5-HTTLPR and anxiety modulate brain-heart covariation

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Abstract

To date, little is known about genes affecting the interplay of brain and heart activity. Because serotonin (5-HT) is involved in cortico-vagal neurotransmission, we tested whether the 5-HT transporter polymorphism 5-HTTLPR affects brain-heart covariation. Further, associations with neuroticism/anxiety (NANX) were tested, as anxiety is related to 5-HT and neurogenic changes of heart period (HP). N = 168 participants performed a time-estimation task while EEG and HP were recorded. Brain-heart covariation was measured using time-lagged within-subject correlations of centromedial feedback-evoked single-trial EEG at 300 ms and subsequent changes of HP. EEG-HP correlations were higher in 5-HTTLPR long-allele carriers. Moreover, after negative feedback, EEG-HP correlations and feedback-related negativity amplitudes independently correlated with NANX. The results indicate that individual differences in brain-heart covariation relate to 5-HT and NANX.

The sheer thought or perception of motivationally meaningful events may change the speed of subsequent heartbeats (Crone et al., 2003; van der Veen, van der Molen, Crone, & Jennings, 2004), presumably involving pathways from different brain structures (e.g. anterior cingulate cortex, insula, amygdala) over midbrain and the sympathetic and parasympathetic branches of the autonomous nervous system down to the sinatrial node of the heart (Benarroch, 1997; Critchley et al., 2003; Wager et al., 2009). One class of stimuli that leads to relatively quick changes in heart period (HP, i.e. the time between two heart beats) is feedback about ones own performance (Crone, et al., 2003; van der Veen, et al., 2004). Specifically, external (Crone, et al., 2003; van der Veen, et al., 2004) and internal (Hajcak, McDonald, & Simons, 2003, 2004) performance feedback has been reliably shown to modulate HP one to several heart beats after feedback presentation / error commission. Because feedback stimuli are often quite abstract and thus likely involve processing at cortical structures, and because such feedback effects on phasic HP are too fast to involve sympathetic neurotransmission (Berntson et al., 1997) these findings strongly suggest, that performance feedback activates communication between the cortex and the vagus.

Consistent with the proposal that feedback evokes coupling of brain and heart activity, it has recently been shown, that the magnitude of a single cardiac response to feedback can be predicted by preceding feedback-evoked brain activity (Mueller, Stemmler, & Wacker, 2010). Specifically, phasic feedback-evoked brain-heart covariation has been demonstrated with cardio-electroencephalographic covariance tracing (CECT), which uses time-lagged within-subject correlations of stimulus-evoked single-trial EEG and HP (Mueller, et al., 2010). Several CECT studies have demonstrated that the EEG signal 300 ms after feedback presentation predicts subsequent reductions in HP: the more positive the EEG signal is after feedback presentation, the more will the heart accelerate at subsequent beats (Mueller, Evers, Wacker, & van der Veen, 2012; Mueller, et al., 2010). This effect has been labeled “N300H”

due to the emerging **n**egative correlation between the EEG signal at **300** ms and **H**P. Note, that the N300H effect is consistent with earlier research, demonstrating that the well known P300 event-related potential (ERP) is elevated in the same conditions that also induce an acceleration of heart rate (Donchin et al., 1984; Gatchel & Lang, 1973; Otten, Gaillard, & Wientjes, 1995). However, it should be emphasized that the N300H is based on within-subject correlations (correlating EEG and HP over trials rather than subjects). The N300H is therefore essentially different from between-subject correlations of P300 and stimulus-evoked HP (Otten, et al., 1995) and is particularly useful to investigate brain-heart covariation at the individual level.

Individual differences in the coupling of brain and heart activity may be of relevance for psychological dispositions (e.g. trait anxiety as described below) and cardiovascular health (Thayer & Lane, 2007). They may be associated with serotonin (5-HT), as this neurotransmitter is relevant for transmitting information from the brain to the heart (Jordan, 2005) e.g. via 5-HT_{1A} receptors at vagal nuclei binding sites and nucleus tractus solitarius (Manaker & Verderame, 1990; Thor, Blitz-Siebert, & Helke, 1992). Of relevance, it has been shown that the pharmacological depletion of tryptophan, a precursor of central 5-HT synthesis attenuates feedback-evoked N300H (Mueller, et al., 2012). Moreover, individual differences in plasma tryptophan predict N300H amplitude such that individuals with presumably reduced 5-HT synthesis capacities show lower brain-heart covariation (Mueller, et al., 2012).

These findings raise the question, whether serotonergic genes contribute to individual differences in brain-heart covariation. Perhaps the most prominent 5-HT related polymorphism is 5-HTTLPR, which exists in short and long allele variants. Carriers of at least one short variant express lower levels of 5-HT transporters. Despite possibly resulting elevations of extracellular 5-HT levels, human and animal studies suggest that the reduction of 5-HT transporters associated with the short allele primarily reduces 5-HT signaling due to attenuated postsynaptic 5-HT_{1A} -receptor sensitivity (David et al., 2005; Li et al., 1999).

Accordingly it could be hypothesized, that short allele carriers show relatively reduced brain-heart covariation due to blunted serotonin signaling. Preliminary support for this hypothesis comes from studies showing that carriers of the short allele display lower cardiac reactivity to mental stress (Brummett, Siegler, Ashley-Koch, & Williams, 2011; Williams et al., 2001; Williams et al., 2008) and reduced respiratory sinus arrhythmia (Ellis, Beevers, Hixon, & McGeary, 2011). However, these findings could also be driven by influences of 5-HTTLPR on mechanisms other than brain-heart coupling (e.g. on overall stress reactivity). Therefore, the primary goal of the present study was to specifically test, whether 5-HTTLPR predicts individual differences in brain-heart covariation as assessed with the previously described CECT method.

The 5-HTTLPR polymorphism has been linked to neuroticism and anxiety (NANX) and the short allele has sometimes been thought of as a vulnerability factor for developing high NANX or affective disorders (Lesch, et al., 1996). However, several meta-analyses indicate that the association between the short allele and elevated NANX measured with different questionnaires is less robust than initially expected (Sen, Burmeister, & Ghosh, 2004; Schinka, Busch, & Robichaux-Keene, 2004), particularly in healthy individuals (Minelli, Bonvicini, Scassellati, Sartori, & Gennarelli, 2011). Moreover, the short allele may predict NANX and related disorders when individuals experienced adverse life-events (Karg, Burmeister, Shedden, & Sen, 2011) while it may even predict reduced affective disturbance if no such events occurred (Wilhelm et al., 2006; Zalsman et al., 2006; Kuepper et al., 2012). Accordingly Homberg & Lesch (2011) recently reconceptualized the functional role of the 5-HTTLPR polymorphism and some have considered the short allele a plasticity marker that may lead to higher or lower risk for affective disturbance than the long allele, depending on further moderators (Belsky, et al., 2009). Thus, although there appears to be no simple bivariate relationship between 5-HTTLPR and NANX, there is converging evidence for a link between 5-HT and NANX, suggesting that individual differences in NANX may be

associated with neurobiological processes which involve 5-HT signaling (Gray & McNaughton, 2000; Graeff, 1994).

Of relevance, brain-heart coupling plays an important role in anxiety and negative affect and individual differences therein (Berntson, Sarter, & Cacioppo, 1998; Friedman, 2007). In conditions of anxiety, rapid neurogenic vagal withdrawal in combination with sympathetic activation is part of the default response to threat, which may be hypersensitive in highly anxious individuals (Thayer & Lane, 2009), who may be characterized by rigid coupling of the central and autonomic nervous system (Friedman, 2007). Furthermore, the perception of increased physiological arousal may contribute to states of panic and anxiety, thereby predisposing individuals with strong brain-heart coupling to experiencing anxiety more often. Because brain-heart coupling could thus be linked to NANX through various mechanisms, the second goal of the present study was to test whether individuals with high NANX are characterized by elevated N300H after feedback. Moreover, NANX-related brain systems (e.g. the so-called Behavioral Inhibition System) may be particularly activated when a mismatch between actual and intended outcomes is detected (Gray & McNaughton, 2000). For example, the feedback-related negativity (FRN, Miltner, Braun, & Coles, 1997), is a frontomedial event-related potential component with a latency of 200 – 300 ms, which is more negative after negative vs. positive feedback and may be increased in high NANX individuals (Santesso, et al., 2012; Sato et al., 2005). Based on this putative hypersensitivity for negative feedback it was further tested, whether NANX would be more strongly correlated with N300H after negative vs. positive feedback.

Methods

Main study

Participants

Data was collected as part of a larger research project in which participants performed three different tasks. From $N = 203$ individuals who participated in this study, datasets had to

be excluded due to missing or insufficient EEG (n = 13; 6.4%), ECG (n = 14; 6.9%) or event-marker data (n = 8; 3.9%) yielding a total sample of N = 168 non-smoking, right-handed young males without illnesses or DSM-IV diagnoses as assessed with a standardized clinical interview (Margraf, 1994). All participants gave written informed consent before participating and received a monetary compensation of 70 EUR (90 USD) for approximately 7 hours involvement in the project. The study protocol was approved by the Ethics Committee of the German Society for Psychology (Deutsche Gesellschaft fuer Psychologie).

Procedure

After arrival at the laboratory participants gave informed consent, were administered the standardized clinical interview and received a light breakfast if no exclusion criteria applied. To test hypotheses unrelated to this report (Mueller, Makeig, Stemmler, Hennig, & Wacker, 2011) half of the participants ingested either a dopamine antagonist (sulpiride, 200 mg) or placebo.[1] Thereafter participants completed personality questionnaires (for a report on the same dataset see: Wacker, Mueller, Hennig, & Stemmler, 2012) and performed a 20-minute signal detection task (Pizzagalli, Jahn, & O'Shea, 2005) which will be reported elsewhere. After the signal detection task participants had a 5-minute break and then started the time-estimation task. After the time estimation task, participants conducted a Flanker task, for which the results have been reported elsewhere (Mueller et al., 2011).

Time Estimation Task

The time estimation task was similar to a previously reported paradigm which has shown associations with NANX (Hirsh & Inzlicht, 2008). A central fixation cross appeared for 250 ms followed by a blank screen. Participants were instructed to press a response button, 1 s after the onset of the fixation cross. A feedback stimulus was provided 2 s after the fixation cross and lasted for 1 s. To allow for the cardiac response to develop the intertrial interval was longer (i.e. variable interval of 5 to 5.5 s) than in the earlier study (Mueller, et al., 2010). Participants completed 10 practice trials and then 4 blocks of 60 trials yielding a total

of 240 trials that were used for analyses. During a short break after the second block participants provided a series of current affect ratings and were asked to relax for 2 min.

The feedback was either positive (a plus sign) or negative (a minus sign) depending on whether the response was given within 1 s +/- x, where x was an adaptively changing time window that was initially 100 ms and that increased 10 ms following an incorrect response and decreased 10 ms following a correct response, ensuring that there was a similar number of correct and incorrect responses throughout the task. Instead of positive or negative feedback, neutral feedback (a question mark) was given on a third of the trials, leaving participants uncertain about their performance.

Physiological Recording

EEG. EEG was recorded at a sampling rate of 512 Hz using an Active Two (BioSemi, Amsterdam, Netherlands) active electrode system with Driving Right Leg and Common Mode Sense as active and passive reference, respectively. Due to changes in equipment availability throughout the course of data collection EEG data of the first n = 86 and remaining n = 82 participants were recorded using 32- and 64-channel configurations, respectively[2]. EEG was re-referenced to average reference, downsampled to 128 Hz, low- (40 Hz) and high- (.1 Hz) pass filtered using two-way least squares finite impulse response filtering implemented in EEGLAB (eegfilt.m; Delorme & Makeig, 2004) and manually screened for data epochs with non-occular artifacts (e.g. due to movement, lose electrodes etc.). Independent Component Analysis (infomax ICA algorithm by Bell & Sejnowski 1995) was then applied to the EEG data and components with clear eye-blink related characteristics (i.e. frontopolar topography and increased activity at blink latencies) were manually selected and subtracted from the EEG. EEG was segmented into intervals from -250 to 500 ms relative to the feedback stimulus and the average amplitude from -250 to 0 ms served as baseline. Each segment was subdivided into 32 baseline plus 64 post-event bins with a duration of 1

sample (7.8 ms) and for each bin, the single-trial signal amplitudes were used for CECT analyses (see below).

Heart Period. The electrocardiogram was assessed using Ag/AgCl surface electrodes (VivoMed, Servoprax, Wesel, Germany) connected to a Biopac MP 100 system with an ECG100c amplifier module (Goleta, CA, USA) and the signal was recorded at a sampling rate of 1000 Hz using Labview-based software. Custom-made MATLAB (Mathworks) scripts merged the ECG and EEG data after bandpass-filtering (1 to 30 Hz; finite impulse response filter implemented in eeglab; Delorme & Makeig, 2004), downsampled the ECG to the same sampling-rate as the EEG (128 Hz) and detected R-spikes to create a continuous heart period (HP) trace where the value at each time-point reflects the temporal distance between the preceding and succeeding R-spike in ms. Together with the EEG and ECG, the HP trace was manually screened for artifacts to remove trials during which a R-spike had not been correctly identified. The HP trace was segmented into intervals from 0 to 5000 ms relative to the feedback stimulus and the HP at the time of the feedback stimulus served as baseline (i.e. was subtracted from each value of the corresponding segment). Each segment was then sub-divided into 10 bins by taking the average HP value for 10 consecutive 500 ms bins (i.e. 0-500 ms, 500-1000 ms, etc). The single-trial HP-values for each bin were used for CECT analyses (see below).

CECT-Analyses

CECTs reflect time-lagged within-subjects correlations between evoked single-trial EEG and evoked single-trial changes in HP. Thus, CECTs were computed at the individual subject level by systematically correlating each of the 64 post-event EEG bins with each of the 10 HP bins over trials, resulting in one 10 x 64 correlation matrix for each type of feedback valence (see Figure 1). In these CECT matrices the value in row 1, column 1 reflects the correlation of the EEG magnitude 0 to 7.8 ms after feedback with HP changes from 0 to 500 ms after feedback, the value in row 2, column 1 reflects the correlation of EEG

magnitude from 0 to 8 ms (i.e. red column in Figure 1B) with HP changes from 500 to 1000 ms (i.e. blue column in Figure 1C), and so on. Accordingly, N300H is reflected in a cluster of negative correlations around column 38, indicating that those trials that are characterized by an increased EEG amplitude around 300 ms are also characterized by subsequent accelerations of heart beat. Note, that these EEG-HP correlations are performed at the individual level in order to quantify cross-trial relationships of EEG and HP, whereas statistical inference is made at the group level. For that purpose, CECT matrices are Fisher transformed and t-tests against zero are computed across individuals for each of the 640 correlation cells (+ 320 cells for the baseline period). Grand average CECTs can also be computed by averaging the individual Fisher-transformed EEG-HP correlation matrices across individuals. Color-coded grand average CECTs and t-test CECTs for the present study are displayed in Figure 2 with blue indicating negative and red indicating positive correlations. To visualize the spread of significant CECT values, thresholded CECTs (i.e. $p <$ .001, uncorrected) are also displayed (Figure 2d).

To test for individual differences in cortico-cardiac covariation N300H values were determined for each individual. In a previous study N300H was maximal from 280 to 340 ms in the EEG and 3500 to 4000 ms in HP time domain at centromedial electrodes (Mueller et al. 2010). In the present study, individual EEG-HP correlations were therefore measured at channel Cz in an *a priori* defined time window from 300 to 350 ms in the EEG and from 3500-4000 ms in the HP time domain. For each individual and feedback valence, all EEG-HP correlations in this time-window were aggregated, resulting in three N300H values for each individual (for positive, negative and neutral feedback) that were used for statistical analyses.

Event-related potentials and evoked HP

To confirm that individual differences in N300H are not driven by individual differences in mean EEG or HP signal amplitude we also measured the mean amplitude in the event-related potential from 300 – 350 ms at channel Cz and the mean evoked HP from 3500

– 4000 ms (both averaged across trials). Moreover, to test if variations in N300H to negative feedback are related to individual differences in the feedback-related negativity event-related potential component, which has been previously associated with NANX (e.g. Sato et al., 2005) we measured the FRN as the mean amplitude of the negative feedback evoked event-related potential from 200 to 300 ms at channel Fz (again averaged across trials).

Genotyping

5-HTTLPR genotypes were determined from buccal cells as previously reported (Osinsky et al., 2008) with the resulting genotype distribution: n = 33, 10, 67, 0, 11, and 47 for S/S, S/L_G, S/L_A, L_G/L_G, L_G/L_A , and L_A/L_A. L_G+ and S+ were grouped based on the almost identical 5-HT transporter expression (Hu et al., 2006) leading to two groups of 47 (L_A/L_A) and 121 (S+,L_G+). To allow comparison with di- rather than tri-allelic classifications of 5-HTTLPR (Lesch, et al., 1996) we also tested S+ (N = 110) vs. L/L (N = 58) carriers. The genotype distribution was in Hardy-Weinberg equilibrium ($X^2 < .8$, $p > .3$).

NANX

To measure individual differences in NANX we used a German translation of the behavioral inhibition system (BIS) scale (Carver & White, 1994) because (a) the BIS, assumed to reflect the neurobiological substrate of anxiety, has been explicitly linked to serotonin and elevated arousal including heart rate (Gray & McNaughton, 2000), and (b) compared to other more heterogeneous NANX scales the items of the BIS scale are strongly focused on the anticipation of and response to negative events and may, thus, be maximally relevant to physiological reactions in the feedback processing paradigm used here (see Results for item examples). In the present sample Cronbach's Alpha of the BIS scale was .78. To test for specificity of associations with the BIS scale we also assessed the BAS score, which reflects interindividual differences in the Behavioral Approach System sensu Gray (Gray & McNaughton, 2000). In addition, the German version of the NEO-PI R Neuroticism scale

(Costa & McCrae, 1992; Ostendorf & Angleitner, 2004) was administered to probe convergent validity with a widely used measure from the NANX domain.

Statistical Analyses

To replicate the finding that *within*-subject correlations between EEG around 300 ms and HP 4 s later are robust *across* subjects (Mueller, et al., 2010), the mean individual N300H amplitudes at channel Cz were tested against zero for each feedback-valence using one-sample t-tests. Further, to confirm the previously reported centromedial topography of N300H, within-subject correlations of EEG at 300-350 ms and HP at 4 s were computed for 32 channels (across feedback valence) and a repeated measures ANOVA with the factor Channel was conducted. To test whether N300H was modulated by 5-HTTLPR, a 5-HTTLPR (S,L_G vs. L_A/L_A) x Feedback Valence (positive vs. negative vs. neutral) ANOVA was conducted on the mean N300H amplitude using the Geisser-Greenhouse correction were applicable. To test whether NANX is associated with enhanced N300H evoked by negative feedback, Pearson correlations between BIS score and N300H for each feedback type were computed. Differences of dependent correlations between positive feedback evoked N300H and BIS vs. negative feedback evoked N300H and BIS were tested using the approach suggested by Meng and Rosenthal (1992). For these tests one-sided significance tests were used because correlations were a priori hypothesized to be larger for negative vs. positive feedback. To make sure that any association with N300H was not driven by individual differences in cardiac acceleration or ERP amplitude per se, analyses controlling for HP and ERP amplitudes (at the same latencies and electrode as N300H) and FRN were conducted.

Re-analysis of Mueller et al. 2010

We reanalyzed a previously published dataset to test for an association between BIS/negative affect and cortico-cardiac covariation. As described in more detail in the original publication (Mueller, et al., 2010), N = 31 participants (14 female; average age: 22.6 years; SD = 3.2 years) performed a gambling-task (Sato, et al., 2005) in which they received

positive or negative feedback after each trial while EEG and ECG were recorded. As reported before, positive and negative feedback evoked robust CECT values between EEG from 280 to 340 ms and changes in HP from 3500 to 4000 ms (see also Figure 3, panel C). Mean CECT values in that time-range showed a centromedial distribution and were maximal at channel C4. All but one participant of this study also filled out the BIS/BAS scales. We tested for a correlation between BIS score and N300H measured as the mean CECT value from 3500 to 4000 ms in the HP time domain and 280 to 340 ms in the EEG time domain at channel C4.

Results

Current Study

N300H. The N300H computed for positive ($t(167) = 3.88$, $p < .0002$), negative ($t(167) = 2.67$, $p < .009$) and neutral ($t(167) = 2.81$, $p < .007$) feedback and the N300H computed across all feedback types ($t(167) = 4.00$, $p < .0002$, see Figure 2), were significantly different from zero indicating reliable *within-subject* correlations of EEG from 300-350 ms and subsequent cardiac acceleration. Importantly, the within-subject correlations of cardiac acceleration at 4s and EEG from 300-350 ms of the subsequent (rather than the same) trials were not significantly different from zero, ($t(167) = 0.67$, $p > .5$). This control analysis confirms that N300H reflects a specific covariation of time-lagged EEG and HP of the same trials and does not result from prolonged fluctuations in EEG and HP signal (e.g. due to changes in arousal, habituation, etc.).

A repeated measures ANOVA involving the factor Channel confirmed the previously reported centromedial topography of N300H computed across all feedback types (Main effect Channel: $F(1,167) = 5.88$, $p < .001$) with maximum absolute N300H values at the neighboring channels Cz, FC1 (difference from zero: $t(167) = 4.49$, $p < .0001$) and FC2 ($t(167) = 4.03$, $p < .0001$). Further control analyses confirmed that the N300H was not correlated with the mean amplitude of the (trial averaged) event-related potential from 300-350 ms at channel Cz, or the (trial averaged) evoked cardiac response at 4s (ps $> .5$) indicating that individual

differences in N300H do not simply capture individual differences in feedback evoked brain or cardiac response but rather the cross-trial coupling of both. Furthermore, the N300H to negative feedback was not correlated with the FRN ($p > .15$) indicating that variations in N300H to negative feedback are not driven by individual differences in FRN.

5-HTTLPR. Importantly, N300H was significantly larger in L_A/L_A vs. S or L_G allele carriers as indicated by a main effect for 5-HTTLPR ($F(1, 166) = 4.1$, $p < .05$) in the 5-HTTLPR x Feedback Valence ANOVA (Figure 3). In addition, there was a marginally significant 5-HTTLPR x Feedback Valence interaction ($F(1, 166) = 2.67$, $p = .07$). As in prior studies (Mueller, et al., 2012; Mueller, et al., 2010) there was no main effect of feedback valence on N300H magnitude ($p > .5$). When the di-allelic rather than tri-allelic classification of 5-HTTLPR (S+ vs. L/L) was used, the main effect for 5-HTTLPR ($F(1, 166) = 3.91$, $p < .05$) was confirmed.

When the FRN was entered as the dependent variable, there were no associations with 5-HTTLPR and only a significant main effect of feedback valence indicating more negative FRN amplitudes to negative vs. positive ($t(167) = 8.82$, $p < .001$), neutral vs. positive ($t(167) = 21.91$, $p < .001$) and neutral vs. negative ($t(167) = 18.07$, $p < .001$) feedback.

BIS. The BIS score was negatively correlated with N300H following negative feedback ($r(168) = -.16$, $p < .05$; outlier shown in Figure 4 removed: $r(167) = -.18$, $p < .05$), indicating that highly anxious subjects have a larger (negative) correlation of EEG and heart activity evoked by negative feedback. There was no significant correlation between anxiety and N300H evoked by neutral or positive feedback ($ps > .4$). Moreover, the correlation of BIS and N300H to negative feedback was significantly different from the correlation of BIS and N300H to positive feedback (Meng et al 1992; $Z = 1.80$, $p < .04$, one-sided, outlier removed). Like the BIS scale, the NEO Neuroticism scale also showed a trend for a negative correlation with N300H after negative feedback ($r(167) = -.14$, $p < .08$). The BAS scale was not correlated with N300H to positive or negative feedback ($ps > .5$).

Consistent with prior reports (e.g. Sato et al., 2005; Santesso, et al., 2012), high NANX also predicted more negative FRN amplitude (BIS: r(168) = -.25, p < .002; NEO-Neuroticism: r(167) = -.19, p < .02). Importantly, the correlation between BIS and N300H remained significant when FRN was partialled (r(165) = -.20, p < .02) indicating that the relationship between NANX and N300H is independent of the previously reported relationship between NANX and FRN.

Relationship between Genotypes and BIS score. There was no significant association between 5-HTTLPR and either the BIS score ($p > .5$) or the NEO neuroticism score (p > .4). However, of potential relevance in light of the plasticity hypothesis of 5-HTTLPR (Belsky, et al., 2009), which would suggest higher interindividual variability of short allele carriers, the Levene's test of variance equality approached significance (p < .07) indicating higher NEO-neuroticism between-subject variation in the group of short vs. the group of long allele carriers.

Reanalysis of previously published dataset

As in the main study there was a significant negative correlation of the BIS score and mean N300H following negative feedback ($r(30) = -.34$, $p < .05$, one-sided; Figure 4). Also in line with the main study, this association was absent after positive feedback ($r(30) = .01$, $p > .9$). Moreover, the difference in correlations approached significance, $Z = 1.5$, $p = .06$, one-sided. The BAS scale was unrelated to N300H after any kind of feedback (all *p*s > .4).

Discussion

The present study investigated interindividual differences in cortico-cardiac covariation using the CECT method. Based on prior work linking the N300H CECT component to serotonin, it was hypothesized that individual differences in N300H are linked to the 5-HT transporter polymorphism 5-HTTLPR. Moreover, it was hypothesized that anxious individuals show increased brain-heart covariation as indicated by N300H, particularly after negative feedback. Consistent with the first hypothesis, it was shown that N300H was significantly increased in long allele (L_A) homozygotes of 5-HTTLPR. This

effect was independent of feedback valence and was significant for both, the diallelic (Lesch, et al., 1996) and the triallelic (Hu, et al., 2006) 5-HTTLPR classification. Consistent with the second hypothesis, self-reported NANX was significantly associated with N300H after negative feedback such that individuals with higher NANX showed a stronger covariation of cortical and cardiac activity. Of relevance, this association was specific to negative feedback and was replicated in a second independent sample. Moreover, the association of NANX and N300H to negative feedback was independent of the previously reported correlation between NANX and the FRN to negative feedback (Santesso et al., 2012), which we replicated in the present study.

The 5-HTTLPR analyses revealed that the long allele was related to significantly larger N300H indicating that feedback-evoked brain-activity in long allele carriers is more strongly connected to subsequent modulations of heart period than in short allele carriers. This finding is consistent with (a) elevated serotonin signaling in long vs. short allele carriers due to higher sensitivity of postsynaptic 5-HT1A receptors (David, et al. 2005), (b) the involvement of 5-HT_{1A} receptors in signal transmission from the brain to the vagus (Jordan, 2005), (c) elevated 5HT1A-mediated physiological responding in wild-type vs. 5-HT transporter deficient mice (Li et al., 1999), and (d) reduced N300H following pharmacological reduction of 5-HT synthesis and in individuals with low plasma levels of the 5-HT precursor tryptophan (Mueller, et al., 2012). Together, these findings indicate that 5-HT signaling is positively related to brain-heart coupling as assessed with the CECT approach.

In addition to its effect on 5-HT neurotransmission, recent work suggests that 5-HTTLPR influences brain morphology. In particular, the long allele has been consistently associated with relatively increased ACC gray matter and increased connectivity between the ACC and the amygdala (Jedema et al., 2010; Pezawas et al., 2005). Of relevance, the centromedial topography of N300H (e.g. Figure 2C, Mueller et al., 2010, 2012) suggests that the ACC may be an underlying cortical source of the N300H phenomenon, and could have its

effect on heart period through indirect projections involving the amygdala and other subcortical structures (Figure 5; see also: Gianaros, Van Der Veen, & Jennings, 2004; Thayer & Lane, 2009). Therefore N300H potentiation in long allele carriers could also be driven by ACC abnormalities with regard to overall morphology and/or connectivity with specific subcortical structures that are involved in central autonomic control.

Of importance, the association of the long allele with increased brain-heart covariation could reflect the underlying mechanism for the previously reported finding that the long allele predicts elevated respiratory sinus arrhythmia (Ellis, et al., 2011; Vulturar, Chis, Ungureanu, & Miu, 2012), which may indirectly indicate central control of the vagus during rest (for limitations see: Grossman & Taylor, 2007; Porges, 1995). Furthermore, the present findings could be relevant for the putative association of the long allele and increased risk for cardiovascular disturbances (Arinami, et al., 1999; Coto et al., 2003; Fumeron et al., 2002) as they suggest that cognitive affective processes may more easily influence peripheral activity in long-allele carriers. Enhanced (sub-) cortical influence on the periphery, in turn, may predispose these individuals, to show elevated cardiovascular reactivity to emotional stressors (Brummett et al., 2011; Williams et al., 2008), which increases the risk for cardiovascular diseases (Matthews, et al., 2006; Light, Dolan, Davis, & Sherwood, 1992; Treiber et al., 2003). Moreover, in conditions of persistent stress, enhanced cortico-vagal covariation could be associated with chronic neurogenic vagal withdrawal, which is another predictor for cardiovascular disease (Thayer & Lane, 2007). Although elevated brain-heart covariation could thus be an intermediate phenotype that links 5-HTTLPR long allele to heightened risk for cardiovascular disease, it should be noted that 5-HTTLPR has pleiotropic impacts on human brain functioning and autonomous nervous system activity (Canli & Lesch, 2007; Cools, Roberts, & Robbins, 2008) and could affect the risk for cardiovascular disease through various mechanisms.

N300H was not only linked to 5-HTTLPR but also to Behavioral Inhibition System (BIS) scores, which served as an indicator for NANX. Individuals with higher BIS values showed enlarged covariation of feedback-evoked brain and heart activity, but only after negative feedback. According to the reinforcement sensitivity theory, the BIS is the neurobiological system of anxiety, and individual differences in BIS sensitivity are responsible for differences in trait anxiety (Gray & McNaughton, 2000). Of relevance, an important output of the BIS is an increase in arousal including elevated heart rate. Moreover, the BIS may be activated when there is a mismatch between expected and actual outcomes, as would be the case for negative feedback (Holroyd & Coles, 2002). Accordingly, links between brain-heart covariation and BIS would be particularly expected after negative but not after positive feedback. Although it could further be speculated that behavioral approach system scores predict N300H to positive feedback, this was not found in the present study, suggesting that N300H may be more relevant for traits associated with negative rather than positive affectivity.

Considering the 5-HTTLPR findings and the NANX findings together, it is worth mentioning that the long allele and NANX were both predictive of higher N300H, even though the long allele has originally been associated with *reduced* levels of anxiety. Although the link between the long allele and low NANX has been replicated in some studies, it was absent in many others (Schinka, Busch, & Robichaux-Keene, 2004) including the present one. Hence the direct association between the s-allele and increased vulnerability for affective disturbance is weak at best (Risch et al., 2009). In fact, in individuals with few negative life-events as in the present healthy student sample the short allele may be even related to reduced vulnerability for affective disturbance (Wilhelm et al. 2006; Zalsman et al., 2006; Kuepper et al., 2012). Taken together, the overall picture linking 5-HTTLPR to NANX involves additional moderators and indicates that while 5-HTTLPR is somehow related to NANX the exact relationship and its mechanisms have yet to be identified (Karg, et al., 2011). Although

the present study did not attempt to solve this issue, it does show that both, 5-HTTLPR and NANX are linked to brain-heart covariation as assessed with N300H. Note however, that 5-HTTLPR affected brain-heart covariation independent of feedback valence, while the association between NANX and N300H was specific for negative feedback. Thus, 5-HTTLPR long allele may alter the feedback-evoked cortico-cardiac covariation in general, for example by enhancing cortico-limbic connectivity (Pacheco, et al. 2009) or by elevating 5-HT1A receptor sensitivity (David, et al. 2005) along the neuraxis displayed in Figure 5. Under the assumption that motivationally meaningful events like performance feedback activate cortico-cardiac coupling, it would be plausible that particularly relevant stimuli (e.g. negative feedback presented to anxious individuals) lead to a highly consistent activation of the underlying pathways thereby inducing a strong covariation of EEG and evoked HP. To test this hypothesis future CECT studies using stimuli with varying degrees of motivational significance (e.g. fear-conditioned stimuli) to evoke N300H are essential.

Although we believe that the pathways displayed in Figure 5 provide one reasonable model for the N300H phenomenon it should be noted that the underlying mechanisms of N300H have not been experimentally tested. We would therefore like to emphasize that N300H is a correlation over time and as such is principally open to alternative explanations regarding its functional significance. First, changes in arousal over the course of the experiment may lead to periods of both, low single-trial P300 amplitudes and reduced cardiac acceleratory response, thereby inducing a cross-trial covariation of EEG and HP. However, under the assumption that fluctuations in arousal should last for several trials, this alternative explanation would predict that EEG responses not only covary with HP changes of the same trial but also of neighboring trials. By demonstrating that N300H does not emerge if EEG is correlated with HP of the preceeding (rather than the same) trial, the arousal-fluctuation explanation was ruled out in our control analyses. As a second alternative explanation, short-term fluctuations in signal to noise ratio, that affect both, EEG and HP, may induce a

covariation of EEG and HP across trials. This explanation would be of high relevance if EEG and HP were sensitive for similar sources of noise. Note, however, that HP is a measure of temporal distance of two r-spikes rather than electrical activity. Because the temporal position of r-spikes can be detected precisely throughout the experiment, electromagnetic artifacts that influence EEG signal to noise ratio, have no impact on HP. Accordingly fluctuations in signal-to noise ratio can hardly explain the N300H phenomenon. Third, changes in HP (or EEG) baseline over trials may affect both, the evoked EEG response and HP response and thereby induce a covariation. However, it was previously demonstrated that N300H is robust against partialling HP at baseline from evoked HP, thereby ruling out this alternative explanation (Mueller et al., 2010). Finally, N300H could reflect cortico-sympathetic rather than cortico-vagal coupling. However, sympathetic responses are slower than vagal responses allowing to remove sympathetic influence on HP by temporally filtering the continuous HP-trace (Berntson, et al., 1997). In support of the cortico-vagal account, it has been shown, that the N300H emerges if sympathetic influences are removed from the HP trace, but disappears if parasympathetic frequencies are removed (Mueller, et al., 2010). In light of the present findings, this interpretation is also consistent with the fact that the serotonergic influence on the central nervous system is assumed to be parasympathetic (Ramage & Villalón, 2008).

Taken together, the current pattern of findings suggests that N300H is more consistent with cortico-vagal coupling than with the alternative explanations reviewed above. However, whether N300H indeed reflects cortical (i.e. top-down) cardiac control should be validated in future experimental studies, for example by testing whether transcranial magnetic stimulation over frontomedial brain regions affects N300H and evoked HP responses.

As an important limitation of the present study we only tested male participants because sexually dimorphic effects of 5-HTTLPR and cardiovascular responses have been reported (Jovanovic et al., 2008; McCaffery, et al., 2003), and would require a larger sample size to be reliably detected. Since gender-differences are also found in NANX or related

disorders and cardiovascular variables (Thayer, Smith, Rossy, Sollers, & Friedman, 1998), follow-up investigations testing female participants are crucial to evaluate whether the present findings generalize to women. As a further limitation, the correlation between NANX and N300H was relatively small within the main (N = 168) dataset, suggesting that N300H explained about 4% of NANX variance. However, in a second sample, using a gambling task instead of a time-estimation task, this correlation was not only replicated but also larger in magnitude. In those CECT studies that we have conducted thus far, studies using a gambling task (e.g. Mueller, et al., 2010) appeared to show larger N300H amplitudes than those using a time-estimation task (e.g. Mueller, et al., 2012). Thus, N300H evoked in gambling tasks may provide a stronger signal for brain-heart covariation and hence show larger correlations with NANX. Future work on the psychoneurometric properties of N300H evoked in different tasks may shed light on this assumption (Patrick, Durbin, & Moser, 2012). Nevertheless it should be emphasized that the correlation between NANX and N300H reached statistical significance in both paradigms, and thus appears to reflect a robust finding. Finally, the herein reported associations between questionnaire measures, genetic makeup and physiology are correlational and only provide limited information with regard to causal links. Future work should therefore clarify whether brain-heart covariation affects dispositional anxiety, whether anxiety affects brain-heart covariation, or whether the reported associations reflect epiphenomena of more relevant mechanisms.

In conclusion, we have shown for the first time that the 5-HTTLPR long-allele is linked to enhanced feedback-evoked covariation of brain and heart activity. Moreover, we have shown that the covariation of brain and heart activity evoked by negative feedback is correlated with NANX in healthy individuals. These findings provide evidence that both, serotonin and anxiety are associated with higher brain-heart covariation as measured with the N300H.

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Footnotes

[1]A power test revealed that testing for small population effects of 5-HTTLPR (partial eta squared = .05, e.g. Ellis et al. 2011) requires a sample size of at least 152 for a power of .80 with alpha =.05. Hence data was collapsed across individuals who received placebo and sulpiride to achieve a sufficient sample size. However, because dopamine may be involved in performance monitoring/feedback processing and may interact with serotonergic processes (Holroyd & Coles, 2002; Mueller, et al., 2011), the factor Substance (Placebo vs. Sulpiride) was included in a set of control analyses. In these control analyses there were no interactions or main effects of Substance (*p*s > .2). Moreover, the reported main effect for 5-HTTLPR (see results) on N300H remained significant in these analyses ($p < .05$). Because we had no explicit a priori hypotheses on dopaminergic effects on brain-heart covariation, the factor Substance was not part of the analyses reported in the Results section.

[2] When the factor Channel-Configuration (32 vs. 64 electrodes) was included in the 5-HTTLPR x Feedback Valence ANOVA, the main effect for 5-HTTLPR remained significant ($F(1,164)=3.98$, $p < .05$) and there were no main effects or interactions involving Channel Configuration (all *p*s > .15). Similarly there was no main effect or interaction involving Channel-Configuration when this factor was entered into the topography-related analyses of N300H (see results section; all *p*s > .15).

Table 1. Sample characteristics and descriptive statistics

	5-HTTLPR S+, L_G+	L_A/L_A	r(BIS)
N	121	47	
Age	24.3(3.4)	23.4(2.4)	.05
$N300H_{all}$	-.01(.01)	-.04(.01)	-.11
$N300H_{pos}$	-.03(.01)	-.04(.01)	-.00
$N300H_{neg}$	-.02(.01)	-.03(.02)	-.16*
$N300H_{neu}$	-.01(.01)	-.06(.01)	-.06
FRN	.45(.14)	.68(.22)	-.25**
BAS	24.7(4.3)	25.7(4.2)	.05
BIS	16.9(3.3)	16.8(3.4)	
NEO N	21.4(3.7)	21.8(2.9)	.60**

Notes: N300H = mean N300H amplitude evoked by all ($N300H_{all}$), positive ($N300H_{pos}$), negative ($N300_{neg}$), neutral ($N300H_{neu}$) feedback. FRN = Feedback-related negativity (evoked by negative feedback). BAS = Behavioral Approach System score; BIS = Behavioral Inhibition System score; NEO N = Neuroticism measured with the NEO PI R scale. Values are given as means. SD (Age, BAS, BIS, NEO N) or SEM (all other variables) are given in parentheses. * $p < .05$, ** $p < .01$,

Figure Captions

Figure 1. Computation of CECTs. (A) Continuous EEG, ECG and heart period data during a gambling task with k trials. Note that heart period changes with every heart beat resulting in a step-like function. The HP value at a given time point reflects the distance in ms between the preceeding and the suceeding r-spike in the ECG. The small boxes in the EEG reflect time-bins of 8 ms and the large boxes in the HP reflect time-bins of 0.5 s. (B) EEG-Matrix containing the baseline-corrected EEG values for the k x 64 time bins of one participant (64 corresponds to the number of EEG bins per trial. Note, that in the present study the number of EEG bins was 96 due to additional 32 bins for baseline which are not shown in this example). (C) HP-Matrix containing the baseline-corrected HP values for the k x 10 (number of HP bins per trial) time bins of one participant. Each EEG-bin is correlated with each HP-bin across trials within subjects (see formula (D)) leading to (E): EEG-HP time-lagged correlation matrix for a single subject (Cardio-Electroencephalographic Covariance Trace). Note that if baseline-corrected EEG voltage at a certain latency relative to a stimulus is linearly related to HP reactions at a certain time-window, this should lead to non-zero values at certain cells of the correlation matrix. (F) Scatterplot of an EEG – HP correlation across trials for a specific EEG bin and a specific HP bin within a single individual (reflecting the correlation value of a single cell in (E)). Note, that each dot in the scatterplot reflects one trial. (G) Cardio-Electroencephalographic Covariance Trace averaged across subjects (after Fisher normalization of correlation values). (H) Color coded Cardio-Electroencephalographic Covariance Trace indicating near-zero values (green), positive values (red) or negative values (blue).

Figure 2. Panel A: Grand Average Cardio-Electroencephalographic-Covariance Trace (CECT) at channel Cz (panel A). Each “pixel” in the coordinate system represents the group average of a within-subject correlation between EEG (x-axis) and HP (y-axis) at one time bin

relative to the feedback stimulus. Negative correlations are plotted blue, positive correlations red (see color bar). The blue spot at 300-350 ms in the EEG time domain and about 1.5 to 4.5 s in the HP time domain reflects the negative time-lagged EEG-HP correlation "N300H" that was previously identified in Mueller et al. (2010). N300H indicates that single trial EEG 300-350 ms after presentation of a feedback stimulus correlates with changes in HP about 1500 to 4500 ms after feedback presentation. Panel B: Temporal positions were within-subject EEG-HP correlations were significantly different from zero across participants ($p < .001$, two-sided). Panel C: Topography of N300H, at the measurement latencies. The position of channel Cz is also indicated.

Figure 3. Group average CECTs at channel Cz for individuals homozygous for the L_G allele (top) and individuals with at least one S or L_A allele (bottom).

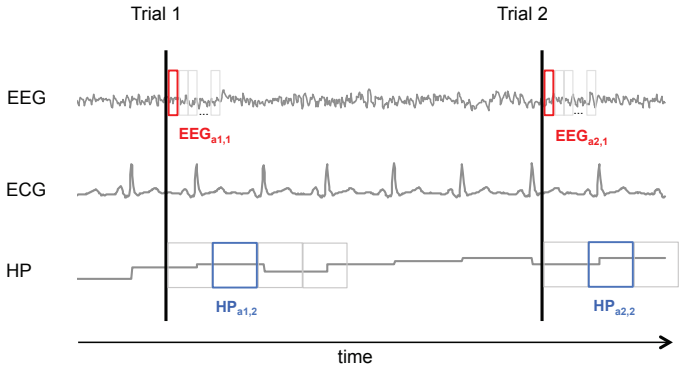
Figure 4. Panel A: Associations between Behavioral Inhibition System (BIS) score and N300H evoked by positive (green; $r = .00$, $p > .9$), neutral (blue, $r = .06$, $p > .4$) and negative (red; $r = -.16$, $p < .02$) feedback in the current study. Panel B: Cardio-electroencephalographic covariance traces (CECTs) evoked by positive and negative feedback (left) in a previously published study (Mueller et al., 2010). The associations between BIS score and N300H evoked by positive (green; $r = .01$, $p > .9$) and negative (red; $r = -.34$, $p < .05$) feedback are also shown. Neutral feedback was not provided in that study.

Figure 5. Possible neural pathway underlying cortico-cardiac covariation after feedback stimuli. (1) A stimulus is presented at time 0. (2) If classified as relevant during processing a cortico-cardiac cascade is initiated which may involve anterior cingulate cortex (producing an EEG response around 300 ms after stimulus presentation,(3)), limbic structures (including amygdala), midbrain, pons, medulla and the vagus nerve. At the nucleus ambiguous (medulla)

vagal nuclei with 5-HT binding sites are located which project to the sinoatrial node of the heart, thereby modulating cardiac acceleration (4) over the next heartbeats. By its effect on 5-HT transmission and/or cortico-limbic pathways 5-HTTLPR may affect how strongly a brain signal is transmitted to the vagus and thereby affect the degree of cortico-cardiac covariation. By specifically enhancing the relevance of negative feedback, neuroticism/anxiety may enhance cortico-cardiac covariation after negative feedback only.

Figure 1

(A) Continuous EEG, ECG and Heart Period (HP) data



(B) EEG-Matrix (single participant)

Bin	Bin 1	Bin 2	Bin c	Bin 64
Time(ms)	*0-8*	*9-16*	*...*	*492-500*
Trial 1	$EEG_{a1,1}$	$EEG_{a1,2}$	EEG_{a1c}	$EEG_{a1,96}$
Trial 2	$EEG_{a2,1}$	$EEG_{a2,2}$	EEG_{a2c}	$EEG_{a2,96}$
Trial i	EEG_{ai1}	EEG_{ai2}	EEG_{aic}	$EEG_{ai,96}$
Trial k	EEG_{ak1}	EEG_{ak2}	EEG_{akc}	EEG_{ak96}

(C) HP-Matrix (single participant)

Bin	Bin 1	Bin 2	Bin d	Bin 10
Time (s)	*0- 0.5 s*	*0.5- 1 s*	*...*	*4.5- 5 s*
Trial 1	$HP_{a1,1}$	$HP_{a1,2}$	HP_{a1d}	$HP_{a1,10}$
Trial 2	$HP_{a2,1}$	$HP_{a2,2}$	HP_{a2d}	$HP_{a2,10}$
Trial i	HP_{ai1}	HP_{ai2}	HP_{aid}	$Hp_{ai,10}$
Trial k	HP_{ak1}	HP_{ak2}	HP_{akd}	HP_{ak10}

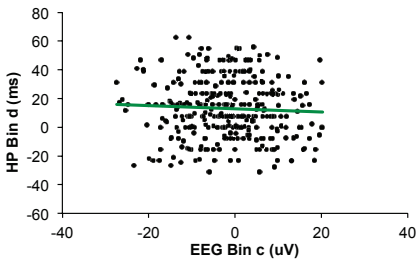
(D) Within-subject correlation formula

$$r_a(d,c) = 1/k \sum_{i=1}^{k} \frac{(EEG_{aic} - \overline{EEG}_{ac})(IBI_{aid} - \overline{IBI}_{ad})}{S_{ac} S_{ad}}$$

(E) Single-subject Cardio-Electroencephalographic Covariance Trace

	Bin_{EEG} 1	Bin_{EEG} 2	Bin_{EEG} c	Bin_{EEG} 64
Bin_{HP} 10	r_a (10,1)	r_a (10,2)	r_a (10,c)	r_a (10,96)
Bin_{HP} d	r_a(d,1)	r_a(d,2)	r_a(d,c)	r_a(d,96)
Bin_{HP} 2	***r_a(2,1)***	r_a(2,2)	r_a(2,c)	r_a(2,96)
Bin_{HP} 1	r_a(1,1)	r_a(1,2)	r_a(1,c)	r_a(1,96)

(F) Single-trial EEG-HP correlation for specified time bins



(G) Grand Average Cardio-Electroencephalographic Covariance Trace

	Bin_{EEG} 1	Bin_{EEG} 2	Bin_{EEG} c	Bin_{EEG} 64
Bin_{HP} 10	*r(10,1)*	*r(10,2)*	*r(10,c)*	*r(10,96)*
Bin_{HP} d	*r(d,1)*	*r(d,2)*	*r(d,c)*	*r(d,96)*
Bin_{HP} 2	*r(2,1)*	*r(2,2)*	*r(2,c)*	*r(2,96)*
Bin_{HP} 1	*r(1,1)*	*r(1,2)*	*r(1,c)*	*r(1,96)*

(H) Color-coded Cardio-Electroencephalographic Covariance Trace

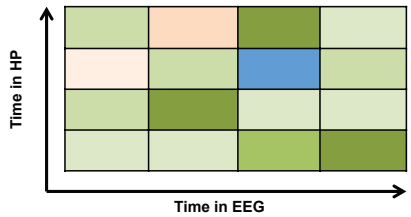


Figure 2

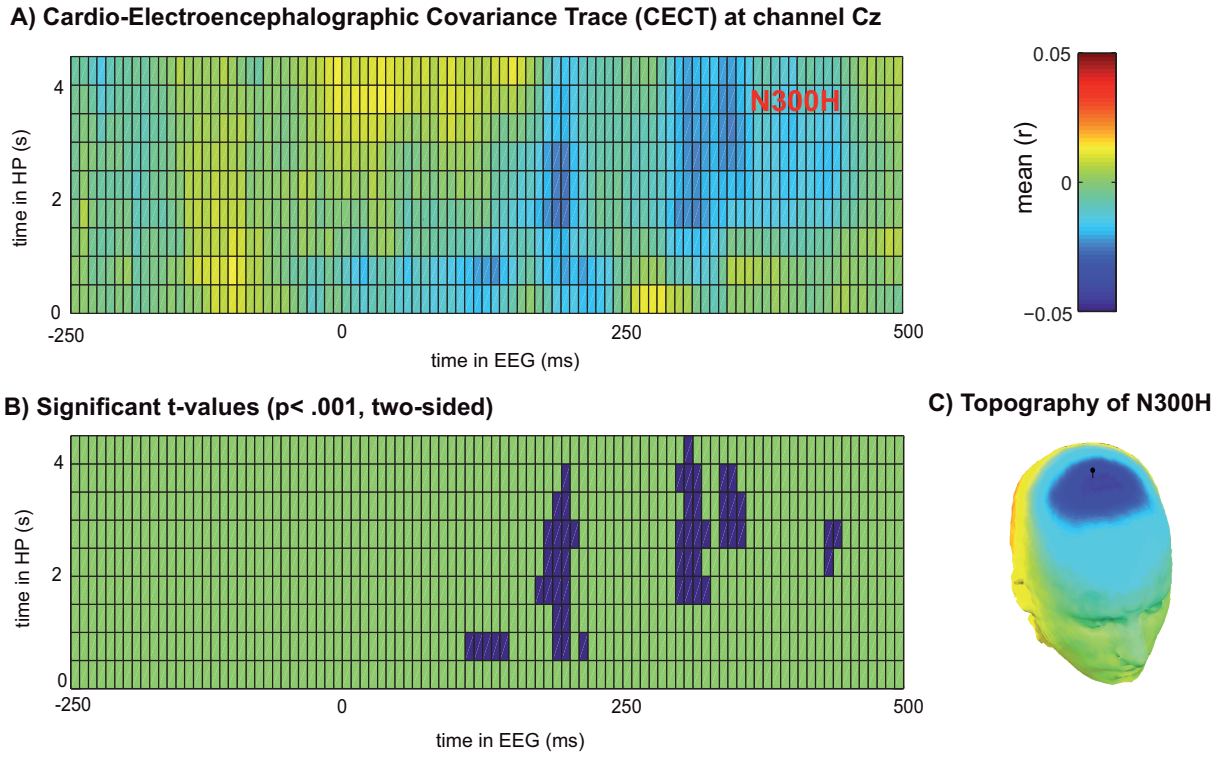

A) Cardio-Electroencephalographic Covariance Trace (CECT) at channel Cz
N300H
time in HP (s)
time in EEG (ms)
mean (r)
0.05
0
−0.05
B) Significant t-values (p< .001, two-sided)
C) Topography of N300H

Figure 3

CECT by 5-HTTLPR genotype

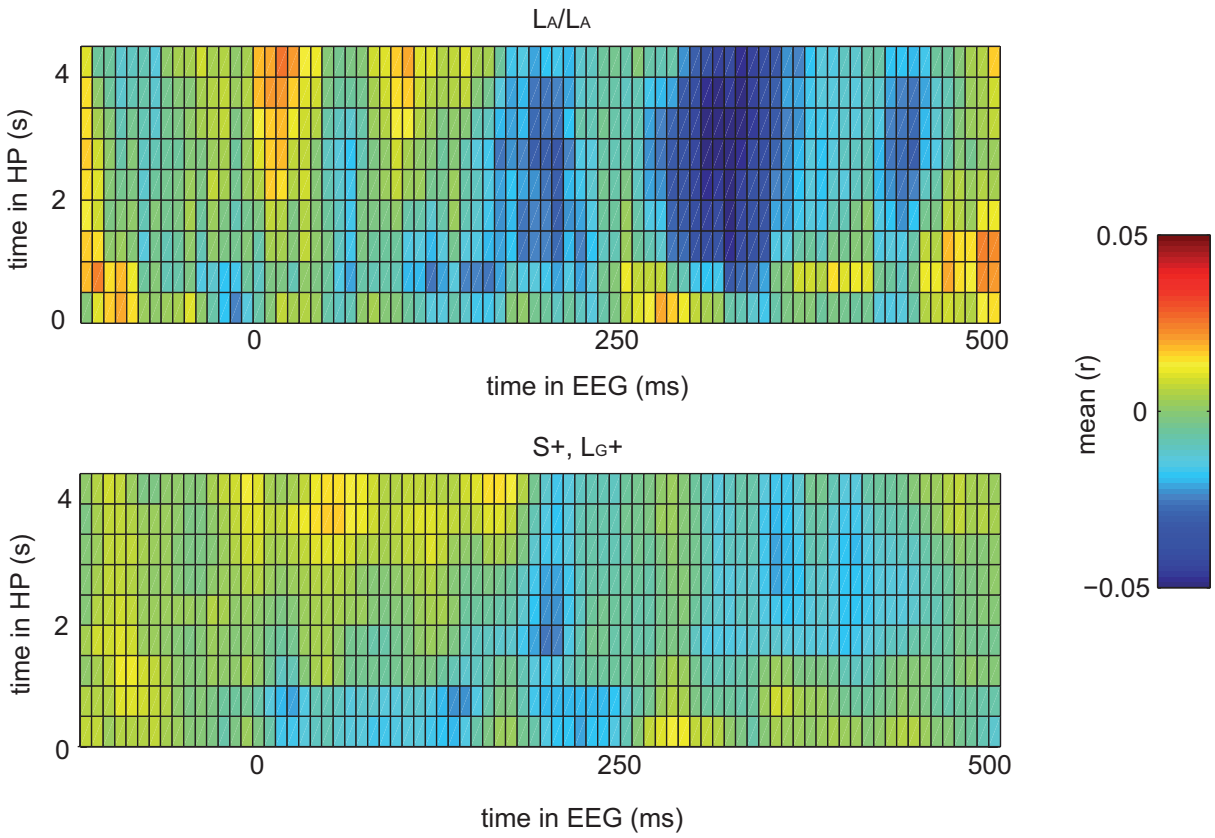
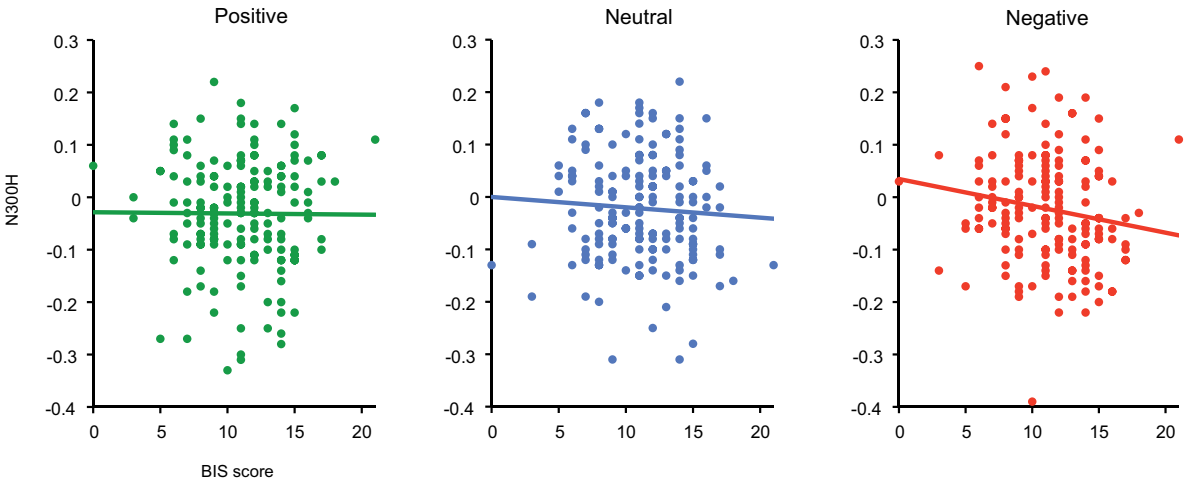


Figure 4

A) Current Study (N =168)



B) Reanalysis of Mueller et al. 2010 (N=31)

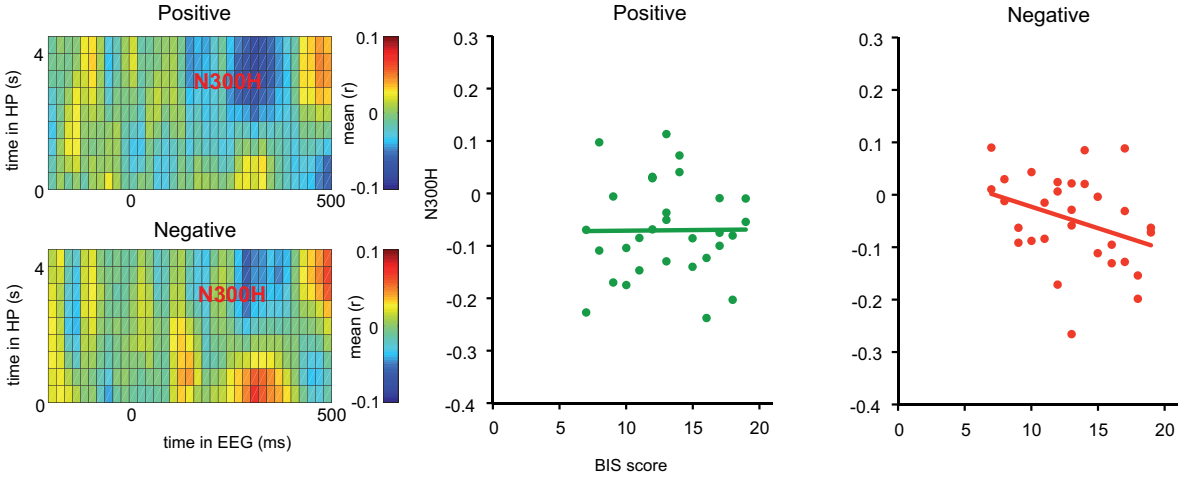
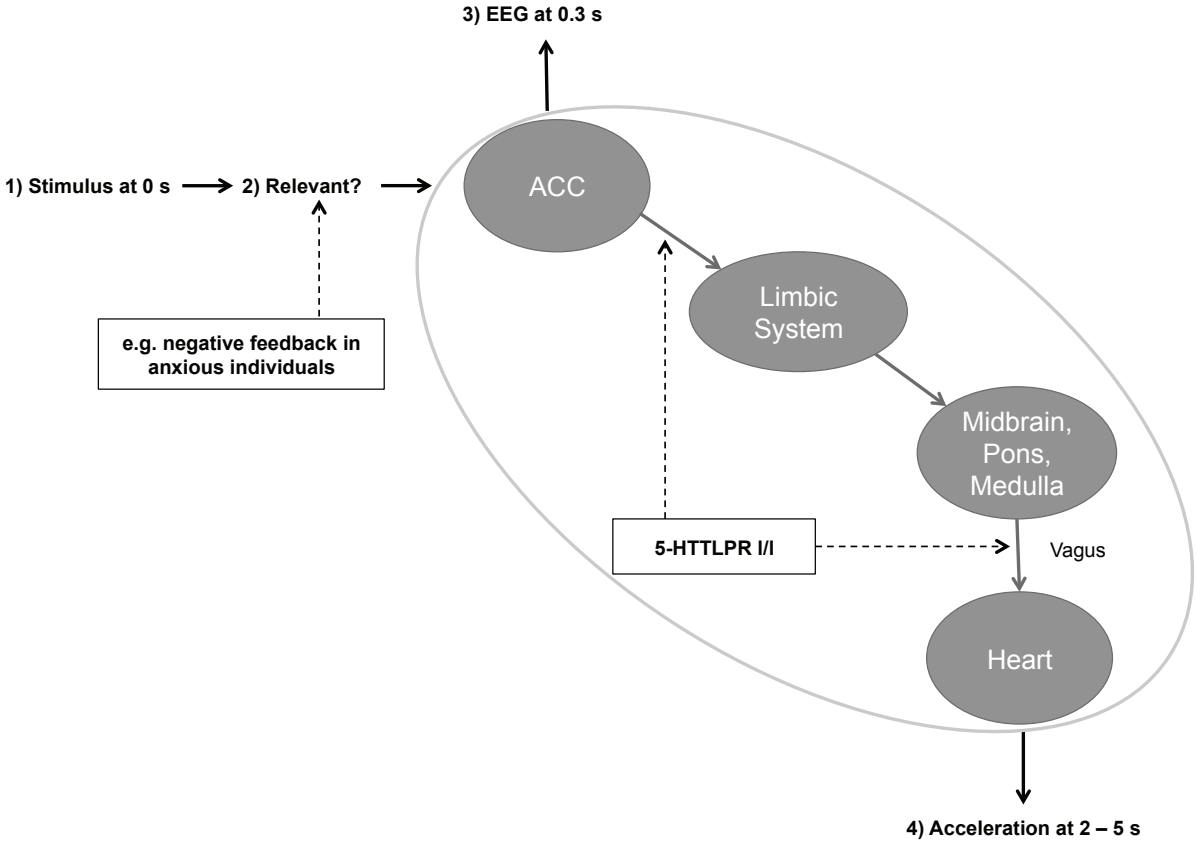


Figure 5


3) EEG at 0.3 s
1) Stimulus at 0 s
2) Relevant?
ACC
Limbic System
Midbrain, Pons, Medulla
e.g. negative feedback in anxious individuals
5-HTTLPR I/I
Vagus
Heart
4) Acceleration at 2 – 5 s