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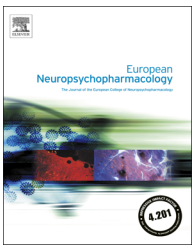

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Effects of serotonin depletion on punishment processing in the orbitofrontal and anterior cingulate cortices of healthy women

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Abstract
Diminished synthesis of the neurotransmitter serotonin (5-HT) has been linked to disrupted impulse control in aversive contexts. However, the neural correlates underlying a serotonergic modulation of female impulsivity remain unclear. The present study investigated punishment-induced inhibition in healthy young women. Eighteen healthy female subjects (aged 20–31) participated in a double-blinded, counterbalanced, placebo-controlled, within subjects, repeated measures study. They were assessed on two randomly assigned occasions that were controlled for menstrual cycle phase. In a randomized order, one day, acute tryptophan depletion (ATD) was used to reduce 5-HT synthesis in the brain. On the other day, participants received a tryptophan-balanced amino acid load (BAL) as a control condition. Three hours after administration of ATD/BAL, neural activity was recorded during a modified Go/No-Go task implementing reward or punishment processes using functional magnetic

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resonance imaging (fMRI). Neural activation during No-Go trials in punishment conditions after BAL versus ATD administration correlated positively with the magnitude of central 5-HT depletion in the ventral and subgenual anterior cingulate cortices (ACC). Furthermore, neural activation in the medial orbitofrontal cortex (mOFC) and the dorsal ACC correlated positively with trait impulsivity. The results indicate reduced neural sensitivity to punishment after short-term depletion of 5-HT in brain areas related to emotion regulation (subgenual ACC) increasing with depletion magnitude and in brain areas related to appraisal and expression of emotions (mOFC and dorsal ACC), increasing with trait impulsivity. This suggests a serotonergic modulation of neural circuits related to emotion regulation, impulsive behavior, and punishment processing in females.

1. Introduction

Dysfunction of the neurotransmitter serotonin (5-HT) plays a decisive role in many neuropsychiatric disorders characterized by inappropriate impulse control, such as attention deficit hyperactivity disorder (ADHD) and borderline personality disorder (BPD) (Kötting et al., 2013; Lis et al., 2007). Previous research suggests an influence of altered serotonergic neurotransmission on inhibition processes, which are closely related to impulse control and inhibited or, with respect to the outlined psychopathologies, disinhibited behavior. With respect to gender, a PET study comparing serotonin synthesis rates, measured as pmol/g/min, within different brain regions of male and female subjects found that female subjects had a 52% lower rate of central 5-HT synthesis compared with male subjects (Nishizawa et al., 1997), suggesting a disproportionately higher vulnerability to central nervous system 5-HT dysfunction in females. There is considerable evidence that sex hormones interact with central 5-HT availability (Carretti et al., 2005; Rubinow, 1998). Likewise, activation of specific brain areas during response inhibition tasks is affected by menstrual cycle phase, e.g., in the dorsolateral prefrontal cortex (dlPFC), the inferior frontal gyrus, the anterior cingulate cortex (ACC) and the insular cortex (Amin et al., 2006; Bannbers et al., 2012; Protopopescu et al., 2005; Roberts et al., 2008). Activation during response inhibition in the ACC and dlPFC was increased during the luteal phase compared with the follicular phase (Amin et al., 2006); activation was also increased in the left insula of healthy controls during the follicular phase and during the luteal phase of patients with premenstrual dysphoric disorder (PMDD) (Bannbers et al., 2012). In an emotional linguistic Go/No-Go task, medial OFC activity was increased for negative words compared with neutral words during the premenstrual and postmenstrual phases; this increase was enhanced in an inhibitory task context (Protopopescu et al., 2005). Activation in the inferior frontal gyrus was significantly reduced during successful inhibitions only during the follicular phase of the menstrual cycle. Brain activation in the ACC was enhanced during inhibitory errors (Roberts et al., 2008).

So far, the available literature is scarce regarding aspects of underlying neural correlates of serotonergic modulation of impulsivity in female subjects. Impulsive behavior and central nervous serotonin function are known to be closely linked. A major dilemma in the field of research on impulsivity is its exact definition. According to the International Society for Research on Impulsivity (ISRI), impulsivity can be defined as a lack of inhibitory control when confronted by negative consequences. Furthermore, the link between the neurotransmitter 5-HT and inhibition appears to be particularly specific to aversive contexts. As shown by Crockett et al. (2009, 2012), acute tryptophan depletion (ATD – a physiological challenge procedure to reduce brain 5-HT synthesis) abolished punishment-induced inhibition, which is defined as "the general suppression of responding in aversive contexts" (Gray and McNaughton, 2000) in a reward- and punishment-implementing Go/No-Go task. After the administration of a control condition, participants' responses were slower under punishment conditions compared with reward conditions; this difference was extinguished after ATD (Crockett et al., 2009). In the present study, we utilized this particular paradigm in an fMRI environment to investigate changes in brain activation related to diminished 5-HT synthesis during response inhibition in aversive contexts. As the relevant behavioral results by Crockett et al. (2009) mainly rely on RTs solely detectable for Go-stimuli, using an fMRI-based set up allows expanding the investigation of punishment-induced inhibition to the neural correlates of the critical inhibition demanding No-Go-stimuli.

One previous study reported that individual differences in trait impulsivity correlated with activation in the dorsal ACC, amygdala and prefrontal cortex (PFC). In particular, trait impulsivity was positively correlated with activity in the bilateral ventral amygdala, parahippocampal gyrus, dorsal anterior cingulate gyrus (BA 32), and bilateral caudate. Conversely, trait impulsivity was negatively correlated with activity in the dorsal amygdala and ventral PFC (BA 47). In another study, ATD strongly impaired the ability to adequately adapt responses to incentive-motivational cues in individuals with high levels of impulsivity (Cools et al., 2005). Together with the aforementioned finding regarding the reduction of punishment-induced inhibition by ATD, we expected that neural activation during the Go/No-Go task would be modulated by trait impulsivity and that these subjects with higher trait impulsivity might be more vulnerable to the effects of ATD, thus showing more brain activity in corticolimbic behavioral arousal areas and less activity in control circuits after ATD utilizing an incentive inhibition paradigm.

A review by Evers et al. (2010), which examined studies that applied ATD to Go/No-Go tasks in fMRI analyses, concluded that ATD attenuates brain activation in the inferior/orbitofrontal cortex, ACC and dorsomedial PFC during response control and negative feedback tasks. This review states that, during emotional processing, ATD modulates blood-oxygen-level dependent

(BOLD) responses in the amygdala primarily by increasing responses in either the right or left amygdala while processing emotional targets. However, the review demanded methodological improvement in future studies in terms of ATD administration and its efficacy to reduce basal 5-HT-release. Here we used the well established Moja-De ATD protocol (Demisch et al. 2002; Kewitz, 2002), which was validated to reduce 5-HT synthesis in two strains of mice as well as in healthy human subjects (Biskup et al., 2012; Dingerkus et al., 2012). Furthermore, the reviewed studies contained notable limitations, e.g., they applied paradigms lacking reinforcement or punishment (Evers et al., 2006; Roiser et al., 2009; Rubia et al., 2005), which did not allow for investigation of serotonergic modulation of inhibition in light of negative consequences. Furthermore, none of these studies considered the importance of sex hormones. We controlled for the substantially influencing variable of menstrual cycle phase. As stated in a recent review article, combining challenge procedures like ATD with neuroimaging techniques can be a valuable tool for translation research in order to study the relationship between a dysfunction of a specific neurotransmitter (in the present study 5-HT) and brain areas related to different behaviors and emotion-related processes (Biskup et al., 2015).

We applied a reward- and punishment-implementing Go/No-Go task in a double-blind, placebo-controlled, within-subjects repeated-measures design. We utilized the improved neurodietary challenge procedure Moja-De (Demisch et al., 2002; Kewitz, 2002), with ATD depleting central nervous system 5-HT synthesis on one of the two test days and a balanced amino acid load (BAL), serving as a control condition, on the other of the two test days. BAL was administered on the first day and ATD on the second day in half the participants and vice versa in the respective other half of the participants. 5-HT depletion is thought to lower sensitivity to punishment, resulting in subjects who are less sensitive to the potential threat of punishment. The differences between BAL and ATD (BAL minus ATD) during No-Go trials under punishment conditions were hypothesized to be positively correlated with the 5-HT depletion magnitude and trait impulsivity in neural areas that are associated with executive control, such as frontal cortex, and neural areas that are associated with emotion regulation and expression, such as limbic regions (i.e., the amygdala, cingulate gyrus and striatal regions). Based on the findings of Crockett et al. (2009), we further hypothesized that there will be a diminished dissociation effect between punishment and reward in terms of less discriminative activation between punishment and reward conditions after ATD compared with BAL.

2. Experimental procedures

2.1. Study design

The fMRI study employed a randomized, placebo-controlled, double-blind, within-subjects repeated measures design, with the administration of a TRP-balanced amino acid load (BAL) as a control or ATD, causing reduced 5-HT synthesis in the brain, on two different study days. A previously published Go/No-Go task (Crockett et al., 2009) using different reward/punishment conditions was applied during a functional event-related MRI measurement to determine the neural activities that underlie impulse control.

2.2. Study sample

Participants were 18 healthy female subjects between 20 and 31 years of age (mean age 24.22 $\pm$ 2.9 years). Sample size was based on a power analysis conducted prior to recruitment with an estimated effect size $f(V)=0.5$. Participants were primarily composed of unmarried students and were screened for and free of neurological and psychiatric disorders (see electronic Supplementary Materials for details). Exclusion criteria consisted of the following: current medical conditions, including migraines, asthma, diabetes, allergies or obesity (mean BMI 23.6 $\pm$ 3.7); any current or past psychiatric or neurological conditions (including head trauma with loss of consciousness); an IQ lower than 85 as assessed by the CFT-20R (Weiß, 2006) (mean IQ 110.3 $\pm$ 10.5); and current or prior pregnancy. Before administration of the ATD/BAL beverages on each study day, urine drug screening and pregnancy tests were conducted to exclude pregnancy and the intake of psychoactive or illicit drugs. This study only included participants who were free of the intake of hormones, such as oral contraceptives (i.e., no hormonal contraceptive use within the last 6 months) or other hormonal medications for hormonal disorders (e.g., metabolic or thyroid diseases). All participants were right-handed and native speakers of the German language. For more details of the study sample, see Supplementary Materials.

The Ethics Committee of the Medical Faculty of the RWTH Aachen University, Germany, assessed and approved the experimental protocol. The study was carried out in accordance with the Declaration of Helsinki. Participants' data were analyzed anonymously. Written and oral informed consent to participate in the study was provided by all participants, who were financially compensated after completion of the study.

2.3. Psychological questionnaires

2.3.1. Eysenck Impulsiveness Questionnaire I7

The Eysenck I7 Impulsiveness Questionnaire (Eysenck et al., 1985) was administered to obtain data regarding trait impulsivity. For additional psychological questionnaires incorporated, see the electronic Supplementary Materials.

2.4. Mood assessment

For mood assessment, a German version of the positive and negative affect schedule (PANAS; Crawford and Henry, 2004) was used.

2.5. Depletion procedure

Due to circadian rhythm-influenced differences in metabolism and synthesis of 5-HT (Sánchez et al., 2008), all participants arrived in the morning. ATD/BAL was administered at 0800 h and the fMRI measurement took place at 1100 h. The Moja-De ATD protocol (Biskup et al., 2012; Demisch et al., 2002; Dingerkus et al., 2012) includes administration of the relevant large neutral amino acids (LNAAs) in accordance with participants' body weights. Regarding the time-point of data acquisition, in a rodent model (Biskup et al., 2012) in all assessed brain regions at the particular time point of assessment used in the present study, 5-HT synthesis was substantially impaired as well as 5-Hydroxyindoleacetic acid (5-HIAA) content (an indirect measure of 5-HT release) was lowered following the administration of the used Moja-De ATD procedure. A recent study conducted in people in a comparable age cohort like the one in the present study cross-validated this procedure showing a robust decrease in total and free TRP influx over the blood-brain barrier after 90 min, remaining stable at this level 180 and 240 min later (Dingerkus et al., 2012). According to pilot data, the TRP-influx into the brain starts rising again after 6 h (Kewitz, 2002); hence, we

ensured to measure in a time slot when TRP levels are still decreasing and not rising back to baseline.

Participants were instructed to arrive after an overnight protein fast and a standard breakfast containing no TRP. In advance of each study day, detailed instructions for such a breakfast were provided to all participants. All participants confirmed that they had followed these recommendations on each study day. Half of the participants ($N=9$) received ATD on their first day, while the other half received ATD on the second study day in a randomized and counterbalanced order. For more detailed information, see the electronic Supplementary Materials.

2.6. Blood samples

Two blood samples were drawn for analysis of TRP influx. The first was drawn at baseline prior to ATD/BAL administration (T0). The second blood sample was drawn after completion of the study day, on average 255 min after challenge intake (T1), to prevent undesired effects of blood withdrawal on task performance. Blood samples were drawn twice during each study day, at the beginning and end of each study day (T1). Therefore, a total of four blood samples were collected per subject. In addition, concentrations of 17-ß estradiol (E2), follicle stimulating hormone (FSH) and luteinizing hormone (LH) were assessed to obtain baseline hormonal status. For more detailed information, see the Supplementary Materials.

2.7. Calculation of total TRP influx into the brain

Concentrations of TRP and competing LNAAs compose the total TRP influx across the BBB, characterized by unidirectional uptake. The Michaelis-Menten equation is a standard procedure that was used to calculate the total TRP influx into the brain (Demisch et al., 2002; Dingerkus et al., 2012; Kewitz, 2002). This equation corrects for multiple substrate competition (Pardridge, 1983; Smith et al. 1987) and provides a valid mathematical model for calculating the unidirectional influx rate of TRP from plasma into the brain (Dingerkus et al., 2012; Kewitz, 2002). Depletion magnitude was calculated as the ratio of TRP/$\sum$LNAAs after BAL baseline minus T1 subtracted from the ratio of TRP/$\sum$LNAAs after ATD at baseline minus T1 ($ATD_{baseline}TRP/\sum LNAAs\text{-}ATD_{T1}TRP/\sum LNAAs)\text{-}(BAL_{baseline}TRP/\sum LNAAs\text{-}BAL_{T1}TRP/\sum LNAAs$).

2.8. Neuropsychological reinforced Go/No-Go paradigm

During fMRI measurements, participants performed a color-coded forced choice reinforced Go/No-Go task. This experimental paradigm has been validated and described in detail by Crockett et al. (2009). Participants were required to press a button ("Go") if a checkerboard that was displayed consisted of a majority of either blue or yellow squares. Participants were rewarded for correct responses or punished for incorrect responses according to four distinct experimental conditions: Reward Go (GR), Reward No-Go (NR), Punish Go (GP) and Punish No-Go (NP). Each experimental condition included 12 easy and 12 difficult Go as well as No-Go trials, 48 in total. In the reward conditions, participants received a reward for correct answers; no points were subtracted in case of errors. In the punishment conditions, participants received a punishment for errors; no gain or loss of points was recorded for correct answers. The amount of the gain or loss (+1, +10, −1, −10) corresponded to the condition applied. For example, in "Reward Go," +10 points were given for a correct Go answer and +1 was given for a correct No-Go answer; in "Reward No-Go," +10 points were given for a correct No-Go answer and +1 was given for a correct Go answer. The corresponding point assignments were similar in the punishment conditions, except with −10 or −1 points. These details are as well depicted in Figure 1 in the article by Crockett et al. (2009) (response-outcome contingencies). For Go stimuli, only no button press during stimulus presentation or the following blank screen was considered incorrect. For No-Go stimuli, any button press during either screen (stimulus and blank) was considered as a lack of impulse control caused by commission errors. Only no button press during the presentation of both screens was considered correct. Blocks were intermitted by blocks of the same Go/ No-Go task without feedback to delete previous learning effects on the following condition blocks. Punishment included a loss of points, the presentation of an angry face and an unpleasant sound. Reward included a gain of points, the presentation of a smiling face and a pleasant sound.

To obtain independent measures of neural activation underlying punishment-induced inhibition processes, the aforementioned Go/ No-Go paradigm was used and adapted to meet the requirements of an event-related fMRI setting. To counteract the convolution effect from rapid event-related fMRI on the hemodynamic response function, inter-trial jitter sequences were added, which ranged from 0 to 4500 ms in 1500 ms increments and were calculated using the optseq2 tool (http://surfer.nmr.mgh.harvard.edu/optseq/, see electronic Supplementary Materials for more detailed information and Figure S1 for the trial sequence used).

2.9. Data Analyses

2.9.1. Behavioral data analysis

Punishment-induced inhibition was assessed following the concepts and strategies outlined in the publication by Crockett et al. (2009), with minor deviations due to diverging results in early data analysis (see the results section). We compared reaction times (RTs) for correct Go responses and commission error rates in punishment conditions to reward and neutral conditions. Faster RTs indicate lower inhibition, as slower responses indicate a more cautious and diligent response style.

2.10. Blood samples

Huynh-Feldt adjusted repeated-measures ANOVA was used to analyze plasma TRP levels.

2.11. Image acquisition and preprocessing

Functional MRIs were acquired using a Siemens Trio 3 T system (Erlangen, Germany) using standard echoplanar imaging (EPI) sequences (TE=30, TR=1500 ms, flip angle=72°, voxel size=3 × 3 mm, matrix size=64 × 64, 24 transverse slices, 5 mm slice thickness, 0.75-mm gap) with a standard 12-channel coil. Functional measurements were followed by high-resolution T1-weighted anatomical images using a magnetization prepared rapid acquisition gradient echo (MPRAGE) sequence (TE=3.6 ms; TR=2250 ms; TI=900; flip angle=9°; FOV=256 × 256 mm^2; 1 mm isotropic voxels; 176 sagittal slices). FMRI data were analyzed using BrainVoyager 2.3.1 software (Brain Innovation, Maastricht, The Netherlands). For more detailed information, see the electronic Supplementary Materials.

2.12. Statistical image analysis

In the underlying GLM matrix in Brain Voyager, all conditions were included, Go and No-Go trials, easy and difficult ones, with and without feedback, in total 64 predictors as well as six other motion predictors. As behavioral data did not show significant results regarding Go stimuli (see the results section), and No-Go stimuli serve as the critical trials in terms of "general suppression" as in the definition of punishment-induced inhibition, analyses included only No-Go trials. Because punishment-induced inhibition is defined as a general suppression in

aversive contexts, we performed an ANOVA between BAL and ATD for punishment conditions (GP and NP). This approach is supported by the finding that 5-HT specifically effects punishment rather than reward-related processing (Blair et al., 2008). Using an ANCOVA, we calculated whole-brain correlation maps using the differences between BAL and ATD during punishment conditions and the following two covariates: (a) depletion magnitude and (b) trait impulsivity. All volume maps were thresholded at $p<0.05$ voxel-wise and then corrected for multiple comparisons using the cluster size level Monte Carlo simulation. Cluster-level approaches assume that true differences in imaging data occur over contiguous tissues or voxels, rather than individual voxels. We employed $n=1000$ iterations because we were interested in the frequency of occurrence of a minimum cluster size of contiguous voxels (voxel intensity $p<0.05$) for 1 in 1000 instances.

We converted the resulting activation maps into region of interest clusters to obtain average β-values and the number of voxels within the clusters. To verify punishment-specific activation differences between BAL and ATD, β-values for reward and punishment after BAL/ATD were analyzed via within subject repeated measures ANOVAs and post-hoc t-tests, if applicable, using SPSS software. Correct or incorrect Go or No-Go trials were not examined due to insufficient trial numbers. Additionally, we did not address feedback for the same reason.

3. Results

3.1. Psychological questionnaires

3.1.1. Eysenck Impulsiveness Questionnaire I7

The participants' mean T-value on the impulsivity scale was 42.93 ± 8.60, indicating low to normal impulsivity. T-values were calculated according to Eysenck et al. (1985), with 95% of values (i.e., normal) ranging from 40 to 60.

3.1.2. Mood assessment

ATD did not affect mood; for more detailed information, see the electronic Supplementary Materials.

3.2. Plasma tryptophan

According to Michaelis-Menten kinetics, plasma TRP influx is calculated in nmol/min/g. A repeated measures ANOVA indicated main effects of challenge procedure ($F[1,17]=320.52$; $p \leq 0.001$; Cohen's $d=6.20$) and time ($F[1,17]=46.56$; $p \leq 0.001$; Cohen's $d=2.36$) and an interaction between time and challenge procedure ($F[1,17]=240.30$; $p \leq 0.001$; Cohen's $d=5.37$). Post-hoc analyses confirmed a significant decrease in total TRP influx into the brain following ATD administration compared to baseline (67.27% decrease, $t_{(17)}=17.5$, $p \leq 0.001$, Cohen's $d=8.75$), and total TRP influx was significantly reduced after ATD administration compared with BAL administration at 255 min after beverage intake ($t_{(17)}=17.25$, $p \leq 0.001$, Cohen's $d=8.63$, see Figure 1).

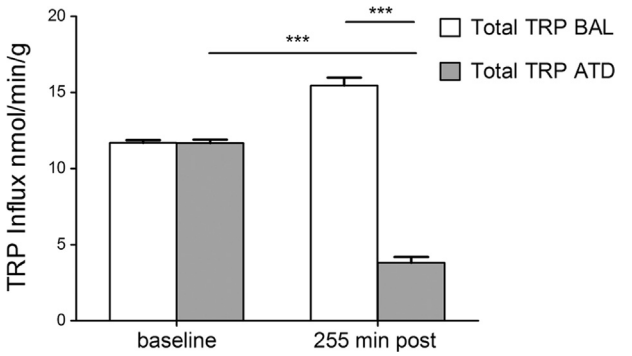

Figure 1 Influx (nmol/min/g brain tissue) of total tryptophan (TRP) across the blood-brain barrier at the time-points T0 (baseline) and T1 (255 min after the intake of an acute tryptophan depletion challenge procedure) and a TRP balanced amino acid load (control). Data are provided as the means ± SD. This figure is slightly modified from a figure in Helmbold et al. (2013), with kind permission of Springer+Business Media.

3.3. Behavioral Go/No-Go paradigm

RTs and commission error rates differed vastly between easy and difficult trials (within-subject repeated-measures ANOVA with factors of challenge administration, condition and difficulty, main effect difficulty: RTs: $F[1,17]=106.844$ ms, $p \leq 0.001$, commission errors: $F[1,17]=319.38$, $p \leq 0.001$). RTs were faster for easy than difficult trials (means ± SD in ms: BAL easy: 563.85 ± 14.33; BAL difficult: 657.55 ± 22.38; ATD easy: 542.56 ± 15.19; ATD difficult: 630.73 ± 20.04). Participants made almost no commission errors during easy trials (highest error percentage rate for easy trials: condition GR: mean ± SD = 0.016 ± 0.25 ms; the highest error rate for difficult trials: condition GR: mean ± SD = 0.275 ± 0.101 ms). According to empirical evidence, easy and hard trials should be separated during discrimination tasks. As shown by Fleming et al. (2010), difficult choices engage a neural basis that is distinct from the neural basis engaged by easy choices. The easy trials in our paradigm may have been solved on a perceptive level, whereas the difficult trials may have potentially involved higher cognitive functions. Therefore, we only utilized difficult trials for analyses, as these trials are likely to evoke decreased impulse control, whereas easy trials appeared to merely describe activation of the task in terms of discrimination ability. All RTs for correct Go responses were normalized to the total mean RT of neutral trials. Data were further analyzed using separate within-subject repeated-measures ANOVAs using SPSS with challenge administration (ATD, BAL) and feedback (reward, punishment) as within-subjects factors. As measured by commission error rates, manipulating 5-HT had no effect on motor response inhibition (main effect of challenge administration, $F[1,17]=242$, $p=0.629$). When participants' responses were biased toward Go (Reward Go, Punish No-Go) they made a higher proportion of commission errors than when biased toward No-Go (Reward No-Go, Punish Go) (main effect of bias, $F[1,17]=23.78$, $p \leq 0.001$). This effect was not altered by challenge administration (interaction between challenge administration and bias, $F[1,17]=0.467$, $p=0.503$); see Supplementary Figure S3. Assessing punishment-induced inhibition, both (a) RT and (b) commission error data did not reveal significant main effects of challenge administration ((a): $F[1,17]=0.169$, $p=0.686$; (b): $F[1,17]=0.242$, $p=0.629$) or feedback ((a): $F[1,17]=0.050$, $p=0.826$; (b): $F[1,17]=1.421$, $p=0.250$), and no interaction between challenge administration and feedback was detected ((a): $F[1,17]=0.946$, $p=0.344$; (b): $F[1,17]=0.274$, $p=0.608$). After BAL intake, participants were not slower to respond in punishment than in reward conditions ($t_{(17)}=0.545$, $p=0.593$) and did not commit fewer commission errors in punishment than

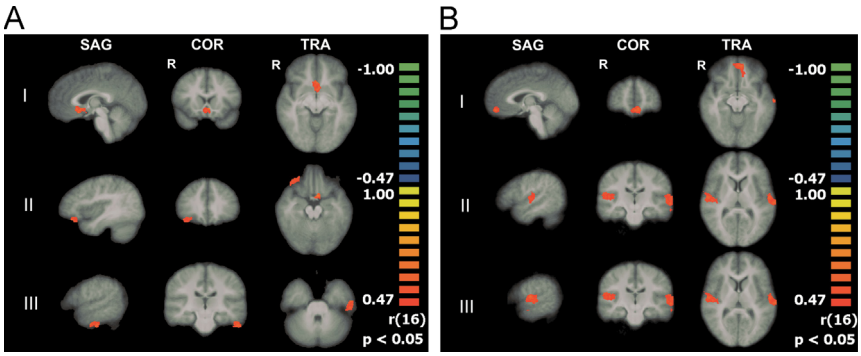

Figure 2 Depicted are the three clusters of the whole brain correlation analysis of the difference between No-Go trials in punishment conditions after BAL and after ATD administration with respect to (A) depletion magnitude: (I) left and right ventral and subgenual ACC, (II) right OFC, (III) left inferior temporal gyrus and (B) with respect to trait impulsivity: (I) right and left OFC and ACC, (II) right superior temporal area, (III) left superior temporal area. Correlations of activations are overlaid on an average of subjects' structural images. A=anterior, R=right hemisphere.

in reward conditions ($t_{(17)}=-0.486$, $p=0.633$). However, participants were slightly faster after ATD than BAL in punishment conditions, as indicated by normalized RTs of correct Go responses, although this was not significant (BAL pun: mean=−0.23 ms, ATD pun: mean=−0.35 ms, SED=0.24 ms; post-hoc *t*-tests BAL (pun) versus ATD (pun) $t_{(17)}=0.896$, $p=0.383$); see Supplementary Figure S4.

3.4. Neuroimaging

3.4.1. Punishment-induced inhibition

As outlined previously, fMRI analyses comprised difficult No-Go trials. An ANOVA that contrasted BAL and ATD during No-Go trials during punishment conditions revealed one cluster with less activation and six clusters with greater activation when BAL was compared to ATD. Less activation was observed after the administration of BAL compared with ATD; this decreased activation was apparent in a widespread cluster that primarily encompassed the bilateral occipital lobe (i.e., peak voxel in Brodmann area 17, the right occipital lobe, and the lingual gyrus). Affected brain regions that had greater activation included the following right hemisphere regions: inferior parietal lobe, ACC, and medial frontal gyrus including the OFC. Affected brain regions that had greater activation included the following left hemisphere regions: medial frontal gyrus, OFC, ACC, culmen, fusiform gyrus, parahippocampal gyrus and middle and superior temporal gyri (Monte Carlo cluster size $k=62$); see Supplementary Table S2.

3.4.2. Punishment-induced inhibition and depletion magnitude

Correlation analyses of the difference between BAL punishment (NP and GP) minus ATD punishment (NP and GP) – all difficult No-Go trials – and depletion magnitude in a whole brain analysis revealed highly significant and strong positive correlations in the right OFC, the ACC (in the right hemisphere, predominantly the subgenual ACC; in the left hemisphere, primarily the ventral ACC) and the left inferior temporal gyrus (Monte Carlo cluster size $k=36$). These regions were also significant in the ANCOVA comparison, indicating that the increased activation during punishment after BAL compared to ATD administration was modulated by depletion magnitude; see Figure 2A and Table 1. The more 5-HT was depleted, the more neural activity dropped after ATD than BAL in these regions. For a detailed overview of anatomical regions, including the number of voxels, see Supplementary Table S3. Extracted β-values of the clusters further revealed a significant main effect between difficult No-Go trials in punishment and reward conditions in the ACC cluster ($F[1,17]=4.634$, $p=0.046$) but no main effect of challenge administration or any interaction between challenge administration and reward versus punishment.

3.4.3. Punishment-induced inhibition and trait impulsivity

Correlation analyses of the difference between BAL punishment (NP and GP) minus ATD punishment (NP and GP) – all difficult No-Go trials – and trait impulsivity in a whole brain analysis revealed highly significant strong positive correlations with the left and right superior temporal gyri, the mOFC and, primarily, the dorsal ACC (Monte Carlo cluster size $k=67$), areas also detected in the ANCOVA comparison, indicating that greater activation during punishment after BAL compared to ATD administration was modulated by trait impulsivity; see Figure 2B and Table 2. The higher participants' trait impulsivity was, the more activity dropped after ATD than BAL in these regions. Another region that yielded a highly significant large-scale correlation not previously detected in the ANOVA comparison was the right insula adjacent to the superior temporal gyrus; for the anatomical regions, including the number of voxels, see supplementary Table S4.

Analysis of β-values identified cluster 1 (mOFC and ACC) as critical for discrimination between punishment and reward. Activation in this cluster exhibits a significant main effect of punishment versus reward conditions, with higher activation during punishment than reward conditions ($F[1,17]=7.39$, $p=0.015$) but no main effect of challenge or any interaction between challenge and reward versus punishment.

4. Discussion

The present investigation examined the serotonergic modulation of impulse control in the face of negative consequences in young adult females, controlling for menstrual

Table 1 Peak-voxel coordinates from the clusters of the whole brain correlation of the difference between No-Go trials in punishment conditions after BAL and after ATD administration with respect to depletion magnitude.

Cluster	Talairach coordinates [mm]			Hemisphere	Anatomical region	Brodmann area	Pearson *r* values	*p* values	Cluster size [mm^3]
1	−4	16	−3	R/L	Anterior cingulate cortex	25	0.79	<0.0001	3108
2	29	31	−9	R	Inferior frontal gyrus - OFC	47	0.68	<0.01	1074
3	−64	−14	−228	L	Fusiform gyrus	20	0.68	<0.01	1074

Table 2 Peak-voxel coordinates from the clusters of the whole brain correlation of the difference between No-Go trials in punishment conditions after BAL and after ATD administration with respect to trait impulsivity.

Cluster	Talairach coordinates [mm]			Hemisphere	Anatomical region	Brodmann area	Pearson *r* values	*p* values	Cluster size [mm^3]
1	−7	49	−6	R/L	Medial frontal gyrus, OFC	10	0.68	<0.01	2024
2	53	−14	12	R	Transverse temporal gyrus	41	0.68	<0.01	2676
3	−55	−11	12	L	Precentral gyrus	43	0.70	<0.01	3194

cycle phase. Reduced brain 5-HT synthesis due to ATD administration significantly affected brain activation during No-Go trials in punishment conditions in young healthy adult females in the early follicular phase of their menstrual cycle. Differences in brain activation under punishment conditions between BAL and ATD (BAL minus ATD) were positively correlated with the degree of depletion magnitude in the right OFC, right subgenual ACC, left ventral ACC and left inferior temporal area; differences were positively correlated with trait impulsivity primarily in the dorsal ACC, and also in the right and left superior temporal area, and the mOFC. Thus, the more central 5-HT was depleted and the higher participants' trait impulsivity was, the more activity dropped after ATD compared to BAL administration in these regions. Because diminished 5-HT is associated with decreased impulse control as outlined in the introduction, decreased activation of these brain areas after ATD, compared with BAL, might indicate lower involvement of these regions during impulse control.

The special finding of this study is the conjunction between changes in serotonergic neurotransmission and trait impulsivity, which might indicate that participants with higher ATD vulnerability are more prone to be impulsive by showing less activation in emotion regulating brain areas and that participants with higher trait impulsivity are more vulnerable to the effects of ATD.

Activation patterns in the OFC and the ACC are consistent with our hypotheses that ATD affects areas that are associated with emotional expression and regulation, which include the following regions: the OFC; the dorsal, ventral and subgenual ACC; and the right insula. The right insula is not apparent in the ANOVA that contrasted BAL and ATD. However, there were no differences in activation detected in the amygdala or striatal regions. During regulation tasks, the ventral ACC/mPFC inhibits negative emotional processing in the amygdala through top-down control (Etkin et al., 2012); this control may explain the absence of differences in amygdalar activation under punishment conditions after BAL compared with ATD in this particular study.

Analysis of β-values of apparent clusters indicated that the OFC and ACC are critical for discrimination between punishment and reward. Previous studies demonstrated that brain activation related to Go/No-Go tasks involved the right inferior PFC, the dorsolateral PFC and the ACC (Braver et al., 2001; Garavan et al., 1999; Garavan et al., 2006; Liddle et al., 2001; Menon et al., 2001; Rubia et al., 2001). A previous fMRI study assessing brain activation after ATD administration during response inhibition revealed serotonergic modulation of the right inferior PFC during inhibitory motor control (Rubia et al., 2005). Macoveanu et al. (2013) showed that activation of the left inferior frontal gyrus (IFG) was greater after the administration of the SSRI citalopram than after an ATD challenge during No-Go trials. No-Go responses were larger after ATD administration in the right IFG in subjects with low neocortical 5-HT_{2A} BP_p receptor binding and reduced in those with high 5-HT_{2A} BP_p receptor binding. These studies, however, applied a Go/No-Go task without reinforcement. In another study using a Go/No-Go task (Evers et al., 2006) ATD decreased BOLD responses in the dorsomedial PFC during performance monitoring but not during response inhibition. Again, this Go/No-Go tasks did not implement reward or punishment, rather simply positive or negative feedback via the presentation of either a green or red screen. In advantage, our participants lost or won 10 points or zero, which they were led to believe would result in a real monetary receipt or

non-receipt at the end of the study. In addition to the cognitive aspect of losing points, on an emotional and sensory level, punishment was accompanied by an angry face and an unpleasant sound. A further study indicated that a region of the ACC at the intersection of its dorsal and rostral subdivisions is associated with emotion and response inhibition. At this particular intersection, greater activation was detected during the inhibition of arousing negative stimuli compared to neutral and arousing positive stimuli (Albert et al., 2012).

During No-Go trials in punishment conditions, participants were not yet punished but were anticipating possible punishment. Reward and punishment processing can be separated into reward anticipation, outcome monitoring and choice evaluation (Liu et al., 2007). One event-related fMRI study revealed that during all three stages, the same areas were activated: the lateral OFC, the anterior insula, the superior temporal pole and the dorsomedial frontal cortex (Liu et al., 2007). Our results also indicated that the lateral OFC and dorsal ACC were areas active during No-Go trials with negative reward anticipation. Therefore, these brain regions are likely to be activated upon losing outcome as well as upon evaluation of wrong choices.

Regarding trait impulsivity, another previous study showed that dispositional impulsivity correlated positively with activation in the parahippocampal gyrus, the dorsal ACC and the ventral amygdala and negatively with the dorsal amygdala and the ventral PFC in inhibitory control paradigms (Brown et al., 2006). Our study revealed similar brain regions. Trait impulsivity correlated positively with activation differences due to 5-HT synthesis and related substrate availability (ATD versus BAL administration) both in the dorsal ACC as well as the mOFC. Controversy to the results by Brown and colleagues, both regions showed a positive correlation between trait impulsivity and neural activation. As outlined in the introduction, pre-existing impulsivity is of high importance when investigating the effects of ATD on corticolimbic behavioral arousal and control circuits. The degree to which ATD affects brain activity in these areas depends on an individual's trait impulsivity and cannot be generalized for all populations because dispositional impulsivity might itself be a result of altered activation in corticolimbic behavioral arousal and control circuits, which is consistent with the results of Brown et al. (2006). The current data suggest the importance of 5-HT in impulse control, especially in populations with high trait impulsivity (e.g., patients with borderline personality disorder).

The OFC has been shown to be strongly involved in emotion-influenced decision-making (Rolls, 1996). The ACC is associated with attentional processes regulating both cognition and emotion (Bush et al., 2000; Devinsky et al., 1995). Both dorsal and rostral subdivisions of the ACC/mPFC make key contributions to emotional processing. Ventral-rostral portions of the ACC/mPFC including the subgenual ACC have been shown to be associated with a regulatory role inhibiting negative emotions via its control on limbic regions involved in generating emotional responses. Dorsal-caudal regions of the ACC/mPFC and thus the OFC are associated with involvement in appraisal and expression of negative emotions (Etkin et al., 2012). In the current study, activation of the ventral and subgenual ACC was shown to be modulated by depletion magnitude and the dorsal ACC was modulated by trait impulsivity after ATD versus BAL intake. One could speculate that the inhibition of punishment associated negative emotions via top down mechanisms can be largely explained by 5-HT synthesis and related substrate availability, whereas the effect of 5-HT on appraisal and expression of negative emotions depends on individual trait impulsivity.

Regarding the scope of the current paper on inhibition processes in aversive contexts, we focused with the fMRI analyses on the punishment conditions. β-values for both punishment and reward conditions were extracted, compared and as stated indicated the OFC and ACC as critical for discrimination between punishment and reward processing during the inhibition task. More investigations could be performed with the current data set. However, examining all trials and condition would exceed the scope of the current paper as well as substantially increase the alpha error due to extensive multiple testing on the same data-set.

The fMRI results were corrected at $p<0.05$ for multiple comparisons using the Monte Carlo simulation. This threshold is commonly used in research studies that combine pharmacological manipulations with fMRI (e.g., Firk et al., 2012; Potkin et al., 2003; Rahm et al., 2014; Ye et al., 2014). Activation correlation analyses showed high Pearson's correlation values (r-values approximately 0.7-0.8) that were highly significant ($p<0.01$); however, a lower p-value in the Monte Carlo simulations would have solidified the results.

The current behavioral data slightly deviate from the results obtained by Crockett et al. (2009). The same tendency in the reaction times is observed, but not to a significant extent. Non-significant punishment-induced inhibition in the behavioral data might be due to some deviations of our study from that of Crockett et al. (2009). We only included women who were controlled for menstrual cycle phase and did not include subjects of both genders, as was done in Crockett et al.'s (2009) study. Furthermore, ATD mixtures differed between the two studies and the possible side effects as well as their magnitudes might differ as well. The mixture used in this study, ATD Moja-De, is adapted to body weight and is known to have a lower magnitude of side effects. We studied a healthy sample low in trait impulsivity. This would be in line with the significant correlations in the fMRI data indicative of less activity after ATD than BAL in emotion regulating brain areas with increasing trait impulsivity. Furthermore, in previous research, disrupting effects of ATD on motivational guidance of goal-directed behavior were highly correlated with trait impulsivity. The strongest effects were observed in highly impulsive individuals (Cools et al., 2005). An acceleration in the normalized RTs in both reward and punishment conditions builds on a general deceleration in neutral, unreinforced trials. However, though not significantly, the behavioral data corresponded to decreased punishment-induced inhibition such that participants responded slightly faster, and were therefore less cautious, to Go stimuli in the punishment conditions after ATD than BAL, implying that punishment conditions are those critical for the impact of ATD on impulse control, which built the foundation for the fMRI analysis strategy.

The current investigation focused on female impulsivity. However, a male comparison cohort would have been necessary to show that these data are female specific. Nevertheless, the methodologically clean and rigorous

approach and controlling for menstrual cycle phase allow an unadulterated insight into the underlying neural mechanisms of female impulsivity.

Notably, ATD administration results in a short-term reduction in central nervous system 5-HT synthesis, which might not exhibit effects equivalent to long-term low 5-HT synthesis that may be detected in specific groups of psychiatric patients. Chronic 5-HT dysfunction might produce more or different effects than a single ATD challenge. Nevertheless, ATD provides a valuable model to examine the effects of altered serotonergic neurotransmission in humans.

After ATD administration and a subsequent decrease in brain 5-HT synthesis, depletion magnitude and trait impulsivity were shown to account for serotonergic modulation of punishment-induced inhibition in neural areas associated with emotion-influenced decision-making. Reduced OFC and ACC activation after ATD administration in the face of punishment might explain the decreased impulse control in the face of negative consequences observed in psychiatric patients likely to exhibit low central nervous system 5-HT levels and high trait impulsivity (Kötting et al., 2013; Lis et al., 2007). By implication, our results regarding ATD effects support the assumption that administration of SSRIs can prevent such effects, by possibly acting on the neurotransmission in the OFC and ACC, which, as mentioned earlier, is of high relevance as SSRIs are a widely accepted interventional strategy for such patients (American Psychiatric Association, 2001). It can be suggested that TRP loading might act somewhat similarly, but this needs to be subject of future research.

Future studies are needed regarding the administration of SSRIs, TRP loading, and both healthy controls and psychiatric patients high in impulsivity and both genders to shed further light on the interplay between 5-HT, gender effects and trait impulsivity. The present data provide a potential avenue for future large-scale studies of psychiatric patients suffering from decreased impulse control in aversive contexts.

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Contributors

Authors Konrad, Habel, Herpertz-Dahlmann, Zepf and Helmbold designed the study.

Author Helmbold wrote the study protocol, conducted the measurements, analyzed the data, managed the literature search and wrote the first draft of the manuscript and incorporated and managed remarks from co-authors.

Author Zvyagintsev wrote the protocol for the fMRI sequences and supervised the fMRI data analyses (together with author Klasen).

Author Dahmen helped with the fMRI analyses and obtained and prepared the blood samples for analyses.

Author Klasen supervised the fMRI data analyses.

Author Gaber supervised the statistical analysis of the behavioral data.

Author Bubenzer helped with conducting the measurements and designing the study.

Author Crockett provided the behavioral paradigm and insights into the concept of ATD and punishment-induced inhibition.

Authors Eisert and Sanchez provided insight and expertise as regards the pharmacological concepts.

Author Zepf supervised the entire study and manuscript.

All authors contributed to and have approved the final manuscript.

Conflict of interest

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.euroneuro.2015.02.007.

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