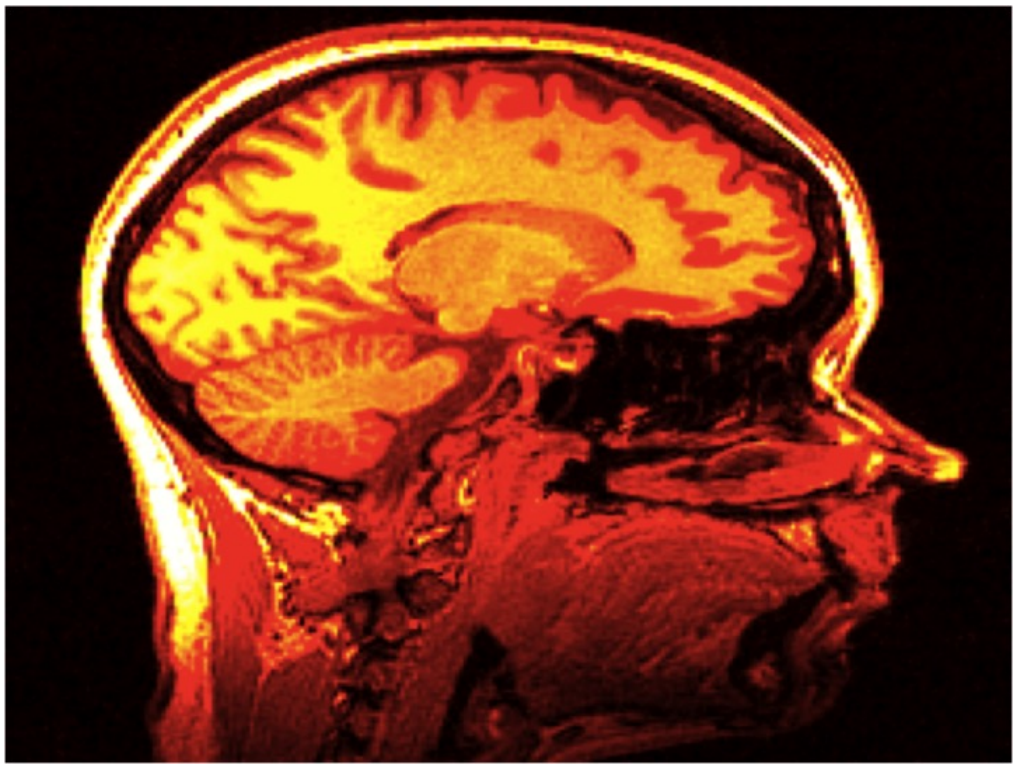
UNIVERSITY OF COPENHAGEN
FACULTY OF HEALTH AND MEDICAL SCIENCES

"Functional brain imaging under serotonergic challenges"



PhD-thesis
by
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Preface

The work presented in this thesis was carried out at the Danish Research Center for Magnetic Resonance (DRCMR), Hvidovre University Hospital, and at the Center for Integrated Molecular Brain Imaging (CIMBI), Copenhagen University Hospital, Rigshospitalet, as part of the Center for Integrated Molecular and Brain Imaging, from November 2006 until May 2012. According to the declaration of Helsinki II, the study was approved by the Copenhagen Ethics Committee ((KF) 01-2006-20).

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I wish to thank all our participants who volunteered their brains for basic research, which will hopefully lead to a better understanding of how to diagnose and treat people with mental disorders.

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Summary

"Functional imaging of the brain under challenge of the serotonergic system"

The study is a part of the Center for Integrated Molecular Biological Imaging (CIMBI) which aims to investigate the connections between personality traits linked to neuroticism and behavior in healthy humans with a main focus on one of the major neurotransmitters, serotonin (5-hydroxytryptamine, 5-HT). In CIMBI genetic markers, biochemical measures, as well as behavior and personality traits has been combined with brain-imaging results from imaging techniques, such as positron emission tomography (PET) and magnetic resonance (MR).

The major aim of the current study was to investigate how the serotonergic system is involved in emotion processing, measured by functional magnetic resonance imaging (fMRI). The neurotransmitter serotonin plays an important role in emotion processing and particularly in fear processing (Bauer, 2015). There is evidence that processing of emotionally salient information in humans involves modulation by 5-HT_{2A} receptors. In this study, brain activation patterns were investigated with fMRI in healthy volunteers while they performed a gender discrimination task on images of fearful, angry, and neutral facial expressions during serotonergic challenges. Our volunteers were therefore not informed about the real purpose of the study, namely to register the unconscious response towards the emotional facial expressions.

Each participant took part in four fMRI scanning sessions, whereof three included a serotonergic challenge, and one did not, but was merely a scanning of the participant's baseline of serotonergic function (control). The three challenges comprised of 1) a gradually increasing blockade of 5-HT_{2A} receptors by ketanserin (KET), given as an intravenous infusion (IV), 2) a global increase in serotonergic tone via a selective serotonin reuptake inhibitor (SSRI), citalopram, also given as an IV, and 3) a global increase of serotonergic tone by acute tryptophan depletion (ATD), obtained by a drink of a tryptophan-free powdered mixture of essential and non-essential amino acids dissolved in water. Prior to the study cerebral 5-HT_{2A} receptor binding (BPp) was measured with ^{18}F-altanserin PET for all participants.

When viewing fearful as compared to neutral faces, 5-HT_{2A} blocking was associated with a significant deactivation in the right medial orbitofrontal cortex (OFC) and left inferior frontal gyrus (IFG). Further, when considering the interaction between the BPp and the receptor occupancy (i.e. the amount of 5-HT_{2A} receptors blocked, O_{KET}) we saw increased activity in the left amygdala as well as changes in functional connectivity between OFC and amygdala. For the SSRI and ATD challenges we did not find any significant results when analyzing the main effect of the serotonergic challenges compared to control. However when using neuroticism score as a covariate we found subgenual cortex to be more active when viewing fearful faces than neutral faces for the SSRI condition compared to ATD. For the ATD condition we found that superior temporal gyrus (STG) was more involved in processing aversive than neutral

faces as compared to control condition. And finally when comparing the two drug sessions SSRI and ATD to each other and not to the control session we found an activation of Heschl's gyrus for viewing aversive faces.

With this study we have shown that structures such as the OFC, amygdala, subgenual cortex, superior temporal cortex as well as Heschl's gyrus play a crucial role in the processing of aversive faces. 5-HT_{2A} receptor mediated signaling increases the sensitivity of OFC to fearful facial expressions and regulates the strength of a negative feedback signal from OFC to amygdala. Furthermore both lowering and increasing the serotonergic tone (SSRI and ATD) of the brain increased the correlation with neuroticism scores when looking at aversive faces. This supports the idea that serotonin and neuroticism are tied together in processing threatening face emotions, and that this influence varies depending on the nature of the threat as well as an individuals personality trait. This finding may represent a neural mechanism for the variable therapeutic effect of SSRI treatment observed in clinical populations.

Dansk resumé

"Funktionel billeddannelse af hjernen under manipulation af det serotonerge system"

Dette studie er en del af Center for Integreret Molekylær Biologisk Billeddannelse (*Center for Integrated Molecular Biological Imaging*, CIMBI). CIMBI har til formål at undersøge sammenhænge mellem personlighedstræk der er koblet til neuroticisme og adfærd hos det raske menneske, med et hovedfokus er på neurotransmitteren serotonin (5-hydroxytryptamine, 5-HT). I CIMBI bliver genetiske markører, biokemiske mål, såvel som adfærd og personlighedstræk kombineret med resultater fra forskellige hjerneskanningsteknikker, så som PET og MR.

Hovedformålet med dette studie var at undersøge hvorledes serotonin systemet er involveret i behandling af emotionelle stimuli, målt ved hjælp af funktionel magnetisk resonans "imaging" (fMRI). I hjernen spiller serotonin en væsentlig rolle ved bearbejdning af emotionelle stimuli især frygtfulde stimuli (Bauer, 2015). Specielt er der indikation for at bearbejdning af emotionel information involverer modulering fra 5-HT_{2A} receptorer. I dette studie er hjerneaktiviteten undersøgt med fMRI på raske forsøgsdeltagere. Under fMRI skanningerne skulle forsøgsdeltagerne kønsbestemme billeder af frygtfulde, vrede eller neutrale ansigtstræk, samtidig med at forsøgsdeltagernes serotonin-niveau blev ændret kortvarig med forskellige medikamenter. Forsøgsdeltagerne var således ikke informeret om det egentlige formål med studiet; vurdering af deres ubevidste reaktion på ansigtsudtrykkene.

Hver forsøgsdeltager deltog i fire fMRI skanninger, hvoraf de tre involverede ændringer (interventioner) i serotonin niveauet, imens den fjerede skanning udelukkende var en kontrol af deltagerens naturlige serotonin niveau. De tre minterventioner udgjordes af: 1) Ketanserin, en intravenøs infusion (IV) der førte til en gradvis blokeringen af 5-HT_{2A} receptorerne. 2) En global forøgelse af serotonin ved en selektiv serotonin genoptagshæmmer (Selective Serotonin Reuptake Inhibitor; SSRI) citalopram, der blokerer serotonin transporteren i den presynaptiske celle, også givet som IV. 3) En akut global reduktion af tryptophan (acute tryptophan depletion; ATD) niveauet i hjernen, fremkaldt ved en drik af tryptophan-fri pulver af essentielle og ikke-essentielle amino-syrer opløst i vand. Inden dette studie blev den cerebrale 5-HT_{2A} receptor binding (BPp) målt med ^{18}F-altanserin PET skanning på alle forsøgsdeltagere.

Når man sammenligner aktivitetsniveauet mellem *at se på frygtfulde* ansigter med *at se på neutrale* ansigter, var blokering af 5-HT_{2A} receptorer associeret med en signifikant deaktivering i højre orbitofrontate hjernebark (OFC) og venstre inferiore frontale gyrus (IFG). Yderligere fandt vi at interaktionen mellem BPp og receptorblokeringen (dvs. den mængde af receptorer der på et givent tidspunkt er blokeret af ketanserin, O_{KET}) viste øget aktivitet i venstre amygdala, såvel som ændringer i den funktionelle kobling mellem OFC og venstre amygdala. For SSRI og ATD udfordringerne fandt vi ingen signifikante resultater da vi analyserede den samlede effekt af medikamenterne sammenlignet med kontrol, men ved at

tage neuroticisme scoren med som kovariat fandt vi at subgenual cortex var mere aktivt ved negative ansigter end neutrale for SSRI udfordringen sammenlignet med ATD. For ATD fandt vi at superior temporal gyrus (STG) var mere involveret i at behandle negative ansigter end neutrale, sammenlignet med kontrol. Og endelig ved at sammenligne SSRI med ATD fandt vi at Heschl's gyrus er involveret i behandlingen af negative ansigter.

Med dette studie har vi vist at strukturer som orbitofrontal cortex (OFC), amygdala, subgenual cortex, superior temporal cortex, samt Heschl's gyrus spiller en væsentlig rolle ved behandling af negative ansigter. Signalering formidlet af 5-HT_{2A} receptorer øger følsomheden af OFC til frygtfulde ansigtsudtryk, og regulerer styrken af den negative feedback fra OFC til amygdala. Desuden øges korrelationen med neuroticisme scoren når man kigger på negative ansigtstræk, både ved en forøgelse samt et fald i den serotonerge tone (SSRI og ATD) i hjernen. Samt styrker ideen om at serotonin og neuroticisme er forbundet i behandlingen af truende ansigtstræk, og at denne indflydelse varierer alt afhængig af typen af truende stimuli, samt den individuelles personlighedstræk. Disse fund kan desuden repræsentere en neural mekanisme for den variable terapeutiske effekt af SSRI behandling observeret i kliniske populationer.

Original manuscripts

Paper1:
Hornboll, B., Macovenau, J., Rowe, J., Elliot, R., Paulson, OB., Siebner, HR., Knudsen, GM. (2013). Acute 5-HT_{2A} receptor blocking alters the processing of fearful faces in orbitofrontal cortex and amygdala. *Journal of psychopharmacology (Oxford, England)*, 27(10), pp.903–914.

Paper2:
Hornboll, B., Macovenau, J., Rowe, J., Elliot, R., Knudsen, GM., Siebner, HR., Paulson, OB. Neuroticism predicts the impact of intravenous citalopram on the neural response of subgenual anterior cingulate cortex to fearful faces. (In prep.)

List of abbreviations

5-HT	5-Hydroxytryptophan (serotonin)
5-HT_{2A}	Serotonin 2A receptor
^{18}F	Fluorine 18 (Radioactive isotope)
AMY	Amygdala
ANOVA	Analysis of Variance
ATD	Acute Tryptophan Depletion
BP_p	Binding potential in plasma
ECG	Electrocardiogram
EEG	Electroencephalography
FWE	Family-Wise Error
FG	Fusiform Gyrus
fMRI	functional Magnetic Resonance Imaging
IFG	Inferior Frontal Gyrus
IV	Intra Venous
KET	Ketanserin
KeV	Kiloelectronvolt
MBq	Megabecquerel
mOFC	medial Orbitofrontal Cortex
mPFC	medial Prefrontal Cortex
MRI	Magnetic Resonance Imaging
NEO-PI-R	Neo Personality Inventory - Revised
OFC	Orbitofrontal Cortex
O_{ket}	Ketanserin occupancy
PET	Positron Emission Tomography
PFC	Prefrontal Cortex
POMS	Profile of Mood States
ROI	Region of interest
RT	Reaction time
SEM	Standard Error of the Mean
sgACC	Subgenual Anterior Cingulate Cortex
SPM5	Statistical Parametric Mapping version 5
SPM12	Statistical Parametric Mapping version 12
SPSS	The Statistical Package for the Social Sciences
SSRI	Selective Serotonin Reuptake Inhibitor
STG	Superior Temporal Gyrus
SVC	Small volume corrected
T	Tesla
$T_{1/2}$	Half life
TRP	Tryptophan

Content

Part I

– The Project

Aims and hypotheses

Serotonin has been implicated in neural processing of emotionally salient information. The aim of this PhD thesis was to identify brain regions involved in emotional processing which rely on serotonergic transmission, and to evaluate the role of serotonin level on emotion processing.

In order of doing so I studied a group of healthy volunteers as they underwent three types of serotonergic challenges, all aiming at transiently changing the serotonergic levels in the participant either by an increase in serotonergic levels (SSRI) or a decrease (ATD, KET). To elucidate serotonins role in processing of fear and anger, healthy individuals were studied with functional magnetic resonance imaging (fMRI).

In the first paper I investigated the effect serotonin 2A receptor (5-HT_{2A}) blockade (KET) has on emotion processing, using PET data from each individual as a covariate to infer about receptor density. Hypothesizing that 5-HT_{2A} receptor blockade would enhance the neural response in the amygdala, while decreasing the response in the OFC.

In the second paper the is was to decipher the different effect the participants neuroticism score have on global increase (SSRI) or decrease (ATD) of serotonergic levels in processing aversive stimuli, as well as to explore the impact SSRI and ATD has on emotion processing.
For this paper it was hypothesized that the neural response to aversive emotional stimuli would be dependent on the interplay between individual neuroticism scores and serotonergic transmission.

Background

Emotion processing

Facial expressions such as happiness, fear, sadness, anger, disgust, and surprise (Figure 1) represent basic human emotional conditions (James, 1884) which are easily decoded by members of all human cultures (Ekman, 1999). In 1997 Kanwisher and colleagues (Kanwisher *et al.*, 1997) discovered an area of the brain, which is more or less dedicated to face processing (fusiform face area, FFA), indicating the importance of faces as a source of information. It is rather important to be fast and efficient in recognizing and analyzing faces, as they provide enormous information about the individual in a complex social situation (Pineda *et al.*, 1993; Trnka *et al.*, 2007; Skerry & Saxe, 2014). Fearful faces represents a social signal about danger, with a distinct neural response (Williams *et al.*, 2006), and fear expressions have been shown to elicit approach behavior in participants (Marsh *et al.*, 2005), whereas the somewhat less ambiguous, but more direct stimuli of anger expressions facilitates avoidance behavior. The ability to appropriately interpret emotional facial expressions is important for our social interactions and impaired emotion processing is associated with an increased risk for affective psychiatric illnesses (Phillips *et al.*, 2003; Mayberg *et al.*, 2005).

Figure 1: The six basic emotions (pictures are from the Karolinska Directed Emotional Faces database (Lundqvist *et al.*, 1998).

Neuroimaging studies in healthy volunteers have identified the amygdala and prefrontal cortex as core-regions of a functional network processing facial emotions (Adolphs, 2002).

The amygdala is a brain structure known to process emotional stimuli fast and efficient. It is presumed that fear activates the amygdala via two neural pathways, a fast non-cognitive covert path, and a somewhat slower cognitive overt path (Morris *et al.*, 1998; Phillips *et al.*, 2004). The latter is critical for automatic processing of facial emotions (de Gelder, 2005). Subconscious sensory input are transported directly from the thalamus to the amygdala, which gives a fast and autonomic response (Williams et al., 2006). A more detailed conscious analyses of the fear signals, depend on the somewhat slower cortical route (LeDoux, 1998) where the amygdala receives visual information about facial emotions via cortical projections from the ventral stream of object processing (Ohman, 2005; Williams *et al.*, 2006). Hereafter the signal is projected to the prefrontal cortex (PFC) (Porrino *et al.*, 1981), which is believed to make a fast evaluation of the emotions of the faces before a complete analyses are done in the visual cortex (Kawasaki *et al.*, 2001). Other studies have shown that subconscious fear creates a larger neural response than conscious fear (Murphy & Zajonc, 1993). Participants who were shown images of fearful faces activated the dorsal part of the amygdala when they were consciously aware of the fear, whereas the basolateral part were activated when the participants were not consciously aware of the fearful faces. Further is has been found that the amygdala has an increased activity when viewing fearful facial expressions compared to neutral as measured with fMRI (Whalen *et al.*, 2001).
The medial prefrontal cortex (mPFC) and orbitofrontal cortex (OFC) are involved in evaluating cognitive aspects such as integrating information about the emotional state of others, derived from face emotion (Bechara *et al.*, 2000; Salzman & Fusi, 2010). OFC and amygdala are strongly interconnected, with OFC exerting inhibitory control over the amygdala (Stein *et al.*, 2007), likely modified by 5-HT signaling as Fisher et al. (2009) reported a positive correlation between amygdala-prefrontal coupling and prefrontal 5-HT_{2A} receptor binding Therefore an effective integration of neuronal activity among these core-regions is likely to be critical for efficient processing of emotions (Fairhall & Ishai, 2006; Liang *et al.*, 2009).

Serotonergic challenges

Serotonin, *Indolealkylamine 5-hydroxytryptoptamine* (5-HT), was originally discovered due to its effect on the smooth musculature. Neurons producing serotonin are found in clusters or groups of cells along the midline of the brainstem, the largest concentration of which are found in the raphe nuclei (Hornung, 2003; Mengod *et al.*, 2015). Serotonin has been associated with a wide range of psychiatric, behavioral, biological and neurological functions and disorders (Cools *et al.*, 2005; Hensler, 2006a) for the current study the focus is on serotonins effect on emotion processing. If serotonin balance is disturbed for a longer period of time, it can lead to an increased risk of developing long lasting structural and functional alterations in brain development in early life (Lauder, 1993) leading to a risk of developing a variety of mental disorders (Harmer, Bhagwagar, *et al.*, 2003; Corchs *et al.*, 2015) such as social phobia, anxiety disorder, obsessive compulsive disorder, and depression later on in life.
From the raphe nuclei the serotonin neurons project to targets throughout the brain, which are participating in regulation of functional systems; especially the limbic and forebrain structures have a high concentration of serotonin projections (Hornung, 2003; Hensler,

2006a). Receptor specific transmission as well as central serotonin availability modulates neural response to both rewarding and aversive stimuli (Macoveanu, 2014).
For example, in fear conditioning paradigms amygdala serotonin concentration increases in response to aversive stimulation (Mo *et al.*, 2008; Yokoyama *et al.*, 2005) and regulation of serotonin release in the mPFC has been identified as a crucial mechanism in rats to deal effectively with stressors and to terminate fear-related behavior (Forster et al., 2006). Previous studies have shown that drugs which targets the serotonergic system modulates the neural processing of emotional faces in healthy individuals: For instance, Harmer et al. (2003) found that acute tryptophan depletion decreases recognition of fearful facial expressions in healthy women, while an acute dose of SSRI enhanced the responsiveness to fear related facial stimuli in healthy volunteers (Harmer *et al.*, 2003).

SSRI

Citalopram (SSRI) is a widely used antidepressant. Chronic treatment with SSRI normalize amygdala activity, and thereby the participants sensitivity for viewing fearful faces, in depressed patients (Sheline *et al.*, 2001). SSRI works by blocking the reuptake of serotonin from the synaptic cleft via the serotonin transporter, thus increasing the levels of free serotonin (Nutt *et al.*, 1999)The long-term effect of the drug is obtained by decreasing the activity of tryptophan hydroxylase and desensitizing the serotonin auto receptors (5-HT_1 and sub-family), whereby their regulatory effects on serotonergic neurotransmission are weakened or lost (Moret & Briley, 1996) These effects are only seen after a prolonged period of treatment, explaining why acute treatment of SSRI has the opposite effect (Harmer, *et al.*, 2003; 2009) and thus increases participants sensitivity towards fearful stimuli (e.g. Browning *et al.*, 2006) by increasing activation in brain areas related to emotion processing such as fusiform gyrus, thalamus, OFC and particularly the amygdala (Del-Ben *et al.*, 2005; Anderson *et al.*, 2007; Bigos *et al.*, 2008).

Ketanserin

The serotonin 2A (5-HT_{2A}) is a postsynaptic receptor (Hariri & Holmes, 2006), which has a high density in areas involved in emotion processing such as the prefrontal cortex and parts of the limbic system including amygdala and hippocampus (Hensler *et al.,* 2006b). Several lines of evidence indicate that the serotonin 2 (5-HT_2) receptor-family (5-HT_{2A}, 5-HT_{2B} and 5-HT_{2C}) is involved in generation and expression of anxiety (Benekareddy *et al.*, 2011; Bauer, 2015) and that the 5-HT_{2A} receptor is the main excitatory serotonin receptor, making it a great candidate for the particular study. In humans, there is accumulating evidence that processing of emotionally salient information is modulated by 5-HT_{2A} receptors and that regional expression of the 5-HT_{2A} receptor in the brain, constitutes a trait related to anxiety (Frokjaer *et al.*, 2008). Density of the 5-HT_{2A} receptor in mPFC has been linked to amygdala activation in response to negative facial expressions (Fisher *et al.*, 2009) even amygdala habituation was linked with mPFC 5-HT_{2A} density, making it easier to cope with aversive facial stimuli the higher the density. Global disruption of 5-HT_{2A} receptor signaling reduces

inhibition in conflict anxiety paradigms in mice (Weisstaub, 2006). Activation of the 5-HT_{2A} receptors in the central nervous system results in an increased body temperature (Hayashi *et al.*, 2004) as well as an increased secretion of neurotransmitter (Nair & Gudelsky, 2004). These autonomic reactions are typical for stimuli signaling danger. Previously ketanserin has mostly been used to treat and study hypertension (e.g. Schmidt *et al.*, 1991; Rossouw *et al.*, 1995; van der Starre & Solinas, 1996). It was to our knowledge novel to use ketanserin for an emotion processing study at the time, but considering that ketanserin is a 5-HT_{2A} antagonist (Millar *et al.*, 1982; Olsen *et al.*, 1992), we expected that treatment with ketanserin would lead to deactivation in response to aversive facial expressions, which we found to be true for OFC (Hornboll *et al.*, 2013) which in return lead to an up regulation of amygdala activation.

Acute Tryptophan Depletion

The amino acid tryptophan (TRP) is a food-derived precursor of serotonin. TRP is available through the diet; especially meat, fish, eggs, milk, bananas, and nuts are rich in TRP. The first step in the production of serotonin is the transportation of the amino acid *L-tryptophan* (trp) across the blood-brain-barrier (BBB). In the brain trp will be converted to *5-hydroxytrypophan* (5-HTP) by tryptophan hydroxylase. After which 5-HTP is converted into serotonin (5-HT) by the enzyme *decarboxylase* (Hensler, 2006b). To enable TRP to reach the brain it has to compete with other large amino acids (valine, leucine, isoleucine, phenylalanine, and tyrosine) (Rao *et al.*, 1994), using the same carrier to cross the BBB. This means that the level of TRP in the brain are not only dependent on the level of TRP in the blood, but also on its concentration compared to other amino acids (Hensler, 2006b). If the level of TRP to enter the brain is reduced this will lead to a reduction of serotonin production (Harmer *et al.*, 2003). This reduction can be obtained experimentally by administering an amino acid mix containing all amino acids using the same transporter to cross the BBB as TRP (Hood *et al.*, 2005). This way only a small amount of TRP will be able to cross the BBB, thus leading to the desired acute depletion of TRP (ATD) in the brain. Whereas an increase of TRP in diet has a positive effect on negative emotional stimuli away from a fearful bias (Mohajeri *et al.*, 2015), also angry faces are rated less negative (intense/arousing) after ATD (Beacher *et al.*, 2010). ATD modulate the processing of emotional faces in cortical and subcortical areas (Fusar-Poli *et al.*, 2009) leading to an enhanced response towards negative facial expressions in the amygdala and fusiform gyrus (Cools *et al.*, 2005) as well as increased neural response to angry faces (Grady *et al.*, 2013), and decrease RT in emotion processing paradigm (Beacher *et al.*, 2010). ATD has also been seen to elicit a decreased recognition of fearful facial expressions in healthy women (Harmer *et al.*, 2003).

Neuroticism

The five factor model of personality traits (Costa & Mccrae, 1992) was developed to test adults without overt psychopathology and describes five distinct personality traits; extraversion, agreeableness, conscientiousness, neuroticism and openness to experience. Neuroticism is believed to have a high influence on affect and mood, and to reflect a stable

disposition that produce a robust association with common mental disorders (Ormel *et al.*, 2013). Making it a risk factor for anxiety and mood disorders (Bienvenu *et al.*, 2001) with an increased tendency to worry and experience psychological distress, accompanied by negative affect and sensitivity to negative cues such as negative facial expressions (Hamann & Canli, 2004). Increased neuroticism scores has been found to be a strong reflection of the liability of developing major depression due to ones genetic profile (Kendler *et al.*, 2006) as well as to higher 5-HT_{2A} receptor binding (Frøkjær *et al.*, 2009). High levels of neuroticism delay overall detection of, and enhance emotional arousal in response to facial expressions (Sawada *et al.*, 2016). Neuroticism may even be reflected in brain anatomy as high neuroticism scores have been linked with increased grey matter volume in the subgenual anterior cingulate cortex (sgACC) in adolescent females, negatively so in males (Blankstein *et al.*, 2009), as well as an inceased functional connectivity of the amygdala with prefrontal regions including sgACC (Madsen *et al.*, 2015). Considering that females have been shown to have a higher prevalence for affective disorders such as depression, this could be indicating a gender specific neuroanatomical correlate in emotion processing. Furthermore higher neuroticism scores has been associated with increased amygdala and sgACC activity in high vs. low emotional conflict trials (Haas *et al.*, 2007), supported by a meta-analysis in which neuroticism was found to be associated with increased activation in the frontal and cingulate regions for negative vs. neutral emotional stimuli (Servaas *et al.*, 2013).

Project set-up

Participants

Twenty-six right-handed healthy adults were recruited for the study from a larger study cohort, of these only 22 (9 females) took part in all 4 scanning days with a mean age 31.8 ±6.5 years. None of the participants had a history of stimulant abuse or any neurological or psychiatric disorders. All participants were naïve for antipsychotics and antidepressants, and all had a normal ECG (taken prior to ketanserin session) as well as neurological examination. Written informed consent was obtained prior to both the MRI and PET scanning sessions, which were performed at two different occasions. The participants underwent functional brain MRI on four separate days, at least one week apart. The fMRI sessions were performed with an average interval of 3.2 years after PET scan, which had been obtained prior to the present study when subject entered the large study cohort, however the data from the PET scan were used in the present study.

Profile of Mood States

Participants completed a Danish version of the Profile Of Mood States (POMS) questionnaire (McNair *et al.,* 1971). The mood questionnaire contained 64 adjectives, of positive and negative value. Most of the words in the questionnaire relate to one of six mood-factors characterizing the mood states; tension/anxiety, depression/dejection, anger/hostility,

vigor/activity, fatigue/inertia, and confusion/bewilderment, enabling us to score a value for each factor to determine self-reported mood state for each of the four scanning sessions. The POMS were given before and right after the MRI scanning, for the SSRI and ATD sessions participants also completed the POMS before drug treatment. For the significant POMS scores we generated contrast images for the relative increase in BOLD signal induced by the emotional faces relative to neutral faces for all three sessions to make sure the mood of our participants did no influence the fMRI results.

NEO-PI-R

The Revised NEO Personality Inventory (NEO-PI-R) is a psychological self-reported personality inventory developed to be used with adults without overt psychopathology (Costa & Mccrae, 1992). It is a 240-item measure of the Five Factor Model that evaluates the broad personality dimensions of: Extraversion, Agreeableness, Conscientiousness, Neuroticism, and Openness to Experience. The Danish translation of the NEO-PI-R has been psychometrically evaluated and normed in a standardization sample of 600 participants (Hansen *et al.*, 2004).
The participant is asked to indicate on a scale from 1 to 5 how well each statement in the test fits his or her personality. Each factor score is derived by adding the scores from assessment of six personality traits (facets) and each trait score is derived by adding the scores on eight items in 0 – 4 Likert format. For this study we were only interested in the neuroticism dimension of the NEO-PI-R questionnaire, wherefore these measures have been used as a covariate in Paper2 (Page 14 of Part II).

Induction of Serotonergic Challenges

This study is a repeated measures type, with all participants having four MR-scannings separated by at least one week to assure a wash out period between administrations of challenges. Three of the four scannings included serotonin challenges (SSRI, ATD, KET) and one scan without drug intervention (control). Drug challenges were assigned in a randomized counterbalanced order to the participants.

Ketanserin

There are several serotonin receptors in the brain (Pithadia, 2009), in this study the 5-HT_{2A} receptor was of interest. Ketanserin blocks the 5-HT_{2A} receptor rendering serotonin unable to bind to this receptor. Ketanserin was administered with a 10 mg bolus at the beginning of the scan session followed by a 6 mg/h intravenous infusion for the duration of the scan approx. 75 min (~ 17.5 mg in total). This infusion schedule was chosen, as it has been shown to lead to a complete blockade of cerebral 5-HT_{2A} receptor binding (Pinborg *et al.*, 2003). Results of the ketanserin challenge are the focus of Paper1 (Page 1 of Part II).

Citalopram (SSRI)

Selective serotonin reuptake inhibitors (*Selective Serotonin Reuptake Inhibitor*, SSRI) works by blocking the presynaptic reuptake of serotonin. The global level of free serotonin in the brain will increase as a result thereof. SSRI's are used in the treatment of several mental diseases such as; social anxiety, panic attacks, obsessive compulsive disorder, and depression (Harmer *et al.*, 2003). Treatment with the SSRI citalopram was given intravenously over a 2 hour period 20 mg/h (total of 40mg) prior to the scanning and 8mg/h during the scanning to maintain plasma concentration in the brain. The results of SSRI along with ATD challenges in relation to the neuroticism scores, are the main focus of Paper2 (Page 14 of Part II).

Acute Tryptophan Depletion

To induce Acute tryptophan depletion (ATD) all subjects were instructed to follow a low protein diet for 24 hours prior to the day of scanning, this was only done prior to this particular scanning day, and the subjects would be notified in advance. Upon arrival all subjects would then drink a 75 g tryptophan-free powdered mixture of essential and non-essential amino acids dissolved in water (XLYS, TRY Glutaridon, SHS International Ltd) 5 hours prior to scanning. The results of ATD along with SSRI challenges in relation to the neuroticism scores are the main focus of Paper2 (Page 14 of Part II).

BOLD fMRI

The main imaging method used in this study was that of blood oxygen level dependent (BOLD) functional magnetic resonance imaging (fMRI). Which we obtained using a Siemens 3T Trio scanner (Siemens, Erlangen, Germany). BOLD fMRI offers good spatial resolution, but poor temporal resolution and a time lag of seconds are common when doing fMRI imaging. The BOLD signal is an indirect measure of neural activity as the signal arises with changes of blood flow to a brain region. fMRI takes advantage of the signal intensity of hemoglobin, which is the protein carrying oxygen to the neurons. Hemoglobin can either be carrying oxygen (oxyhemoglobin) or be depleted of oxygen (deoxyhemoglobin). Investigating oxygen metabolism over time serves as an indirect measure of neural activity in the brain. When a brain area becomes activated it requires more energy than in its resting state, which leads to an initial decrease of oxygen in the area with a simultaneous increase in deoxyhemoglobin. However an increased supply of oxygen follows within seconds of activation onset as the brain vasculature responds to the local oxygen depletion, leading to an overcompensation of oxygen to the activated area (Attwell & Iadecola, 2002). Deoxyhemoglobin is slightly paramagnetic, which influences the protons in surrounding water molecules, and thus allows investigation of changes in blood flow, i.e. increased neural activity, as this will cause a change to the oxy- to deoxyhemoglobin ratio in the activated area. This change in blood flow in response to activation is the BOLD effect, which is the basis for the fMRI signal.

^{18}F-altanserin PET scanning

Positron Emission Tomography (PET) is an imaging technique based on the radioactive decay of positron-emitting isotopes that are injected into the bloodstream of a participant. The radioisotope has a specific half-life ($T_{½}$) leading it to decay at a specific time rate (^{18}F has a half life of 109,7 min). As the isotope decays it produces positrons, an antimatter to an electron. When a positron and an electron collide an annihilation event occurs creating two 511keV gamma photons shooting off in opposite direction (Haselman *et al.*, 2009). They travel through the body to be detected by scintillator crystals in the PET scanner to measure gamma rays, or PET signal (Melcher, 2000). The ^{18}F-altanserin PET data that were used in this study were acquired by the neurobiological research unit for CIMBI (Knudsen *et al.*, 2015) in an eighteen-ring GE-Advance scanner operating in 3D-acquisition mode (GE, Milwaukee, Wisconsin, USA) as previously described by (Pinborg *et al.*, 2003). ^{18}F-altanserin was administrated as a combination of a bolus injection followed by continuous infusion to obtain steady state of the tracer in blood and tissue; the participants received a maximum dose of 3.7 MBq/kg bodyweight (see original manuscript for further description of acquisition and analyses of PET data, page 1 of Part II).

To assess the effects of ketanserin a model with two exponentials were generated to infer O_{KET} which is the fraction (%) of a receptor population being occupied by ketanserin: one representing the ketanserin binding and release of the radioligand from the receptor with a half life of $T_{k1/2}$ and the other representing the diffusion of free radioligand out of the brain tissue into the blood with a half life of $T_{r1/2}$. The time dependent estimation of O_{KET} was based on a previous PET study also including ketanserin infusion (Pinborg *et al.*, 2003); when ketanserin is infused into the bloodstream it diffuses to the receptor to which it then binds thereby releasing radioligand from the receptor into the bloodstream. By applying this model to our data an excellent fit was obtained when $T_{k1/2}$ and $T_{r1/2}$ values both were in the range of 5–10 min and the sum of $T_{k1/2}$ and $T_{r1/2}$ amounted to roughly 15 min. This enabled us to estimate the minimum and maximum $T_{k1/2}$ values corresponding to two O_{KET} outcomes termed O_{KET5} and O_{KET10} (referred to as O_{KET} covariates, see also Figure 3). Using this model t-tests were computed for the emotion contrast images from the ketanserin session only, including three covariates: average neocortical BP_P, O_{KET} covariates and the product of the two covariates (BP_P times O_{KET}). This made it possible to identify brain regions where ketanserin induced changes in emotional face processing depending on the three above-mentioned covariates.

Activation Paradigm

The paradigm was a gender discrimination task integrating blocks of neutral, fearful and angry faces from the Karolinska Directed Emotional Faces database (Lundqvist *et al.*, 1998). An equal number of female and male faces were used in the paradigm. Fearful and angry faces were presented once whereas neutral faces were presented twice throughout the paradigm. Each block consisted of four face stimuli of the same emotion (Fear, anger, neutral) and two null events (fixation cross), which were pseudo-randomly intermixed within the blocks.

Throughout the session, 16 blocks of neutral (N) was alternated with 8 blocks of fear (F) and 8 blocks of angry (A) faces (N-A-N-F-N-A-N-F-.....) were shown to the participants (Figure 2). Each image was presented in the middle of the screen for 1800ms with a 200ms inter trial interval. The task was run twice with a short break in between. Face images were shown in color, no actions were taken to alter the luminance of the models hair or makeup, in order to make the images seem as realistic as possible. Since the participants were told only to discriminate between the genders of the faces, the emotional processing was implicit and was assumed not to depend on voluntary or attentional processing.

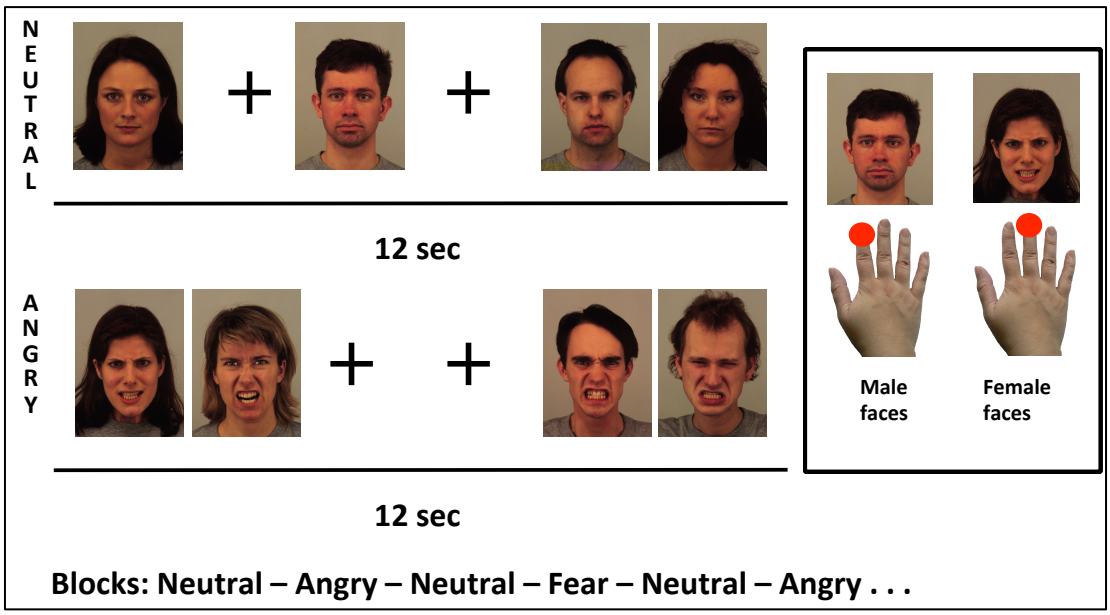

Figure 2: Illustration of activation paradigm using images from the Karolinska Directed Emotional Faces database. Participants responded by pressing one of two buttons, to indicate the gender of the faces.

Statistical analysis of fMRI data

The paradigm was analyzed as an event-related design with three event types (neutral, angry, fearful faces). Each event was modeled with the onset of the appearance of the cue. To begin with images were realigned, to account for head movement in the scanner, and normalized to an MNI (Montreal Neurological Institute) stereotactic space using transformation parameters derived from segmentation of the structural MRI. Then first-level analyses were conducted to identify the brain areas that were activated in response to the gender discrimination task for each individual participant. Each event (neutral, angry, fear faces) was modeled as a delta function with onset coinciding with the appearance of the cue. Then the covariates (neutral, angry, fearful faces) were convolved using a standard canonical hemodynamic response function (HRF) as the BOLD signal is an indirect measure of neural activity with a time lag in the range of seconds. The HRF then models this delay and the relationship that is assumed to exist between the BOLD signal and the underlying neuronal activity. The effects of physiological noise was controlled for by including in the statistical model regressors related

to heart beat, respiration, and head movements recorded during the scan (40 nuisance regressors in total) (Glover *et al.*, 2000; Lund *et al.*, 2006).

To assess the variation in activation between subjects a random effects model was created, modeling the fMRI runs of the four drug sessions. In the Ketanserin study, contrast images were generated for each subject to assess the relative increase in the Blood Oxygen Level Dependent (BOLD) signal induced by the emotional faces relative to neutral faces in both the control and ketanserin sessions. These were then applied to a group level analyses in which individual contrast images were entered into separate paired t-test models thereby indicating the brain areas involved in emotion processing and how those areas respond to ketanserin.
Similarly, for the SSRI and ATD study, contrast images for SSRI and ATD were analyzed at group level with a mixed-effect ANOVA model with three factors; challenge (ATD, SSRI, control), emotion (angry, fear, neutral) and subject as a random as a random factor (22 subjects). This statistic model also included the individual neuroticism scores from the "NEO-PI-R" as subject-specific covariates. A statistical test is applied to all voxels in the brain, leading to a high risk of false positives, which must be corrected for, therefore clusters were considered significant at $p<0.05$, and an extend threshold of $p<0.01$, for SSRI and ATD analyses, after Family-Wise Error (FWE) correction for multiple non-independent comparisons.

We expected amygdala's neuronal activity to change directly in response to aversive faces and to correlate with the occupancy levels of the ketanserin (O_{KET}) and binding potential ($BP_{P)}$ values as obtained with PET. Therefore we performed FWE correction using small volume correction (SVC) for fear, angry, and aversive contrasts in the amygdala voxels, to try to enhance the signal changes from this small anatomical region, by using predefined regions of interest (ROI) (Fusar-Poli *et al.*, 2009). Functional connectivity were also assessed, to identify ketanserin-induced changes in OFC connectivity that could be explained by O_{KET} , BP_p or an interaction between the two factors (BP_P times O_{KET}) during the processing of fearful faces. Further details about the connectivity analyses can be found in Paper1 (Page 1).

Software for imaging data analyses

Our fMRI data were all preprocessed and analyzed using SPM version 5 (http://www.fil.ion.ucl.ac.uk/spm/software/spm5) (referred to as SPM5 from hereon) and SPM version 12 (http://www.fil.ion.ucl.ac.uk/spm/software/spm12) (SPM12 from hereon). The specifics to the analyses including pre-processing, have been described in greater detail in the original manuscript with addendum (Pages 14 and 32 of Part II).

Summary of key findings

Paper1: *Acute serotonin 2A receptor blocking alters the processing of fearful faces in the orbitofrontal cortex and amygdala.*

For this study the focus were on the effects of 5-HT_{2A} blocking by ketanserin compared to control. On the day of the ketanserin scan, participants were given a 10 mg bolus injection of ketanserin, followed by a 6 mg/h maintenance IV infusion for the duration of the scan, leading to a gradual increase in 5-HT_{2A} receptor occupancy (O_{KET}) reaching ~100% occupancy within an hour (Pinborg et al., 2003).

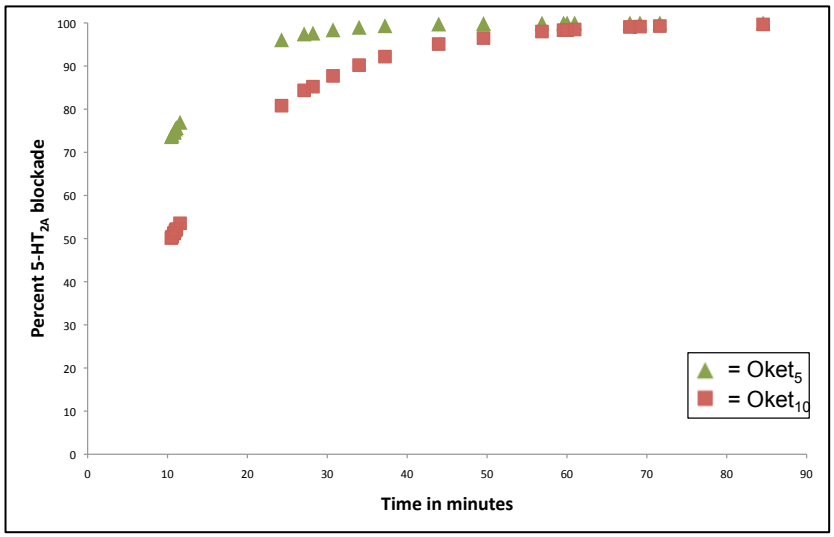

Figure 3: Estimated levels of serotonin 2A (5-$HT_{2A)}$ receptor occupancy (O_{KET}) blocking over time for each subject shown for both O_{KET5} (triangles) and O_{KET10} (squares) (Please see page 10 or 1 for an explanation of O_{KET5} and $O_{KET10)}$

Participants were asked to make a gender judgment task during the fMRI scans (Figure 2). In looking at the reaction times (RT) of these judgments faces with an aversive emotion delayed the gender-judgment in both sessions relative to neutral faces resulting in longer RT. Compared to the control session without medication, ketanserin treatment was associated with longer RT of approximately 5% compared to the control session this increase was consistent across all facial expressions (Figure 4). When differing between the aversive faces, and looking at the RT for fearful and angry faces by them selves RT was longer for both facial expression in ketanserin as well as control sessions. However RT were only significantly different between the two facial expressions anger and fear in the control session. There was no difference found in the error rates between control and ketanserin session for any of the three facial expressions (neutral, anger, fear) indicating that the 5% increase in RT during the ketanserin session was not paralleled by a change in accuracy.

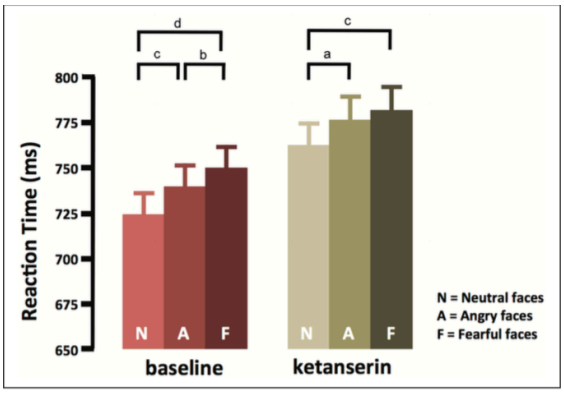

Figure 4: Mean reaction time (RT) recorded during the gender-judgment task based on facial expressions in the control and ketanserin functional magnetic resonance imaging (fMRI) sessions. Data are presented as mean ± standard error of the mean (SEM), $^{a}p<0.1$, $^{b}p<0.05$, $^{c}p<0.01$, $^{d}p<0.001$.

To assess the current mood of each participant on scanning days, a POMS questionnaire was completed right before and right after the scan for both control and ketanserin sessions. For the ketanserin scans the participants reported significant decreases in the mood factor *vigor/activity* and increases in *fatigue/inertia* as compared to the responses prior to the ketanserin infusion. Conversely, the scores for *anger/hostility* were significantly lower at the end of the control session relative to scores obtained before the session. None of these mood changes correlated significantly with the fMRI activation patterns. The relative increase in RT in the ketanserin session correlated with the ketanserin-induced decrease in self-report on vigor, but no correlation was found with the reported increase in fatigue. The functional neuroimaging data revealed that gender judgment of angry and fearful faces, compared to neutral faces, consistently activated the amygdala bilaterally, as well as clusters in the fusiform gyrus (Figure 5).

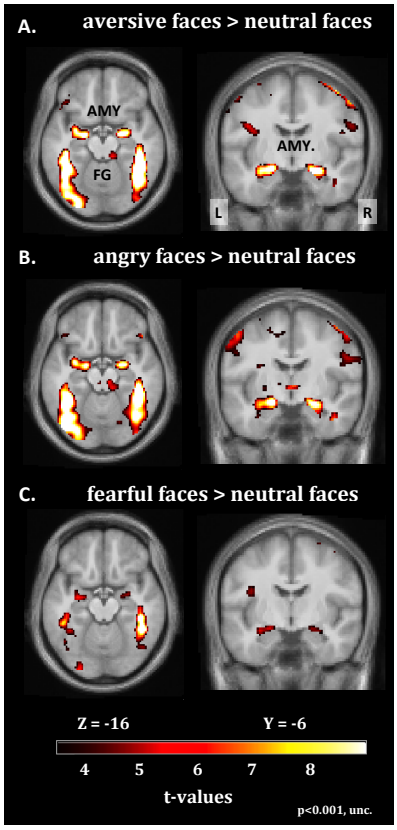

Figure 5: Statistical parametric maps (SPMs) showing brain areas, which are activated by aversive facial expressions relative to neutral faces, as reflected by an increase in blood oxygen level dependent (BOLD) signal. The SPMs are thresholded at $p<0.001$ (uncorrected). (a) Activation maps for the contrast aversive faces > neutral faces. (b) angry faces > neutral faces. (c) fearful faces > neutral faces.

Compared to the control session ketanserin reduced the neural response to aversive facial expressions (angry and fear), but mostly so for fearful faces in the mOFC while increasing the response to neutral faces (Figure 6). The change in emotion-specific activity found in the amygdala during ketanserin challenge was predicted by the interaction of the two covariates BP_P and O_{KET} (i.e. BP_P times O_{KET}) and only for fearful faces. The ketanserin related facilitation of the amygdala response to fearful faces was stronger the more 5-HT_{2A} receptors were occupied with ketanserin. When pooling the response from both angry and fearful faces we saw a bilateral trend in amygdala activation with the O_{KET5} covariate

Our connectivity analyses showed that the neural activity of the OFC region where ketanserin reduced the response to fearful faces the most, had a strong influence on the activity in the left amygdala. This correlation was dependent on the degree of 5-HT_{2A} receptors that had been blocked by ketanserin. The more receptors blocked, the stronger the increase in functional connectivity between OFC and left amygdala (**Figure 7**).

Concluding that the strength of a negative feedback signal from OFC to amygdala during processing of fearful faces is regulated by 5-HT_{2A} receptor mediated signaling increases the sensitivity of OFC to fearful facial expressions.

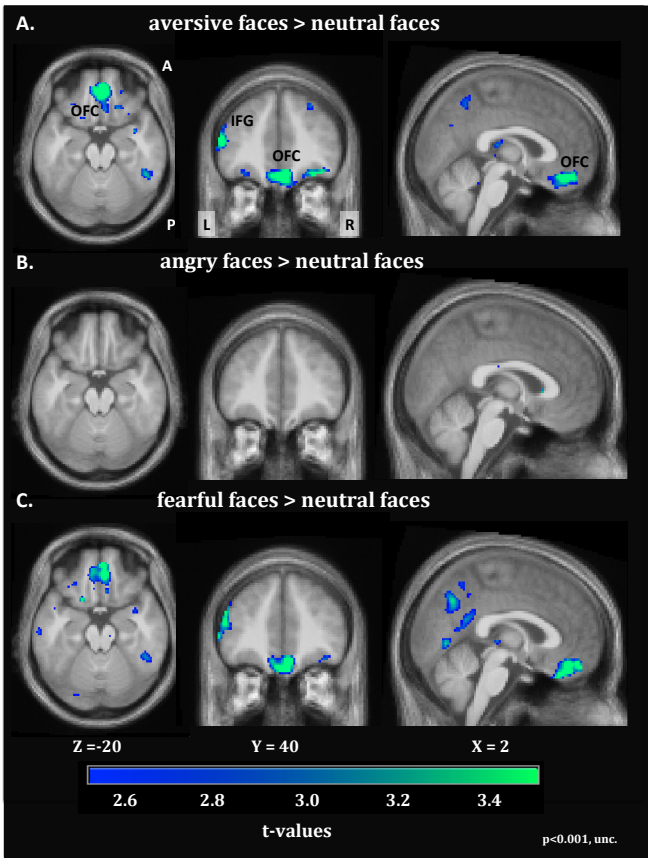

Figure 6: Statistical parametric maps (SPMs) showing brain regions, which show a decrease in activation for aversive face expressions relative to neutral faces during the ketanserin challenge as opposed to baseline (control session). The SPMs indicate decreases in BOLD signal and are thresholded at $p<0.001$ (uncorrected). (a) Depicts decreases in regional responsiveness to aversive (angry, fearful) faces under ketanserin treatment. (b) angry faces > neutral faces. (c) fearful faces > neutral faces.

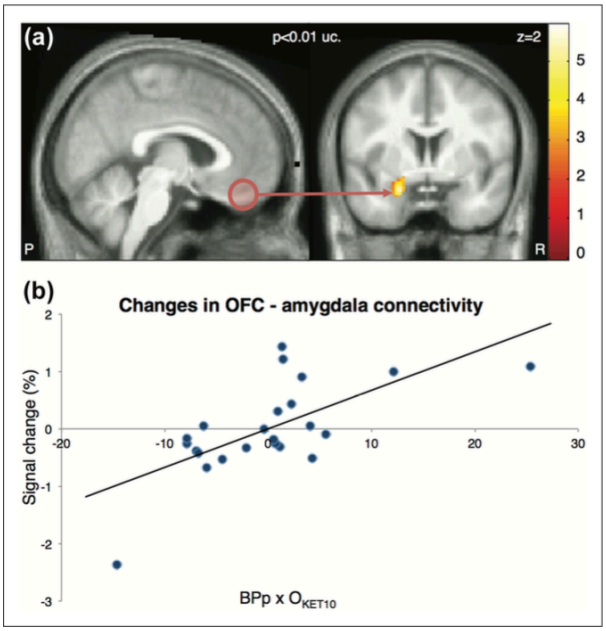

Figure 7: The figure summarizes the connectivity analysis exploring ketanserin related changes in OFC-amygdala connectivity during fear events. (a) SPMs showing the changes in coupling between the OFC seed region and the amygdala following acute 5-HT_{2A} blockade. (b) Positive correlation between the OFC-amygdala connectivity and the interaction between 5-HT_{2A} receptor binding potential (BP_p) and ketanserin-induced 5-HT_{2A} receptor occupancy (O_{KET10}). The higher the O_{KET} and the higher the BP_P, the stronger the individual increase in connectivity between the OFC and left amygdala. Values are mean normalized. The extent threshold of the SPMs is set at $p<0.01$ (uncorrected).

Paper2: *Neuroticism predicts the impact of intravenous citalopram on the neural response of subgenual anterior cingulate cortex to fearful faces.*

With extract of findings from SPM5 analyses.

For this manuscript the main focus were on the two serotonergic challenges selective serotonin reuptake inhibitor (SSRI; citalopram) and acute tryptophan depletion (ATD). We wanted to explore how the effect on emotion processing of these two challenges differed from that of the control session, as well as each other. On two separate scanning days our participants were given SSRI intravenously over a 2hour period prior to the scanning followed by a maintenance dose during the scanning. For the ATD challenge participants were given a tryptophan-free powdered mixture of essential and non-essential amino acids dissolved in water (XLYS, TRY Glutaridon, SHS International Ltd) 5 hours prior to scanning to obtain acute tryptophan depletion (ATD).

POMS scores were compared across experimental conditions (SSRI, ATD, control) using a 3x3 ANOVA, which yielded a significant effect of time (*arrival, before, and after scan*) for the mood factor *Anger/Hostility* with lower scores at the end of the scanning session. POMS scores indicated that the participants did not remain in a high arousal state throughout the scan. None of the POMS scores correlated significantly with the fMRI activation patterns. Because the fMRI analyses focused on the differential effects of ATD and SSRI, we also set up a second ANOVA model with 2x3 factors including the two 5-HT interventions (*ATD and SSRI*) and time (*arrival, before, and after scan*). Here we found a main effect of time, with a decrease in Vigor/Activity scores after both pharmacological interventions, but neither a main effect of the type of intervention nor an intervention × time interaction for any of the mood states.

RT measures were obtained for both ATD and SSRI sessions. For the ATD session, mean RT were longer for both angry and fearful than for neutral faces. However for the SSRI session mean RT were only longer for angry compared to neutral faces. In an ANOVA analyses we found a main effect of emotion on RT. Using standard t-tests we found that error rates were overall decreased for all three facial expressions in both ATD and SSRI sessions with a mean error rate reduction of 2% compared to an error rate of 4% in the control session. Prolactin measures showed an overall decrease by 75% of plasma tryptophan during the ATD challenge, indicative of reductions in central tryptophan bioavailability. An ANOVA of prolactin levels revealed no main effect of drug or time within session from baseline to scanning.

As mentioned previously imaging data for the SSRI and ATD challenges has both been analyzed using SPM5 (http://www.fil.ion.ucl.ac.uk/spm/software/spm5) as well as SPM12 (http://www.fil.ion.ucl.ac.uk/spm/software/spm12). The specifics to the analyses including pre-processing, have been described in greater detail in the original manuscript with addendum (Page 14 and 32 of Part II). There is a large overlap in the results between SPM5 and SPM12 (Tabel 1), however some differences were also found. Which will be explained further below, all illustrations are from SPM5 analyses.

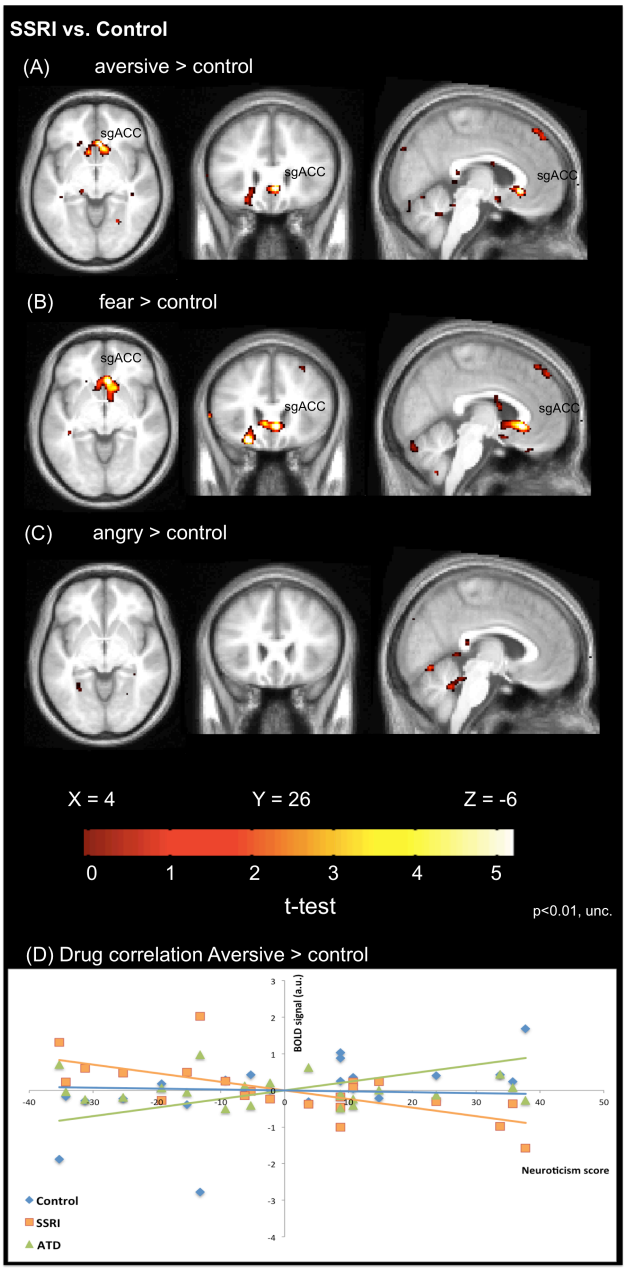

Figure 8: Statistical parametric maps (SPMs) showing changes in activation for aversive face expressions relative to neutral faces during the SSRI challenge compared to baseline (control) in the sgACC. The SPMs indicate changes in BOLD signal and are thresholded at $p<0.001$ (uncorrected). (a) Depicts decreases in regional responsiveness to aversive (angry, fearful) faces under SSRI treatment. (b) angry faces > neutral faces. (c) fearful faces > neutral faces. (d) panel shows the correlation between the individual neuroticism scores and the BOLD response in the sgACC for each of the three drug challenges (SSRI, ATD, Control) (SPM5 analyses).

Similar for SPM5 and SPM12 when comparing the effects of SSRI with ATD we found that neither the main effect of challenge (SSRI and ATD) nor interaction between challenge and emotion (angry, fearful, and neutral) revealed any significant results (see **Figure 2** from addendum page 32 of Part II). However in right sgACC, the individual neuroticism scores predicted the impact of the two 5-HT challenges (SSRI and ATD) on the neural response to fearful faces (Figure 8B). Individual neuroticism scores were negatively correlated with the impact of SSRI. The higher the neuroticism score, the stronger the reduction of the sgACC response to fearful faces with SSRI relative to ATD condition. No such relationship was seen for angry faces. Post hoc correlational analyses confirmed that the association between neuroticism and fear related regional activity had opposing directions for the two 5-HT challenges in SPM5 (Figure 8D). The fear related BOLD response in the sgACC correlated negatively with individual neuroticism scores during the SSRI challenge.

Furthermore exclusively to the SPM5 (Tabel 1) analysis we found an ATD induced increase in neural response in the left superior temporal gyrus (STG), to aversive facial expressions relative to the control session, correlated positively with individual variations in neuroticism scores (Figure 9A). Post hoc analysis yielded a positive linear relationship between neuroticism and the neural response to aversive faces for the ATD condition in left superior temporal gyrus, but a negative relationship for the control session (Figure 9B). No significant effects emerged when considering angry or fearful faces separately.
For right Heschl's gyrus, neuroticism was associated with an increased responsiveness to aversive faces in the ATD relative to the SSRI condition. Post hoc analysis revealed a positive linear relationship between neuroticism scores and neural activity evoked by aversive facial expressions in the ATD session, and a trend towards a negative relationship in the SSRI condition. This effect only was found when pooling the aversive facial expressions, but not when the response to angry or fearful faces was analyzed separately.

SPM12			Peak coordinates			
			x	y	z	p_{FWE}
SSRI>ATD	fearful>neutral					
		sgACC	9	26	-4	0.015
SPM5						
			x	y	z	p_{FWE}
SSRI>ATD	fearful>neutral					
		sgACC	6	24	-6	0.03
ATD>Control	aversive>neutral					
		STG	-58	-4	-4	0.003
ATD>SSRI	aversive>neutral					
		Heschl's gyrus	42	-22	4	0.002

Tabel 1: Overview of the findings in SPM12 and SPM5 respectively

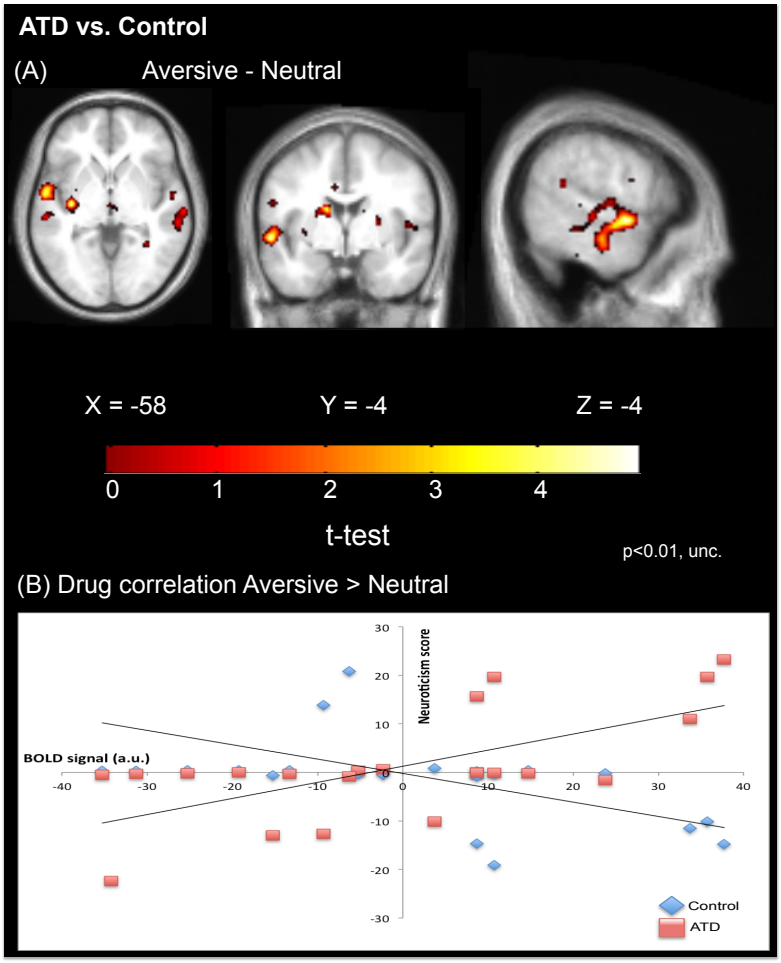

Figure 9: Statistical parametric maps (SPMs) showing changes in activation for aversive face expressions relative to neutral faces during the SSRI challenge compared to baseline (control) in the superior temporal gyrus (STG). The SPMs indicate changes in BOLD signal and are thresholded at $p<0.001$ (uncorrected). (A) Depicts activation in response to aversive (angry, fearful) faces under ATD condition. (B) Shows the correlation between the individual neuroticism scores and the BOLD response in the STG for control and ATD (SPM5 analyses).

Concluding that the personality trait neuroticism may predict the impact of 5-HT challenges on fear processing in sgACC. As both an increase as well as a lowering of serotonergic tone in the brain increased the impact of 5-HT challenges on fear processing in the sgACC. This finding may represent a neural mechanism for the variable therapeutic effect of SSRI treatment observed in clinical populations. As well as the idea that serotonin and neuroticism are tied together in processing threatening face emotions and that this influence varies depending on the nature of the threat.

General discussion[1]

The current study set out to investigate the role of the serotonergic system on emotion processing. We did so by altering the serotonergic system by use of drugs capable of manipulating the serotonergic levels in the brain in three different ways. We included one drug (ketanserin) that targets a specific serotonin receptor (5-HT_{2A}) in the brain. Whereas the remaining two challenges (SSRI and ATD) both caused a global change in the serotonergic levels in opposite directions, with SSRI increasing the global serotonin levels and ATD decreasing it.

The main findings of this study showed that acute 5-HT_{2A} receptor blockade 1) suppressed the neural response to fearful faces in medial OFC, 2) thereby modulating the functional connectivity between medial OFC and amygdala as a function of 5-HT_{2A} receptor blockade, and 3) increased the functional coupling between medial OFC and left amygdala in response to fearful faces. Additionally for our global serotonin manipulations (SSRI, ATD) we found that changes to serotonin levels affected how neuroticism is correlated with brain activity in the sgACC, the higher the neuroticism score, the lower the sgACC activity for SSRI compared to ATD, indicating that SSRI treatment becomes more effective in subjects rating high in neuroticism scores. However when not considering neuroticism scores, we found that the overall difference between SSRI and ATD challenges did not reveal any significant effect on neural activation, neither compared to each other or to control. Despite this we do see an increase of accuracy following both ATD and SSRI, indicating that the drugs do have some effect on emotion processing.

It has been postulated (Drevets *et al.*, 2008) that emotion processing, are associated with a circuitry involving the sgACC together with other limbic structures including the medial OFC and amygdala, suggesting that abnormal synaptic connections between these areas may contribute to abnormalities in emotion processing or regulation leading to e.g. anxiety for which neuroticism is a risk factor (Bienvenu *et al.*, 2001). Our findings of a reduced response of the OFC to fearful faces following acute pharmacological 5-HT_{2A} receptor blocking, as well as a negative correlation between neuroticism scores and activation in sgACC for SSRI condition relative to ATD is in support of the notion of high levels of 5-HT involved signaling in cortical processing of fearful facial expressions in the frontal regions.

Furthermore we saw that the ketanserin-induced increase in functional connectivity between medial OFC and left amygdala during the processing of fearful faces, was related to the degree of 5-HT_{2A} receptor blockage. The more 5-HT_{2A} receptors were blocked, the stronger the increase in functional connectivity between OFC and left amygdala. Considering that OFC exerts inhibitory control over the amygdala (Stein *et al.*, 2007; Fisher *et al.*, 2009) it can be assumed that these OFC-to-amygdala projections are under the control of orbitofrontal 5-HT_{2A} related neurotransmission and blocking of 5-HT_{2A} receptors enhances the impact of OFC on amygdala responsiveness to fearful faces.

[1] Detailed discussion of the specific findings can be found in each of the manuscripts

Blocking the 5-HT_{2A} receptors attenuated the OFC response to fearful faces and enhanced the response to neutral faces. This differential effect of 5-HT_{2A} receptor blockade indicates a shift in preferential processing towards non-threat related face features in OFC. A possible scenario is that acute 5-HT_{2A} blockade reduced excitatory signaling in OFC circuits which compute fear related information while increasing neural processing in circuits processing other social stimuli features.

When considering the product between the magnitude of receptors (BP_P), and the relative proportion of 5-HT_{2A} receptors blocked by ketanserin (O_{KET}) it becomes evident that there is a ketanserin-induced effect on OFC-to-amygdala coupling and that the efficiency of 5-HT_{2A} receptor blocking (as indexed by the proportion of blocked receptors) had the strongest impact in individuals with a high density of neocortical binding sites. If this observation can be replicated in future studies, it will have a large impact on the current view of assessment of receptor drug occupancy as the single most important measure for prediction of drug efficacy. These results suggest an important general implication showing that individual variations in regional receptor binding might determine individual susceptibility to drug-induced manipulation of receptor function.

The main findings of our global manipulations of the serotonergic tone, are that not only did changes to serotonin levels affect how neuroticism is correlated with brain activity in the subgenual cortex, but also in the superior temporal gyrus (STS) and Heschl's gyrus. We found a negative correlation between neuroticism scores and activation in subgenual cortex for SSRI condition, as well as for superior temporal gyrus in ATD condition compared to control. For Heschl's gyrus we found a positive correlation between neuroticism scores and neural activation for ATD condition, but a negative correlation for SSRI condition.

It should be noted that the activation we see in Heschl's gyrus is positively correlated with neuroticism scores, so that with a lower neuroticism score the stronger the involvement of Heschl's gyrus. Heschl's gyrus has mostly been recognized for its involvement in auditory processing (see Uppenkamp & Röhl, 2014) for a review), however auditory cortex has substantially been shown to be involved in classical Pavlovian fear conditioning as well as extinction learning (Grosso *et al.*, 2015). This could be indicative of our findings of activation in Heschl's gyrus being positively correlated with neuroticism scores, as fear learning becomes more heightened in individuals with affective disorders, hence a lower neuroticism score would inevitably lead to less fear learning. Our results along with previous studies reporting Heschl's gyrus involvement in emotion processing provides a stronger foundation for including Heschl's gyrus as part of an emotion processing network.

In conclusion the study found support for high serotonin involvement in emotion processing. Both lowering and increasing the serotonergic tone of the brain increased the correlation with neuroticism scores. Indicating subgenual cortex, superior temporal cortex as well as Heschl's gyrus as important structures in emotion processing. Additionally we found further support for a functional connectivity between frontal cortical regions and lower limbic structures such as the amygdala both when blocking the 5-HT_{2A} receptors, but also indirectly by observing a change in sgACC activity linked with the SSRI challenge. As well as the idea

that serotonin and neuroticism being tied together in processing threatening face emotions, and that this influence varies depending on the nature of the threat.

Limitations to the study

A more thorough discussion of the potential limitation of the each sections of the study can be seen in the manuscripts (Page 1 and 14 of Part II), however one of the more obvious limitations that apply to the general setup of the study was that the pharmacological challenges were not double-blinded. In addition, when the study was designed, it was believed that a full placebo control of the oral ATD solution and the IV infusions for both ketanserin and SSRI sessions, would be too excessive for a within-subject design, meaning that each subject would have to go through three protocols on each session: oral solution for the ATD protocol, possibly including the low protein diet the day before scanning, 2 hours of IV infusion followed by a bolus injection right before scanning. Despite that a placebo control would be advantageous in several respects, and prevent the need to consider placebo effects or effects of IV versus no manipulations, the no-drug condition without blinded placebo IV/oral solution was seen as an acceptable choice. Although subjects were made aware of potential side effects of each challenge, they were not made aware of the specifics to the administration of each, neither were they informed about the expected effect from the individual challenges, and were therefore considered naïve. Therefore the specific effects observed with our fMRI protocol cannot be accounted for by a simple placebo effect or a lack of blinding.

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Part II

– The manuscripts

PAPER 1

Original Paper

Acute serotonin 2A receptor blocking alters the processing of fearful faces in the orbitofrontal cortex and amygdala

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Abstract

Background: The serotonin 2A (5-HT_{2A}) receptor has been implicated in neural-processing of emotionally salient information. To elucidate its role in processing of fear and anger, healthy individuals were studied with functional magnetic resonance imaging (fMRI) after 5-HT_{2A} receptor blockade, while judging the gender of neutral, fearful and angry faces.

Methods: 5-HT_{2A} receptors were blocked with ketanserin to a variable degree across subjects by adjusting the time between ketanserin-infusion and onset of the fMRI protocol. Neocortical 5-HT_{2A} receptor binding in terms of the binding potential (BP_p) was assessed prior to fMRI with ^{18}F-altanserin positron emission tomography (PET) and subsequently integrated in the fMRI data analysis. Also functional connectivity analysis was employed to evaluate the effect of ketanserin blocking on connectivity.

Results: Compared to a control session, 5-HT_{2A} receptor blockade reduced the neural response to fearful faces in the medial orbitofrontal cortex (OFC), independently of 5-HT_{2A} receptor occupancy or neocortical 5-HT_{2A} receptor BP_p. The medial OFC also showed increased functional coupling with the left amygdala during processing of fearful faces depending on the amount of blocked 5-HT_{2A} receptors.

Conclusions: 5-HT_{2A} receptor mediated signaling increases the sensitivity of the OFC to fearful facial expressions and regulates the strength of a negative feedback signal from the OFC to amygdala during processing of fearful faces.

Keywords

Functional magnetic resonance imaging, positron emission tomography, emotion, fearful faces, serotonin, serotonin 2A receptors, ketanserin

Introduction

Facial expressions such as happiness, fear, sadness, anger, disgust, and surprise represent basic human feelings that are readily decoded by members of all human cultures (Ekmann, 1999). The ability to appropriately interpret emotional facial expressions is important for our social interactions, and impaired emotion-related processing is associated with an increased risk for affective psychiatric illnesses (Mayberg, 2003; Phillips et al., 2003). Neuroimaging studies in healthy volunteers have identified the amygdala and prefrontal cortex as core regions of a functional network processing facial emotions (Adolphs, 2002). Evidence indicates that the amygdala receives visual information about facial emotions via cortical projections from the ventral stream of object processing, and from a fast subcortical pathway. The latter pathway includes the superior colliculus and pulvinar as the only relays and is critical for automatic processing of facial emotions (De Gelder et al., 2005). The medial prefrontal cortex (mPFC) and orbitofrontal cortex (OFC) are involved in evaluating cognitive aspects such as integrating information about the emotional state of others, derived from face emotion (Bechara et al., 2000; Salzman and Fusi, 2010). The OFC and amygdala are strongly interconnected, with the OFC exerting inhibitory control over the amygdala during processing of emotional faces (Stein et al., 2007). Therefore, an effective integration of neuronal activity among these core regions is likely to be critical for efficient processing of emotions (Fairhall and Ishai, 2007; Liang et al., 2009).

Serotonin (5-HT) signaling plays an important role in the processing and regulation of emotions (Cools et al., 2007). For example, regulation of 5-HT release in the mPFC in response to aversive stimuli has been identified as a crucial mechanism in rats to deal effectively with stressors and to terminate fear-related behavior (Forster et al., 2006). Previous studies have also shown that serotonergic drugs modulate the neural processing of emotional faces in healthy individuals. For instance, Harmer et al.

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(2003) found that acute tryptophan depletion decreases recognition of fearful facial expressions in healthy women, while Passamonti et al. (2012) found that acute tryptophan depletion modulated the interactions between the PFC and amygdala while viewing emotionally salient faces. Further, a single dose of the selective serotonin reuptake inhibitor (SSRI), citalopram, a widely used antidepressant, increased the neural response of amygdala to happy but not to fearful faces in healthy individuals (Norbury et al., 2009).

Several lines of evidence indicate that the serotonin 2 (5-HT_2) receptor-family (5-HT_{2A}, 5-HT_{2B} and 5-HT_{2C}) is involved in generation and expression of anxiety. Global disruption of 5-HT_{2A} receptor signaling reduces inhibition in conflict anxiety paradigms in mice (Weisstaub et al., 2006). In humans, there is accumulating evidence that processing of emotionally salient information is modulated by 5-HT_{2A} receptors and that regional expression of the 5-HT_{2A} receptor in the brain, constitutes a trait related to anxiety (Frokjaer et al., 2008). Recently, Fisher et al. (2009) showed that inter-individual variations in 5-HT_{2A} receptor density in mPFC correlated inversely with the activation of right amygdala by angry or fearful faces in a face-matching task compared to a control task. They also reported a positive correlation between amygdala-prefrontal coupling and prefrontal 5-HT_{2A} receptor binding. These neuroimaging data suggest a regulation of amygdala reactivity via feedback inhibition from the PFC, which is more pronounced in individuals with greater neocortical 5-HT_{2A} receptor density.

Motivated by these reports, we adopted a multimodal neuroimaging strategy to explore the relation between 5-HT_{2A} receptor signaling and emotional face processing in amygdala and OFC. Our experimental approach integrated pharmacological functional magnetic resonance imaging (fMRI) and positron emission tomography (PET) of 5-HT_{2A} receptor binding. We performed blood oxygen level dependent (BOLD) fMRI while healthy participants made gender-judgments on photographs of male or female faces with fearful, angry or neutral expressions. 5-HT_{2A} receptors were acutely blocked with intravenous ketanserin infusion. By varying the relative timing between drug intake and the onset of fMRI, we adjusted the relative magnitude of acute 5-HT_{2A} receptor blockage across subjects. In addition, 5-HT_{2A} receptor binding as measured with PET, was used as trait marker of neocortical 5-HT_{2A} receptor dependent neurotransmission. This novel study design enabled us for the first time to study the impact of a gradually increasing 5-HT_{2A} receptor blockage on emotional face processing and to investigate its relation to 5-HT_{2A} receptor binding and occupancy.

We hypothesized that the individuals' cerebral 5-HT_{2A} receptor binding would have differential effects on neural processing of negative face emotions and that pharmacological blocking of 5-HT_{2A} receptors would suppress neural response in the OFC while enhancing neural response in the amygdala. We further predicted that the pharmacologically induced activity changes during emotional face processing would depend on the 5-HT_{2A} receptor occupancy level.

Methods

Participants

Twenty-three right-handed adults (nine females), mean age 31.8±6.5, were recruited from a larger cohort of healthy volunteers who had previously undergone ^{18}F-altanserin PET (Erritzoe et al., 2009). All subjects were re-interviewed prior to inclusion in the fMRI study. None of the participants reported a history of stimulant abuse or other psychiatric or neurological disorders. All participants were naïve to antipsychotics and antidepressants. They had a normal neurological examination, heart rate and electrocardiogram. Participants completed a modified Danish version of the Profile of Mood States (POMS) questionnaire (McNair et al., 1971) to assess current mood. On each fMRI session, participants completed the mood questionnaire twice, prior to the start of the fMRI scan (and drug infusion) and immediately after the fMRI scan. Written informed consent was obtained prior to both MRI and PET scanning according to the declaration of Helsinki II. The study was approved by the Ethics Committee of Copenhagen and Frederiksberg, Denmark (KF 01-2006-20).

Behavioral task

During fMRI, participants performed a gender-judgment task on face stimuli taken from the Karolinska Directed Emotional Faces database (Lundqvist et al., 1998). Unmasked colored photographs of a male or female face were presented in the middle of the screen for 1800 ms, with an inter-trial interval (ITI) of 200 ms. Faces were shown from a frontal perspective and had a neutral, fearful or angry expression. Subjects responded by pressing one of two buttons as quickly as possible with their right index or middle finger.

We employed a mixed fMRI design with alternating blocks showing neutral or aversive male and female faces in equal proportion (NEUTRAL-ANGRY-NEUTRAL-FEARFUL-NEUTRAL…). Each block comprised six events; three to five face stimuli (average of four), and one to three (average of two) null events (fixation cross), which were pseudo-randomly intermixed. In total, 32 blocks of neutral, 16 blocks of fear and 16 blocks of angry faces were presented over two fMRI runs, separated by a short break. All neutral faces were presented twice in total, whereas aversive faces were only presented once. Stimulus presentation and response recordings were performed using E-prime (Psychological Software Tools, Pittsburgh, Pennsylvania, USA).

Acute blockage of 5-HT_{2A} receptors

Subjects took part in four fMRI sessions. These sessions included ketanserin as below, a control condition with no pharmacological intervention (referred to as the control session throughout), and two other pharmacological interventions; intravenous treatment with the SSRI, citalopram, as well as acute tryptophan depletion (ATD). The order of the drugs were fully counter-balanced across subjects. Apart from the intravenous (IV) line and drug infusion administered while in the scanner, the scanning protocol was identical for the control and ketanserin sessions. To test for drug-related changes in the neural response that depended on the receptor occupancy, we systematically varied the interval between the onset of ketanserin administration and the onset of fMRI measurements across subjects. The time interval ranged from 5–75 min, leading to a blockade of 5-HT_{2A} receptors of variable degree across subjects (Figure 1). Ketanserin was administered intravenously as a 10 mg bolus (time 0) followed by 6 mg/h for the duration of the fMRI scan. This infusion schedule results in a

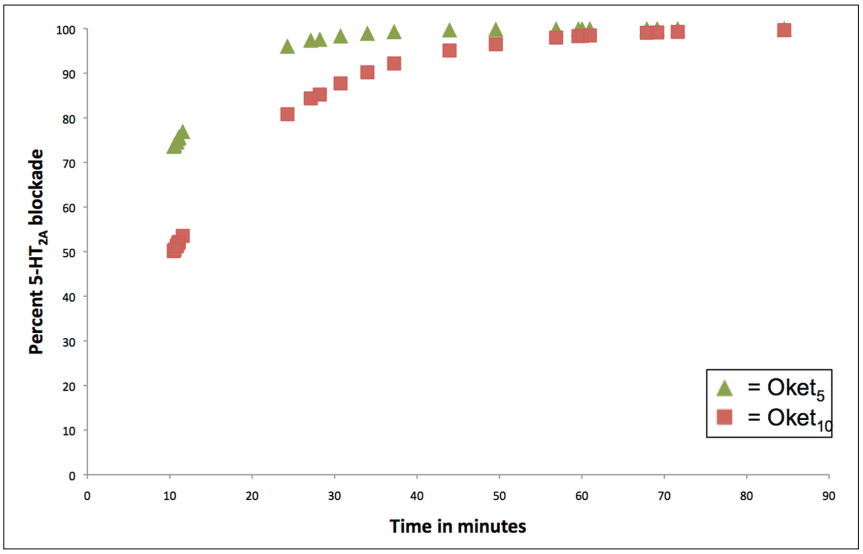

Figure 1. Estimated levels of serotonin 2A (5-HT_{2A}) receptor occupancy (O_{KET}) blocking over time for each subject shown for both O_{KET5} (triangles) and O_{KET10} (squares).

gradual increase in 5-HT_{2A} receptor occupancy (O_{KET}) reaching ~100% occupancy within an hour (Pinborg et al., 2003). O_{KET} is defined as the fraction (%) of a receptor population that is occupied during treatment with an unlabelled drug. The time-dependent estimation of O_{KET} was based on data from our previous PET study with acute ketanserin infusion (Pinborg et al., 2003). First, ketanserin enters the brain from the blood stream and diffuses to the receptor to which the drug then binds, thereby liberating radioligand, which then diffuses back into the bloodstream. To describe this process, we generated a model with two exponentials: one representing the ketanserin binding and liberation of the radioligand from the receptor with a half life of $T_{k½}$ and the other representing the diffusion of free radioligand out of the brain tissue into the blood with a half life of $T_{r½}$. By applying this model to experimental data of time-dependent 5-HT_{2A} receptor occupancy following ketanserin injection (Pinborg et al. 2003), an excellent fit was obtained when the following two conditions were met: $T_{k½}$ and $T_{r½}$ values both were in the range of 5–10 min and the sum of $T_{k½}$ and $T_{r½}$ amounted to roughly 15 min. This enabled us to estimate the minimum and maximum $T_{k½}$ values corresponding to two O_{KET} outcomes termed O_{KET5} and $O_{KET10.}$ In the absence of actual single subject occupancy measurements we tested the robustness of any observed relation between occupancy and fMRI data using both the estimated maximum and minimum values, O_{KET5} and O_{KET10}.

The study reported here was designed to investigate the effect of 5-HT_{2A} receptor blockade on emotion processing, and not to investigate the effects of increasing or decreasing overall serotonin levels in the brain. Results from the ATD and SSRI sessions that address global serotonin changes have been reported elsewhere (Grady et al. 2013). The fMRI sessions were performed on four different scanning days at least one week apart to ensure a proper wash-out period, with session order counterbalanced across subjects. Apart from the pharmacological manipulation, the experimental procedure was the same for all sessions. The study design with four different serotonergic challenges did not make full placebo control practical. We therefore controlled for non-specific pharmacological effects of ketanserin administration (e.g. IV line present during scan) and indirect effects of drug (e.g. via induced side effects) by directly contrasting the ketanserin behavior and functional data with the behavior and functional data acquired during the SSRI session, which had a similar administration protocol with IV administration during the entire MRI session (at a rate of 8 mg/h). The expected neurophysiological effect of citalopram is increased general serotonergic transmission compared with ketanserin that specifically reduces 5-HT_{2A} receptor transmission. Further, the subjects were unaware of the expected effects of the 5-HT manipulations, the differences in probabilities of side effects between the different drug interventions and the degree of 5-HT_{2A} blockade during ketanserin administration.

Measurements of cerebral 5-HT_{2A} receptor binding

^{18}F-altanserin PET was undertaken as described by Pinborg et al. (2003). In short, ^{18}F-altanserin was administered as a combination of a bolus injection followed by continuous infusion to obtain steady state of the tracer in blood and tissue resulting in a maximum dose of 3.7 MBq/kg bodyweight. PET studies were conducted between 1200 and 1800 hrs. Individual ^{18}F-altanserin PET had been acquired on average 3.2±1.7 years before the fMRI experiment. Test-retest studies have found that, in healthy individuals, cerebral 5-HT_{2A} receptor binding remains relatively stable over two years (Marner et al., 2009), showing that ^{18}F-altanserin

PET can be considered a stable trait marker for neocortical 5-HT_{2A} receptor binding.

PET data were acquired in 3D using an eighteen-ring GE-Advance scanner (GE, Milwaukee, Wisconsin, USA). ^{18}F-altanserin PET images and the structural T1-weighted MR images were co-registered and the PET images were then normalized to the same anatomical template as that used for MR images (Pinborg et al., 2003). Volumes of interest (VOIs) were automatically delineated on each individual transaxial MRI slice in a strictly user-independent fashion (Svarer et al., 2005). Given the extensive co-variation of neocortical 5-HT_{2A} receptor binding across neocortical areas a global neocortical region was defined for each participant as described in Erritzoe et al. (2010). PET images were partially volume-corrected using the segmented MRI. A two-tissue model based on gray matter, white matter, and cerebrospinal fluid was used (Muller-Gartner et al., 1992; Quarantelli et al., 2004). The binding potential (BP_p) of specific binding relative to plasma was calculated as:

$$BP_p = V_T - V_{ND} = \frac{C_T - C_{ND}}{C_P} \quad (1)$$

C_T and C_{ND} being the radioactive concentration in each region of interest and in the reference region, V_T and V_{ND} being the distribution volumes in each regions of interest and in the reference region, and C_P being the metabolite corrected plasma [^{18}F]altanserin. The cerebellum was used as the reference region, as it represents non-displaceable uptake only (Pinborg et al. 2003).

MRI

As for the PET scans, all MRI measurements were carried out between 1200–1800 hours. Images were acquired on a 3T Trio scanner with an eight-channel head array coil (Siemens, Erlangen, Germany). BOLD fMRI uses a T2*-weighted gradient echo spiral echo-planar imaging (EPI) sequence with a repetition time of 2.5 s, echo time of 26 ms, flip angle of 90°, and 41 slices with a slice thickness of 3 mm and 25% gap between slices.

The EPI sequence was optimized for signal recovery in the OFC by tilting slice orientation from a transverse toward a coronal orientation by about 30° and the use of a preparation gradient pulse (Deichmann et al., 2003). A total of 128 whole-brain volumes were acquired in each of the two sessions (total 12.8 min). We additionally acquired a high-resolution 3D structural brain scan using a T1-weighted spin echo sequence (TI/TE/TR=800/3.93/1540 ms, flip angle 9°, 1×1×1 mm isotropic resolution).

Analysis of the fMRI data

Data were preprocessed and analyzed using SPM5 (Wellcome Trust Centre for Neuroimaging, http://www.fil.ion.ucl.ac.uk/spm/software/spm5). Images were realigned and normalized to MNI (Montreal Neurological Institute) stereotactic space using transformation parameters derived from segmentation of the structural MRI. The normalized images were smoothed using a symmetric 8 mm Gaussian kernel. None of the subjects had head motions at any time that exceeded 3 mm (voxel size) in any direction. We tested for differences in head movement between the drug sessions by calculating the root mean square of the movement parameters in *x, y* and *z* direction and included the individual values in a repeated measures analysis of variance (ANOVA) with drug session and movement direction as within-subject factors.

The paradigm was analyzed in an event-related fashion with three event types defined at subject level, corresponding to presentation of neutral, angry, or fearful faces. Each event was modeled as a delta function with onset coinciding with the appearance of the cue. Covariates were then convolved with a canonical hemodynamic response function. A two-stage random effects model was created for each subject, modeling the fMRI runs of the ketanserin and control sessions. Each fMRI run was modeled with the three covariates described above together with a mean (constant) term over scans for each run in order to model the main effects of runs. The within-subject model also included 40 nuisance regressors to account for variance caused by physiological noise, including heart beat (10 regressors), respiration (6 regressors), and head movements (24 regressors) (Glover et al., 2000; Lund et al., 2006).

Parameter estimates for each covariate were calculated and statistical parametric maps of the *t*-statistics (SPM{*t*}) resulting from linear contrasts of covariates were generated for each subject. Thus, we generated contrast images for the relative increase in BOLD signal induced by the emotional faces relative to neutral faces in both the control and ketanserin sessions.

At the group level, individual contrast images were entered into separate paired *t*-test models testing the difference in BOLD response of emotional faces relative to neutral faces in the ketanserin relative to the control session. Additional analog group level models were set up by substituting the control contrasts with the equivalent contrasts of the SSRI session.

We also computed one-sample *t*-tests for the emotion contrast images from the ketanserin session only, including average neocortical BP_P, time-dependent O_{KET} (O_{KET5} and O_{KET10}), and in order to look at the linear relationship between the two covariates we calculated the product (i.e. $BP_P \times O_{KET}$). BP_P values were time-corrected to comply with the delay between PET and fMRI scannings according to Erritzoe et al. (2009) This model enabled us to identify brain regions where ketanserin-induced changes in emotional face processing that varied depending on the neocortical BP_P, O_{KET}, or the product of the two, and the analysis was performed for both O_{KET} covariates. In order to test for correlations with mood state, a separate analysis was performed using POMS factor scores (anger/hostility, vigor/activity, and fatigue/inertia) as covariates.

We used the psychophysiological interaction (PPI) method described by Friston et al. (1997) to identify ketanserin-induced changes in OFC connectivity during the processing of fearful faces that can be explained by O_{KET}, BP_p or an interaction between the two factors.

In the first stage, we defined a spherical region of interest (ROI), 8 mm in diameter and centered in the peak OFC region (MNI (*x, y, z*)=(4,38,−24)) showing an attenuation of the BOLD response in the ketanserin session vs control during perception of fearful faces and we extracted the time-course of the BOLD response from this region. A PPI term was calculated by multiplying the estimated deconvolved time-course from the OFC ROI with the fear vs neutral contrast. We then computed new subject-specific statistical models (SPMs) where the calculated PPI term and the time-course of the seed region were added as regressors to the initial first level subject model which included the three task regressors (neutral,

angry and fearful faces). Individual contrasts based on the PPI term were entered in a second level multiple regression model with BP_p, O_{KET} (O_{KET5} and O_{KET10}), and the product of the two (i.e. $BP_p \times O_{KET}$) as covariates. Linear contrasts were specified and SPM{*t*} based on one-tailed *t*-statistics were generated.

As general significance level, we used a threshold of $p<0.01$ on a voxel-wise level and considered clusters significant at $p<0.05$ after family-wise error (FWE) correction for multiple non-independent comparisons. All imaging results are reported by the *Z* score and stereotactic MNI coordinates of the regional maxima. We expected the amygdala's neuronal activity to change directly in response to aversive faces. To define the amygdala, we delineated spherical ROIs with a radius of 8 mm (the size of the smoothing kernel) centered in the maximum activation likelihood estimations from the Fusar-poli et al. (2009) meta-analysis for fearful faces. We first converted the estimated voxels for fear vs neutral contrasts from Talairach to MNI space according to (Lancaster et al., 2007) and then used the resulting coordinates ((−23, −4, −15) and (22, −4, −20)) to perform FWE correction for the voxels using small volume correction (SVC) for fear contrasts as well as angry and aversive.

Analysis of task performance

Behavioral data were analyzed using SPSS (version 18, Chicago, Illinois, USA). Individual scores on mood questionnaires were analyzed using a three-way repeated measures ANOVA with the within-subject factors session (ketanserin versus control), mood factors of the POMS (six levels), and time of assessment relative to fMRI (before versus after). Reaction time differences were assessed using a two-way repeated measures ANOVA with within-subject factors session (ketanserin versus control, or ketanserin versus SSRI) and emotion of the face stimuli (neutral, anger, fear). The Greenhouse-Geisser method was used to correct for non-sphericity if appropriate. Conditional on significant *F*-values in the ANOVA, post-hoc paired *t*-tests were performed. Error rates were analyzed using nonparametric Wilcoxon signed-rank tests, comparing each facial expression from the control session with the same facial expression from the ketanserin session. Behavioral data are given as mean±standard deviation (SD).

Results

Mood assessment

The effect of ketanserin on mood was evaluated by comparing POMS scores collected before ketanserin was given as well as right after completion of the fMRI session. Compared to the control session, acute ketanserin challenge had a specific effect ($F(1.7; 30.9)=44.1$; $p<0.001$). In the ketanserin session, participants reported significant decreases in vigor/activity (1.79±0.62 versus 1.36±0.80, $t(21)=4.6$; $p<0.001$), and increases in fatigue/inertia (0.5±0.51 versus 0.91±0.61, $t(21)=-3.7$; $p=0.001$) compared to the responses prior to ketanserin infusion. Conversely, in the control sessions the scores for anger/hostility were significantly lower at the end of the session relative to scores at the beginning of the session (0.26±0.23 versus 0.20±0.13), $t(18)=2.6$; $p=0.02$). None of these mood changes correlated significantly with the fMRI activation patterns.

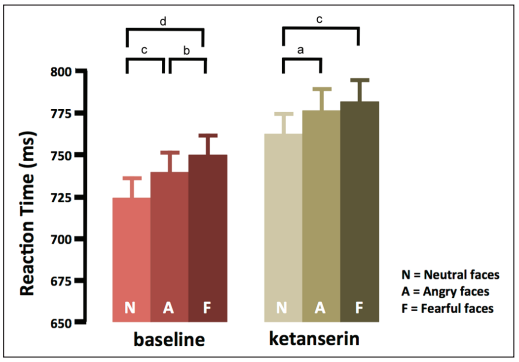

Figure 2. Mean reaction time (RT) recorded during the gender-judgment task based on facial expressions in the control and ketanserin functional magnetic resonance imaging (fMRI) sessions. Control session: neutral faces; 724 ±23.55, angry faces; 739±22.23, fearful faces; 750±23.20. Ketanserin session: neutral faces; 762±23.99, angry faces; 776±23.99, fearful faces; 781±25.70. Data are presented as mean ± standard error of the mean (SEM), $^{a}p<0.1$, $^{b}p<0.05$, $^{c}p<0.01$, $^{d}p<0.001$. Faces with an aversive emotion delayed the gender-judgment in both sessions relative to neutral faces. Compared to the control session without medication, ketanserin treatment was associated with longer RT.

Task performance

Mean reaction time (RT) was longer when subjects judged the gender of a fearful or angry face relative to a neutral face, showing that gender-judgment was delayed when faces showed an aversive emotion ($F(1.8; 38.7)=16.53$; $p<0.001$). The delay in RT induced by an aversive facial emotion was comparable in size with or without ketanserin treatment (Figure 1). Mean RT was longer for fearful than for neutral faces in both the ketanserin session ($t(23)=2.80$; $p=0.011$) and control session ($t(23)=6.35$; $p<0.001$). The same was true for RTs when comparing trials with angry faces versus neutral faces for both ketanserin session ($t(23)=2.47$; $p=0.022$) and control session ($t(23)=3.,53$; $p=0.006$). In the control session, mean RTs were also longer in trials with fearful compared to angry faces ($t(23)=-2.77$; $p=0.011$).

Ketanserin treatment prolonged mean RT, with an overall increase of approximately 5% compared to the control session (ketanserin session: 774 ms±118.6, control session: 741 ms±105.5, $F(1;23)=18.10$; $p=0.001$). Post-hoc paired *t*-tests showed this increase was consistent across all facial expressions ($p<0.001$). Ketanserin had the same effect on RT responses to neutral and aversive faces (Figure 2) and also the ANOVA revealed no interaction between facial emotion and intervention. The relative increase in RT in the ketanserin session correlated with the ketanserin-induced decrease in self-report on vigor (Pearson's $r=0.387$, $p=0.037$), whereas no correlation was found with the reported increase in fatigue (Pearson's $r=0.05$, $p=0.411$).

Error rates did not differ between control and ketanserin session for any of the three facial expressions (neutral faces: $p=0.186$, angry faces: $p=0.903$, fearful faces: $p=0.613$). This shows that the overall slowing of RT found during the ketanserin session was not paralleled by a change in accuracy.

fMRI results

Judging the gender of angry or fearful faces, relative to neutral faces, consistently activated the expected set of brain regions involved in face and emotional processing (Figure 3, Table 1). The amygdala showed a bilateral increase in neural activity when responses to angry and fearful faces were pooled together (Figure 3(a)) or considered separately (Figure 3(b) and 3(c)). Additional bilateral clusters in the fusiform gyrus and visual cortex displayed increases in activity when angry or fearful faces were presented relative to neutral faces (Figure 3(a)–(c)).

Effect of ketanserin on face processing

OFC. Ketanserin attenuated the regional neuronal response to aversive facial emotions relative to the control session in medial OFC (Figure 4; peak reduction at (*x, y, z*)=(4,40,−18), *Z*=4.12, p_{FWE}=0.005). This attenuation was mainly driven by a reduced responsiveness of the OFC to fearful faces (peak reduction at (*x, y, z*)=(4,38,−24), Z=4.03, p_{FWE}<0.001). Inspection of the regional response profile in the OFC revealed an interaction with ketanserin having an opposite effect on OFC activity depending on the emotional content. Ketanserin attenuated the OFC response to aversive faces, especially fearful faces (Figure 4(d)). The ketanserin-related effects on OFC activity were not correlated with inter-individual variations in neocortical BP_P. The region in OFC where ketanserin reduced the response to fearful faces also had a stronger influence on the coupling with left amygdala, when the interaction of the two covariates BP_P and O_{KET} (i.e. $BP_P \times O_{KET}$) was considered. The strength of OFC-to-amygdala connectivity correlated positively with $BP_P \times O_{KET}$, meaning that the higher number of 5-HT_{2A} receptors blocked by ketanserin, the stronger the coupling between OFC and the left amygdala for both O_{KET5} and O_{KET10} (peaked at MNI coordinates; O_{KET5}: (*x, y, z*)=−(22,4,−18), *Z*=3.92, p_{FWE}=0.007. O_{KET10}: (*x, y, z*)=(−20,4,−18), Z=4.38, p_{FWE}=0.002, corrected within the amygdala ROI, Figure 5(a) and (b)). This also held true when excluding the two extreme values from the analysis. Figure 5(b) shows the correlation analysis between the BOLD response in left amygdala and the $BP_P \times O_{KET10}$ interaction. There was no significant difference in the magnitude of head movements during the fMRI acquisitions between the control and ketanserin sessions (*F*(1;22)=0.388; *p*=0.540).

Amygdala. Ketanserin did not change the overall amygdala response to aversive faces, and this was also the case when considering inter-individual variations in either neocortical BP_P or any of the O_{KET} covariates (O_{KET5} and O_{KET10}). When looking at the linear relationship between the two covariates BP_P and O_{KET} (i.e. $BP_P \times O_{KET}$) we saw a trend towards an increase in activation in the left amygdala when processing fearful or aversive faces when using the O_{KET5} covariates (peak modulation for fearful faces at −24,−6,−8, *Z*=2.76, p_{FWE} = 0.06, for aversive faces at −26,−6,−10, Z=2.71, *p*=0.06).

Comparison between the ketanserin and SSRI sessions. In order to control for non-specific effects that could have been induced by administering ketanserin we did a post hoc validation of the observed changes by contrasting the ketanserin session with the SSRI session acquired in the same subjects following a similar IV administration protocol.

Mean RTs from the SSRI session did not differ significantly to the control session (control session: 741 ms±105.46, SSRI session: 745±110.9, *F*(1.0; 21)=16.622; *p*=0.760), thus the longer mean RTs found in the ketanserin session compared to control session were also found in the SSRI session (ketanserin session: 774 ms±118.6, SSRI session: 745±110.9, *F*(1.0; 21)=4.078; *p*=0.056).

Compared to the ketanserin session, SSRI data confirmed our initial results showing decreased BOLD response in the OFC during aversive (peak reduction at (*x, y, z*)=(2,34,−26), *Z*=4.30, p_{FWE}<0.001) and fearful face presentation (peak reduction at (*x, y, z*)=(2,34,−26), *Z*=3.70, p_{FWE}<0.001).

Discussion

Acute 5-HT_{2A} receptor blockade with ketanserin-modulated emotional face processing in the medial OFC and in amygdala, has lead to two main findings. First, ketanserin suppressed the neural response to fearful faces in the medial OFC. Second, the more 5-HT_{2A} receptors that were blocked, the stronger the functional coupling between the medial OFC and left amygdala in response to fearful faces.

Effect of 5-HT_{2A} blockade on face processing in orbitofrontal cortex

Acute pharmacological 5-HT_{2A} receptor blocking reduced the regional response of the OFC to fearful and to a lesser degree angry faces (Figure 4) supporting the view that 5-HT_{2A} receptor signaling is involved in cortical processing of fearful facial expressions. This result corroborates the notion that OFC provides an interface between cognitive and emotional functions (Paulmann et al., 2010). The ability to change behavior based on facial expressions relies partly on the OFC (Kringelbach and Rolls, 2003). Patients with uni- or bilateral OFC lesions show an inability to respond appropriately to other people's emotions and an impaired recognition of emotional features in face and voice (Hornak et al., 1996, 2003). This is probably not related to a failure to recognize facial expressions per se but is rather caused by a deficit in using this social information to guide appropriate actions or decisions (Willis et al., 2010).

The OFC did not express an emotion-specific response pattern in the control session when participants judged the gender of neutral, angry or fearful faces. The lack of a specific response to aversive as opposed to neutral faces suggests that the OFC automatically processes a wealth of facial features relevant to social interaction including face identity, gender, and emotional state. Blocking the 5-HT_{2A} receptors attenuated the OFC response to fearful faces and enhanced the response to neutral faces (Figure 4). This differential effect of the 5-HT_{2A} receptor blockade indicates a shift in preferential processing towards non-threat related facial features in the OFC.

The distribution of the 5-HT_{2A} receptors in the cerebral cortex would allow for such a shift in the relative weight of complementary processing routes within the OFC. Immunocytochemical studies in the cortex of macaques have shown that excitatory 5-HT_{2A} receptors are not only expressed in the apical dendritic field proximal to the pyramidal cell soma, but also in gamma-aminobutyric (GABA)ergic interneurons known to specialize in the perisomatic inhibition of pyramidal cells (Jakab and Goldman-Rakic, 1998, 2000). A possible scenario is that acute 5-HT_{2A} blockade reduced excitatory signaling in OFC circuits which compute fear-related

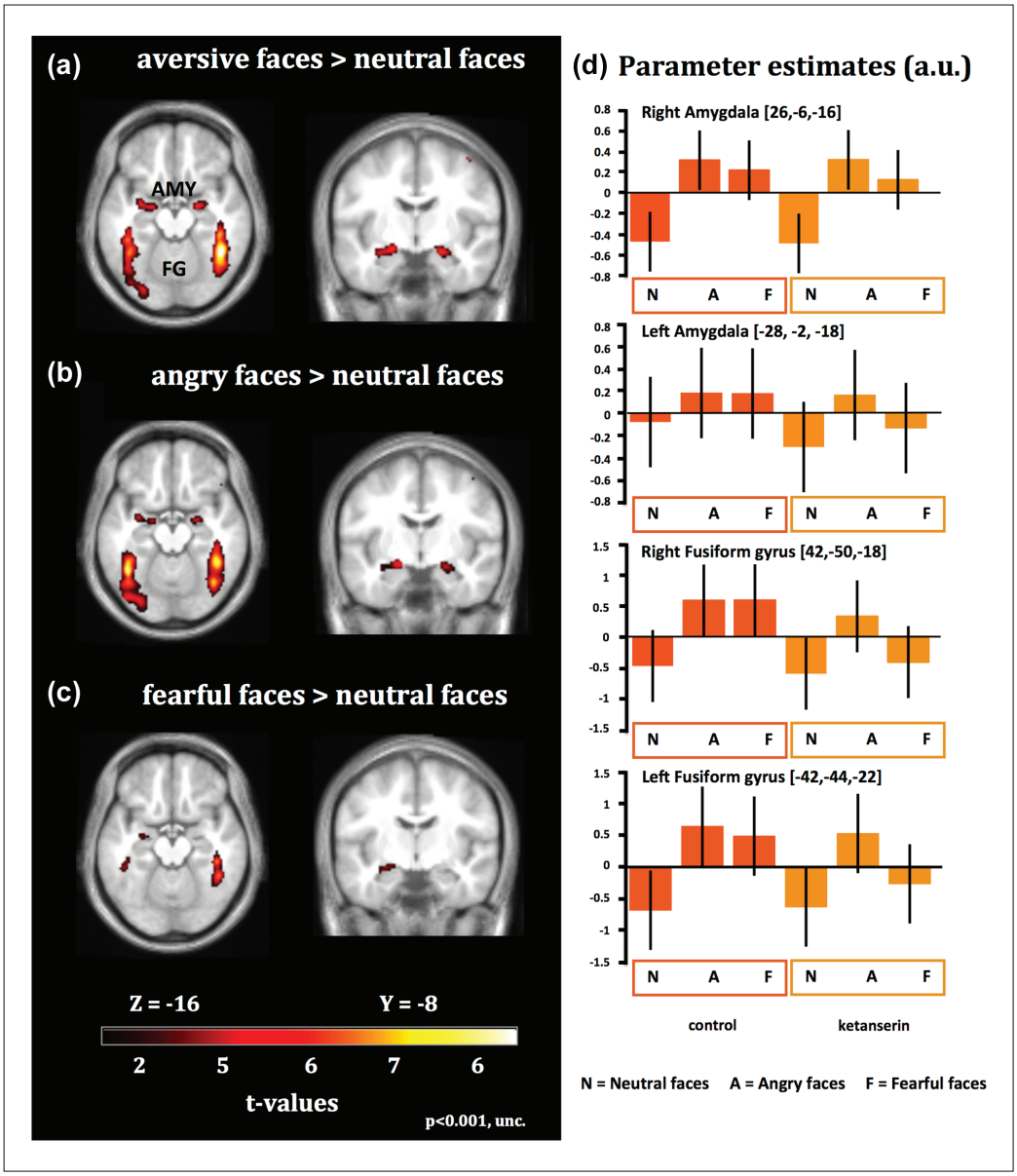

Figure 3. Statistical parametric maps (SPMs) showing brain areas, which are activated by aversive facial expressions relative to neutral faces, as reflected by an increase in blood oxygen level dependent (BOLD) signal. The SPMs are color coded in yellow and red indicating increases in activity, and are thresholded at $p<0.001$ (uncorrected). (a) The upper left panel gives the activation maps for the contrast aversive faces > neutral faces. The middle and lower panel on the left display the activation maps for the two facial emotions separately: (b) angry faces > neutral faces; (c) fearful faces > neutral faces. (d) The bar graphs presented in the right panel give statistical estimates (arbitrary units) of face related activity levels in the amygdala and fusiform gyrus for the control session (left column) and ketanserin session (right column). The parameter estimates are taken from the regional maxima showing the strongest increase in regional activity for aversive faces relative to neutral faces. The error bars represent the 90% confidence intervals of the mean.

Table 1. Coordinates and Z-scores of the voxel showing a peak increase in blood oxygen level dependent (BOLD) signal when viewing aversive (angry/fearful) faces relative to neutral faces.

		Aversive > Neutral				Fear > Neutral				Angry > Neutral			
		Area		(*x*, *y*, *z*)	*Z*-score	Area		(*x*, *y*, *z*)	*Z*-score	Area		(*x*, *y*, *z*)	*Z*-score
A	**Main effect of task**												
		Amygdala	R	22, −8, −16	3.71[a]	Amygdala	R	30, −4, −20	2.86[a]	Amygdala	R	24, −6, −16	3.17[a]
			L	−20, −10, −12	3.96[b]		L	−24, −10, −14	2.92[a]		L	−18, −10, −12	3.71[a]
		Fusiform gyrus[b]	R	40, −52, −14	5.54	Fusiform gyrus	R	42, −34, −18	4.39 n.s.	Fusiform gyrus[b]	R	42, −46, −18	5.10
			L	−42, −42, −22	4.83		L	−46, −44, −24	3.96 n.s.		L	−42, −54, −14	5.20
		Visual cortex[b]	R	28, −90, 4	5.44	Visual cortex	R	26, −88, −2	3.49 n.s.	Visual cortex[b]	R	28, −90, 4	5.76
			L	−26, −90, −6	6.85		L	−26, −88, −8	4.69		L	−28, −92, −2	6.54
										IFG	R	54, 30, 6	3.11 n.s.
											L	−46, −34, 0	3.18 n.s.
B	**Effect of ketanserin**												
				OFC	R	4, 40, −18	4.12[a]	OFC	R	4, 38, −24	4.03[a]		
				IFG	L	−50, 34, 22	3.60 n.s.	IFG	L	−50, 34, 22	3.35 n.s.		
C	$BP_P \times O_{KET5}$						Amygdala	L	−24, −6, −8	2.80[c]			
D	PPI_{OFC}						Amygdala	L	−20, 2, −20	4.22			

BP_P: binding potential; IFG: inferior frontal gyrus; O_{KET}: receptor occupancy; OFC: orbitofrontal cortex; PPI: psychophysiological interaction.
[a]Family-wise error (FWE) corrected within predefined region of interest (ROI); [b]part of the same cluster; [c]only significant for O_{KET5}, n.s.: only trend significance ($p<0.001$).

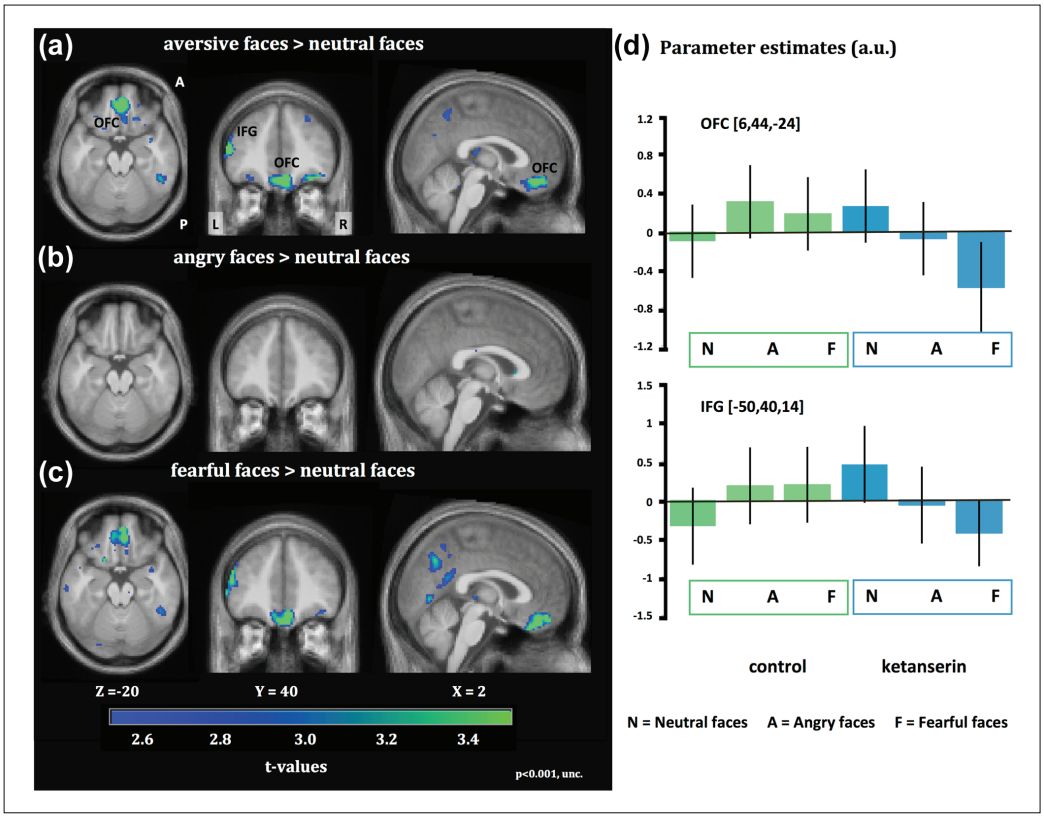

Figure 4. Statistical parametric maps (SPM{*t*}) showing brain regions, which show a decrease in activation for aversive face expressions relative to neutral faces in the ketanserin session as opposed to baseline (control session). The SPM{*t*} are color coded in green and blue indicating decreases in BOLD signal and are thresholded at $p<0.001$ (uncorrected). (a) The upper left panel depicts decreases in regional responsiveness to aversive (angry, fearful) faces under ketanserin treatment. The middle and lower panel on the left present the corresponding SPM{*t*} for (b) angry and(c) fearful faces. (d) The bar graphs plot the statistical estimates (arbitrary units) of face-related activity levels in the orbitofrontal cortex (OFC) and inferior frontal gyrus (IFG) for the control session (baseline) and ketanserin session. The parameter estimates are taken from the regional maxima showing the strongest decrease in the regional response to aversive faces relative to neutral faces. The error bars equal the 90% confidence intervals of the mean. OFC: orbitofrontal cortex.

information while increasing neural processing in circuits involved with other social stimuli features.

The individual change in emotion-related activity in OFC during acute 5-HT_{2A} receptor blocking was not correlated with individual *BPp*, O_{KET} or the product of the two. These findings suggest a non-linear relationship between 5-HT_{2A} receptor-related signaling and the neural responsiveness of the OFC to fearful facial expressions, with a rapid attenuation of the response to threatening facial features as a result of even a relatively small reduction of 5-HT_{2A} receptor signaling.

Effect of 5-HT_{2A} blockade on OFC-amygdala connectivity

The same OFC region in which ketanserin-modified facial expression related neuronal activity also showed a stronger correlation with neural activity in the left amygdala during the processing of fearful faces. The ketanserin-induced increase in functional connectivity between the medial OFC and left amygdala was related to how many 5-HT_{2A} receptors had been blocked. The more 5-HT_{2A} receptors were blocked, the stronger was the increase in functional connectivity between the OFC and left amygdala. Since Stein et al (2007) showed that the OFC exerts inhibitory control over the amygdala, we now propose that the OFC-to-amygdala projections are under the control of orbitofrontal 5-HT_{2A} related neurotransmission and blocking of 5-HT_{2A} receptors enhances the impact of the OFC on amygdala responsiveness to fearful faces. Experimental evidence from animal and human studies supports our hypothesis. Forster et al. (2006) showed that, in rats, fear correlated negatively with 5-HT levels in the OFC. Moreover, Fisher et al (2009) showed an inverse relationship between a greater level of 5-HT_{2A} receptors in the OFC, and reduced amygdala activity, as well as a functional

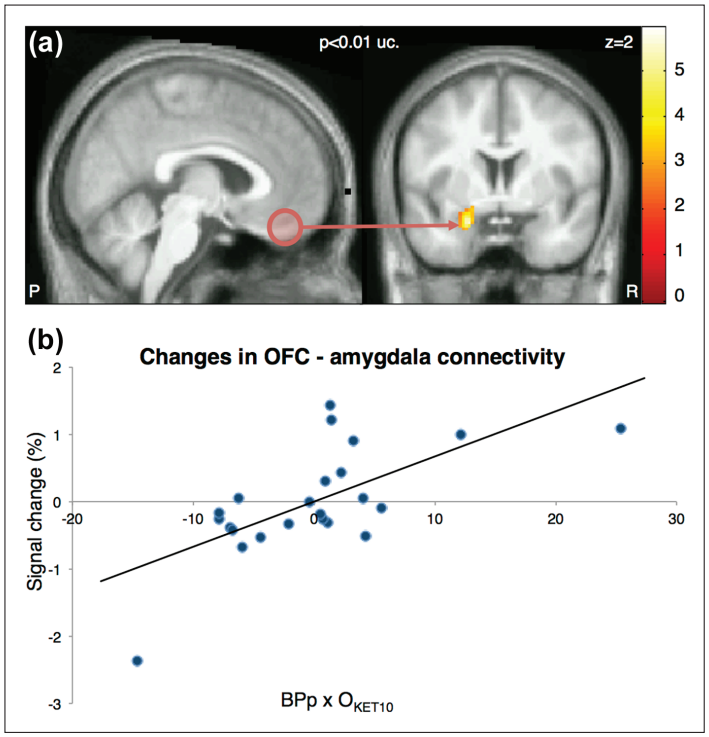

Figure 5. The figure summarizes the results of the psychophysiological interaction (PPI) connectivity analysis exploring ketanserin related changes in orbitofrontal cortex (OFC)-amygdala connectivity during fear events (for details see methods section). (a) Maps showing the changes in coupling between the OFC seed region and the amygdala following acute serotonin 2A (5-HT_{2A}) blockade. (b) Positive correlation between the OFC-amygdala connectivity and the interaction between neocortical 5-HT_{2A} receptor binding potential (BP_P) and ketanserin-induced 5-HT_{2A} receptor occupancy (O_{KET10}). The higher the O_{KET} and the higher the neocortical BP_P, the stronger was the individual increase in connectivity between the OFC and left amygdala. Values are mean normalized. The extent threshold of the SPMs is set at p<0.01 (uncorrected).

coupling between the habituation of amygdala responses with prefrontal regulatory regions. This was supported by Passamonti et al. (2012) who found an altered connectivity between the amygdala and PFC during acute tryptophan depletion. We infer that the efficiency of 5-HT_{2A} receptor blocking (as indexed by the proportion of blocked receptors) had the strongest impact in individuals with a high density of neocortical binding sites, as the ketanserin-induced effect on OFC-to-amygdala coupling only became evident when the product between the magnitude of receptors (BP_P), and the relative proportion of 5-HT_{2A} receptors blocked by ketanserin (O_{KET}) was considered. These results suggest an important general implication showing that individual variations in regional receptor binding might determine individual susceptibility to drug-induced manipulation of receptor function. If this observation can be replicated in future studies, it will have a large impact on the current view of assessment of receptor drug occupancy as the single most important measure for prediction of drug efficacy.

Methodological considerations

Ketanserin caused a general slowing in RT in the gender-judgment task. This effect on RT accords with the known effects of ketanserin. When given orally, 20 mg of ketanserin may reduce sustained attention (Wingen et al., 2007) or alertness (Koudas et al., 2009), although the clinical effect of ketanserin on arousal is not profound (Herrmann and Baumgartner, 1986). Further, at this dose, ketanserin does not significantly effect cerebral blood flow (Olsen et al., 1992). Participants reported a decrease in vigor and increased fatigue, confirming the known effects of the drug. Importantly, the relative RT cost associated with the gender-judgment of angry or fearful faces relative to neutral faces was not altered by ketanserin. Moreover, individual changes in RT or mood state did not correlate with drug-induced changes in task-related activation or connectivity as revealed by fMRI. Therefore, we argue that effects of ketanserin on task performance and mood state did not account for the observed changes in activation patterns.

Receptor occupancy was not continuously monitored in each individual. Rather, the receptor occupancy was estimated on the basis of time elapsed from the beginning of ketanserin infusion. However, even if the absolute occupancy levels were not precise, the occupancy term nonetheless approximates the normal distribution required for statistical parametric mapping. Further, the robustness of the estimates was supported by the use of two

different estimates for the time-dependent occupancy. Another potential limitation of the study was that the pharmacological challenge was not double-blinded. A placebo control would be advantageous in several respects, and prevent the need to consider placebo effects or effects of IV versus no manipulations. However, when the study was designed, and approved by the ethics committee, it was felt that a full placebo control of the oral ATD solution and the IV infusion for ketanserin and SSRI sessions, would be too excessive for a within-subject design. Given the heterogeneity of 5-HT_{2A}, and other genetic or personality factors relevant to inhibition, a between subjects design might have been compromised differently, by uncertainty over the cause of differences between groups and imperfect matching. The no-drug condition without blinded placebo IV/oral solutions was seen as an acceptable choice. Although subjects were made aware of potential side effects within the study, they were not made aware of the specific differences between the interventions. However, the drug effects on neural activity were specific to fearful relative to neutral faces and the changes in amygdala activity depended on the magnitude of 5-HT_{2A} receptor blockade and the individual 5-HT_{2A} receptor density. Given that the volunteers had no prior information about expected effects of the drug given, nor the degree of their individual blocking, these specific effects cannot be accounted for by a simple placebo effect or a lack of blinding. Furthermore, we confirmed the effects of ketanserin by contrasting against both the control session and the SSRI session, the latter sharing the intravenous infusion.

One must also consider potential effects of ketanserin arising from receptors other than 5-HT_{2A}. Ketanserin has some affinity for the 5-HT_{2C}, α1 adrenergic and histamine receptors (Korstanje et al., 1986). However, the affinity of ketanserin is approximately 14-fold higher for the 5-HT_{2A} relative to the 5-HT_{2C} receptor (Glennon et al., 2002). Our hypothesis was entirely based on ketanserin's modulation of the serotonergic system by blocking the 5-HT_{2A} receptors. We therefore specifically studied the interaction between the estimated 5-HT_{2A} receptor blockade and 5-HT_{2A} receptor density as measured by PET. We consider it therefore unlikely that α1 adrenergic, histamine, or 5-HT_{2C} receptor pharmacological effects could have a significant impact on our observations.

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Conflict of interest

None of the authors has any biomedical financial interests or potential conflicts of interest in relation to the study.

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PAPER 2

- With addendum[2]

[2] The following manuscript is exceeded by an extract of the findings from the SPM5 analyses, emphasizing analyses, results and illustrations.

Neuroticism predicts the impact of 5-HT challenges on fear processing in subgenual anterior cingulate cortex

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Abstract

Background: The personality trait neuroticism is associated with an increased vulnerability to anxiety and mood disorders, conditions linked with abnormal serotonin (5-HT) neurotransmission and emotional processing. The interaction between the personality trait neuroticism and 5-HT tone during emotional processing is however not understood. Here we investigate how individual neuroticism scores impact the neural response to negative emotional faces depending on the 5-HT tone.

Methods: Twenty young healthy participants performed an emotional face task under functional MRI at three occasions: increased 5-HT tone following a single dose of the 5-HT selective reuptake inhibitor (SSRI) citalopram, decreased 5-HT tone following an acute tryptophan depletion (ATD) protocol, and an investigation with no 5-HT challenge (control). During the task the participants judged the gender of neutral, fearful and angry facial expressions.

Results: Individual variations in neuroticism scores were associated with the neural response of subgenual anterior cingulate cortex to fearful but not angry facial expressions. The association was however opposite for the two 5-HT challenges. The fear-related response in this region and individual neuroticism scores correlated negatively during the SSRI session and positively during the ATD session.

Conclusions: The personality trait neuroticism may predict the impact of 5-HT challenges on fear processing in subgenual anterior cingulate cortex. This finding may represent a neural mechanism for the variable therapeutic effect of SSRI treatment observed in clinical populations.

Introduction

Facial expressions such as happiness, fear, sadness, anger, disgust, and surprise represent basic human emotions that are readily decoded by members of all human cultures (Ekman 1999). The ability to appropriately interpret emotional facial expressions is important for our social interactions, and impaired emotion-related processing is associated with an increased risk for affective psychiatric illnesses (Phillips et al. 2003; Mayberg et al. 2005). Human and animal studies have provided cumulating evidence that serotonergic (5-hydroxytryptamine, 5-HT) neurotransmission plays a key role in processing and regulation of emotions (Cools et al. 2007, 2008). In rats, the administration of corticotropin-releasing factor to the serotonin cell body regions of the dorsal raphe nucleus gave rise to a delayed and prolonged increase in 5-HT release in the medial prefrontal cortex (mPFC) which was associated with the termination of fear-related freezing behavior (Forster et al. 2006). In healthy individuals, functional brain imaging has shown that serotonergic challenges alter the neural processing of facial expressions (Passamonti et al. 2012; Grady et al. 2012; Hornboll et al. 2013). Recent neuroimaging studies have revealed that serotonin challenge may differentially influence the neural response to different emotions such as anger and fear (Fusar-Poli et al. 2009; Vytal and Hamann 2010; Grady et al. 2012). While both, fearful as well as angry faces, do imply threat, the type of threat is different for the two types of emotion. Whereas anger represents a more direct threat to the viewer and elicits avoidance behaviors, fear represents a more ambiguous threat and may at times elicit approach behaviors (Marsh et al. 2005).

In healthy individuals, acute (one dose) and chronic treatment with the selective serotonin reuptake inhibitor (SSRI) citalopram, were shown to attenuate amygdala activation in response to negative facial expressions, relative to neutral faces (McKie et al. 2005; Del-Ben et al. 2005a; Harmer et al. 2006; Grady et al. 2012) and to reduce attentional shifts away from aversive faces (Kerestes et al. 2008)., Acute SSRI was further shown to increase the neural response to happy but not to fearful faces in the amygdala (Norbury et al. 2009) as well as facilitate the recognition of fearful faces (Harmer et al. 2003a).

The ingestion of an amino acid mixture depleted of L-tryptophan, but containing large neutral amino acids yields a reversibly decrease of central nervous 5-HT synthesis (Acute Tryptophan Depletion, ATD). ATD has shown to enhance the neural response to angry faces in a widespread neural network including frontal regions (Grady et al. 2013), to elicit a decreased recognition of fearful facial expressions in healthy women (Harmer et al. 2003b).

Neuroticism is characterized by a tendency to worry and be anxious and to experience negative affect (Watson et al. 1988). This personality trait is associated with increased vulnerability to anxiety and mood disorders such as social affective disorders (SAD) and depression (Bienvenu et al. 2001). Positron emission imaging (PET) revealed an association between individual neuroticism scores and 5-HT_{2A} receptor binding in frontolimbic areas pointing towards a link between 5-HT signaling and neuroticism (Frokjaer et al. 2008, 2009). This association may reflect an increased sensitivity to environmental stress in individuals with high frontolimbic 5-HT_{2A} receptor binding, although the personality facets “angry hostility” and “impulsiveness” did not contribute to the correlation between high frontolimbic 5-HT_{2A} receptor binding and neuroticism. Functional brain imaging studies have further shown that the degree of neuroticism is associated with amygdala-prefrontal connectivity in response to viewing negative facial expressions scales with (Cremers et al. 2010). Furthermore neuroticism has been found to correlate positively with amygdala and sgACC

activation during trials of high emotional conflict, compared with low emotional conflict trials (Haas et al. 2007) and a higher sgACC activation has been found in s-allele carriers of the 5-HTTLPR in response to fearful faces (O'Nions et al. 2011). SSRI treatment with fluoxetine decreased the activation level significantly in the sgACC and amygdala which has been found to be significantly higher in major depressed youths compared to normal controls for fearful compared to neutral facial expressions (Tao et al. 2012). Contradictory Hall et al. (2014) found that brain activity in adolescents with major depression, show an negative correlation between activity in the sgACC and the severity of depression. Subgenual prefrontal gray matter volume was reduced in depressed subjects compared to normal controls as measured with PET (Drevets et al. 1997).

In the present study we explore whether and how the individual personality trait neuroticism predicts the impact of fluctuations in central 5-HT levels on emotional processing. To address this question, we assessed how the neuronal response to negative emotional stimulation is modulated by the interplay between 5-HT level and individual neuroticism scores. We studied a group of healthy subjects with blood oxygen level dependent (BOLD) functional MRI (fMRI). The fMRI measurements of the brain were performed in a state of decreased 5-HT transmission induced by ATD, a state of increased 5-HT transmission caused by the SSRI citalopram and a normal state without 5-HT challenge while they discriminated the gender of male or female faces with fearful, angry or neutral expressions. We chose these expressions because, although research suggests that they both imply threat, the behavioral response is different for the two facial expressions. Whereas anger may elicit avoidance behavior, fearful faces tend to elicit approach behavior (Marsh et al. 2005). This along with numerous other studies suggesting a different neural response to angry and fearful faces (see review by Fusar-Poli et al. 2009) it can be assumed that serotonin challenges may differentially impact the brains response to these two emotions.

Methods

Participants

We recruited twenty-six right-handed healthy adults (17 males) for participation in the study. Twenty participants (13 males) with a mean age of 31.5 ± 6.2 years were included in the final analysis. Three participants were excluded as they did not complete all three sessions of the study and three participants were excluded due to excessive movement during MRI scan acquisition. None of the participants reported a history of stimulant abuse or other psychiatric or neurological disorders, nor had they ever been prescribed antipsychotic or antidepressant medication. All participants had a normal neurological examination prior to inclusion. Written informed consent was obtained prior to study inclusion according to the declaration of Helsinki II. The study was approved by the Ethics Committee of Copenhagen and Frederiksberg, Denmark (KF 01-2006-20).

Behavioral task

Upon arrival and immediately after scanning, participants completed a modified Danish version of the Profile of Mood States (POMS) questionnaire (McNair et al. 1971) to assess

current mood. For the ATD and SSRI sessions, participants also completed the POMS right before the MRI scan.

During fMRI, participants performed a gender judgment task on face stimuli taken from the Karolinska Directed Emotional Faces database (Lundqvist et al. 1998). Participants were instructed to button press with their right index or middle finger according to the gender of the face as quickly as possible. The face stimuli were unmasked color photographs shown from a frontal perspective with neutral, fearful or angry expressions.

The images were presented in the middle of the screen for 1800ms, with a 200ms inter-trial-interval (ITI).

We employed a mixed fMRI design with alternating emotional blocks (NEUTRAL-ANGRY-NEUTRAL-FEARFUL-NEUTRAL…) showing male and female faces in equal proportion. Each block comprised of six events which were pseudo-randomly intermixed: three to five face stimuli (average of four), and one to three (average of two) null events (fixation cross). In total, 32 blocks of neutral, and 16 blocks of each fear and angry faces, were presented over two fMRI runs separated by a short break. Each neutral face stimulus was presented twice and aversive face stimuli were presented once. Stimulus presentation and response recordings were performed using E-prime 1.2 (Psychological Software Tools, Pittsburgh, PA, USA).

Serotonergic challenges

Participants took part in four experimental days with the challenges: SSRI, ATD, ketanserin, and a control. The results from the ketanserin challenge have been reported elsewhere (Hornboll et al. 2013). Each challenge was performed on four different days at least one week apart. The order of the challenges were randomized and counterbalanced across participants. All MRI measurements were carried out between noon and 6 pm. Participants were informed about potential side effects of the challenges, but not about any expected effects of the challenges.

The SSRI challenge was initiated prior to fMRI acquisition with a two hour intravenous infusion of citalopram at a dose of 20 mg/h to ensure a stable and sufficient transporter blocking throughout the MRI scan (others have used shorter infusion times, see e.g. (Del-Ben et al. 2005b; Bigos et al. 2008). The initial infusion was followed by a maintenance dose during fMRI acquisition of 8 mg/h (~50 mg in total). To assess serum prolactin as a proxy for cerebral 5-HT level changes, blood samples were collected three times: before citalopram administration, right before scanning start and right after the MRI scan.

For the ATD challenge, upon arrival on the scanning day, subjects ingested within a maximum period of 10 minutes 75 g tryptophan-free powdered mixture of essential and non-essential amino acids dissolved in water (XLYS, TRY Glutaridon, SHS International Ltd) after having kept a low protein diet the day before. fMRI acquisition was performed five hours after ingestion. To assess plasma amino acid levels, blood samples were collected before ingestion and right before scanning (5h after ingestion of the amino acid drink).

Because of considerable differences in the mode of drug administration across the challenges, placebo was not used during the control scan. However, apart from the pharmacological manipulation, the experimental procedure was the same.

Neuroticism

The Revised NEO Personality Inventory (NEO-PI-R) were obtained on average 3.2 ± 1.7 years prior to the fMRI scans, as an individual neuroticism scores, which were the main variable of interest. The NEO-PI-R is based on the five factor model of personality and provides metrics for broad personality dimensions of extraversion, agreeableness, conscientiousness, neuroticism, and openness to experience. It is a psychological self-reported personality inventory which consists of 240 items. Participants indicated on a scale from 1 to 5 how well each statement fits his or her personality. The inventory was developed to test adults without overt psychopathology (Costa and Mccrae 1992). Participants completed the Danish version of the 240-item NEO-PI-R self-report personality questionnaire. The Danish translation of the NEO-PI-R has been psychometrically evaluated and normed in a standardization sample of 600 subjects (Hansen et al. 2004; 2004).

Each factor score is derived by adding the scores from assessment of six personality traits (facets) of each of the five personality factors and each trait score is derived by adding the scores on eight items in 0 – 4 Likert format.

Magnetic resonance imaging

MR images were acquired on a 3T Trio scanner with an eight-channel head array coil (Siemens, Erlangen, Germany). Blood oxygen level dependent (BOLD) fMRI using a T2*-weighted gradient echo spiral echo-planar (EPI) sequence with a repetition time of 2.5 s, echo time of 26 ms, flip angle of 76°, and 41 slices with a slice thickness of 3 mm and 25% gap between slices. The EPI sequence was optimized for signal recovery in orbitofrontal cortex by tilting slice orientation from a transverse toward a coronal orientation by about 30° and the use of a preparation gradient pulse (Deichmann et al. 2003). A total of 156 whole-brain volumes were acquired in each of the two sessions (total 13 min). Physiological measurements of pulse (monitored with an infrared finger clip) and respiration (monitored with a chest belt) were obtained during fMRI acquisition. B0 field maps were acquired either before or after fMRI acquisition (TR = 488 ms; TE1 = 5.19 ms, TE2 = 7.65 ms; flip angle = 60°; distance factor = 25%; FOV = 240 mm; 41 slices; slice thickness = 3 mm). We additionally acquired a high-resolution 3D structural brain scan using a T1-weighted spin echo sequence (TI/TE/TR =800/3.93/1540 ms, flip angle 9°, 1 x 1 x1 mm isotropic resolution). We further performed a six-minute measurement of regional blood perfusion of the brain. Perfusion measurements used arterial spin labeling (ASL) lasting six minutes. This enabled us to examine whether differences in the regional BOLD signal between ATD, SSRI and control sessions were caused by a mere difference in baseline blood perfusion levels. ASL-based perfusion measurements used FAIR Q2TIPS (Luh et al. 1999) sequences with 3D GRASE (Günther et al. 2005) single-shot readout with background suppression (TR = 3.4s, TE = 19.3 ms, TI = 200, 400, 600, 800, 1000, 1200, 1400, 1600, 1800, 2000, 2200, 2400, 2600, 2800, 3000 ms, 2 averages per TI, Q2TIPS saturation duration = 150 ms, 26 slices, voxel size = 5.0 x 5.0 x 4.0 mm, FOV = 320 x 160 x 104 mm, vessel suppression with bipolar gradients, b = 6 s/mm2). ASL images were calculated using FABBER with spatial priors (www.fmrib.ox.ac.uk/fsl/fabber) and permutations testing for differences between drug conditions.

Analysis of the fMRI data

The design of the study was as described above a block design. But, each block was mixed with null events allowing the design to also be viewed as an event related design. Consequently, the data were first analyzed using an older version of spm5 (http://www.fil.ion.ucl.ac.uk/spm/software/spm5) and subsequently using spm12 (http://www.fil.ion.ucl.ac.uk/spm/software/spm12). Data were analyzed as an event related design for both of the two SPM versions. These two modes of analysis revealed essentially the same findings. Therefore, we are in the following only reporting the results obtained with the newest model of analysis; spm12.

On visual inspection of the fMRI data, artifacts (possibly due to radio frequency interferences) were notably bad in some slices for seven of the fMRI scan sessions (over six subjects). We therefore applied to the seven sets of functional images the slice repair utility within the ArtRepair toolbox for SPM12 (http://spnl.stanford.edu/tools/ArtRepair), which automatically detects bad slices and repairs by interpolation from slices of previous and subsequent volumes. In order to correct for B0 field inhomogeneities, the acquired B0 fieldmap was used to create a voxel displacement map (VDM) with the Fieldmap toolbox integrated within SPM12. The resulting VDM was used to unwarp the functional images during the realignment procedure for each session. Realignment was to the first functional volume and a mean functional image was created. The mean functional image was co-registered to the T1-weighted anatomical image and co-registration parameters were applied to all other functional images. The T1-weighted anatomical image was segmented using standard tissue priors and normalized to MNI (Montreal Neurological Institute) stereotactic space. The resulting deformation field image of the non-linear warping parameters for normalization from native space to MNI template space was then applied to the functional images. Lastly, normalized functional images were smoothed with a 6 mm Gaussian kernel.

The functional data from all three scan days were analyzed in a single event-related general linear model as separate sessions. Onsets of each task event, Neutral, Angry, and Fear, were modeled as separate regressors. Each individual's realignment parameters and their derivatives were modeled to account for head movement and subsequent spin effects (Andersson et al 2001). Physiological noise was modeled with RETROICOR (Pinborg et al. 2003; Lund et al. 2006) to obtain six respiration and four pulse regressors. The first-level contrasts for each challenge day's of Neutral, Angry, and Fear events were entered into a second-level flexible factorial design to investigate the within-group effects of challenge and emotional face processing. There were three factors modeled: "Subject", "type of pharmacological Challenge" (3 levels: ATD, SSRI, and control), and "Emotion" (3 levels: fearful, angry and neutral) . In order to verify that subjects responded to emotional stimuli, we ran t-contrasts for Fear > Neutral, Anger > Neutral, and Aversive (Fear and Anger) > Neutral. We tested F-contrasts for the main effect of Challenge and the interaction effects of Emotion x Challenge. Significant findings in these were followed up, post-hoc, by their respective t-contrasts.

In the analysis of association with neuroticism scores, we ran one-sample t-tests of contrasts between challenges for aversive faces, i.e. Aversive faces (Fear and Anger) for ATD>control, SSRI>control, and SSRI>ATD. Neuroticism scores for each individual were entered as a

covariate of interest. These analyses were conducted on 19 participants since neuroticism data was lacking for one participant.

In all tests, the significance threshold was set to $p < 0.05$, corrected for multiple comparisons with family wise error (FWE) at the peak-level, and an extend threshold of $p<0.01$. All imaging results are reported by the Z-score and stereotactic MNI coordinates of the regional maxima.

Analysis of task performance

Behavioral data were analyzed using SPSS (version 20, Chicago, Illinois, USA). Individual scores on mood questionnaires were analyzed using a three-way repeated measures ANOVA with the within-subject factors *session* (ATD, SSRI, control), *mood factors* of the POMS (6 levels), and *time* of assessment relative to fMRI (arrival, before and after scan). Reaction time changes were assessed using a two-way repeated measures ANOVA with within-subject factors *session* (ATD, SSRI, control) and *emotion* of the face stimuli (neutral, angry, fear). The Greenhouse-Geisser method was used to correct for non-sphericity if appropriate. Conditional on significant F-values in the ANOVA, post-hoc paired t-tests were performed. Error rates were analyzed using nonparametric Wilcoxon signed-rank tests, comparing each facial expression from the control session with the same facial expression from the relative drug session. Behavioral data are given as mean ± standard deviation.

Results

Behavioral data

The 3x3 ANOVA of the RT showed a main effect of emotion ($F(1.9,40.8)=25.5$, $p<0.001$), but no main effect of drug ($F(1.8,37.7)=0.91$; $p=0.894$) and no interaction between drug and emotion ($F(2.7,56.2)=0.568$; $p=0.619$). Mean reaction time (RT) was longer when subjects judged the gender of a fearful or angry face relative to a neutral face ($F(37.7; 40.8) = 25.5$; $p<0.001$; **Figure 1**). For the ATD session, mean RT was longer for angry than for neutral faces ($t(21) = 2.68$, $p=0.014$) the same was true for fearful faces (fear: $t(21) = 2.98$, $p=0.007$). Mean RT was longer for angry than neutral faces in the SSRI session ($t(21)=5.33$, $p<0.001$).

With respect to mean error rates, a 3x3 ANOVA with the factors 5-HT challenges (*Control, ATD and SSRI*) and emotions (*angry, fear and neutral*) showed a main effect of emotion ($F(1.7, 37.7)=34.89$, $p<0.001$) and drug ($F(1.1, 23.9)=6.12$, $p=0.018$), but no interaction between challenge and emotion. Simple t-tests revealed that error rates were overall decreased in SSRI session with a mean error rate of 2% compared to the control with a mean error rate of 4% for all three facial expressions (angry faces: $p=0.018$, fearful faces: $p=0.026$, neutral faces: $p=0.026$). A comparable reduction in error rates was also found in the ATD condition with a mean error rate of 2% (fearful faces: $p=0.013$, neutral faces: $p=0.053$; angry faces: $p=0.051$).

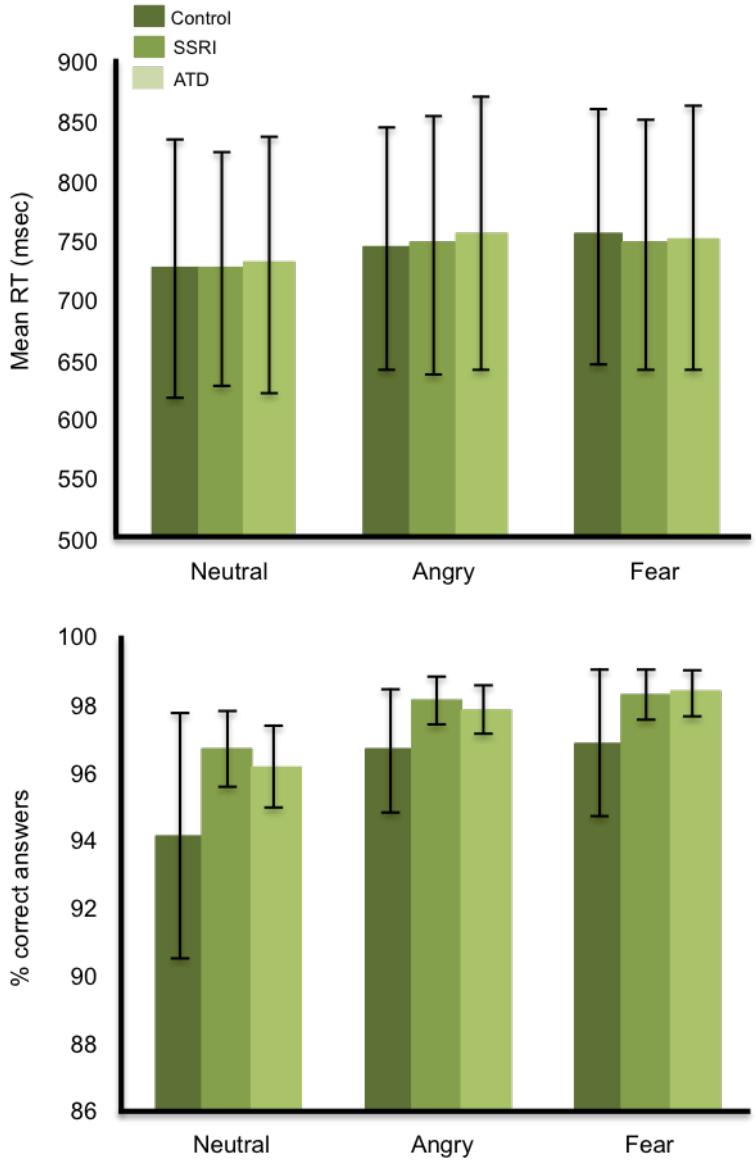

Figure 1: Mean reaction time (RT) recorded during the gender-judgment task based on facial expressions in the control, SSRI and ATD functional magnetic resonance imaging (fMRI) sessions. Control session: neutral faces; 727 ± 109.5, angry faces; 744±102.5, fearful faces; 752±107.4. SSRI session: neutral faces; 730±109.5, angry faces; 755±115, fearful faces; 751±111.6. ATD session: neutral faces; 727±99.5, angry faces; 747±109.3, fearful faces; 746±104.9.Data are presented as mean ± standard deviation of RT. We found a global 2% decrease in error rates for both SSRI and ATD challenges compared to control.

The ANOVA of the POMS yielded a significant effect of time for Anger/Hostility with lower scores at the end of the scanning session as compared to pre-scanning baseline ($F(12)=6.98$, $p=0.022$). Indicating that the subjects did not remain in a high arousal state throughout the scan. Importantly, there was no significant intervention × time interaction in any of the reported mood states. Because the fMRI analyses focused on the differential effects of ATD and SSRI, we also set up a second ANOVA model with 2x3 factors including the two 5-HT interventions (*ATD and SSRI*) and time (*arrival, before, and after scan*). Here we found a main

effect of time, with a decrease in Vigor/Activity scores after both phamacological interventions ($F(22)=6.61$, $p=0.009$), but neither a main effect of the type of intervention nor an intervention × time interaction for any of the mood states.

Biochemical data

Baseline prolactin levels correlated highly between sessions ($r= 0.80$, $n = 19$, $p<0.001$). An ANOVA of prolactin levels revealed no main effect of drug ($F<1$) or time within session from baseline to scanning ($F1, 17 = 2.86$, ns). The ATD protocol reduced the plasma ratio of tryptophan by 75% (paired t-test: before $M=49.2$, $SD=10.0$; after $M=12.3$, $SD=12.7$; $t(21)=11.2$, $p<0.001$) indicating reductions in central tryptophan bioavailability (Williams et al. 1999; Blokland et al. 2002).

Neuroimaging data

Across all challenge conditions (ATD, SSRI, and control), the right inferior frontal gyrus as well as bilateral clusters of middle temporal gyrus stretching to the middle occipital gyrus and bilateral fusiform gyrus and amygdala showed increased activity for aversive faces relative to neutral faces. When separating the emotional faces, a similar pattern was found for activity that was greater for fearful than neutral faces. However, there lacked amygdala activity increases for angry faces compared to neutral faces. Neither the main effect of drugs (SSRI and ATD) nor interaction between drugs and Emotion (angry, fearful, and neutral) revealed any significant results.

Impact of neuroticism on the responsiveness to drug challenges

Individual neuroticism scores were negatively associated with the impact of citalopram compared to ATD (SSRI > ATD) on the neural response of the right subgenual anterior cingulate cortex (sgACC) to fearful faces. The higher the neuroticism score, the lower the sgACC activity increase with SSRI compared to the ATD condition (x,y,z=9,26,-4, $Z=5.0$, $p_{FWE}=0.015$), No such relationship was seen for angry faces.

Post hoc correlational analyses revealed a positive linear relationship between neuroticism scores and sgACC activity evoked by fearful facial expressions in the ATD session (Pearsons correlation; $r=0.551$, $p=0.014$). In contrast, we found a negative relationship between neuroticism scores and sgACC response (Pearsons correlation; $r=-0.611$, $p=0.005$).

Regional brain perfusion at rest

Whole-brain analysis of the ASL data revealed no significant differences in regional cerebral perfusion when perfusion levels during the SSRI or ATD sessions when contrasted with brain perfusion measured in the control session (FWE $p<0.05$ corrected). Additionally, no difference in regional brain perfusion was found when contrasting the ASL data of the SSRI and ATD sessions. Critically, we found no pharmacologically induced changes in regional brain perfusion within the subgenual cortex ROI (FWE $p<0.05$ corrected within the ROI or at uncorrected threshold $p<0.001$).

Discussion

The main findings of this study showed that changes to serotonin levels affected how neuroticism is correlated with brain activity in the sgACC, the higher the neuroticism score, the lower the sgACC activity for SSRI compared to ATD condition. Post Hoc we found a negative correlation for SSRI condition, and a positive correlation for ATD condition between neuroticism scores and activation in sgACC for fearful faces. This indicate that SSRI treatment becomes more effective in subjects rating high in neuroticism scores, which is in accordance with previously published material showing increased neuroticism scores to be a strong reflection of the liability of developing major depression due to ones genetic profile (Kendler et al. 2006) as well as to higher 5-HT_{2A} receptor binding (Frøkjær et al. 2009). Furthermore neuroticism scores has been found to increase functional connectivity of the amygdala with prefrontal regions (Madsen et al. 2015). Neuroticism is a risk factor for anxiety and mood disorders (Bienvenu et al. 2001) which has been associated with a circuitry involving the subgenual cortex together with parts of the orbitomedial PFC, amygdala, hippocampus, striatum and thalamus (Drevets et al. 2008) . Furthermore hypothalamus, raphe, and periacuaductal gray has been shown to be anatomically connected with the subgenual cortex in the monkey brain (Ongür et al. 1998), suggesting that abnormal synaptic connections between these areas and the subgenual cortex may contribute to abnormalities in emotion processing or regulation thereoff. Hall et al. (2014) found activation in subgenual cortex to be correlated with the severity of depressive symptoms. A recent PET study found a positive correlation between serotonergic transporter (SERT) binding and cortisol awakening response (CAR) in subgenual cortex (Frokjaer et al. 2013).

Although the current study does not investigate the genetic profile of included subjects, previous studies has shown a link between carriers of the low expressing variant, the “short” allele (s-allele), of the 5-HTTLPR (serotonin transporter linked polymorphic region) and an increased risk for developing major depression when exposed to severe stress (Caspi 2003). In a study by Pezewas et al. (2005) this polymorphism has further been associated with reduced grey matter volume in the subgenual cortex, reduced functional connectivity between the amygdala and the subgenual cortex in healthy s-allele carriers. Further Madsen et al. (2015) did a post hoc psychophysiological interaction (PPI) between the amygdala and the sgACC as reported by Pezewas et al. (2005) for s-allele carriers showed a higher interaction. This increased interaction between the amygdala and the sgACC may be maladaptive under severe stress, potentially underlying the increased risk for developing depression within the context of stress, for s-alleles carriers (Caspi 2003).

Synthesis of serotonin (5-HT) is dependent on the precursor L-tryptophan (TRP). We included only healthy subjects and found that neuroticism scores enhance the effect of ATD as compared to SSRI. This is in accordance with previous findings, e.g. Neumeister et al. (2004) showing that patients with major depressive disorder showed increased BOLD fMRI activation in the orbitofrontal cortex, anterior and posterior cingulate cortices, in response to ATD. A high proportion, 50-60% of patients with major depressive disorders experience depressive like symptoms in response to ATD, whereas healthy controls seem unaffected (Booij et al. 2002).

The overall difference between elevated levels of free serotonin due to SSRI, and the global lowering of serotonin levels due to ATD did not reveal any main effect of drug when combining all facial expressions (neutral, angry, fear). However we have previously shown

that an increase of serotonin levels due to acute citalopram infusion, abolished differential brain activity across aversive emotional expressions (angry and fear), whereas a reduction of serotonin levels due to ATD specifically enhanced the neural response towards angry faces, making it indistinguishable from the response to fearful faces (Grady et al. 2012). While we were not able to detect any significant effect of either of the 5-HT challenges on neural activation compared to the control session, we found an increased accuracy following both ATD and SSRI interventions, indicating that the 5-HT challenges do affect emotion processing,. Further, in our previous study in the same group of subjects exploring the effects of 5-HT challenges on the neural response to emotional faces (Grady et al. 2012), we found a significant impact of ATD and SSRI on the selectivity on neural response to fearful faces, however the selectivity depended on the serotonin effect. An enhancement of the brains response to angry faces was seen with the ATD condition making the neural response similar to that of fearful faces, whereas the SSRI abolished differential brain responses across all three facial emotions (neutral, angry, fear). It is notable however, that the previous study implemented a multivariate data model in contrast with the univariate statistical analysis implemented here. Also, we see a significant effect of task across drugs, dismissing the notion that our facial stimuli could be a confounding factor for the lack of significant findings when contrasting the SSRI and ATD sessions with the control session.

We found no effect of 5-HT challenges on performance of the face task in terms of RT. Neither did we see any differences between drug challenges with blood perfusion measures (ASL). Therefore, the effects observed in the brain can be attributed to the interactions of serotonin challenge and the neural processing of face emotions, and not to drug effect on behavior or mood.

Although participants reported themselves to be more angry/hostile on scanning days in general, however as POMS scores did not correlate with any of the activation measures, we found no evidence to suggest that the changes of mood scores had any influence on the observed brain activity.

In conclusion both lowering and increasing the serotonergic tone of the brain increased the correlation with neuroticism scores. The personality trait neuroticism seems to predict the impact of 5-HT challenges on fear processing in subgenual cingulate cortex. This finding may represent a neural mechanism for the variable therapeutic effect of SSRI treatment observed in clinical populations. As well as the idea that serotonin and neuroticism are tied together in processing threatening face emotions and that this influence varies depending on the nature of the threat.

Limitations

One limitation to the present study was the necessary differences in the protocols of the two 5-HT challenges (SSRI, ATD), specifically the different ways of administering the challenges including the time between drug administration and scanning as well as it not being double-blinded. A placebo control would have been advantageous in several respects, and prevent the need to consider placebo effects or effects of IV versus no manipulations. However, when the study was designed, it was felt that a full placebo control of the oral ATD solution and the IV infusion for SSRI session, would be too excessive for a within-subject design. Given the heterogeneity of 5-HT_{2A}, and other genetic or personality factors relevant to inhibition, a between subjects design might have been compromised differently, by uncertainty over the cause of differences between groups and imperfect matching. The no-drug condition without

blinded placebo IV/oral solutions was seen as an acceptable choice. Although subjects were made aware of potential side effects within the study, they were not made aware of the specific differences between the interventions or expected effects of these. Given that the volunteers had no prior information about expected effects of the drug given; these specific effects cannot be accounted for by a simple placebo effect, a lack of blinding, or even an expectation or anticipation of the action of either SSRI or ATD. The only effects of the pharmacological challenges on mood, as obtained from the POMS, were observed on anger/hostility scores, but these scores were not significantly related to any of the activity measures. This would appear to rule out any general influence of the drug challenges or scanning procedures on brain activity, and as previous mentioned we also did not see any differences in perfusion measures between drug challenges. Although we cannot rule out some influence of these procedural differences, we think that the uniqueness of this dataset, allowing for the assessment of different serotonin challenges on the brain's response to emotion in the same individuals, outweighs these limitations.

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Addendum from SPM5 analyses

Analysis of the fMRI data

As previously mentioned, data were first analyzed using SPM5, and later also in SPM12. As the article in its present form build on the SPM12 analysis this addendum gives the additional information including figures from the SPM5 analysis of relevance for the present PhD thesis.

For the SPM5 analyses, data were preprocessed and analyzed using version 5 of the Statistical Parametric Mapping program (Wellcome Trust Centre for Neuroimaging, http://www.fil.ion.ucl.ac.uk/spm/software/spm5). Images were realigned and normalized to an MNI (Montreal Neurological Institute) stereotactic space using transformation parameters derived from segmentation of the structural MRI. The normalized images were smoothed using a symmetric 8-mm Gaussian kernel.
The paradigm was analyzed in an event-related fashion with three event types defined at subject level (first level), corresponding to presentation of neutral, angry, or fearful faces. Each event was modeled as a delta function with onset coinciding with the appearance of the cue. Covariates were then convolved with a canonical hemodynamic response function. Three first-level models were created for each subject, modeling the fMRI runs of the drug and control sessions. Each fMRI run was modeled with the three covariates described above together with a mean (constant) term over scans for each run in order to model the main effects of runs. The within-subject model also included 40 nuisance regressors to account for variance caused by physiological noise, including heart beat (10), respiration (6), and head movements (24) (Lund et al. 2006; Pinborg et al. 2003). Group statistical analysis used a mixed-effect second-level ANOVA model with three factors: "type of pharmacological challenge" (3 levels; ATD, SSRI, and control), "emotion" (3 levels: fearful, angry and aversive) and "subject" as random factor (22 levels). The statistic model also included the individual neuroticism scores from the "NEO-PI-R" as subject-specific covariates.
Parameter estimates for each covariate were calculated and statistical parametric maps of the t-statistic (SPM {t}) resulting from linear contrasts of covariates were generated for each subject. Thus, we generated contrast images for the relative increase in BOLD signal induced by the emotional faces relative to neutral faces for all three sessions. In order to test for correlations with mood state, a separate analysis was performed using the significant POMS factor scores (Anger/Hostility and Vigor/Activity) as covariates. The general significance level was set at $p<0.05$ after Family-wise Error (FWE) correction for multiple non-independent comparisons at the cluster level and an extent threshold of $p<0.01$, all imaging results are reported by the Z-score and stereotactic MNI coordinates of the regional maxima.

Results

Neuroimaging data

When pooling the emotional faces; angry and fearful across all serotonergic conditions, the right middle temporal gyrus and inferior frontal gyrus as well as bilateral fusiform gyrus and amygdala showed increased activity for aversive faces relative to neutral faces (**Figure 2**A, see Table 1 for peak coordinates). When separating the emotional faces, fearful faces activated the fusiform gyrus and amygdala bilaterally as well as left inferior occipital cortex and superior parietal lobule (**Figure 2**B, see Table 1 for peak coordinates). For angry faces, the activated

areas included right middle temporal gyrus, the right and left fusiform gyrus, and right inferior frontal gyrus (**Figure 2**B, see Table 1 for peak coordinates).

Main effect of task					
Aversive	**MNI**	Area	Z-score	T-score	P-score
	48,-40,4	R. Midl. Temp. Gyrus	INF	16,44	0,000
	42,-46,-18	R. Fusiform Gyrus	INF	13,28	0,000
	-42,-46,-18	L. Fusiform Gyrus	7,57	11,43	0,000
	-22,-10,-14	L. Amygdala	6,29	8,30	0,000
	22,-8,-16	R. Amygdala	6,29	8,30	0,000
	56,30,4	R. IFG	5,89	7,49	0,001
FEAR					
	42,-36,-18	R. Fusiform Gyrus	7,31	10,70	0,000
	-42,-46,-22	L. Fusiform Gyrus	5,10	6,10	0,000
	-28,-88,-10	L. Inf. Frontal Gyrus	6,55	8,86	0,000
	-22,-10,-12	L. Amygdala	6,06	7,82	0,001
	20,-8,-16	R. Amygdala	5,94	7,59	0,001
	-20,-56,46	L. Sup. Parietal Lobule	5,04	6,00	0,003
ANGRY					
	48,-40,4	R. Midl. Temp. Gyrus	INF	12,52	0,000
	-42,-46,-22	L. Fusiform Gyrus	6,39	8,50	0,000
	44,-46,-18	R. Fusiform Gyrus	6,60	8,97	0,000
	54,30,4	R. IFG	4,47	5,13	0,037
		AMYGDALA not significant in angry			

Table 1: Table of peak coordinates found when regarding the main effect of task for aversive, fearful and angry faces respectively, when pooling all fMRI data together for all three scanning sessions (control, SSRI, ATD).

Neither ATD nor SSRI altered the response to angry or fearful faces relative to neutral faces as compared to control. However trend increases in regional activation were seen in left hippocampus for all aversive faces compared to neutral faces (aversive: x,y,z=-18,-16,-16, Z= 3.97, PFWE= 0.683, angry: x,y,z=-18,-18,-18, Z=3.34, P_{FWE}=0.998, fearful: x,y,z=-18,-16,-16, Z=3.23) for the SSRI session compared to the control session.

A simple t-test based on the contrast image obtained during the control session did not reveal a significant linear relationship between neuroticism and neural activity for either of the emotional faces (angry, fear), but a trend towards a positive linear relationship was found in right caudate nucleus (x,y,z=4,22,.2, Z=4.02, P_{FWE}=0.625) and right fusiform gyrus (x,y,z=32,-48,-4, Z=4.25, P_{FWE}=0.367) for fearful faces.

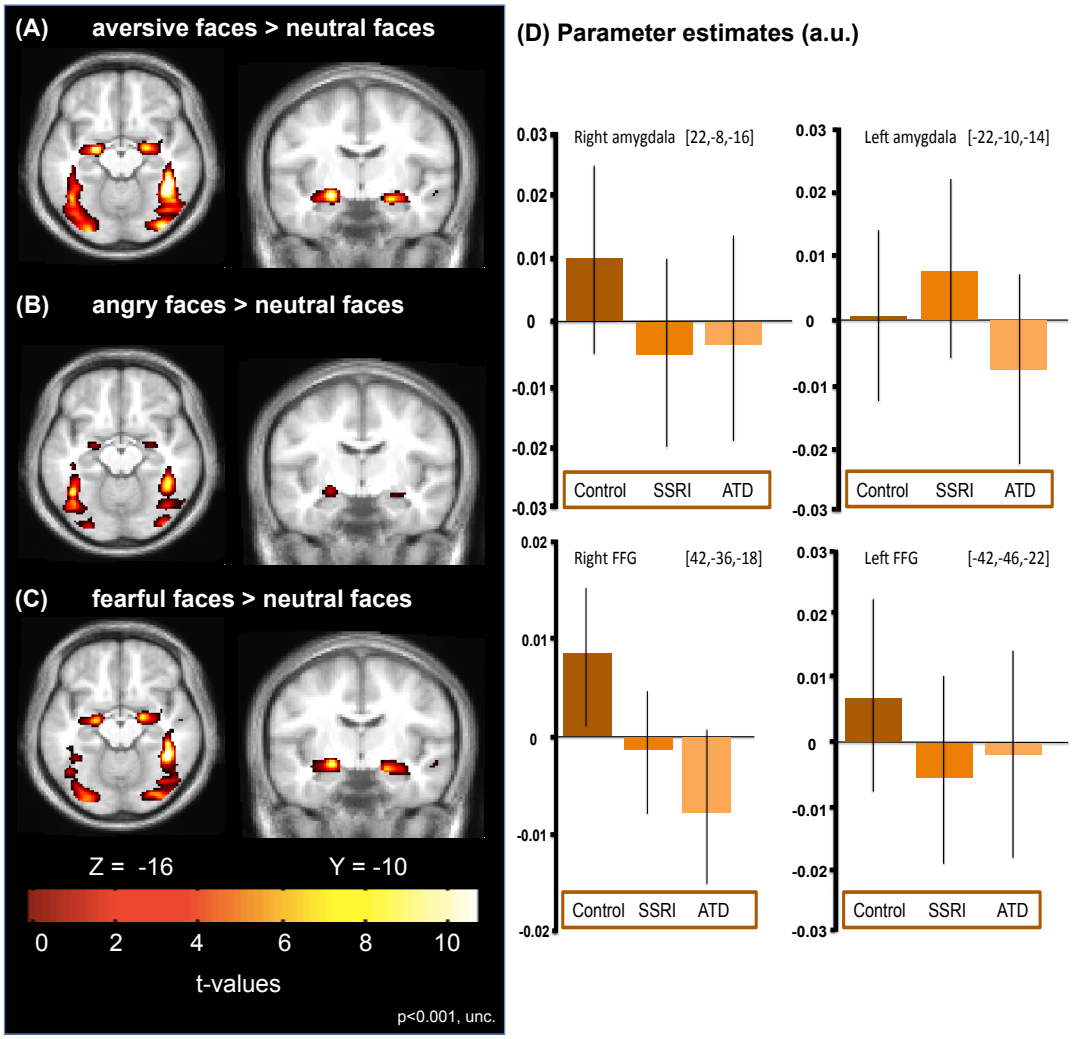

Figure 2: Statistical parametric maps (SPMs) showing brain areas, which are activated by aversive (A), fearful (B) or angry facial (C) expressions relative to neutral faces, as reflected by an increase in blood oxygen level dependent (BOLD) signal. The SPMs are color coded in yellow and red indicating increases in activity, and are thresholded at $p<0.001$ (uncorrected). Panel A gives the activation maps for the contrast aversive faces > neutral faces. B panel is for angry faces > neutral faces; finally the C panel shows the activation maps for fearful faces > neutral faces. (D) The bar graphs presented in the right panel give statistical estimates (arbitrary units) of face related activity levels in the amygdala and fusiform gyrus (FFG) for the control (left column) SSRI (middle column) and ATD (right column). The parameter estimates are taken from the regional maxima showing the strongest increase in regional activity for aversive faces relative to neutral faces. The error bars represent the 90% confidence intervals of the mean.

Impact of neuroticism on the responsiveness to drug challenges

Individual neuroticism scores predicted the impact of citalopram on the neural response of the right subgenual cingulate cortex (sgACC) to fearful faces (Figure 3). The higher the neuroticisom score, the stronger the reduction of the subgenual response to fearful faces in the SSRI condition relative to the control session (x,y,z=6,24,-6, Z=4.07, p_{FWE}=0.03), No such

relationship was seen for angry faces. Post hoc correlational analyses revealed a positive linear relationship between neuroticism scores and subgenual activity evoked by fearful facial expressions in the control session (Pearsons correlation; r=0.566, p=0.006). This relationship flipped from a positive to a negative correlation in the SSRI session (Pearsons correlation; r=-0.678, p=0.001).

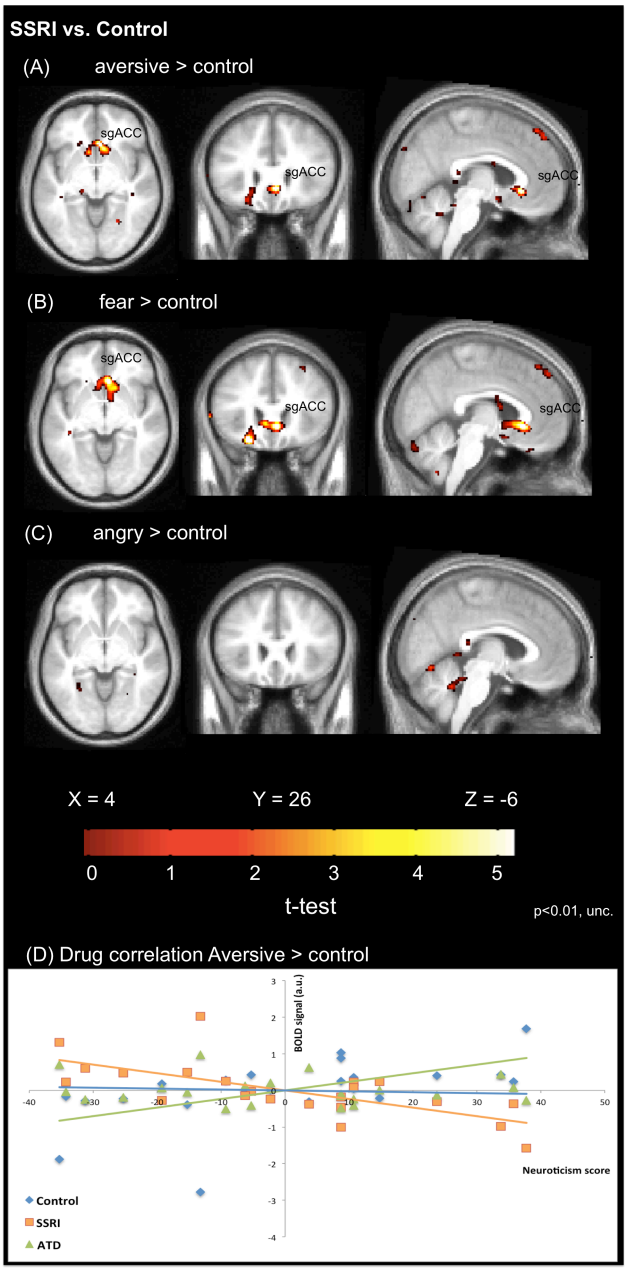

Figure 3: Statistical parametric maps (SPMs) showing changes in activation for aversive face expressions relative to neutral faces during the SSRI challenge compared to baseline (control) in the sgACC. The SPMs indicate changes in BOLD signal and are thresholded at $p<0.001$ (uncorrected). (a) Depicts decreases in regional responsiveness to aversive (angry, fearful) faces under SSRI treatment. (b) angry faces > neutral faces. (c) fearful faces > neutral faces. (d) panel shows the correlation between the individual neuroticism scores and the BOLD response in the sgACC for each of the three drug challenges (SSRI, ATD, Control) (SPM5 analyses).

In the left superior temporal gyrus (STG), the ATD-induced increase in neural response to aversive facial expressions relative to the control session correlated positively with individual variations in neuroticism scores (Figure 4, peak effect at x,y,z=-58,-4,-4, Z=3.84, p_{FWE}=0.003). Post hoc analysis yielded a positive linear relationship between neuroticism and the neural response to aversive faces for the ATD condition in left superior temporal gyrus (Figure 4D,Pearsons correlation; r=0.655, p=0.001), but a negative relationship for the control session (Figure 4D, Pearsons correlation; r= -0.584, p=0.004). No significant effects emerged when considering angry or fearful faces separately.

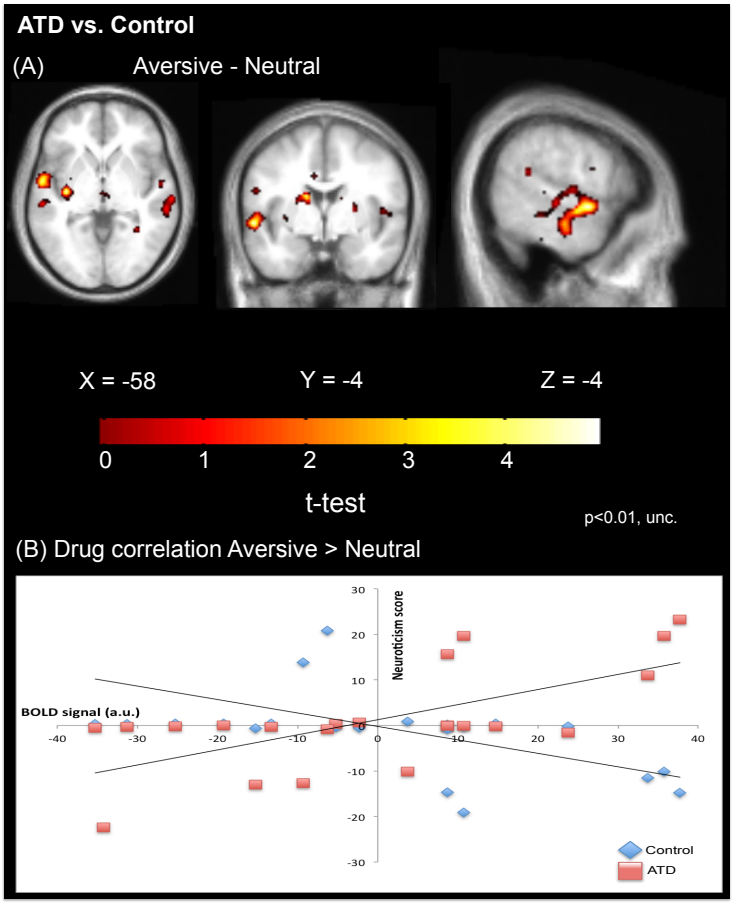

Figure 4: Statistical parametric maps (SPMs) showing changes in activation for aversive face expressions relative to neutral faces during the SSRI challenge compared to baseline (control) in the superior temporal gyrus (STG). The SPMs indicate changes in BOLD signal and are thresholded at $p<0.001$ (uncorrected). (A) Depicts activation in response to aversive (angry, fearful) faces under ATD condition. (B) Shows the correlation between the individual neuroticism scores and the BOLD response in the STG for control and ATD.

In right Heschl's gyrus, neuroticism was associated with an increased responsiveness to aversive faces in the ATD relative to the SSRI condition (peak effect at p value 0.005 x,y,z=42,-22,4, Z=3.82, p_{FWE}=0.002, not illustrated) . Post hoc analysis revealed a positive linear relationship between neuroticism and neural activity evoked by aversive facial expressions in the ATD session (Pearsons correlation; r= 0.760, p<0.001, not illustrated), and a trend

towards a negative relationship in the SSRI condition (Pearsons correlation; r= -0.345, p= 0.115, not illustrated). This effect only was found when pooling the aversive facial expressions, but not when the response to angry or fearful faces was analyzed separately.

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