity with the ventral network than the CMA (t=3.707, p<.001, Cohen's d= .507) before stress induction (figure 6). These results partly confirm observations from the study by Roy et al. (2009).

Amygdala-frontal connectivity: changes due to stress induction

Next we investigated changes due to stress induction in the brain, by contrasting resting-state scan one and two (before and after stress induction) for each amygdala subnucleus. BLA-based whole-brain connectivity analysis revealed significantly increased connectivity after stress bilaterally in the supramarginal gyrus, posterior and anterior cingulate gyri as well as the right precuneus and superior frontal gyrus and left angular gyrus after stress induction (FWE p<.05 corrected) (Table 1). CMA-based whole-brain connectivity analysis revealed significant increased connectivity bilaterally in the precentral gyrus and the left post central gyrus after stress induction (FWE p<.05 corrected) (Figure 5C, Table 1).

The opposite contrast did not yield any significant connections, there were no areas showing more connectivity before stress induction than after stress induction. To specifically test our hypotheses regarding the connectivity of the amygdala subnuclei with the frontal ROIs, an RM ANOVA with ROI (dorsal/ventral network) and time (resting-state scan 1/resting-state scan 2) as within subject factors was conducted. Connectivity of the BLA increased due to stress induction ($F_{(1,68)}$= 18.440, p<.001 and η_p^2=.213). The interaction effect time*ROI was not significant, indicating similar increases of BLA connectivity with the dorsal as with the ventral network. Connectivity of the CMA did not increase due to stress induction (see figure 6). There was no significant interaction effect of time*ROI. Thus, only the BLA showed increased connectivity in response to stress induction with both the dorsal and ventral network.

Amygdala – frontal connectivity: after stress induction

BLA-based whole-brain connectivity analysis revealed significant widespread connectivity throughout the cortex, now including the dorsal medial prefrontal area as well. CMA-based whole-brain connectivity analysis revealed significant widespread connectivity again in the temporal and frontal regions, including more laterally located areas as well (FWE p<.05 corrected) (Figure 5B). An RM ANOVA with seed region (BLA/CMA) and ROI (dorsal/ventral network) as within-subject variables was conducted using the cross correlation connectivity parameters instead of the partial correlation connectivity parameters. The BLA showed stronger connectivity overall ($F_{(1,68)}$=16.133, p<.001 and η_p^2=.192), paired-samples t-tests were performed to further elucidate the significant interaction effect seed*ROI ($F_{(1,68)}$=20.188, p<.001 and η_p^2=.229). Contrasting the connectivity of the subnuclei for each ROI revealed that the BLA showed stronger connectivity with the dorsal network than the CMA (t=2.603, p=.011, d=.314) as well as with the ventral network (t=4.207, p<.001, d=.511). In addition, contrasting the connectivity of the ROIs for each subnucleus revealed that the BLA showed stronger connectivity with the ventral than the dorsal network after stress induction (t=-3.987, p<.001, d=-.509). The CMA showed stronger connectivity with the dorsal than the ventral network after stress induction (t=3.116, p=.003, d=.380) (figure 6).

Figure 5

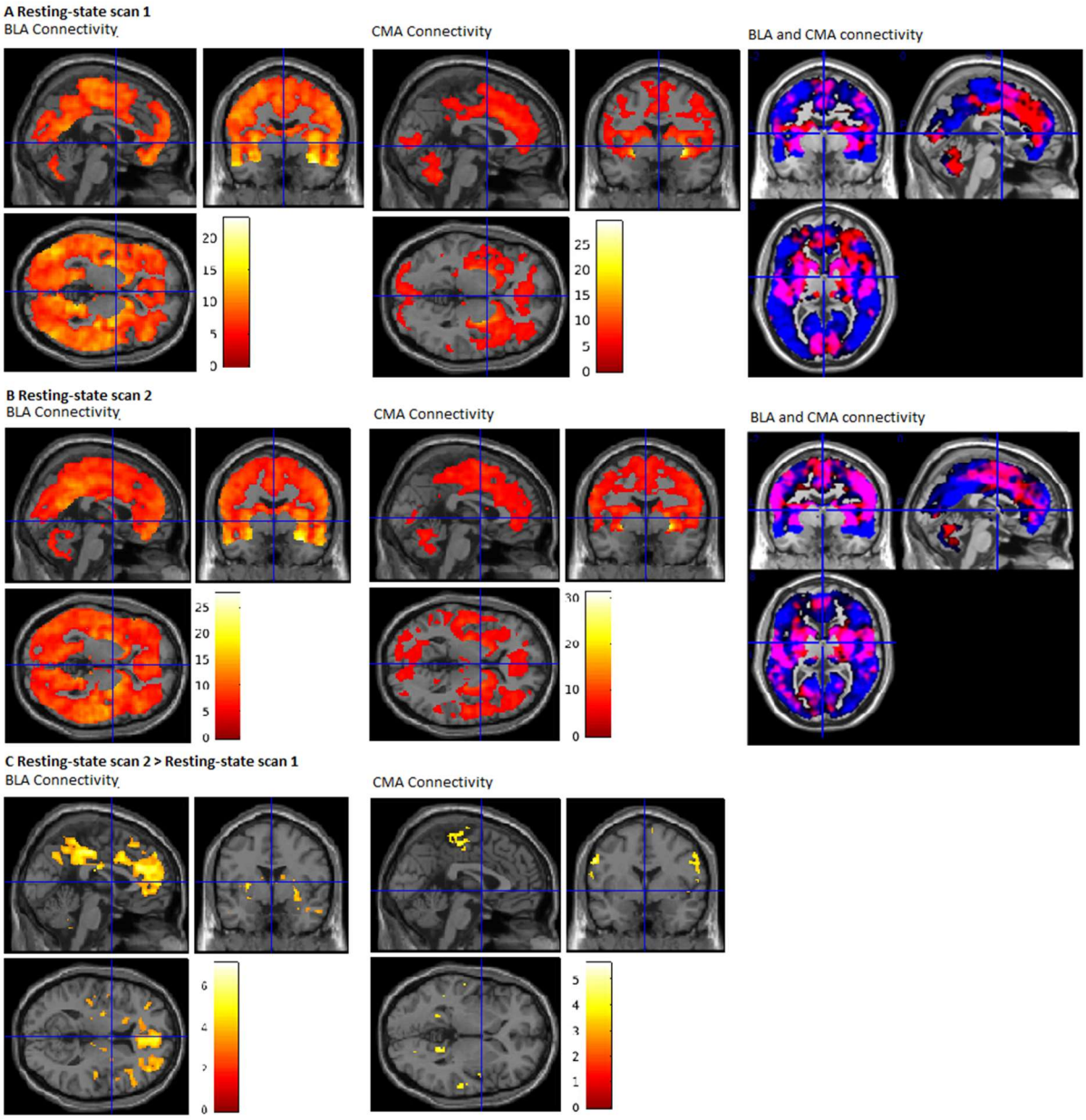


Figure 5: Left and middle: BLA/CMA-based connectivity maps, colors indicate t-statistics, right: BLA whole brain connectivity is portrayed in blue, CMA whole brain connectivity in red and purple indicates the overlap of two A) before stress induction. Image shows FWE (p<.05) corrected connectivity, thresholded at t=5.22. B) after stress induction both seeds show enhanced connectivity throughout the cortex. Image shows FWE (p<.05) corrected connectivity, thresholded at t=5.22. C) contrast after>before. Image shows uncorrected p<.001 connectivity.

Table 1
fMRI coordinates before < after stress induction

	BLA			
Region	MNI (x, y, z in mm)			t
Bilateral				
Supramarginal Gyrus	52	-44	34	7.08
	-58	-48	32	5.88
Anterior Cingulate Gyrus	2	34	24	6.54
	-2	40	-2	5.56
Posterior Cingulate Gyrus	4	-42	24	5.71
	-12	38	38	6.44
Middle Frontal Gyrus	30	56	22	5.94
	-28	38	44	6.39
Right hemisphere				
Superior Frontal Gyrus	2	54	8	5.73
Thalamus	6	-20	6	5.47
Precuneus	8	-46	36	5.42
Left hemisphere				
Angular Gyrus	-52	-54	48	5.26
	CMA			
ROI	MNI (x, y, z in mm)			t
Bilateral				
Precentral gyrus	6	-28	58	5.42
	-58	2	34	5.38
Left				
Postcentral gyrus	-54	16	36	5.49

Figure 6

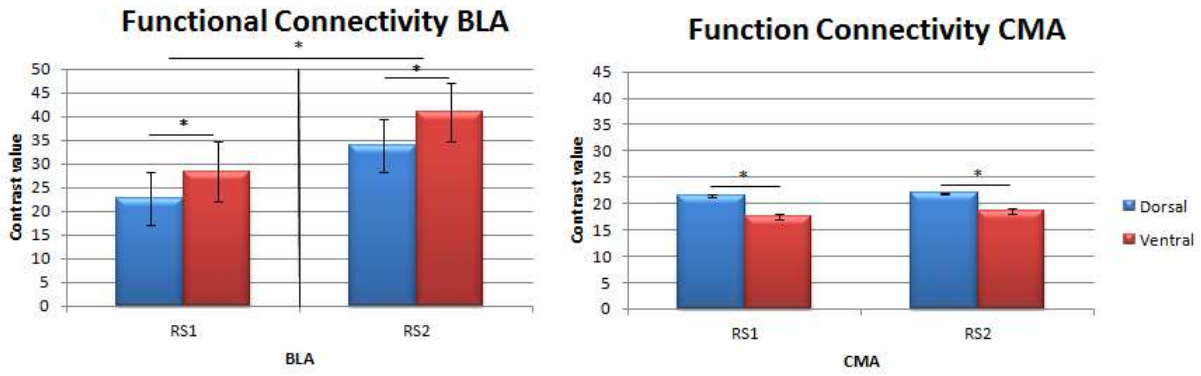
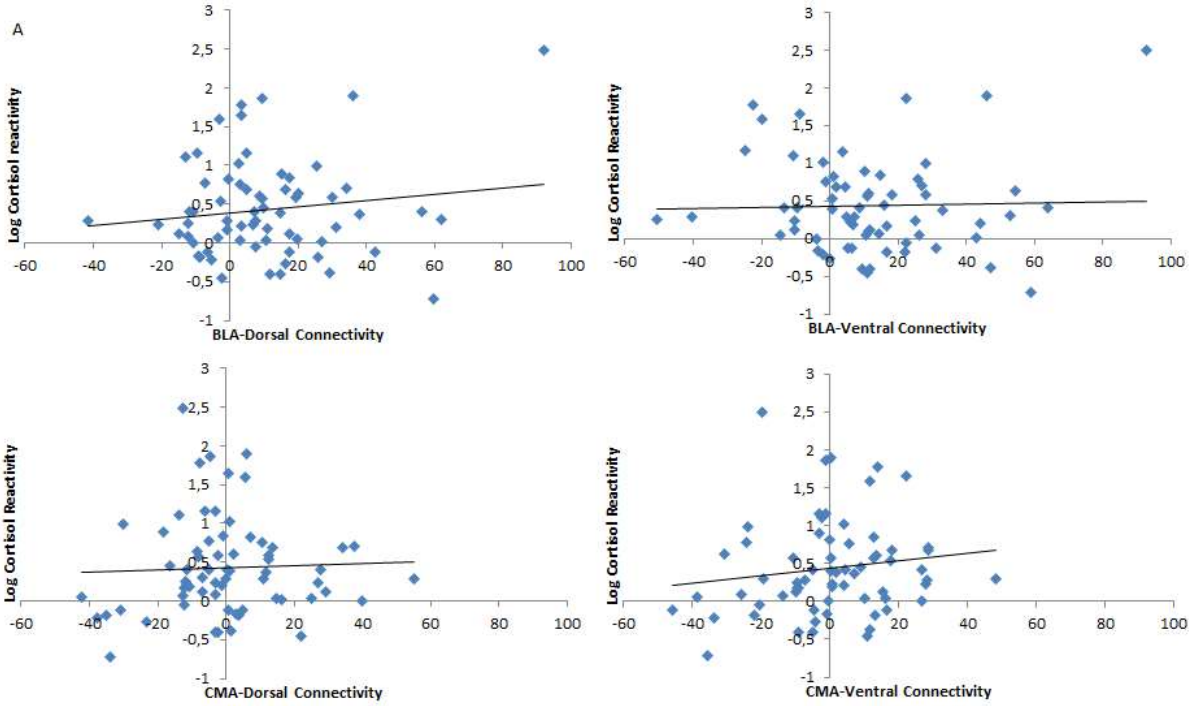


Figure 6: A) Functional connectivity for the BLA increased after stress induction in a similar fashion with the dorsal network as with the ventral network. At both scans the BLA showed stronger connectivity with the ventral network than with the dorsal network. B) Functional connectivity for the CMA did not increase and at both scans the CMA showed stronger connectivity with the dorsal network than with the ventral network. Error bars denote standard error.

Relationship between stress induced brain connectivity changes and real-life stress

Stress induction check: are the connectivity changes related to stress?

To verify that the connectivity differences found between the amygdala and frontal networks are due to stress induction we performed Spearman's correlations on the connectivity coefficients with stress related increases in cortisol, negative affect and unpleasantness of the cold water. Negative affect increase correlates positively with BLA-dorsal increase at trend (r=.240, *p*=.047) and BLA-ventral increase (r=.272, *p*=.024). Cortisol reactivity and unpleasantness was not related to any connectivity parameters. See figure 7A-C for scatterplots.



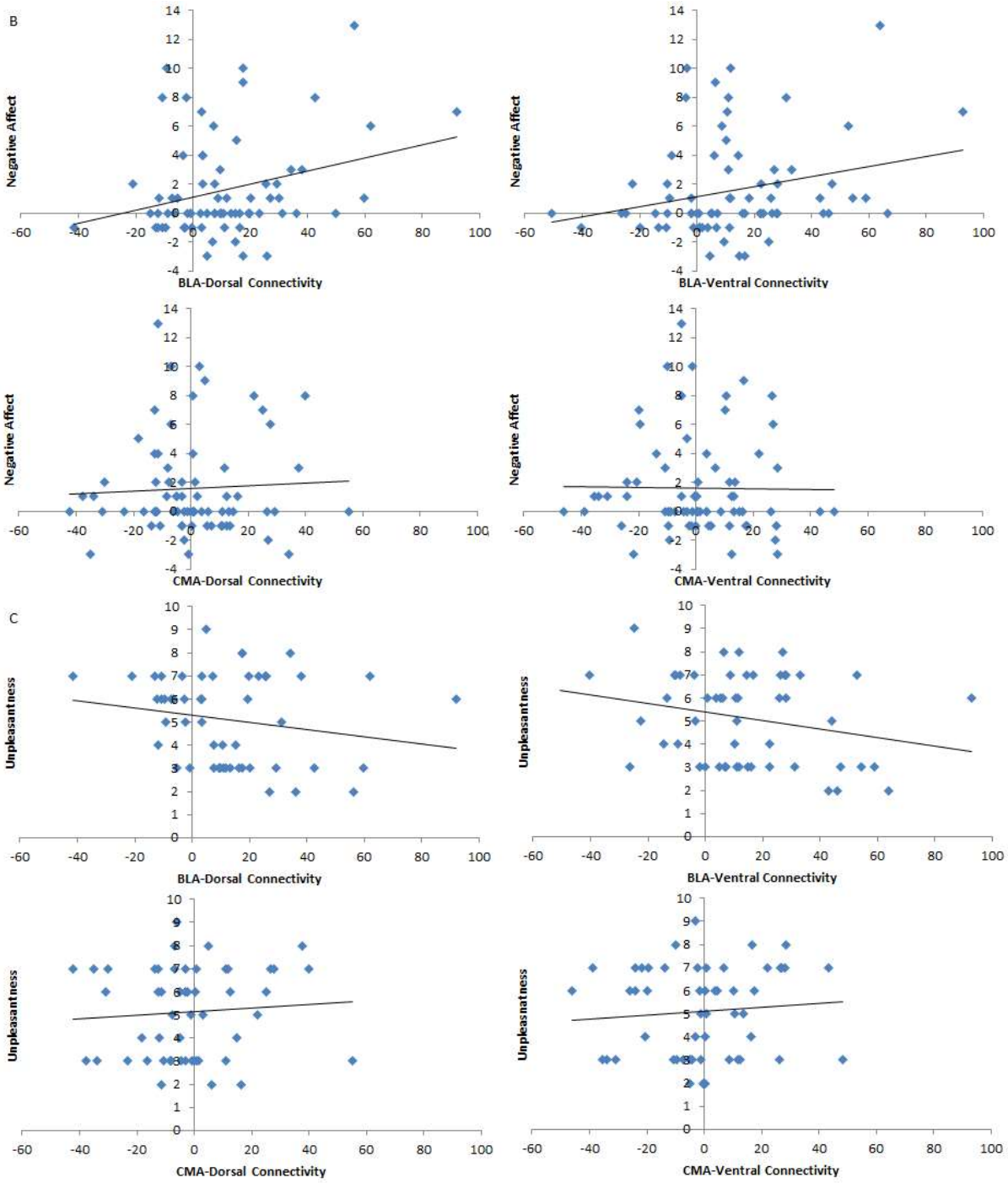


Figure 7: Scatterplots for connectivity coefficients with A) Cortisol reactivity B) Negative affect and C) Unpleasantness

Do stress induced amygdala-frontal connectivity changes predict later stress?

In the current study, we found connectivity increases from the amygdala subnuclei to the medial prefrontal regions due to stress induction, as we expected. We further investigated if those network changes could predict later real-life perceived stress as measured by the PSS (Cohen et al., 1983). Overall, the mean scores of the PSS in our sample were lower than expected based on the numbers reported by Cohen et al. (1983): midtwenties (23-26) and standard deviations hovering around seven

(6-8). As we expected the four months between PSS W1 and PSS IQ to be qualitatively different between subgroups, below we report results separately for police recruits and controls. For police recruits, means and standard deviations were 12.6 (6.4) and 15.4 (6.7) for PSS W1 and PSS IQ respectively and this increase in perceived stress from PSS W1 to PSS IQ was significant (t= -2.195 and p=.036, d=-.385). For controls means were 20.5 (8.2) and 20.9 (8.0) for PSS W1 and PSS IQ respectively, this increase was not significant (t=.- 266 and p=.772, d=-.052). The interaction effect time*group however was not significant (p=.18 and η_p^2= .020). It seems that police recruits experienced increased stress levels after performing four months of emergency aid, but controls did not.

The difference score of the Perceived Stress Scale (PSS IQ- PSS W1) was then utilized as the dependent variable in a regression analysis with connectivity increases, group, gender and the Dutch version of the Youth Trauma Questionnaire – short list (JTV; Bernstein et al, 2003) as predictors. Separate regression models were conducted for the dorsal and ventral network respectively. The regression analysis for the dorsal network revealed that the proposed model with these predictors was only marginally significantly better at predicting the dependent variable than a null model containing the mean of all predictors ($F_{(5,60)}$=1.78, p=.131, Adjusted R^2= .057). However upon inspection of the separate predictors, only group was found to be significant as a predictor of PSS difference score (b^*=-.282, t=-2.168 p=.033). Additionally, JTV showed a marginally significantly predictive effect (b^*=.244, t=1.879 p=.065). The regression analysis for the ventral network revealed a similar pattern although the JTV predictor did not contribute to the PSS difference score. As early trauma can influence amygdala connectivity patterns, most notably of the BLA (see for example Qin, Young, Duan, Chen, Supekar and Menon, 2014). We repeated the regression analysis without the JTV, results remained similar although group was no longer a significant predictor. This may be partly explained by the fact that controls have experienced significantly more trauma than recruits (Mann Whitney U, z=-2.706, p=.007, d=-.710).

Group differences in the relationship between connectivity and perceived stress

In addition to their differential response to the stress induction, police recruits do show an increase in perceived stress while controls do not. Therefore, we repeated the regression models above for police recruits (N=31) and control (N=35) participants separately. For police recruits in both regression models, again considering the dorsal and ventral network separately, none of the predictors was significant. Even though on average there was no increase in perceived stress, the range and variance for the difference score (PSS IQ minus PSS W1) was similar for recruits and controls. For controls, both regression models did not contain any significant predictors. However, at trend, the BLA-dorsal increase (b^*= .298, t=1.855, p=.073) and CMA-dorsal increase (b^*= -.303, t=-

1.952, *p*=.060) after stress induction were predictive of the PSS difference score, as well as the JTV score (*b**=.291, t=1.91, *p*=.066). Thus, some connectivity coefficients might aid in the prediction of perceived stress.

Discussion

In the current study we investigated whether specific stress induced amygdala (BLA/CMA) – frontal (dorsal/ventral) connectivity changes were predictive of perceived stress at a later moment in time. This was assessed in three steps whereby we first explicated a model of the amygdala – frontal connectivity based on Roy et al. (2009). Then we established how the connectivity coefficients changed as a consequence of induced stress. Finally, we related the changes in connectivity coefficients to levels of perceived stress after a four month period. This was done utilizing a resting-state fMRI procedure by contrasting two resting-state scans with a well-established stress induction protocol in between for two groups of participants, police recruits and controls.

Our findings partly confirmed the proposed amygdala-frontal connectivity model. As expected, the BLA demonstrated stronger connectivity with the ventral network than the CMA at baseline (i.e. before stress induction). However, there was no difference between the BLA and CMA with regards to their connectivity to the dorsal network. Although strong reciprocal connections to both the dorsal and ventral network from the BLA could be accounted for by the fact that the BLA is the main input node for information coming from the medial prefrontal regions (Bear, Connors and Paradiso, 2007), this does not explain why the CMA shows equally strong connectivity to the dorsal network as the BLA. Animal literature points to a differential connectivity pattern from the BLA to the CMA, the BLA can both excite and inhibit the CMA depending on which cell cluster is activated through external input from the mPFC. The infralimbic inputs (arising from the ventral part of the mPFC) excite the ITC neurons that inhibit the CMA and the prelimbic inputs (arising from the more dorsal part of the mPFC) excite the glutamatergic cells that excite the CMA (Pape and Paré, 2010). Activation from the dorsal network could thus have activated the glutamatergic cells in the BLA that in turn activated the CMA, clarifying the similar connectivity patterns for the BLA and the CMA with the dorsal network.

Furthermore, as expected, we found that the BLA showed stronger connectivity to the ventral network than to the dorsal network; the CMA showed stronger connectivity to the dorsal network than the ventral network. This is consistent with the idea that the ventral network is involved in regulation, by providing input to the BLA, and that the dorsal network is involved in expression, whereby it is activated by output of the CMA (Bear, Connors and Paradiso, 2007; Etkin, Büchel and Gross, 2015).

Secondly, we investigated the change of the connectivity coefficients in response to acute stress induction. Connectivity of the BLA with the dorsal and ventral network was strengthened as a

result of stress induction, as expected (Van Marle et al., 2010; Veer et al., 2011). Connectivity of the CMA with either the dorsal or ventral network was not affected. The increased connectivity of the BLA with the dorsal network could indicate increased expression of stress, through excitation of the glutamatergic cells that project to the CMA to trigger the physiological stress response (Bear, Connors and Paradiso, 2007; Pape and Paré, 2010). Subsequently, the increased connectivity of the BLA with the ventral network could be seen as a regulatory reaction to this increased stress response.

After stress induction the BLA showed stronger connectivity with both the dorsal and ventral network than the CMA. Thereby partially replicating the results from Vogel et al. (2014), who, in contrast, found that the CMA presented stronger connectivity with the dorsal network than the BLA after stress induction. Even though Vogel et al.'s (2014) connectivity results were task-based (emotional face matching task), we expected that the amygdala-frontal connectivity pattern found, may be indicative of internal stress regulation, due to the functions (expression vs. regulation) of the regions involved (Etkin, Büchel and Gross, 2015). On the basis of our results, we postulate that the BLA is more responsive to acute stress induction than the CMA and could serve as a regulatory node within the amygdala. Its extensive projections from cortical areas (Bear, Connors and Paradiso, 2007) and its different inhibitory/excitatory clusters in connection to the CMA (Pape and Paré, 2010), make the BLA able to both up- and downregulate the physiological stress response.

Additionally, even though the BLA showed stronger connectivity to both frontal regions, it exhibited stronger connectivity with the ventral than with the dorsal network after stress induction. This suggests that the stress response could be inhibited through excitation of the inhibitory ITC neurons in the BLA, as we expected on the basis of animal literature and human resting-state fMRI (Lihtik et al., 2005; 2008; Jovanovich & Ressler, 2010; Roy et al., 2009; Etkin, Büchel and Gross, 2015). The stronger connectivity of the CMA with the dorsal region than with the ventral region suggests that the CMA is mainly involved in expression of the stress response (Etkin, Büchel and Gross, 2015) as it is regarded the main output nucleus of the amygdala (Bear, Connors and Pardiso, 2007).

Finally, predictive validity of the amygdala – frontal connectivity model was assessed using regression analysis to predict perceived stress at a later stage. Contrary to our expectations, increased functional connectivity of the BLA was associated with PTSD symptoms (Brown et al., 2014) and higher anxiety in children (Qin et al., 2014), we did not find any connectivity coefficients to be predictive of later perceived stress. For the whole sample only group and JTV were shown to be (marginally) significantly predictive of the PSS. When groups were investigated separately, in the regression analysis for recruits none of the connectivity coefficients reached significance. Recently researchers found that amygdala – frontal connectivity was not related to the amount of stress exposure per se, but to perceived threat during stress exposure instead (Van Wingen, Geuze,

Vermetten & Fernandez, 2011). As the recruits did not show enhanced negative affect we speculate that they may not have perceived the stress induction as particularly threatening. This may be partly explained by their training at the police academy, police recruits are required to practice stress relieving/mental strength behaviour, such as adjusting breathing patterns (www.politieacademie.nl). Several studies have found that for example mindfulness and yogic breathing can aid in coping with life events (Astin, 1997; Brown and Gerbag, 2005). In addition, these techniques can lower basal cortisol levels (Matousek, Dobkin and Pruessner, 2010), possibly also explaining the baseline difference in cortisol levels of police recruits and controls. Although there have not yet been any studies investigating the relationship between mindfulness based training and acute stress induction, it is suggested the training may have beneficial effects on stress responsivity (Matousek, Dobkin and Pruessner, 2010). Police recruits may respond differently to stressful life events than control participants. More research is needed to elucidate how mental strength exercises (such as yogic breathing or mindfulness) may influence brain connectivity and possible mediating effects in perception of (acute) stress.

Even though the control participants, on average, did not increase in perceived stress from PSS W1 to PSS IQ, the variance and range of the difference score was similar to the police recruits. The connectivity coefficients of BLA-dorsal and CMA-dorsal responsivity to acute stress induction were marginally significant predictors of PSS score for controls, suggesting they could be of use as an indicator of perceived stress. BLA-dorsal and CMA-dorsal connectivity responsivity may indicate that the amount of expression (dorsal network), instead of regulation (ventral network) (Etkin, Gross and Büchel, 2015), may be indicative of perceived stress. We must remain cautious, however, as none of the predictors reached the significance threshold of $p<.05$, which could be due to the small sample size. For prediction with a multiple linear regression analysis the sample size we employed may be too small. There is an ongoing debate about minimum sample size (from 30 participants to 1 predictor to 10 participants to 1 predictor), what is becoming clear, is that the purpose of the multiple regression analysis and the size of the effect measured can be leading as to decisions on sample size (Knofczynski & Mundfrom, 2007). With our sample size around 30 for each subgroup, effects must be of considerable size to allow for enough power to detect them. Including more participants will benefit the detection of predictive validity.

Apart from sample size, a further limitation of this project is the absence of a control task for the stress induction protocol. In absence of a control task we reverted to correlating connectivity coefficients to known stress markers. Although suboptimal, correlation does not imply causation, it is plausible that the connectivity changes found arise from stress induction and are not due to other factors (e.g. time/practice effect). The stress induced changes in brain connectivity patterns were found to be positively correlated to stress increase markers e.g. increased cortisol and negative

affect. Unexpectedly, heartrate did not increase due to stress induction, underlying reasons could be that, due to time constraints, only a small sample was analysed (N=27). In addition, by averaging over the entire resting-state scan (as is common in similar studies, e.g. Vogel et al. (2015)) we may not have been able to discern small heartrate variations during the scan. Further research could, for example, use one minute bins instead of an average over the resting-state scan. In addition to methodological improvements, future research may look into the predictive effect of the amygdala – frontal connectivity pattern on symptomatology of other stress/mood/anxiety-related disorders.

Concluding, the current paper shows for the first time in a targeted manner that there are consistent differences in amygdala – frontal connectivity patterns regarding their (functionally different) sub regions. In addition, we show that these connectivity patterns rapidly adapt to induced acute stress and that they appear related to subjective stress levels, immediately after stress induction and later in response to real life stress. This demonstrates that amygdala – frontal connectivity and its response to induced acute stress could be of aid in predicting how individuals react to stress, which could aid in screening and perhaps early intervention for vulnerable individuals in high risk professions.

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