Testing antidepressant compounds in a neuropsychological model of drug action
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Testing antidepressant compounds
in a neuropsychological model of drug action

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Sevgili anneannem'e

Contents

Chapter 1

General Introduction

Background

The efficacy of antidepressants is tested in randomized controlled trials (RCTs). RCTs report rather modest remission rates when it comes to the efficacy of serotonin reuptake inhibitors (SSRIs) (Keller *et al.*, 1998; Trivedi *et al.*, 2006). For instance, an RCT with the SSRI sertraline in chronically depressed patients yielded a response rate of 22% (i.e. reduction of symptoms of at least 50%) and a remission rate of 36% (i.e. symptom reduction below the threshold of disorder) after 12 weeks (Keller *et al.*, 1998). The response rate of outpatients with major depressive disorder (MDD) to treatment with the SSRI citalopram in an observational study was 47%, while the remission rate was only 28-33% after 8 weeks of treatment (Trivedi *et al.*, 2006). Overall, this leaves ± 50% of MDD patients who do not respond to antidepressants (Keller *et al.*, 1998; Trivedi *et al.*, 2006). Meta-analysis of RCTs have also indicated that antidepressants improve the symptoms of depression, but the difference with placebo is small and may only be clinically worthwhile in severely depressed patients (where placebo is less effective) (Khan *et al.*, 2002; Kirsch *et al.* 2008; Fournier *et al.*, 2010).

A complementary tool to predict efficacy of novel antidepressant drug

Although much research effort has been put into the development of new antidepressant drugs, the process of developing a drug often fails at the stage of large RCTs in which an initially promising compound appears to lack efficacy after all (Harmer *et al.*, 2011). Several experimental medicine models have been proposed as preclinical tools in order to predict drug efficacy before the stage large RCTs (Dawson *et al.*, 2011). Experimental medicine models focusing on antidepressant drug development tap into a range of biomarkers underlying the defective processes seen in MDD patients including behavioural measures, electroencephalogram (EEG), Magnetic Resonance Imaging (MRI) and sleep physiology (Pangalos *et al.*, 2007; Dawson *et al.*, 2011).

Among the various experimental medicine models, the cognitive neuropsychological model has been proposed as a complementary tool to predict the efficacy of antidepressant drug even before the stage of large scale and expensive RCTs (Harmer *et al.*, 2009; 2011). Despite the fact that antidepressants may take weeks to show a clinical effect in depressed patients, they do have immediate effects on emotional information processing, even after single doses in healthy volunteers (Harmer *et al.*, 2003; Harmer *et al.*, 2008; Arnone *et al.*, 2009; Murphy *et al.*, 2009; Rawlings *et al.*, 2010). The neuropsychological model of drug action aims to measure early antidepressant effects by detecting the shift from a negative to a more positive bias in different domains of emotional information processing such as attention, memory and processing of emotional facial expressions (reviewed by Harmer *et al.*, 2009). Some markers of depression that are present in remitted patients are also detectable through pharmacological challenges in healthy individuals (Harmer *et al.*, 2003; Harmer *et al.*, 2008; Arnone *et al.*, 2009; Murphy *et al.*, 2009; Rawlings *et al.*, 2010). Furthermore, healthy individuals do not have a current or history of psychiatric disorders and their response to

antidepressants is not contaminated by earlier pharmacological treatment. These attributes of healthy individuals makes them suitable for assessing the effects of interventions in experimental psychological settings.

Cognitive neuropsychological model of drug action

Antidepressants may take 6-12 weeks to elicit a clinically observable reduction of depressive symptoms in MDD patients (Frazer and Benmansour 2002). Antidepressants do have immediate biological effects as several processes (e.g. increasing the amount of serotonin in the synaptic cleft by blockade of transporters) are directly initiated at the molecular level (Berton and Nestler 2006; Stahl *et al.,* 2013). Already in the early stage of antidepressant administration these direct biological effects lead to the initiation of downstream neuro-adaptive processes but also to a shift from a negative to a positive bias in information processing. According to the neuropsychological model of drug action, these induced changes in information processing interact with emotional and social cues from the environment, resulting over the course of time in an effect on mood states (Figure 1). This gives an alternative (complementary) explanation for the delay in clinically observable antidepressant effects. The early shift towards positive emotional processing is proposed to be associated with neural fine-tuning in subcortical and cortical circuits (Review, Harmer *et al.,* 2009), which will be discussed in the following paragraphs.

a)

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┌──────────────┐     ┌──────────────────┐     ┌──────────────┐     ┌──────────────┐
│ Increased 5-HT│ ──▶│    Downstream    │ ──▶│   Improved   │ ──▶│   Change in  │
│              │     │neuroadaptive effects│    │     mood     │     │emotional bias│
└──────────────┘     └──────────────────┘     └──────────────┘     └──────────────┘
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b)

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┌──────────────┐  Delay ┌──────────────────┐
│ Increased 5-HT│ ──────▶│    Downstream    │
│              │        │neuroadaptive effects│
└──────┬───────┘        └────────┬─────────┘
       │                         │
       │                         ▼
       │              ┌──────────────────┐  Delay ┌──────────────┐
       └─────────────▶│Change in emotional│ ──────▶│ Improved mood│
                      │       bias        │        │              │
                      └──────────────────┘        └──────────────┘
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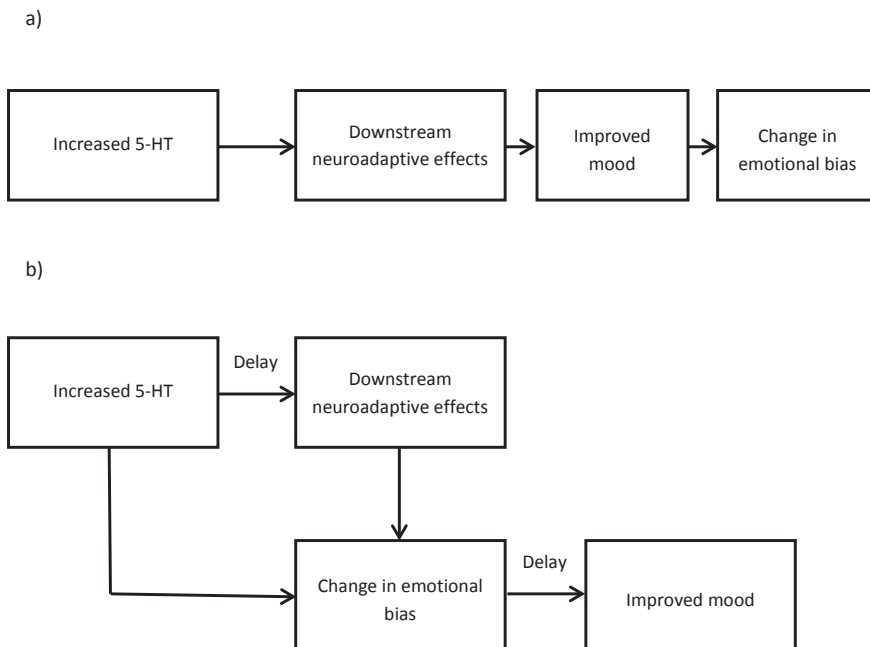
Figure 1. a) Mechanisms underlying the delay in antidepressant drug action as it is generally accepted; b) Mechanisms underlying the delay of drug action as it described in the neuropsychological model of drug action by Harmer et al., 2009.

Validation of the cognitive neuropsychological model of drug action

The cognitive neuropsychological model of drug action has been validated in healthy volunteers by means of behavioural tasks and neural measures, assessing different domains of emotional information processing following administration of conventional antidepressant drugs which act on different neurotransmitter systems (Harmer *et al.*, 2003; Harmer *et al.*, 2008; Arnone *et al.*, 2009; Norbury *et al.*, 2007; Murphy *et al.*, 2009; Rawlings *et al.*, 2010).

Drug administration and early behavioural changes

Two hours following a single dose of the tetracyclic antidepressant mirtazapine (15 mg), healthy volunteers were less accurate in recognizing fearful faces during a facial expression recognition task (FERT) without affecting reaction times (Arnone *et al.*, 2009). Mirtazapine increased reaction times to self-referential words in general, and specifically improved the recall of positive self-referential words during an Emotional Categorization and Memory Task, which is another component of the Emotional Test Battery (ETB) (Arnone *et al.*, 2009).

In another study a single dose of the noradrenergic antidepressant reboxetine (4 mg) improved the recall of happy facial expressions only 2 hours after administration in healthy volunteers (Harmer *et al.*, 2003). In addition, the reboxetine group had a faster reaction time towards positive *vs* negative self- referential words, while this difference was much smaller in the placebo group during the Emotional Categorization Task (Harmer *et al.*, 2003).

The ETB was also completed by healthy volunteers 6 hours after administration of a single dose of the serotonin-norepinephrine reuptake inhibitor duloxetine (60 mg) (Harmer *et al.*, 2008). Duloxetine improved the recognition of both disgust and happy facial expressions without affecting reaction times (Harmer *et al.*, 2008).

These findings provide evidence for early changes in the behavioural response to emotional stimuli following antidepressant drugs administration, however, the interpretation of the changes found on the behavioural measures may vary.

Drug administration and early neural changes

Next to the early detection of changes in emotional processing and biases by means of behavioural measures following antidepressant treatment, early changes at a neural level can also be detected by means of neuroimaging techniques measuring neuronal activity. Neuronal activity is accompanied by an increase in oxygenation metabolism, cerebral blood flow and volume. MRI is an imaging technique that measures these changes in blood flow and provides an indirect measure of neuronal activity in the brain.

A single dose of mirtazapine (15 mg) *vs* placebo was associated with a differential neural response to emotional faces in healthy volunteers two hours after administration (Rawlings *et al.*, 2010). Mirtazapine decreased activation to fearful faces and increased activation to happy

faces in brain regions involved in emotional processing such as the amygdala, hippocampus and fronto-striatal cortex (Rawlings *et al.*, 2010).

Reboxetine administration (8 mg daily for seven days) in healthy volunteers was associated with decreased neural response to subliminally presented fearful faces in the right amygdala and with increased activation in the right fusiform gyrus to subliminally presented happy faces (Norbury *et al.*, 2007).

Furthermore, a single dose of the SSRI citalopram (20 mg) reduced the neural response to fearful faces in the amygdala of healthy individuals, while it did not affect the response to happy and neutral facial expressions (Murphy *et al.*, 2009).

Thus, the findings on the neural response to emotional stimuli following antidepressant drugs administration seems to be more unequivocal compared to the findings on the behavioural response.

The majority of the aforementioned studies investigated the acute effect of various antidepressants on emotional information processing (i.e. on behaviour and neural responses) and reported differences between the antidepressant *vs* placebo groups in absence of changes in mood or subjective state (Harmer *et al.*, 2003; Norbury *et al.*, 2007; Murphy *et al.*, 2009; Rawlings *et al.*, 2010). The findings indicate a positive shift in emotional information processing. These observations may be interpreted as evidence for the cognitive neuropsychological model which hypothesizes that early changes in emotional bias precede the improvement in mood following antidepressant treatment (Harmer *et al.*, 2009). Since single or short-term administration of antidepressant drugs already have a detectable effect in healthy volunteers, the ETB has been applied as a tool to investigate whether new compounds such as GSK424887 (NK1 antagonist and serotonin reuptake inhibitor) (Harmer *et al.*, 2013) and erythropoietin (Miskowiak *et al.*, 2007a; Miskowiak *et al.*, 2007b) may have antidepressant effects. As mentioned above, this approach implements a potential new step in the development of new antidepressant drugs. This might improve the efficiency of the registration process, as initially promising compounds in animals often fail to be effective in depressed patients (Review, Harmer *et al.*, 2011).

In addition to the altered neural and cognitive responses to emotional information, altered resting-state connectivity is associated with MDD and may contribute to its pathophysiology. Several pharmacological studies have investigated the effect of conventional antidepressant drugs on connectivity within the affective networks associated with MDD (Anand *et al.*, 2005; McCabe and Mishor, 2011; van Wingen *et al.*, 2014). These studies have mainly focussed on abnormalities in the cortico-limbic mood regulating circuit (MRC), the default-mode network (DMN) and the task-positive network (TPN) as these have been reported to be altered in depressed patients (reviewed by Wang *et al.*, 2012). Pharmacological resting-state studies

conducted with conventional antidepressant drugs in healthy individuals reported reduced connectivity within the cortico-limbic network (McCabe and Mishor *et al.*, 2011) and reduced connectivity in DMN and TPN, the latter two networks are thought to be increased in MDD patients (Greicius *et al.*, 2007; Wang *et al.*, 2012).

Structure of this dissertation

The studies presented in this thesis concern two projects. In both projects we applied the cognitive neuropsychological model of drug action to test antidepressant effects of a compound in healthy volunteers. In the second project, concerning a well-known compound (L-tryptophan), we further investigated the model by tapping into HPA-axis reactivity and social decision making as additional outcomes, and investigated their interaction with a genetic marker. In the first project, concerning a novel compound (ARA290), we used not only behavioural/cognitive measures but also neuroimaging to detect antidepressant effects.

Pharmacological compound ARA290

Erythropoietin (EPO) is an endogenous protein of which the primary function is to regulate the proliferation of hematopoietic stem cells into red blood cells (erythropoiesis). Next to its role in erythropoiesis, EPO plays a role in tissue protection and repair through anti-inflammatory actions (Brines and Cerami, 2005). EPO administration in animal models of clinical conditions involving tissue damage such as experimental brain damage (Brines *et al.*, 2000) and neonatal stroke (Gonzalez *et al.*, 2009) have shown that EPO crosses the blood brain barrier (BBB) and exerts neurotrophic actions. In humans, administration of EPO improved cognitive performance of patients with multiple sclerosis and schizophrenia (Ehrenreich *et al.*, 2007a; 2007b).

Based on these beneficial effects of EPO on brain tissue and cognition, a series of studies have been conducted to investigate the antidepressant-like effects of EPO, using the neuropsychological model of drug action (Miskowiak *et al.*, 2007a; Miskowiak *et al.*, 2007b). The first studies were in healthy volunteers and later in patients with MDD (Miskowiak *et al.*, 2009; 2010). Although the results of these studies did suggest that a single dose of EPO administration may have antidepressant effects, its utilization as antidepressant drug is rather limited due to hematopoietic side effects of repetitive EPO administration (Wolf *et al.*, 1997; Stohlawets *et al.*, 2000). However, the hematopoietic and tissue protective roles of EPO are regulated by two distinct receptor systems (Figure 2). ARA290 is an 11-amino acid, linear peptide developed as an EPO analogue, which solely acts on a specific receptor (i.e. Innate Repair Receptor; IRR) that initiates tissue protective and anti-inflammatory actions. The IRR consist of a β-common receptor (βCR) subunit (CD131) coupled to an EPOR and its activation initiates multiple signalling pathways resulting tissue protective actions, without initiating hematopoietic effects (Figure 2) (Review, Brines and Cerami, 2012). ARA290 has an

elimination half-life of approximately 2 minutes following i.v. administration (Niesters *et al.*, 2013). Despite the short elimination half-life, ARA290 is suggested to elicit durable effects due to the activation of IRR which regulates the innate tissue-protective response in various stages over a period from hours to days (Brines *et al.*, 2008a; Brines and Cerami, 2012).

The lack of hematopoietic effects makes ARA290 suitable for repetitive administration and - if similar antidepressant-like effects are found as was reported with EPO administration – more promising to develop into an effective antidepressant drug. In Chapter 2 and 3, the first studies testing potential antidepressant-like effects of ARA290 in humans will be described. Specifically, effects of ARA290 on behavioural measures (emotional information processing) and neural measures (task-related and resting-state fMRI) will be presented.
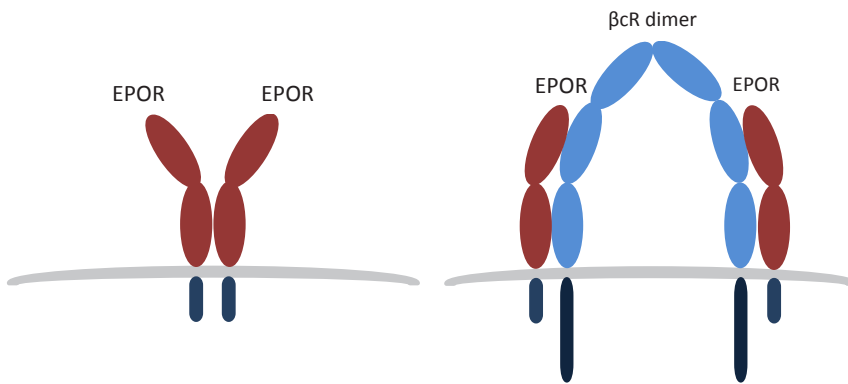

Figure 2. ARA290 is acting on the EPO-R-CD131 receptor to initiate tissue protection (adapted from Brines and Cerami, 2012, The receptor that tames the innate immune response).

Dietary compound: L-tryptophan

Reduced serotonergic activity is involved in the pathophysiology of depression (reviewed by Nestler *et al.*, 2002). Experimental manipulations of serotonin in humans, for instance by means of acute tryptophan depletion, have been extensively used in order to shed light on its role in depression (Review, Booij *et al.*, 2003). Acute Tryptophan Depletion (ATD) is an dietary intervention in which deprivation of L-tryptophan (the amino acid precursor of serotonin) causes a transient state of reduced serotonergic activity. ATD affects mood and cognition in a subgroup of remitted patients treated with SSRIs and recovered depressed patients in a manner that is consistent with a depressogenic effect (Review, Booij *et al.*, 2003). Since reducing levels of tryptophan may induce (transient) depressive symptoms, dietary interventions that increase tryptophan have been used in order investigate whether opposite effects on mood and cognition can be achieved (Booij *et al.*, 2006; Markus *et al.*, 2008). This approach has been applied in depression vulnerable populations defined by, for instance,

high neuroticism scores, high cognitive reactivity scores or s-carriers of the 5-HTTLPR genotype (Markus *et al.,* 1998;Firk and Markus, 2009; Markus and Firk, 2009). One of the multiple dietary interventions to increase plasma Tryptophan/Large Neutral Amino Acids ratio (TRP/LNAA) – an index of central serotonin availability – is to administer pure L-tryptophan (Tryptophan) capsules (Markus *et al.,* 2008). Tryptophan is an amino acid present in nutrients and is daily ingested as part of our diet (World Health Organization, 2002). Besides being the precursor of serotonin, tryptophan is metabolized in various tissues in the periphery and has multiple physiological functions (Le Floc'h *et al.,* 2011). Approximately 90% of ingested tryptophan found in blood and plasma is bound to albumin (Madras *et al.,* 1974; Pardridge 1979). The remaining 10% of free tryptophan in plasma is available for transport through the BBB into the extracellular fluid and cerebrospinal fluid of the central nervous system (CNS) (Le Floc'h *et al.,* 2011). Transport through the BBB is facilitated by transporters embedded in the capillaries of the BBB. These transporters are shared with Large Neutral Amino Acids (LNAA), therefore, the transportation of tryptophan into the CNS is under influence of the TRP/LNAA ratio in plasma (Le Floc'h *et al.,* 2011). Once tryptophan has crossed the BBB it is taken up by cells within the CNS, and is used for i.e. serotonin synthesis as depicted in Figure 3. The synthesis of serotonin is dependent on the amount of tryptophan entering the brain as the rate limiting enzyme, Tryptophan hydroxylase is not saturated at physiological levels of tryptophan in the brain (Le Floc'h 2011; Silber and Schmitt 2010).

Chapter 4 and 5 describes two studies based on the premise that increasing dietary tryptophan intake will increase TRP/LNAA ratio and as a consequence serotonin availability in the brain. The effect of increasing serotonin availability in a genetically vulnerable (i.e. 5-HTTLPR genotype) group was expected to attenuate the physiological response to social stress (i.e. HPA-axis response) and to attenuate the regulation of emotions in response to unfairness (i.e. social-emotional decision making).
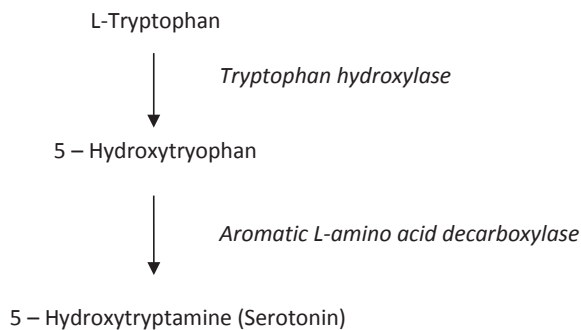
L-Tryptophan

Tryptophan hydroxylase

5 – Hydroxytryophan

Aromatic L-amino acid decarboxylase

5 – Hydroxytryptamine (Serotonin)

Figure 3. Serotonin synthesis with the precursor L-tryptophan (adapted from Le Floc'h *et al.,* 2011)

Serotonin transporter polymorphism and vulnerability to depression

One of the genetic variations found to be related to depression which has been studied extensively is an allelic variation in the promoter region of the serotonin transporter gene: the serotonin transporter polymorphism (Heils *et al.*, 1996). The serotonin transporter polymorphism is a repetitive sequence in the promoter region consisting of two alleles which can be either long or short. The long (L) variant is associated with higher, whereas the short (S) variant is associated with lower transcription activity. Depending on the combination of the two variants of alleles (i.e. L/L, L/S or S/S) more or less serotonin transporters are expressed in the membrane of the presynaptic neurons. The serotonin transporter regulates the availability of serotonin in the synaptic cleft by removing and recycling serotonin and therefore influences serotonin neurotransmission (Canli and Lesch, 2007).

The serotonin transporter polymorphism (5-HTTLPR) has been associated with personality traits related to anxiety and depression (Lesch *et al.*, 1996). Meta-analysis, however, has reported no consistent direct effects of 5-HTTLPR genotype on the anxiety-related personality traits such as harm avoidance and neuroticism (Munafo *et al.*, 2009).

Apart from the direct effect of 5-HTTLPR genotype, its interaction with adverse childhood events was associated with the probability of depression and with suicide risk (Caspi *et al.*, 2003). Despite the mixed results of meta-analyses and reviews on 5-HTTLPR as a vulnerability gene for depression (Munafo *et al.*, 2009; Risch *et al.*, 2009; Belsky *et al.*, 2009; Uher & McGriffin *et al.* 2010; Karg *et al.*, 2011) the literature on Gene x Environment (G x E) interactions and the modulation of vulnerability markers related to depression by 5-HTTLPR genotype continues to expand (Favaro *et al.*, 2014).

One of these vulnerability markers is the regulation of neuroendocrine responses to stress. The association between 5-HTTLPR genotype and HPA-axis reactivity has been of special interest due to the interaction of the serotonin system and HPA axis (Fuller, 1990; Porter *et al.*, 2004) in regulating neuroendocrine responses. The effect of 5-HTTLPR genotype on HPA-axis reactivity has been studied by inducing acute (social) stress through exposure to a public speaking task and assessing levels of the stress hormone cortisol (Gotlib *et al.*, 2008; Way and Taylor 2010). Some studies showed that homozygous S- allele carriers have an increased cortisol response to acute stress (Gotlib *et al.*, 2008; Way and Taylor., 2010), whereas Mueller *et al.* (2011) reported the opposite: homozygous L-carriers showed an increased cortisol response compared to individuals carrying at least one S allele. A few other studies did not find an effect of 5-HTTLPR genotype on HPA-axis reactivity (Wüst *et al.*, 2009; Verschoor and Markus, 2011). These inconsistent findings may have been caused by confounding effects of gender, type of stressor, time of testing during the day and the use of hormonal contraceptives. Despite the inconsistent findings, however, the association between 5-HTTLPR genotype and HPA-axis reactivity to stress, with homozygous s-carriers exhibiting an increased cortisol response has been confirmed in a meta-analysis (Miller *et al.*, 2013). However, the association had a rather small effect size (d = 0.27) (Miller *et al.*, 2013).

Chapter 4 of this dissertation describes a study in which the association between 5-HTTLPR genotype and cortisol response, as a measure of HPA-axis reactivity, has been investigated. We carefully identified and excluded methodological limitations of previous studies that investigated the association between 5-HTTLPR genotype and HPA-axis activity, but reported inconsistent findings. Specifically, we a) pre-selected participants based on genotype b) exposed every participant to the stressor only once to avoid anticipation) c) and excluded possible confounders of the cortisol response (e.g. use of hormonal contraceptives). The strict study design and in- and exclusion criteria were expected to facilitate the detection of the distinct effect of 5-HTTLPR genotype on the cortisol response. Additionally, we investigated whether the hypothesized elevated cortisol response in homozygous s-allele carriers can be treated by tryptophan supplementation.

Next to HPA-axis reactivity, the 5-HTTLPR genotype has also been associated with emotional processing as a behavioural endophenotype (Perez-Edgar *et al.*, 2010; Antypa *et al.*, 2011; Koizumi *et al.*, 2013). Furthermore, the association between serotonin availability and the regulation of emotions in social decision making has been shown by studies with serotonergic manipulations in healthy individuals (Crockett *et al.*, 2008; 2010). Since variation in 5-HTTLPR genotype is associated with altered processing of emotional and socially relevant information, and serotonin availability is involved in decision making processes in which people have to overcome an emotional reaction, we investigated whether 1) 5-HTTLPR genotype is associated with altered regulation of emotions in response to unfair offers made by others and whether 2) the regulation of emotions in response to unfair offers can be altered by tryptophan supplementation (Chapter 5).

Outline of this thesis

- Chapter 2 describes a placebo-controlled randomized clinical trial, testing the effect of a novel pharmacological intervention, ARA290, on the behavioural and neural measures related to emotional information processing in healthy individuals.

- Chapter 3 describes a study on the effect of ARA290 versus placebo on resting-state connectivity in the same healthy individuals.

- Chapter 4 describes a placebo-controlled randomized clinical trial, testing the effect of a dietary intervention, tryptophan supplementation, on HPA-axis reactivity in healthy carriers of the 5-HTTLPR genotype variants.

- Chapter 5 describes the effect of tryptophan versus placebo on social decision making in healthy carriers of the 5-HTTLPR genotype variants.

References

Anand A, Li Y, Wang Y, Wu J, Gao S, Bukhari L *et al* (2005). Antidepressant Effect on Connectivity of the Mood-Regulating Circuit: An fMRI Study. *Neuropsychopharmacology* 30: 1334-1344.

Antypa N, Cerit H, Kruijt A, Verhoeven F, Van der Does A (2011). Relationships among 5-HTT genotype, life events and gender in the recognition of facial emotions. *Neuroscience* 172: 303-313.

Arnone D, Horder J, Cowen PJ, Harmer CJ (2009). Early effects of mirtazapine on emotional processing. *Psychopharmacology* 203: 685-691.

Belsky J, Jonassaint C, Pluess M, Stanton M, Brummett B, Williams R (2009). Vulnerability genes or plasticity genes? *Mol Psychiatry* 14: 746-754.

Berton O, Nestler E J (2006). New approaches to antidepressant drug discovery: beyond monoamines. *Nat Rev Neurosci* 7: 137-151.

Booij L, Merens W, Markus CR, Van der Does AJW (2006). Diet rich in α-lactalbumin improves memory in unmedicated recovered depressed patients and matched controls. *J Psychopharmacol* 20: 526-535.

Booij L, Van der Does AJW, Riedel WJ (2003). Monoamine depletion in psychiatric and healthy populations: review. *Mol Psychiatry* 8: 951-973.

Brines M, Cerami A (2005). Emerging biological roles for erythropoietin in the nervous system. *Nat Rev Neurosci* 6: 484-494.

Brines M, Cerami A (2012). The receptor that tames the innate immune response. *Mol Med* 18: 486-496.

Brines ML, Ghezzi P, Keenan S, Agnello D, de Lanerolle NC, Cerami C *et al* (2000). Erythropoietin crosses the blood–brain barrier to protect against experimental brain injury. *Proc Natl Acad Sci USA* 97: 10526-10531.

Brines M, Patel NS, Villa P, Brines C, Mennini T, De Paola M et al (2008a). Nonerythropoietic, tissue-protective peptides derived from the tertiary structure of erythropoietin. *Proc Natl Acad Sci USA* 105: 10925-10930.

Canli T, Lesch KP (2007). Long story short: the serotonin transporter in emotion regulation and social cognition. *Nat Neurosci* 10: 1103-1109.

Caspi A, Sugden K, Moffitt TE, Taylor A, Craig IW, Harrington H *et al* (2003). Influence of Life Stress on Depression: Moderation by a Polymorphism in the 5-HTT Gene. *Science* 301: 386-389.

Crockett MJ, Clark L, Hauser MD, Robbins TW (2010). Serotonin selectively influences moral judgment and behavior through effects on harm aversion. *Proc Natl Acad Sci USA* 107: 17433-17438.

Crockett MJ, Clark L, Tabibnia G, Lieberman MD, Robbins TW (2008). Serotonin modulates behavioral reactions to unfairness. *Science* 320: 1739-1739.

Dawson GR, Dourish CT, Goodwin GM (2011). Special issue on CNS experimental medicine. *Journal of Psychopharmacology* 25: 1145-1147.

Ehrenreich H, Fischer B, Norra C, Schellenberger F, Stender N, Stiefel M *et al* (2007a). Exploring recombinant human erythropoietin in chronic progressive multiple sclerosis. *Brain* 130: 2577-2588.

Ehrenreich H, Hinze-Selch D, Stawicki S, Aust C, Knolle-Veentjer S, Wilms S *et al* (2007b). Improvement of cognitive functions in chronic schizophrenic patients by recombinant human erythropoietin. *Mol Psychiatry* 12: 206-220.

Favaro A, Manara R, Pievani M, Clementi M, Forzan M, Bruson A (2014). Neural signatures of the interaction between the 5-HTTLPR genotype and stressful life events in healthy women. *Psychiatry Res: Neuroimaging: in press.*

Firk C, Markus CR (2009). Mood and cortisol responses following tryptophan-rich hydrolyzed protein and acute stress in healthy subjects with high and low cognitive reactivity to depression. *Clin Nutr* 28: 266-271.

Fournier JC, DeRubeis RJ, Hollon SD, Dimidjian S, Amsterdam JD, Shelton RC *et al* (2010). Antidepressant drug effects and depression severity: A patient-level meta-analysis. *JAMA* 303: 47-53.

Frazer A, Benmansour S (2002). Delayed pharmacological effects of antidepressants. *Mol psychiatry* 7: S23-28.

Fuller RW (1990). Serotonin receptors and neuroendocrine responses. *Neuropsychopharmacology* 3: 495-502.

Gonzalez FF, Abel R, Almli CR, Mu D, Wendland M, Ferriero DM (2009). Erythropoietin Sustains Cognitive Function and Brain Volume after Neonatal Stroke. *Dev Neurosci* 31: 403-411.

Gotlib IH, Joormann J, Minor KL, Hallmayer J (2008). HPA Axis Reactivity: A Mechanism Underlying the Associations Among 5-HTTLPR, Stress, and Depression. *Biol Psychiatry* 63: 847-851.

Greicius MD, Flores BH, Menon V, Glover GH, Solvason HB, Kenna H *et al* (2007). Resting-State Functional Connectivity in Major Depression: Abnormally Increased Contributions from Subgenual Cingulate Cortex and Thalamus. *Biol Psychiatry* 62: 429-437.

Harmer CJ, Cowen PJ, Goodwin GM (2011). Efficacy markers in depression. *J of Psychopharmacol* 25: 1148-1158.

Harmer CJ, Dawson GR, Dourish CT, Favaron E, Parsons E, Fiore M *et al* (2013). Combined NK1 antagonism and serotonin reuptake inhibition: effects on emotional processing in humans. *J Psychopharmacol* 27:435-443.

Harmer CJ, Goodwin GM, Cowen PJ (2009). Why do antidepressants take so long to work? A cognitive neuropsychological model of antidepressant drug action. *Br J Psychiatry* 195: 102-108.

Harmer CJ, Heinzen J, O'Sullivan U, Ayres RA, Cowen PJ (2008). Dissociable effects o acute antidepressant drug administration on subjective and emotional processing measures in healthy volunteers. *Psychopharmacology* 199: 495-502.

Harmer CJ, Hill SA, Taylor MJ, Cowen PJ, Goodwin GM (2003).Toward a neuropsychological theory of antidepressant drug action: increase in positive emotional bias after potentiation of norepinephrine activity. *Am J Psychiatry* 160: 990-992.

Heils A, Teufel A, Petri S, Stöber G, Riederer P, Bengel D *et al* (1996). Allelic Variation of Human Serotonin Transporter Gene Expression. *J Neurochem* 66:2621-2624.

Karg K, Burmeister M, Shedden K, Sen S (2011). The Serotonin Transporter Promoter Variant (5-HTTLPR), Stress, and Depression Meta-analysis Revisited: Evidence of Genetic Moderation. *Arch Gen Psychiatry* 68: 444-454.

Keller MB, Gelenberg AJ, Hirschfeld RM, Rush AJ, Thase ME, Kocsis JH *et al* (1998). The treatment of chronic depression, part 2: a double-blind, randomized trial of sertraline and imipramine. *J Clin Psychiatry* 59: 598-607.

Khan A, Leventhal RM, Khan SR, Brown WA (2002). Severity of Depression and Response to Antidepressants and Placebo: An Analysis of the Food and Drug Administration Database. *J Clin Psychopharmacol* 22: 40-45.

Kirsch I, Deacon BJ, Huedo-Medina TB, Scoboria A, Moore TJ, Johnson BT (2008). Initial severity and antidepressant benefits: a meta-analysis of data submitted to the Food and Drug Administration. *PLoS medicine* 5: e45.

Koizumi A, Kitagawa N, Kondo HM, Kitamura MS, Sato T, Kashino M (2013). Serotonin Transporter Gene-Linked Polymorphism Affects Detection of Facial Expressions. *PLoS ONE* 8: e59074.

Le Floc'h N, Otten W, Merlot E (2011). Tryptophan metabolism, from nutrition to potential therapeutic applications. *Amino Acids* 41: 1195-1205.

Lesch KP, Bengel D, Heils A, Sabol SZ, Greenberg BD, Petri S *et al* (1996). Association of Anxiety-Related Traits with a Polymorphism in the Serotonin Transporter Gene Regulatory Region. *Science* 274: 1527-1531.

Madras BK, Cohen EL, Messing R, Munro HN, Wurtman RJ (1974). Relevance of free tryptophan in serum to tissue tryptophan concentrations. *Metabolism* 23: 1107-1116.

Markus CR, Firk C (2009). Differential Effects of Tri-Allelic 5-HTTLPR Polymorphisms in Healthy Subjects on Mood and Stress Performance After Tryptophan Challenge. *Neuropsychopharmacology* 34: 2667-2674.

Markus CR, Firk C, Gerhardt C, Kloek J, Smolders GF (2008). Effect of different tryptophan sources on amino acids availability to the brain and mood in healthy volunteers. *Psychopharmacology* 201: 107-114.

Markus CR, Panhuysen G, Tuiten A, Koppeschaar H, Fekkes D, Peters ML (1998). Does Carbohydrate-rich, Protein-poor Food Prevent a Deterioration of Mood and Cognitive Performance of Stress-prone Subjects when Subjected to a Stressful Task? *Appetite* 31: 49-65.

McCabe C, Mishor Z (2011). Antidepressant medications reduce subcortical–cortical resting-state functional connectivity in healthy volunteers. *Neuroimage* 57: 1317-1323.

Miller R, Wankerl M, Stalder T, Kirschbaum C, Alexander N (2013). The serotonin transporter gene-linked polymorphic region (5-HTTLPR) and cortisol stress reactivity: a meta-analysis. *Mol Psychiatry* 18: 1018-1024.

Miskowiak KW, Favaron E, Hafizi S, Inkster B, Goodwin GM, Cowen PJ *et al* (2009). Effects of erythropoietin on emotional processing biases in patients with major depression: an exploratory fMRI study. *Psychopharmacology* 207: 133-142.

Miskowiak KW, Favaron E, Hafizi S, Inkster B, Goodwin G, Cowen PJ *et al* (2010). Erythropoietin modulates neural and cognitive processing of emotional information in biomarker models of antidepressant drug action in depressed patients. *Psychopharmacology* 210: 419-428.

Miskowiak K, O'Sullivan U, Harmer CJ (2007a). Erythropoietin Reduces Neural and Cognitive Processing of Fear in Human Models of Antidepressant Drug Action. *Biol Psychiatry* 62: 1244-1250.

Miskowiak K, O'Sullivan U, Harmer CJ (2007b). Erythropoietin enhances hippocampal response during memory retrieval in humans. *J Neurosci* 27:2788-2792.

Mueller A, Armbruster D, Moser DA, Canli T, Lesch KP, Brocke B *et al* (2011). Interaction of Serotonin Transporter Gene-Linked Polymorphic Region and Stressful Life Events Predicts Cortisol Stress Response. *Neuropsychopharmacology* 36: 1332-1339.

Munafò MR, Freimer NB, Ng W, Ophoff R, Veijola J, Miettunen J *et al* (2009). 5-HTTLPR genotype and anxiety-related personality traits: A meta-analysis and new data. *Am J Med Genet B Neuropsychiatr Genetic* 150B: 271-281.

Murphy SE, Norbury R, O'Sullivan U, Cowen PJ, Harmer CJ (2009). Effect of a single dose of citalopram on amygdala response to emotional faces. *The British Journal of Psychiatry* 194: 535-540.

Nestler EJ, Barrot M, DiLeone RJ, Eisch AJ, Gold SJ, Monteggia LM (2002). Neurobiology of Depression. *Neuron* 34: 13-25.

Niesters M, Swartjes M, Heij L, Brines M, Cerami A, Dunne A et al (2013). The erythropoietin analog ARA 290 for treatment of sarcoidosis-induced chronic neuropathic pain. *Expert Opin Orphan Drugs* 1: 77-87.

Norbury R, Mackay CE, Cowen PJ, Goodwin GM, Harmer CJ (2007). Short-term antidepressant treatment and facial processing: Functional magnetic resonance imaging study. *Br J Psychiatry* 190: 531-532.

Pangalos MN, Schechter LE, Hurko O (2007). Drug development for CNS disorders: strategies for balancing risk and reducing attrition. *Nat Rev Drug Discov* 6: 521-532.

Pardridge WM (1979). Tryptophan transport through the blood-brain barrier: in vivo measurement of free and albumin-bound amino acid. *Life Sci* 25: 1519-1528.

Pérez-Edgar K, Bar-Haim Y, McDermott JM, Gorodetsky E, Hodgkinson CA, Goldman D *et al* (2010). Variations in the serotonin-transporter gene are associated with attention bias patterns to positive and negative emotion faces. *Biol Psychol* 83: 269-271.

Porter RJ, Gallagher P, Watson S, Young AH (2004). Corticosteroid-serotonin interactions in depression: a review of the human evidence. *Psychopharmacology* 173: 1-17.

Rawlings, N., Norbury, R., Cowen, P., & Harmer, C. (2010). A single dose of mirtazapine modulates neural responses to emotional faces in healthy people. *Psychopharmacology, 212*(4), 625-634.

Risch N, Herrell R, Lehner T, Liang KY, Eaves L, Hoh J *et al* (2009). Interaction Between the Serotonin Transporter Gene (5-HTTLPR), Stressful Life Events, and Risk of Depression – a meta-analysis. *JAMA* 301:2462-2471.

Silber BY, Schmitt JA (2010). Effects of tryptophan loading on human cognition, mood, and sleep. *Neurosci Biobehav Rev* 34: 387-407.

Stahl S, Lee-Zimmerman C, Cartwright S, Ann Morrissett D (2013). Serotonergic drugs for depression and beyond. *Current drug targets* 14: 578-585.

Stohlawetz PJ, Dzirlo L, Hergovich N, Lackner E, Mensik C, Eichler HG *et al* (2000). Effects of erythropoietin on platelet reactivity and thrombopoiesis in humans. *Blood* 95: 2983-2989.

Trivedi MH, Rush AJ, Wisniewski SR, Nierenberg AA, Warden D, Ritz L *et al* (2006). Evaluation of outcomes with citalopram for depression using measurement-based care in STAR* D: implications for clinical practice. *Am J Psychiatry* 163: 28-40.

Uher R, McGuffin P (2010). The moderation by the serotonin transporter gene of environmental adversity in the etiology of depression: 2009 update. *Mol Psychiatry* 15: 18-22.

Verschoor E, Markus CR, (2011). Effects of acute psychosocial stress exposure on endocrine and affective reactivity in college students differing in the 5-HTTLPR genotype and trait neuroticism. *Stress* 14: 407- 419.

Van Wingen GA, Tendolkar I, Urner M, van Marle HJ, Denys D, Verkes RJ *et al* (2014). Short-term antidepressant administration reduces default mode and task-positive network connectivity in healthy individuals during rest. *Neuroimage* 88: 47-53.

Wang L, Hermens DF, Hickie IB, Lagopoulos J (2012). A systematic review of resting-state functional-MRI studies in major depression. *J Affec Disord* 142: 6-12.

Way BM, Taylor SE (2010). The Serotonin Transporter Promoter Polymorphism Is Associated with Cortisol Response to Psychosocial Stress. *Biol Psychiatry* 67: 487-492.

Wolf RF, Peng J, Friese P, Gilmore LS, Burstein SA, Dale GL (1997). Erythropoietin administration increases production and reactivity of platelets in dogs. *Thromb Haemost* 78: 1505-1509.

World Health Organization (WHO) 2002. Protein and amino acid requirements in human nutrition: report of a joint FAO/WHO/UNU expert consultation. WHO technical report series; no. 935. WHO, Geneva.

Wüst S, Kumsta R, Treutlein J, Frank J, Entringer S, Schulze TG *et al* (2009). Sex-specific association between the 5-HTT gene-linked polymorphic region and basal cortisol secretion. *Psychoneuroendocrinology* 34: 972-982.

Chapter 1

Chapter 2

Testing the Antidepressant Properties of the Peptide ARA290
in a Human Neuropsychological Model of Drug Action

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Abstract

Studies on the cognitive and neural effects of Erythropoietin (EPO) indicate that EPO may have antidepressant effects. Due to its hematopoietic effects, EPO may cause serious side-effects with repeated administration if patients are not monitored extensively. ARA290 is an EPO-analogue peptide without such hematopoietic side-effects but may have neurotrophic and antidepressant effects. The aim of this study was to investigate the possible antidepressant effects of ARA290 in a neuropsychological model of drug action.

Healthy participants (N=36) received ARA290 (2 mg) or placebo in a double-blind, randomized, parallel-group design. Neural and cognitive effects were assessed one week after administration. Primary outcome measures were the neural processing of fearful *vs* happy faces and the behavioural recognition of emotional facial expressions.

ARA290-treated individuals displayed lower neural responses to happy faces in the fusiform gyrus. ARA290 tended to lower the recognition of happy and disgust facial expressions. Although ARA290 was not associated with a better memory for positive words, it was associated with faster categorization of positive **vs** negative words. Finally, ARA290 increased attention towards positive emotional pictures. No effects were observed on mood and affective symptoms.

ARA290 may modulate some aspects of emotional processing, however, the direction and the strength of its effects do not unequivocally support an antidepressant-like profile for ARA290. Future studies may investigate the effects of different timing and dose.

Registration Clinical Trial:

Introduction

Despite the large number of pharmacological treatment options for depression, many patients show partial or no recovery, and a significant time-lag for the onset of clinical effects. Furthermore, the majority of patients experience side effects such as weight gain and sexual dysfunction (Masand and Gupta, 2002). There is a need for the development of treatments that target different molecular pathways.

Erythropoietin (EPO) is a glycoprotein that regulates erythropoiesis. EPO also crosses the blood brain barrier (BBB) and has neuroprotective and neurotrophic effects when delivered in high doses (Brines *et al.*, 2000; Shingo *et al.*, 2001; Gonzalez *et al.*, 2009). The finding that cells in ischemic/hypoxic brain tissue express EPO and its receptor (EpoR) (Sirén *et al.*, 2001) has led to studies investigating the role of EPO in different types of brain injuries (reviewed by Brines and Cerami, 2005).

In humans, long-term administration of high dose EPO improved cognitive functions in patients with schizophrenia (Ehrenreich *et al.*, 2007a) and multiple sclerosis (Ehrenreich *et al.*, 2007b), whereas lower-dose EPO produced no cognitive benefits (Ehrenreich *et al.*, 2007b). The effects were not correlated with changes in haematological parameters, suggesting that they were mediated by a mechanism other than that of red blood cell increase. In patients with treatment-resistant depression, eight weeks of weekly EPO versus saline infusions as augmentation treatment had no effect on clinician-rated depression severity (primary outcome), but did have small positive effects on self-rated depressive symptoms, quality of life, psychosocial function and cognition (Miskowiak *et al.*, 2014). Earlier research in healthy volunteers had shown that one dose of EPO *vs* saline down-regulates neural response to fearful faces in the fusiform gyrus and reduces the recognition of fearful facial expressions (Miskowiak *et al.*, 2007a). EPO increased bilateral hippocampus activation during a picture memory task one week after administration (Miskowiak *et al.*, 2007b), but not three days after administration (Miskowiak *et al.*, 2007c). EPO also caused transient improvements in self-reported mood in healthy volunteers which lasted for three days (Miskowiak *et al.*, 2007a, Miskowiak *et al.*, 2008). These studies use a neurocognitive model of antidepressant drug action (Harmer *et al.*, 2009) which is based on the finding that various established antidepressants have immediate effects (after a single dose or short-term treatment) on emotional information processing in healthy individuals (reviewed by Harmer *et al.*, 2009, 2010). These early neurocognitive changes induced by antidepressants or by other monoaminergic manipulations may be related to subsequent mood changes (Tranter *et al.*, 2009; Booij and Van der Does, 2011).

In summary, there is evidence to suggest that the effects of EPO mimic antidepressant actions both at a behavioural and at a neural level. However, the human proof-of-concept studies were conducted in relatively small samples. Another limitation is the limited clinical potential of EPO to treat depressive symptoms in non-anemic patients, due to the hematopoietic actions of EPO with repeated administration (Wolf *et al.*, 1997; Stohlawets *et al.*, 2000).

The 11-amino acid, linear peptide ARA290 is an EPO-derivative that exerts tissue-protective effects but is not a hematopoietic stimulant (Brines *et al.*, 2008). ARA290 exerts anti-inflammatory actions in animals (Swartjes *et al.*, 2011; Pulman *et al.*, 2013). In humans, ARA290 had beneficial effects on neuropathic symptoms in patients with sarcoidosis (Heij *et al.*, 2012; Dahan *et al.*, 2013; Niesters *et al.*, 2013). The anti-inflammatory actions of ARA290 are mediated by the innate repair receptor (IRR). (Niesters *et al.*, 2013). Activation of the IRR initiates multiple signalling pathways initiating tissue-protective actions, one of which is the inhibition of inflammation-induced apoptosis (Brines and Cerami, 2012). The effects of ARA290 on neurocognitive processing of emotional information relevant to depression have not yet been assessed.

The aim of this study was to investigate whether ARA290 produces antidepressant-like effects. We carried out a double-blind, randomized placebo-controlled clinical trial in healthy volunteers. Primary outcome measures were the accuracy and speed of recognition of facial expressions of emotions and the neural processing of emotions, in particular amygdala, hippocampal and ventromedial prefrontal cortex (vmPFC) response to viewing fearful versus happy facial expressions. These effects were measured one week after administration of ARA290.

Methods

Participants

Participants were recruited via advertisements at various sites within Leiden University. Inclusion criteria were: Dutch-speaking males and females (not pregnant and not breastfeeding); age 18-35 years; right-handedness; BMI of 18-33kg/m². Exclusion criteria were major physical illness; current or past psychiatric disorder (Mini International Neuropsychiatric Interview; M.I.N.I.; Sheehan *et al.*, 1997; Van Vliet *et al.*, 2000); current use of medication (including over the counter medication); lifetime use of hard drugs; any use of nicotine products in the past week; use of soft drugs in the past three months; use of more than 14 alcohol units per week and more than 4 units on any day during the past week; general MRI contraindications. Participants received €90 for the whole study. This study was approved by the Medical Ethics Committee (METC) of Leiden University Medical Centre (LUMC).

Instruments

Affective symptoms and mood states

Participants filled out the 20-item Positive and Negative Affectivity Schedule (PANAS; state version) (Watson *et al.*, 1988), the 14-item Hospital Anxiety and Depression Scale (HADS; Zigmond and Snaith, 1983; Spinhoven *et al.*, 1997) and single-item Mood States Scales (MSS; 0-10) (Sadness, Annoyance, Tension, Cheerfulness, Energy). The time frame of the HADS was changed into 'the past three days'. IQ was estimated with the National Adult Reading Test (Nelson, 1982; Schmand *et al.*, 1992).

fMRI tasks

E-prime v1.0 was used for presentation of fMRI tasks and recording of responses. Task stimuli were back-projected on a screen located at the end of the scanner bore, which participants could see through a mirror. Description and results of the secondary measure (Picture Recognition Task) and Visual Stimulation Condition are described in the Supplementary Material.

Facial Expression Processing

Pictures of fearful and happy facial expressions were taken from the Radboud Faces Database (Langner *et al.*, 2010) and were presented in blocks. Eight blocks of fearful (4 blocks) and happy (4 blocks) were presented (48 sec/block) in fixed order interspersed with 30 seconds of fixation cross. Each block consisted of 16 faces (8 male and 8 female, randomly presented within a block) presented for 100ms. After presentation of each face a black screen with a white "X" was presented for 2900ms. Responses were only recorded within the first 2000ms

after onset of a trial. The starting block (fearful or happy) was randomized, stratified for sex. Participants performed a simple gender discrimination task. The task took 11 minutes.

fMRI data acquisition

Imaging data were acquired on a Philips 3.0-T Achieva MRI scanner using a 32-channel SENSE head coil for radiofrequency reception (Philips Healthcare, Best, Netherlands).

Whole-brain fMRI data sets were acquired using T2*-weighted gradient echo planar imaging with the following scan parameters: 301 (Facial Expression Processing Task)/170 (Picture Recognition Task)/ 137 (Visual Stimulation Condition) volumes; 38 axial slices scanned in ascending order; repetition time (TR)= 2200ms; echo time (TE)= 30ms; flip angle= 80˚; FOV= 220 x 220 mm; 2.75 mm isotropic voxels with a 0.275 mm slice gap.

A high-resolution anatomical image (T1- weighted ultra-fast gradient-echo acquisition; TR= 9.76ms; TE= 4.59ms; flip angle= 8˚;140 axial slices; FOV= 224 x 177.33 mm; in-plane resolution= 0.875 mm x 0.875 mm; slice thickness= 1.2 mm), and a high-resolution T2*-weighted gradient echo EPI scan (TR= 2.2 s; TE = 30ms; flip angle= 80˚; 84 axial slices; FOV= 220 x 220 mm; in-plane resolution= 1.96 x 1.96 mm, slice thickness= 2 mm) were acquired for registration and normalization to standard space.

fMRI data pre-processing

Prior to analyses, all fMRI data sets were submitted to a visual quality control check to ensure that no gross artefacts were present in the data. Next, data were analysed using FSL Version 4.1.6 (FMRIB's Software Library, www.fmrib.ox.ac.uk/fsl).

The following pre-processing steps were applied to the EPI data sets: motion correction (Jenkinson *et al.*, 2002), non-brain removal (Smith, 2002), spatial smoothing using a Gaussian kernel of 6 mm full width at half maximum (FWHM) for the Facial Expression Recognition and Picture Recognition tasks and 8mm FWHM for the Visual Stimulation Condition, grand-mean intensity normalization of the entire 4D dataset by a single multiplicative factor, a high-pass temporal filter of 165 sec. (i.e., 0.006 Hz) for the Facial Expression Processing Task, 60 sec. (i.e., 0.017 Hz) for the Picture Recognition Task and 40 sec. (i.e., 0.025 Hz) for the Visual Stimulation Condition. Time-series statistical analysis was carried out with local autocorrelation correction (Woolrich *et al.*, 2001). fMRI EPI datasets were registered to the high resolution EPI image, the high resolution EPI image to the T1-weighted image, and the T1-weighted image to the 2 mm isotropic MNI-152 standard space image (T1-weighted standard brain averaged over 152 subjects; Montreal Neurological Institute, Montreal, Canada) (Jenkinson and Smith, 2001; Jenkinson *et al.*, 2002).

fMRI data analysis

For each participant, two explanatory variables (EVs) were included in a general linear model, representing the Fearful and Happy facial expression blocks. Besides the main effects of both expression separately, two other contrasts of interest were defined: Fearful > Happy, Happy > Fearful.

The first-level images of the contrasts of parameter estimates and their corresponding variances were registered to standard space and fed into a second level mixed effects group analysis (Woolrich *et al.*, 2004). We tested for group main effects (one-sample *t*-tests) and between group effects (independent *t*-tests).

The resulting statistic images were cluster corrected for multiple comparisons using an initial cluster forming threshold of z>2.0, and a corrected cluster significance of p<.05. For clusters or regions where significant effects were observed, mean z-scores were extracted from those clusters for each individual participant to create bar graphs for illustration of the effects. The Harvard-Oxford cortical and subcortical atlases, available in FSL, were used for anatomical reference.

Region of interest (ROI) analysis:

The bilateral amygdala was selected for the Facial Expression Processing, using the Harvard-Oxford Subcortical Probability atlas. All regions were thresholded at a 50% probability, binarized, and used as pre-threshold masks in the respective group level mixed effects analyses. Cluster correction was also used within the ROI (z>2.0, p< .05).

Emotional Test Battery

Facial Expression Recognition

The facial expression recognition task (FERT) used a different set of facial stimuli than the one used in the scanner. Pictures of faces from Ekman and Friesen (1976) were presented sequentially on a computer screen in randomized order for 500ms. Faces expressed one of five emotions: happiness, sadness, fear, anger, or disgust. Participants were instructed to identify the emotion by pressing the corresponding key on the response box as quickly and accurately as possible. Emotional expressions had been morphed between two standard images, 0% (neutral) and 100% (full emotion) in 10% steps. Four examples of every emotion at each intensity level (40/Emotion) were presented together with neutral expressions (4 trials) making a total of 204 stimuli presentations. Accuracy, reaction time for correct choices, and misclassifications were recorded.

Emotion categorization and memory

– Categorization. Sixty personality characteristics generally considered to be disagreeable (e.g., untidy, hostile) or agreeable (e.g., honest, optimistic) were presented on the computer screen for 500ms. Positive and negative words were matched in terms of word length, ratings of usage frequency. Volunteers were asked to categorize these words as quickly as possible. Specifically, they were asked to indicate whether they would be pleased or upset if they overheard someone else referring to them as possessing this characteristic. Reaction times to positive and negative traits were computed.

– Free Recall. Fifteen minutes after completion of the categorization task, participants were asked to recall as many of the personality traits as possible within two minutes. Hits (correct responses) and intrusions (false responses) were analysed.

– Recognition. The 30 disagreeable and 30 agreeable characteristics were intermixed with an equal number of distracters that were not presented previously. The number of hits (correct recognitions) and reaction times were computed. Sensitivity (d') and response bias (β) were computed as in Tranter *et al.,*2009.

Visual-Probe

Biased attention was assessed using the visual-probe task. Stimulus pictures were selected from the International Affective Picture Set (Lang *et al.,*2005). Sixteen of these had a negative valence, 16 a positive valence and 32 were neutral. The categories negative and positive valence were matched on arousal ratings (*M*= 5.7) and differed in valence (*M*=7.3 for positive, *M*=2.4 for negative). Mean arousal and valence ratings for the neutral pictures were 3.2 and 5.1, respectively. All stimulus pictures were presented in grayscale. The probes consisted of images of either one or two black dots, four pixels wide and high.

The task started with a number of practice trials, in which a separate set of neutral pictures was used. Practice trials continued until six consecutive trials were correctly answered with a minimum of eight trials. The actual assessment was based on 192 trials, divided in six cycles of 32 trials. Within each cycle, each positive and negative image was randomly selected once and randomly paired with one of the 32 neutral images. Within trials of each valence (positive-neutral, negative-neutral), the location of the emotional stimulus, the probe identity and the congruency were counterbalanced and randomized.

The stimulus display showed two pictures in a horizontal arrangement. Timing of a trial was as follows: cross (500ms)- stimuli (500ms)- probe (until response)- inter trial interval (750ms). A short self-paced break was offered every 30 trials. Participants were instructed to respond as accurately and as fast as possible.

Design

Randomized, double-blind placebo-controlled, parallel-group study. Randomization was carried out in blocks of six, and was stratified for sex. The study was conducted at the LUMC and randomization was carried out by the LUMC pharmacy, Leiden. The study included two lab visits separated by 6 or 7 days.

Procedure

First Lab Visit

Participants who showed interest were provided with information by email and underwent a brief telephone screening. Upon arrival at the laboratory, participants provided written informed consent after the study had been fully explained. Participants underwent a screening procedure including the M.I.N.I. interview and a physical examination. Screening for alcohol use was done by means of a breath test, drugs screening (QD1 220 Drug card-Quantum diagnostics,UK) and a pregnancy test (QuickVue-Quidel Corporation San Diego,USA) were done by urine tests. Next, participants completed questionnaires and the IQ test. After ARA290 or placebo (i.v. 2 mg) administration, the participant was monitored for 10 minutes. At the end of the session participants were given written and oral instructions for the coming week: no nutritional supplements, no drugs or tobacco and limitation of alcohol use to 4 units/day with a maximum of 14 units/week. Caffeine intake was forbidden within one hour before the second lab visit. Participants received a diary in which they were asked to record any violations of these instructions.

Second Lab visit

Upon arrival the participant handed in the diary and was interviewed to check for compliance. Following screening for alcohol use, drug use and pregnancy, participants completed questionnaires and the Picture Encoding Task (Supplementary Material). Participants underwent the MRI scanning session (60 minutes) and completed the Emotional Test Battery afterwards.

Results

Sample characteristics

Of the 45 individuals who were invited to an intake session, two did not show up (illness (N=1); did not provide a reason (N=1)). Five participants did not meet in- and exclusion criteria (use of soft drugs (N=1); excess alcohol use (N=1); history of MDD (N=2); impaired vision (N=1). One eligible participant could not be included due to miscommunication among staff (no certified person available to administer treatment). One participant dropped out after the first lab visit for personal reasons unrelated to the project. Thirty-six participants completed the whole study (Figure 1). The analyses were conducted on 36 participants, except for the fMRI data for which 34 participants were included. fMRI data of two participants were excluded due to head motion (>3mm or 3° in any direction). Groups did not differ in age, IQ, sex distribution and clinical characteristics (Table 1).
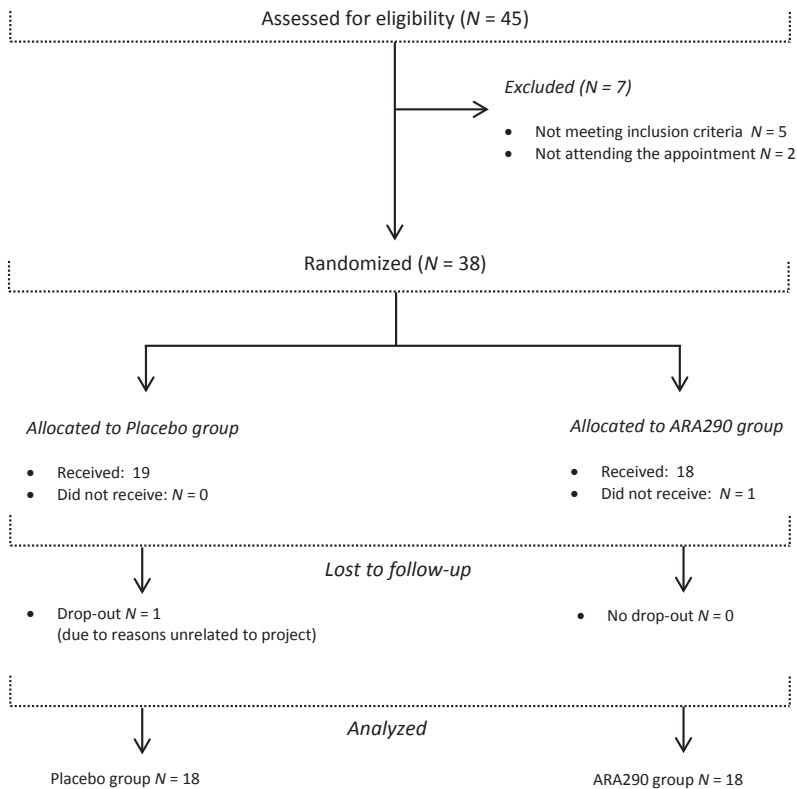

Figure 1. Numbers of participants assessed and included in the data analysis of the double-blind, randomized placebo-controlled clinical trial.

Table 1. Demographics and Characteristics

	ARA290 (*M±SD*)	PLC (*M±SD*)
Sex (f/m)	9/9	9/9
Age	21.3 ± 3.5	20.5 ± 2.0
IQ	104.4 ± 7.9	104.8 ± 7.8
PANAS-S *Positive*		
Pre-treatment	31.5 ± 5.0	33.8 ± 6.0
Post-treatment	31.4 ± 5.6	31.7 ± 6.3
PANAS-S *Negative*		
Pre-treatment	12.6 ± 2.3	13.2 ± 2.6
Post-treatment	12.7 ± 1.6	12.1 ± 1.8
HADS *Anxiety*		
Pre-treatment	2.4 ± 1.8	4.0 ± 2.7
Post-treatment	2.9 ± 1.3	4.0 ± 2.8
HADS *Depression*		
Pre-treatment	1.4 ± 1.2	2.0 ± 2.0
Post-treatment	2.2 ± 1.9	2.1 ± 2.2

PLC = Placebo; f = female; *M* = Mean; *SD* = Standard Deviation

Effects on affective symptoms and mood states

RM-ANOVA with Time (pre-post) as within-subject factor and Treatment (ARA290-Placebo) as between-subject factor was conducted separately on the Anxiety and Depression scale scores of the HADS. No main effects of Time were found in either analysis. A main effect of Treatment was found at trend level on the Anxiety scale ($F(1, 34)=4.10$, $p=0.051$), with lower anxiety levels in the ARA290 group (Table 1). No interactions effects were found. RM-ANOVAs on the Positive and Negative Affect scores revealed no main effect of Time, Treatment, or interaction effect (Table 1).

A 6x5x2 RM-ANOVA with Time (pre-treatment, day 1-5 post-treatment) and mood state (5 scales) as within-subject factors and Treatment (ARA290-Placebo) as between-subject factor was conducted on 30 participants (ARA290, *N*=13; Placebo, *N*=17) because

eight participants had missing MSS scores on one or more days. A significant main effect of Scale (F(1.74,48.67)=142.19;p<0.01) and a trend-level interaction effect of Time x Scale (F(8.37,234.24)=1.85,p=0.066) were found. No main or interaction effects involving Treatment were found (Table S1).

BOLD Response-Facial Expression Processing

Whole brain- main effects of emotion. In both the ARA290 and Placebo group, the processing of fearful (Table S2a) and happy (Table S2b) facial expressions activated multiple regions within the occipital cortex, the precentral gyrus and motor cortex. No differences were found between the two groups for each of the emotions separately.

Whole brain- Intervention x Emotion interaction. A between groups difference on the contrast Fear > Happy was found in the lateral occipital cortex, supramarginal gyrus and temporal occipital fusiform cortex (Table 2; Figure 2). The mean *z*-scores of the clusters for each condition (happy and fear) are presented in Figure S1. The interaction of Intervention x Emotion in the fusiform gyrus is mainly driven by the difference in response of the ARA290 and Placebo groups to happy faces. Specifically, processing of happy faces resulted in reduced activation in the ARA290 group compared to Placebo in the bilateral fusiform gyrus, whereas processing of fearful faces elicited increased activation in the right fusiform gyrus in the ARA290 group compared to placebo. A small volume correction applied within the bilateral amygdala mask did not reveal any differences between the two groups.
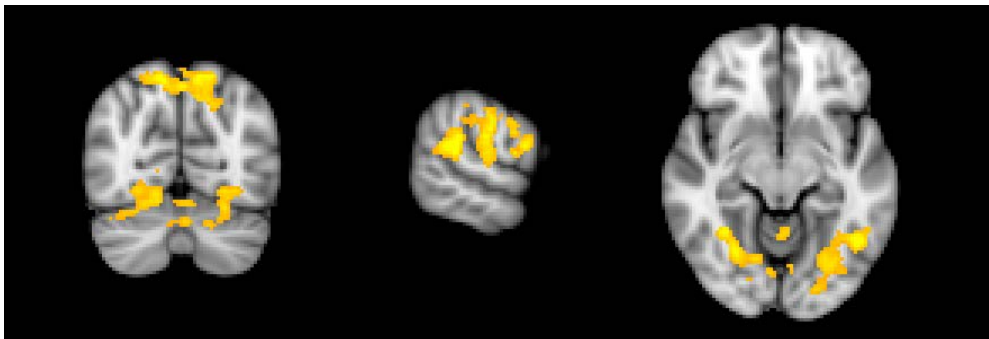

Figure 2. BOLD response during Facial Expression Processing task. Contrast Fear vs. Happy blocks, in clusters where BOLD response by ARA290 is greater than by placebo (x = 62 y = -68 z = -10) *(z>2.0, corrected p<.05).*

Table 2. Contrast Fear > Happy for ARA290 > Placebo

Region	Hemisphere	Cluster size 2 mm	Peak voxel coordinates			Z-value
ARA290 > Placebo			X	Y	Z	
Lateral occipital cortex	R	1461	12	-70	56	3.20
	L		-12	-70	56	3.04
Precuneus	R		4	-80	54	3.05
Cuneus	L		-6	-82	42	2.92
Fusiform gyrus	R	1174	34	-52	-12	3.25
Lingual gyrus	R		24	-62	-4	3.13
Cerebellum	L		-2	-62	-28	2.86
Supramarginal gyrus	R	1026	62	-36	16	3.26
Postcentral gyrus	R		64	-14	28	3.21
Precentral gyrus	R		62	6	18	2.92
Inferior temporal	L	947	-44	-56	-8	3.20
Cerebellum	L		-22	-60	-30	2.88
Fusiform gyrus	L		-30	-72	-10	2.62

z>2.0 and a corrected cluster significance threshold of p=.05

Behavioural response-Facial Expression Processing

Groups did not differ in accuracy of gender discrimination during emotional face processing. Overall accuracy scores were high (95% correct) on both happy and fearful faces. A 2x2 RM-ANOVA on reaction times with Emotion as within-subjects factor and Treatment as between-subjects factor revealed no significant main or interaction effects. This allows us to asses any neural differences unconfounded by differences in performance of the task.

Effects on Emotional Test Battery

- Facial Expression Recognition

RM-ANOVA was conducted with Emotion (six facial expressions) as within-subjects factor and Treatment (ARA290-Placebo) as between-subjects factor on total accuracy scores of each emotion. The main effect of Emotion was significant (F(3.27,111.41)=240.08,p<0.001).

A main effect of Treatment on total accuracy was found (F(1,34)=5.15,p=0.030) with reduced performance in the ARA290 group, but no significant interaction effect was found (F(3.27,111.41)=0.99,p=0.402) (Figure S2;Table S3).

Separate RM-ANOVAs were conducted for each emotion of the FERT, with Intensity (10 levels) as within-subjects factor and Treatment (ARA290-Placebo) as between-subjects factor on accuracy scores. The main effect of Intensity was significant for each emotion. Differences between groups were only found on the emotions "Happy" and "Disgust". A main effect of Treatment on the emotion "Happy" was found at trend level (F(1,34)=4.07,p=0.052), but no interaction effects were found. This was driven by worse relative performance in the ARA290 compared to the placebo group. A main effect of Treatment (F(1,34)=5.10,p=0.031) and a trend-level Intensity x Treatment interaction (F(5,169.99)=2.13,p=0.065) were also found on the emotion "Disgust". Overall, the emotion of "Disgust" was recognized less accurately by the ARA290 (M=24.4, SD=4.4) than the Placebo-treated group (M= 27.1, SD= 2.3).

Separate RM-ANOVAs were conducted with Emotion (6 levels) as within-subjects factor and Treatment (ARA290-Placebo) as between-subjects factor on reaction times, target sensitivity (d') and response bias (β) (Table S3). There were no effects of Treatment or interaction effects on the outcomes reaction times or target sensitivity. Only a main effect of Treatment on response bias (β) was found (F(1,34)=4.59,p=0.039), but no significant interaction effect (F(1.82,61.9)=0.90,p=0.40). Independent-samples t-tests revealed that the ARA290-treated group had a higher β value i.e. fewer false alarms (M=0.94, SD=0.070) than the Placebo-treated group (M=0.89, SD=0.08) for the sad faces (t(34)=2.11,p=0.043) (Table S3).

- Emotion categorization

A 2x2 RM-ANOVA on the reaction times, with Valence (Positive-Negative) as within-subjects factor and Treatment (ARA290-Placebo) as between-subjects factor revealed a trend effect of Valence (F(1,34)=3.06,p=0.09) and a Valence x Treatment interaction effect (F(1,34)=5.61,p=0.024). This was driven by increased speed to positive (M=770, SD=134.9) vs negative (M=831, SD=156.8) words following ARA290 (t(17)= 3.50,p=0.003) (Table 3). There was no main effect of Treatment on reaction times (F(1,34)=0.261),p=0.613). The same analyses on the accuracy scores revealed no main or interaction effects (Table 3).

- Emotional Memory -Free Recall

A 2x2 RM-ANOVA on the recall scores with Valence (Positive-Negative) as within-subjects factor and Treatment (ARA290-Placebo) as between-subjects factor revealed no main or interaction effects (Table 3). The same analysis on Intrusive memory scores revealed only a main effect of Valence (F(1,34)=30.3,p<0.001). This was driven by increased false recalls (intrusions) in positive vs negative words in both groups (Table 3).

- Emotional Memory -Recognition

A 2x2 RM-ANOVAs on Hits with Valence (Positive-Negative) as within-subjects factor and Treatment (ARA290-Placebo) as between-subjects factor revealed only a main effect of Valence ($F(1,34)=36.50,p<0.001$). This was driven by increased Hits in positive vs negative words in both groups (Table 3).

The same analysis on reaction time (for hits) revealed only a main effect of Valence ($F(1,34)=8.94,p=0.005$). This effect was driven by longer reaction times to negative ($M=1038.9$, $SD=254.6$) vs positive ($M=975.5$, $SD=252.1$) words in the Placebo group ($t(17)=2.75,p=0.014$.

Separate RM-ANOVAs were conducted with Valence (Positive-Negative) as within-subjects factor and Treatment (ARA290-Placebo) as between-subjects factor on target sensitivity (d') and response bias (β). These analyses revealed only a main effect of Valence on response bias. In both groups conservative response (i.e., a higher β value) was given to negative compared to positive words.

Visual-Probe

RM-ANOVA with Valence (Negative-Positive Bias Index) as within-subject factor and Treatment (ARA290-Placebo) as between-subject factors revealed no main effect of Valence ($F(1,34)=2.89,p=0.098$) or interaction effect. A main effect of Treatment ($F(1,34)=6.82,p=0.013$) was found. The ARA290 group had higher bias indexes for both valences (Figure 3).
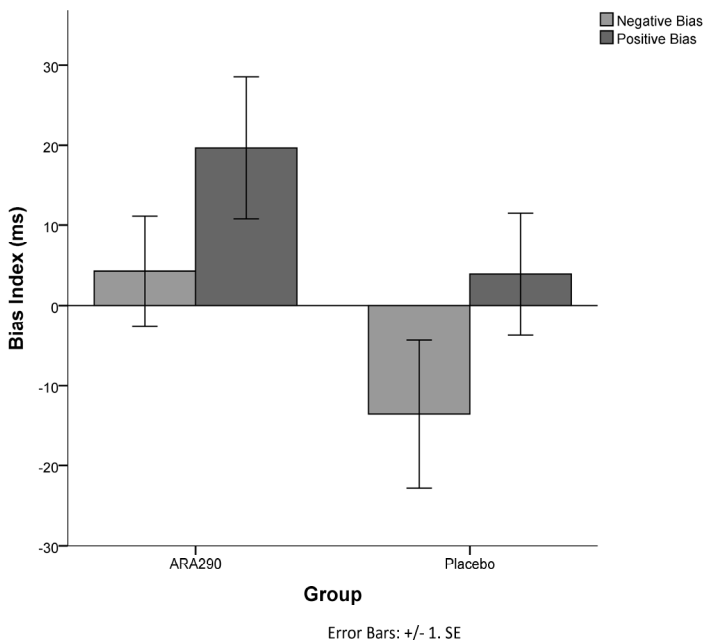

Error Bars: +/- 1. SE

Figure 3. Attentional Bias for positive and negative pictures.

Table 3. Emotion Categorization and Memory (Free Recall and Recognition)

			ARA290 (M ± SD)	Placebo (M ± SD)
Categorization	Hits (# correct out of 30)	Positive	28.0 ± 2.1	28.1 ± 1.9
		Negative	28.3 ± 1.2	26.7 ± 6.2
	Reaction Time (ms)	Positive	770 ± 134.9*	834 ± 232.2
		Negative	831 ± 156.8*	825 ± 166.5
Free Recall	Hits (# correct recall)	Positive	3.8 ± 2.6	4.3 ± 1.5
		Negative	3.8 ± 1.9	3.4 ± 1.6
	Intrusions (# false recall)	Positive	2.0 ± 1.7	1.9 ± 1.8
		Negative	0.3 ± 0.6	0.6 ± 0.7
Recognition	Hits (# correct out of 60)	Positive	24.2 ± 4.1	25.7 ± 2.5
		Negative	20.4 ± 4.4	19.9 ± 5.9
	Reaction Time (ms)	Positive	947.6 ± 232.4	975.5 ± 252.1
		Negative	998.1 ± 254.3	1038.9 ± 254.6

Target sensitivity (*d'*)	*Positive*	0.851 ± 0.102	0.841 ± 0.162
	Negative	0.866 ± 0.072	0.859 ± 0.101
Response bias (*β*)	*Positive*	-0.148 ± 0.437	-0.173 ± 0.384
	Negative	0.393 ± 0.354	0.370 ± 0.318

* Valence x Treatment interaction effect (*p*=0.024). ACC = Accuracy; RT = Reaction Times; ms = milliseconds; *M* = Mean; *SD* = Standard Deviation

Discussion

We used a cognitive neuropsychological model of antidepressant drug action to investigate antidepressant-like effects of ARA290, a derivative of EPO. Based on the literature on EPO, a single dose of ARA290 or placebo was administered to healthy volunteers and the effects were measured one week after administration.

On the primary outcome measures, we observed two small trend-level differences between ARA290- and placebo-treated individuals. The recognition of both happy and disgusted facial expressions in the behavioural task tended to be lower after ARA290 than after placebo. ARA290 had no effect on the neural processing of facial stimuli (positive *vs* negative expressions) in the amygdala, hippocampus or vmPFC in healthy individuals. However, ARA290-treated individuals did show reduced neural responses to happy *vs* fearful faces in the fusiform gyrus, a region involved in face- specific processing (McCarthy *et al.*, 1997).

On secondary outcomes, we found faster categorization of positive (self-referential) words compared to negative words in the ARA290-treated group, but not a better memory for positive words. Furthermore, ARA290-treated individuals had a higher positive attentional bias score than placebo-treated individuals.

Unlike EPO (Miskowiak *et al.*, 2007b), ARA290 did not increase memory-relevant neural response in the hippocampus during a picture recognition task. Finally, ARA290 did not have an effect on self-reported mood states or affective symptoms.

We hypothesized that ARA290 would be associated with reduced recognition of fearful and/or increased recognition of happy facial expressions. The observed trend-level effects on happy and disgust expressions were both in the same direction, which cannot be interpreted as an antidepressant-like effect. The reduced neural response to happy faces in the fusiform gyrus found in the ARA290 group, is the same region as reported by Miskowiak *et al.* (2007a) for EPO. However the direction of this effect was opposite: in Miskowiak *et al.* (2007a), EPO reduced the response to fearful *vs* neutral faces. In our study the difference was mainly driven by the effect of ARA290 on the happy faces which resulted in lower activation in the ARA290 group in the bilateral fusiform gyrus. Processing of fearful faces elicited increased activation in the right fusiform gyrus in the ARA290 group compared to placebo.

In contrast with the previous demonstration of enhanced bilateral hippocampus response in healthy participants after EPO (Miskowiak *et al.*, 2007b), ARA290 did not reliably increase the neural response in the hippocampi during the same picture recognition task. A methodological difference between the current study and the EPO study is that our participants completed the encoding task outside the scanner, meaning that the encoding task was completed one hour before recognition. The longer time frame between the two related tasks may have weakened the hippocampus response.

Consistent with a possible anti-depressant effect, ARA290 was associated with faster categorization of positive *vs* negative self-referential words. This is in line with the effects of single dosages of conventional antidepressants in healthy individuals (Harmer *et al.*, 2003; 2004) and could suggest that ARA290 may lead to a shift from negative towards more positive information processing, particularly regarding self- image. However, ARA290 had no effect on memory (i.e., recall and recognition) of positive *vs* negative self-referential words. Although there are no EPO studies with this specific outcome, we would expect improvement in memory for positive *vs* negative words, based on studies conducted with conventional antidepressant drugs (Harmer *et al.*, 2004; Arnone *et al.*, 2009). ARA290 did increase the attention for positive stimuli similar to the effect of citalopram on attention in healthy volunteers (Browning *et al.*, 2007; Murphy *et al.*, 2009).

Taken together, on our primary outcomes we found that ARA290 tended to lower the recognition of both positive (happy) and negative (disgust) faces. ARA290 also elicited a differential neural response compared to placebo during processing of facial expressions, though in an unexpected direction. However, on the secondary outcomes of emotional categorization and attention ARA290 did show antidepressant-like effects. Our findings show that while ARA290 modulates some aspects of emotional processing the direction and the strength of its effects is overall not congruent with the biomarker model of early antidepressant effects (Harmer *et al.*, 2009, 2010), in which antidepressants produce a marked shift from a negative towards a positive emotional processing bias.

A strength of our study is that we had a relatively large sample size. Therefore, there is little risk that the present negative findings are due to type II error. However, the study has some limitations which need to be addressed. Except for measures related to mood, affective symptoms, we did not include baseline measurements prior to ARA290 administration. The reason for this is that all other measures include emotional stimuli which are subjective to learning and habituation effects. Therefore, all measurements related to emotional processing were completed one week after administration of ARA290 or placebo. Since we had to limit the baseline measurements we cannot be certain whether the several small effects found in this study are due to a pre-existing difference between the groups (i.e., randomization failure) or due to the ARA290 treatment. However, we did control for changes in mood and subjective states and participants were matched for age, sex and IQ.

We based our current study design on findings with EPO (Miskowiak *et al.*, 2007a; 2007b), as data published on ARA290 in healthy populations is still sparse. Therefore, we might not have been able to examine the effect of ARA290 with the highest effective dose and the time point on which ARA290 is the most effective. Administration of this dose (2 mg i.v.) to somatic patients (i.e., neuropathic pain patients) raised no safety concerns (Heij *et al.*, 2012; Niesters *et al.*, 2013). Since this is the first study assessing the antidepressant properties of ARA290 in humans, a single dose of 2 mg might have been too low to exert an antidepressant-like effect in healthy participants and/or the effect of ARA290 may have lasted shorter than one week.

ARA290 acts on the receptor that initiates tissue-protective and anti-inflammatory actions. ARA290 might exert its beneficial effects on mood and cognition by decreasing inflammation in the central nervous system, and therefore it may be interesting to look at the effect of ARA290 in a subtype of depression, namely depressive patients with high inflammation biomarkers in their blood.

As a first step towards a clinical trial in patients, we tested the effects of a single dose in healthy volunteers on the cognitive and neural processing of emotions one week after ARA290 administration. The model we have used has been validated with various registered antidepressants (reviewed by Harmer *et al.*, 2009). Although ARA290 does not seem to cause a marked shift from negative to positive emotional processing, it does have an effect on emotional processing in general. Our study needs replication and future studies may benefit from: a) higher doses and/or repeated administration of ARA290; and b) earlier measurement of the effects of ARA290 after its administration.

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References

Arnone D, Horder J, Cowen PJ, Harmer CJ (2009) Early effects of mirtazapine on emotional processing. *Psychopharmacology* 203: 685-691.

Birn RM, Cox RW, Bandettini PA (2002) Detection versus estimation in event-related fMRI: choosing the optimal stimulus timing. *NeuroImage* 15: 252–264.

Booij L, Van der Does AJW (2011) Emotional processing as a predictor of symptom change: An acute tryptophan depletion study in depressed patients. *Eur Neuropsychopharmacol* 21: 379-383.

Brines ML, Ghezzi P, Keenan S, Agnello D, de Lanerolle NC, Cerami C, Itri LM, Cerami A (2000) Erythropoietin crosses the blood–brain barrier to protect against experimental brain injury. *Proc Natl Acad Sci USA* 97: 10526-10531.

Brines M, Cerami A (2005) Emerging biological roles for erythropoietin in the nervous system. *Nat Rev Neurosci* 6: 484-494.

Brines M, Patel NSA, Villa P, Brines C, Mennini T, De Paola M, Erbayraktar Z, Erbayraktar S, Sepodes S, Thiemermann C (2008) Nonerythropoietic, tissue-protective peptides derived from the tertiary structure of erythropoietin. *Proc Natl Acad Sci USA* 105, 10925-10930.

Brines M, Cerami A (2012) The Receptor That Tames the Innate Immune Response. Mol Med 18: 486–496.

Browning M, Reid C, Cowen PJ, Goodwin GM, Harmer CJ (2007) A single dose of citalopram increases fear recognition in healthy subjects. *J Psychopharmacol* 21: 684-690.

Dahan A, Dunne A, Swartjes M, Proto PL, Heij L, Vogels O, van Velzen M, Sarton E, Niesters M, Tannemaat MR (2013) ARA 290 Improves Symptoms in Patients with Sarcoidosis-Associated Small Nerve Fiber Loss and Increases Corneal Nerve Fiber Density. *Mol Med* 19: 334-345.

Ehrenreich H, Hinze-Selch D, Stawicki S, Aust C, Knolle-Veentjer S, Wilms S, Heinz G, Erdag S, Jahn H, Degner D, Ritzen M, Mohr A, Wagner M, Schneider U, Bohn M, Huber M, Czernik A, Pollmacher T, Maier W, Siren AL *et al.* (2007a) Improvement of cognitive functions in chronic schizophrenic patients by recombinant human erythropoietin. *Mol Psychiatry* 12: 206-220.

Ehrenreich H, Fischer B, Norra C, Schellenberger F, Stender N, Stiefel M, Siren AL, Paulus W, Nave KA, Gold R, Bartels C (2007b) Exploring recombinant human erythropoietin in chronic progressive multiple sclerosis. *Brain* 130: 2577-2588.

Ekman P, Friesen WV (1976) Pictures of Facial Affect. Consulting Psychologists Press: Palo Alto, CA.

Gonzalez FF, Abel R, Almli CR, Mu D, Wendland M, Ferriero DM (2009) Erythropoietin sustains cognitive function and brain volume after neonatal stroke. *Dev Neurosci* 31: 403-411.

Hariri AR, Goldberg TE, Mattay VS, Kolachana BS, Callicott JH, Egan MF, Weinberger DR (2003) Brain-derived neurotrophic factor val66met polymorphism affects human memory-related hippocampal activity and predicts memory performance. *J Neurosci* 23: 6690–6694.

Harmer CJ, Hill SA, Taylor MJ, Cowen PJ, Goodwin GM (2003) Toward a neuropsychological theory of antidepressant drug action: increase in positive emotional bias after potentiation of norepinephrine activity. *Am J Psychiatry* 160: 990–992.

Harmer CJ, Shelley NC, Cowen PJ, Goodwin GM (2004) Increased positive versus negative affective perception and memory in healthy volunteers following selective serotonin and norepinephrine reuptake Inhibition. *Am J Psychiatry* 161: 1256-1263.

Harmer CJ, Goodwin GM, Cowen PJ (2009) Why do antidepressants take so long to work? A cognitive neuropsychological model of antidepressant drug action. *Br J Psychiatry* 195: 102-108.

Harmer CJ, Cowen PJ, Goodwin GM (2010) Efficacy markers in depression. *J Psychopharmacol* 25: 1148-1158.

Heij L, Niesters M, Swartjes M, Hoitsma E, Drent M, Dunne A, Grutters JC, Vogels O, Brines M, Cerami A (2012) Safety and efficacy of ARA 290 in sarcoidosis patients with symptoms of small fiber neuropathy: A randomized, double-blind pilot study. *Mol Med* 18: 1430-1436.

Jenkinson M, Smith S (2001) A global optimisation method for robust affine registration of brain images. *Med Image Anal* 5: 143-56.

Jenkinson M, Bannister P, Brady M, Smith S (2002) Improved optimization for the robust and accurate linear registration and motion correction of brain images. *NeuroImage* 17: 825-841.

Lang PJ, Bradley MM, Cuthbert BN (2005) International affective picture system (IAPS): affective ratings of pictures and instruction manual. Technical report A-6, University of Florida, Gainesville, FL.

Langner O, Dotsch R, Bijlstra G, Wigboldus DHJ, Hawk ST, van Knippenberg A (2010) Presentation and validation of the Radboud Faces Database. Cogn & Emot 24: 1377-1388.

Masand PS, Gupta S (2002) Long-term side effects of newer-generation antidepressants: SSRIS, Venlafaxine, Nefazodone, Bupropion, and Mirtazapine. *Ann of Clin Psychiaty* 14: 175-182.

McCarthy G, Puce A, Gore JC, Allison T (1997) Face-specific processing in the human fusiform gyrus. J of Cognitive Neurosci 9: 605-610.

Miskowiak K, O'Sullivan U, Harmer CJ (2007a) Erythropoietin reduces neural and cognitive processing of fear in human models of antidepressant drug action. *Biol Psychiatry* 62: 1244-1250.

Miskowiak K, O'Sullivan U, Harmer CJ (2007b) Erythropoietin enhances hippocampal response during memory retrieval in humans. *J of Neurosci* 27: 2788-2792.

Miskowiak K, Inkster B, Selvaraj S, Goodwin G, Harmer C (2007c) Erythropoietin has no effect on hippocampal response during memory retrieval 3 days post-administration. *Psychopharmacology* 195: 451-453.

Miskowiak K , Inkster B, Selvaraj S, Wise R, Goodwin GM, Harmer CJ (2008) Erythropoietin improves mood and modulates the cognitive and neural processing of emotion 3 days post administration. *Neuropsychopharmacology* 33: 611- 618.

Miskowiak KW, Vinberg M, Christensen EM, Bukh JD, Harmer CJ, Ehrenreich H, Kessing LV (2014) Recombinant human erythropoietin for treating treatment-resistant depression: A double-blind, randomized, placebo-controlled phase 2 trial*. Neuropsychopharmacology;* e-pub ahead of print 10 December 2013. doi: 10.1038/npp.2013.335.

Murphy SE, Yiend J, Lester KJ, Cowen PJ, Harmer CJ (2009) Short-term serotonergic but not noradrenergic antidepressant administration reduces attentional vigilance to threat in healthy volunteers. *Int J Neuropsychooharmacol* 12: 169-179.

Nelson HE (1982) National Adult Reading Test. Windsor, UK: NFER-Nelson.

Niesters M, Swartjes M, Heij L, Brines M, Cerami A, Dunne A, Hoitsma E, Dahan A (2013) The erythropoietin analog ARA 290 for treatment of sarcoidosis-induced chronic neuropathic pain. *Expert Opin on Orphan Drugs* 1: 77-87.

Pulman KGT, Smith M, Mengozzi M, Ghezzi M, Dilley A (2013) The erythropoietin-derived peptide ARA290 reverses mechanical allodynia in the neuritis model. *Neuroscience* 233: 174–183.

Schmand B, Lindeboom J, Van Harskamp F (1992) De Nederlandse Leestest voor Volwassenen. [The Dutch adult reading test.] Lisse: Swets and Zeitlinger.

Shingo T, Sorokan ST, Shimazaki T, Weiss S (2001) Erythropoietin regulates the in vitro and in vivo production of neuronal progenitors by mammalian forebrain neural stem cells. *J Neurosci* 21: 9733-9743.

Sheehan DV, Lecrubier Y, Harnett Sheehan K, Janavs J, Weiller E, Keskiner A, Schinka J, Knapp E, Sheehan MF, Dunbar GC (1997) The validity of the Mini International Neuropsychiatric Interview (MINI) according to the SCID-P and its reliability. *Eur Psychiatry* 12: 232-241.

Sirén AL, Knerlich F, Poser W, Gleiter CH, Brück W, Ehrenreich H (2001) Erythropoietin and erythropoietin receptor in human ischemic/hypoxic brain. *Acta Neuropathologica* 101: 271-276.

Smith SM (2002) Fast robust automated brain extraction. Hum Brain Mapp 17: 143- 155.

Spinhoven PH, Ormel J, Sloekers PPA, Kempen GIJM, Speckens AEM, Van Hemert AM (1997) A validation study of the Hospital Anxiety and Depression Scale (HADS) in different groups of Dutch subjects. *Psychol Med* 27: 363-370.

Stern CE, Corkin S, Gonzalez RG, Guimaraes AR, Baker JR, Jennings PJ, Carr CA, Sugiura RM, Vedantham V, Rosen BR (1996) The hippocampal formation participates in novel picture encoding: evidence from functional magnetic resonance imaging. *Proc Natl Acad Sci USA* 93: 8660–8665.

Stohlawetz PJ, Dzirlo L, Hergovich N, Lackner E, Mensik C, Eichler HG, Kabrna E, Geissler K, Jilma B (2000) Effects of erythropoietin on platelet reactivity and thrombopoiesis in humans. *Blood* 95: 2983-2989.

Swartjes M, Morariu A, Niesters M, Brines M, Cerami A, Aarts L, Dahan A (2011) ARA290, a peptide derived from the tertiary structure of erythropoietin, produces long-term relief of neuropathic pain: an experimental study in rats and β-common receptor knockout mice. *Anesthesiology* 5: 1084–1092.

Tranter R, Bell D, Gutting P, Harmer C, Healy D, Anderson IM (2009) The effect of serotonergic and noradrenergic antidepressants on face emotion processing in depressed patients. *J of Affect Disord* 118: 87-93.

Van Vliet IM, Leroy H, Van Megen HJM (2000) De MINI-Inter-nationaal Neuropsychiatrisch Interview. Leiden University Medical Center, Leiden, NL.

Watson D, Clark LA, Tellegen A (1988). Development and validation of brief measures of positive and negative affect: the PANAS scales. *J Pers Soc Psychol* 54: 1063-1070.

Wolf RF, Peng J, Friese P, Gilmore LS, Burstein SA, Dale GL (1997) Erythropoietin administration increases production and reactivity of platelets in dogs. *Thromb Haemost* 78: 1505-9.

Woolrich MW, Ripley BD, Brady M, Smith SM (2001) Temporal autocorrelation in univariate linear modeling of FMRI data. *NeuroImage* 14: 1370–1386.

Woolrich MW, Behrens TE, Beckmann CF, Jenkinson M, Smith SM (2004) Multilevel linear modelling for FMRI group analysis using Bayesian inference. *NeuroImage* 21: 1732–1747.

Zigmond AS, Snaith RP (1983) The hospital anxiety and depression scale. *Acta Psychiat Scand* 67: 361-370.

Chapter 2

Supplemental Material

Methods

fMRI tasks

Picture Encoding and Recognition Task: The picture encoding task was completed before scanning in a quiet room. One hour later participants completed the picture recognition task inside the scanner. The encoding and recognition tasks were selected because of hippocampal engagement in encoding and recognition of complex visual scenes (Stern *et al.*, 1996; Hariri *et al.*, 2003), and were the same as the ones used by Miskowiak *et al.* (2007b). Pictures were matched for emotional valence, arousal, and visual complexity, and were presented in a blocked paradigm to maximize sensitivity for hippocampal blood oxygenation level dependent (BOLD) signal change (Birn *et al.*, 2002; Miskowiak *et al.*, 2007b). In both tasks, each of the eight picture blocks (24 sec.) were preceded by brief instructions (2 sec.) and presented interleaved with 20 sec. of fixation cross, resulting in a total task duration of 6 min. Blocks consisted of 6 pictures presented serially for 3 sec. interleaved with a 1 sec. fixation cross. The encoding and recognition tasks contained an equal number of pictures representing indoor and outdoor scenes. In addition, the recognition task contained an equal number of old (i.e., previously encoded) and new pictures. During encoding, volunteers determined whether the picture represented an "indoor" or "outdoor" scene, while in the subsequent recognition task they needed to determine whether the picture was "old" or "new".

Visual Stimulation Condition: To explore whether drug-related effects observed during the Facial Expression Processing and Picture Recognition tasks were attributable to global effects of ARA290 on the BOLD signal, a control visual stimulation condition was used. A flashing checkerboard (frequency, 8 Hz) was presented in blocks of 15 sec. alternating with 15 sec. of a fixation cross for a total of 10 cycles (total duration of 5 min.). Participants were instructed to passively view the screen.

fMRI data analysis

Picture Recognition Task: One explanatory variable (EV) was included in a general linear model, representing the recognition blocks during which participants had to respond whether they had seen the picture before or not. Contrasts were made for task-related activation and deactivation.

Visual Stimulation Condition: One explanatory variable (EV) was included in a general linear model, representing the visual stimulation block during which participants had to view the flickering checkerboard. One contrast of interest was made for task-related activation.

The first-level images of the contrasts of parameter estimates and their corresponding variances were registered to standard space and fed into a second level mixed effects group analysis (Woolrich *et al.*, 2004). We tested for group main effects (one-sample *t*-tests) and between group effects (independent *t*-tests). The resulting statistic images were cluster corrected for multiple comparisons using an initial cluster forming threshold of z>2.0, and a corrected cluster significance of p<.05.

Region of interest (ROI) analysis

The bilateral hippocampus was selected for the Picture Recognition Task, and area V1 for the Visual Stimulation Condition. To define the hippocampus ROI's the Harvard-Oxford Subcortical Probability atlas was used, for area V1 the Juelich Histological atlas. All regions were thresholded at a 50% probability, binarized, and used as pre-threshold masks in the respective group level mixed effects analyses. Cluster correction was also used within the ROI (z>2.0, p<0.05).

Supplemental Material

Results

BOLD Response- Picture Recognition Task

Whole brain - main effect of task. In the ARA290 group, recognition of in- or outdoor scenes activated the visual cortex and a cluster in the paracingulate gyrus and supplementary motor cortex (Table S4). A similar pattern of activation was observed in the Placebo group (Table S4). The ARA290 group did not differ from the Placebo group in neural response during recognition of pictures, neither when the analysis was reduced to the ROI comprising voxels within the bilateral hippocampus.

Behavioural Response- Picture Recognition Task

Picture Encoding:

Independent-samples t- tests revealed no significant differences between the ARA290-treated group (M = 44, SD = 10.8) and the Placebo-treated group (M = 47, SD = 1.9) on accuracy (t (33) = -1.084, p = 0.29). The same analysis revealed no differences between the ARA290-treated group (M = 762, SD = 157.5) and the Placebo-treated group (M = 784, SD = 157.9) on reaction times (t (33) = -0.418, p = 0.68).

Picture Recognition:

Independent-samples t- tests on the four categories (misses, false alarms, correct rejections and hits) with Treatment as grouping variable revealed no differences between the two groups. Independent-samples t- tests on Target sensitivity (d') and Response bias (β) with Treatment as grouping variable revealed a trend for response bias (p = 0.058). There was a trend toward a higher β value i.e. fewer false alarms in ARA290-treated (M = 0.13, SD = 0.15) vs Placebo-treated group (M = 0.04, SD, = 0.13) (t (34) = 1.963, p = 0.058).

BOLD Response- Visual Stimulation Condition

Both the whole brain and ROI analysis (within area V1) revealed no differences between the two groups in response to the visual stimulation condition.

Table S1 Mood State Scale

	Group	Pre (M ± SD)	D1 (M ± SD)	D2 (M ± SD)	D3 (M ± SD)	D4 (M ± SD)	D5 (M ± SD)
Sadness	ARA290	0.5 ± 0.8	1.5 ± 1.3	1.5 ± 1.9	1.6 ± 1.7	1.7 ± 1.5	1.2 ± 1.7
	Placebo	0.6 ± 0.9	1.6 ± 2.1	1.7 ± 2.1	1.6 ± 1.8	0.6 ± 0.9	0.9 ± 1.3
Annoyance	ARA290	0.2 ± 0.4	1.5 ± 1.5	1.8 ± 2.2	1.7 ± 1.7	1.2 ± 1.3	1.5 ± 1.6
	Placebo	0.8 ± 1.3	1.4 ± 1.6	2.1 ± 2.3	1.8 ± 2.3	1.2 ± 1.3	1.5 ± 2.3
Tension	ARA290	2.0 ± 1.4	2.1 ± 2.0	2.1 ± 2.2	2.5 ± 2.3	1.9 ± 1.7	2.0 ± 1.5
	Placebo	2.5 ± 1.5	2.2 ± 2.3	2.5 ± 1.7	2.5 ± 1.7	2.4 ± 2.0	2.4 ± 2.3
Cheerfulness	ARA290	5.6 ± 1.7	5.8 ± 1.5	4.9 ± 2.3	5.1 ± 1.8	5.4 ± 1.5	5.8 ± 2.0
	Placebo	6.5 ± 1.2	5.5 ± 1.8	5.5 ± 1.8	5.3 ± 1.8	6.1 ± 1.4	5.3 ± 2.1
Energetic	ARA290	5.4 ± 1.3	5.8 ± 1.9	5.3 ± 2.1	5.3 ± 1.9	5.3 ± 1.7	5.7 ± 1.8
	Placebo	6.3 ± 1.5	5.3 ± 2.1	5.5 ± 1.5	5.1 ± 2.2	6.1 ± 1.3	5.4 ± 2.2

M = Mean; SD = Standard Deviation

Table S2A BOLD response during fear blocks - ARA290 and placebo groups

Region	Hemisphere	Cluster size 2 mm	Peak voxel coordinates			Z-value
ARA290			X	Y	Z	
Occipital pole	L	31647	-14	-100	-4	6.27
Lateral occipital cortex	R		42	-78	-14	6.07
Fusiform gyrus	R		38	-54	-26	5.88
Lingual gyrus	R		8	-76	-16	5.62
Postcentral gyrus	L	6314	-40	-26	56	6.35
Precentral gyrus	L		-38	-8	64	5.19
Supplementary motor	L	1527	-4	2	52	5.04
	R		10	4	46	4.48
Anterior cingulate	L		-10	18	30	2.33
Superior frontal gyrus	R		8	14	54	2.25
Placebo						
Supplementary motor	L	41478	-8	8	50	5.96
Cerebellum	R		24	-52	-24	5.64
Occipital pole	R		22	-100	-4	5.28
Fusiform gyrus	R		38	-58	-18	5.22
Inferior frontal gyrus	R		50	12	6	5.03
Precentral gyrus	L		-38	-20	68	4.80

z>2.0 and a corrected cluster significance threshold of p=0.05

Table S2B BOLD response during happy blocks - ARA290 and Placebo groups

Region	Hemisphere	Cluster size 2 mm	Peak voxel coordinates			Z-value
ARA290			X	Y	Z	
Fusiform gyrus	L	33197	-34	-78	-16	6.25
	R		36	-60	-22	5.91
Lateral occipital cortex	R		42	-76	-14	6.20
Occipital pole	L		-20	-92	0	5.80
Cerebellum	R		36	-52	-28	5.68
Precentral gyrus	R	1999	46	-2	46	4.68
Placebo						
Cerebellum	R	53450	26	-52	-24	5.89
Lateral occipital cortex	R		38	-76	-20	5.36
Occipital pole	R		22	-100	-4	5.30
	L		-14	-100	-4	5.26
Fusiform gyrus	R		38	-58	-20	5.26
Supplementary motor	R		10	2	50	5.18

z>2.0 and a corrected cluster significance threshold of p=0.05

Chapter 2

Table S3 Facial Expression Recognition Task

	ARA290 (*M ± SD*)	PLC (*M ± SD*)
Accuracy (Hits)		
Anger	18.1 ± 5.1	20.6 ± 4.2
Disgusted	24.4 ± 4.4	27.1 ± 2.3
Fearful	24.9 ± 2.6	25.7 ± 2.8
Happy	26.9 ± 3.8	29.2 ± 2.7
Sad	17.0 ± 3.8	19.1 ± 7.6
Neutral	3.9 ± 0.3	3.7 ± 0.7
Reaction time (ms)		
Anger	1275.2 ± 299.5	1236.5 ± 297.8
Disgusted	1028.9 ± 433.5	991.9 ± 219.2
Fearful	1016.1 ± 309.4	1133.1 ± 317.2
Happy	866.1 ± 250.4	810.6 ± 198.0
Sad	1154.0 ± 239.6	1148.5 ± 391.3
Neutral	788.0 ± 387.3	882.3 ± 393.0
Target sensitivity (*d'*)		
Anger	0.84 ± 0.04	0.87 ± 0.03
Disgusted	0.88 ± 0.05	0.91 ± 0.02
Fearful	0.89 ± 0.02	0.90 ± 0.02
Happy	0.92 ± 0.02	0.93 ± 0.02
Sad	0.85 ± 0.02	0.86 ± 0.05
Neutral	0.90 ± 0.04	0.88 ± 0.07
Response Bias (*β*)		
Anger	0.83 ± 0.15	0.90 ± 0.10
Disgusted	0.71 ± 0.21	0.78 ± 0.15
Fearful	0.75 ± 0.13	0.83 ± 0.15
Happy	0.96 ± 0.06	0.98 ± 0.04
Sad	0.94 ± 0.07*	0.89 ± 0.08
Neutral	-0.89 ± 0.31	-0.76 ± 0.46

PLC = Placebo, *M* = Mean; *SD* = Standard Deviation

Table S4 Main Effects Picture Recognition task (activation)

Region	Hemisphere	Cluster size 2 mm	Peak voxel coordinates			Z-value
			X	Y	Z	
ARA290						
Lingual gyrus	L	60522	-4	-88	-8	7.37
Temporal occipital fusiform cortex	R		28	-54	-10	7.32
Occipital pole	L		-26	-90	6	7.18
Lateral occipital cortex	R		26	-86	16	6.96
Lateral occipital cortex, superior	R		30	-84	14	6.94
Paracingulate gyrus	L	2975	-6	12	46	6.50
	R		2	22	44	5.79
Supplementary motor	L		-6	4	52	5.74
Placebo						
Parahippocampal	L	74570	-22	-40	-16	6.87
Temporal occipital fusiform cortex	R		30	-48	-12	6.85
Occipital pole	L		-8	-94	-8	6.75
	R		20	-98	6	6.53
Lingual gyrus	L		-20	-48	-12	6.64

z>2.0 and a corrected cluster significance threshold of p=0.05

Chapter 2

Supplemental Figures
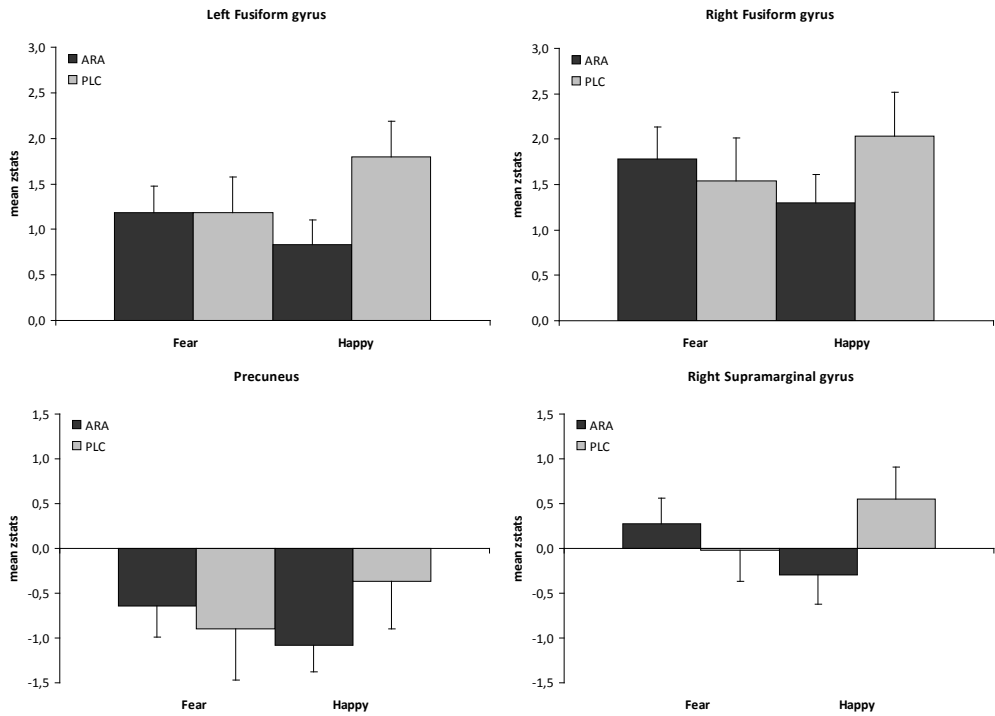
Figure S1. Mean z-stats (SE) for Fear and Happy conditions separately of the clusters resulting from contrast Fear > Happy (ARA = ARA290; PLC = Placebo).
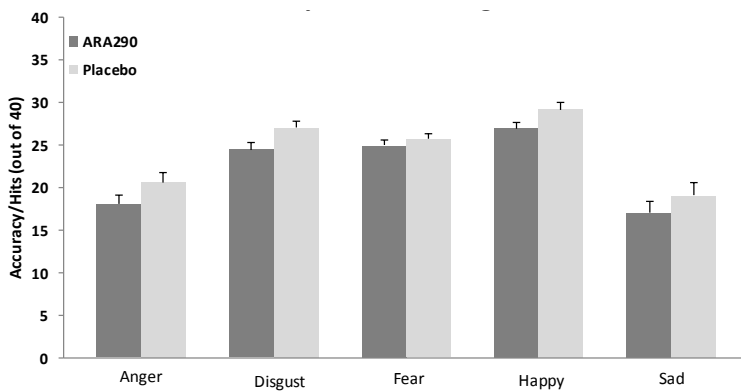
Figure S2. Accuracy scores (SE) on the Facial Expression Recognition task.

Chapter 3

The Effects of ARA290, an Erythropoietin Analogue,
on Resting State Networks Associated with Depression:
a randomized placebo-controlled trial.

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Abstract

Studies on the cognitive and neural effects of Erythropoietin (EPO) indicate that EPO may have antidepressant effects. ARA290 is an EPO-analogue peptide without dangerous hematopoietic side-effects, but it may still have neurotrophic and antidepressant effects. In a previous report, we found that ARA290 may modulate some aspects of cognitive and neural processing. The first aim of this study was to investigate whether ARA290 affects connectivity in the brain, in relation to eight standard networks. The primary networks of interest were the default mode network (DMN) and the executive salience network, known to be involved in MDD. The second aim of this study was to investigate whether ARA290 affects connectivity of the hippocampus, amygdala and/or the fusiform gyrus with other brain regions.

Healthy participants (N=36) received ARA290 (2 mg) or placebo in a double-blind, randomized, parallel-group design. Effects on functional connectivity (resting-state fMRI) were assessed one week after administration. Whole brain analysis revealed that ARA290 did not affect connectivity in relation to the eight standard networks, nor in relation to the seed regions (hippocampus/amygdala and fusiform gyrus) in the current sample of healthy volunteers.

We did not find evidence for an effect of ARA290 on MDD related networks, and thus could not confirm our hypothesis that the effects of ARA290 on functional connectivity contribute to its putative antidepressant effects. Future studies may benefit from higher dosage of ARA290 administration, a shorter time lag between administration and measurements, and a crossover design.

Introduction

The need for new pharmacological treatment options for depression has led to development of compounds that target different molecular pathways. One such compound is Erythropoietin (EPO), a glycoprotein that regulates erythropoiesis. EPO also crosses the blood brain barrier (BBB) and has neuroprotective and neurotrophic effects when delivered in high doses (Brines *et al.*, 2000; Shingo *et al.*, 2001; Gonzalez *et al.*, 2009). Studies have found beneficial effects of EPO on cognitive functions in patients with schizophrenia (Ehrenreich *et al.*, 2007a), multiple sclerosis (Ehrenreich *et al.*, 2007b) and depression (Miskowiak *et al.*, 2010).

The 11-amino acid, linear peptide ARA290 is an EPO analogue and exerts tissue-protective effects but is not a hematopoietic stimulant (Brines *et al.*, 2008). ARA290 exerts anti-inflammatory actions by acting on the innate repair receptor (IRR) (Niesters *et al.*, 2013). Specifically, activation of the IRR initiates multiple signalling pathways initiating tissue-protective actions, one of which is the inhibition of inflammation-induced apoptosis (Brines & Cerami 2012). More importantly, ARA290 does not stimulate erythropoiesis and does not initiate hematopoietic actions, even not after repeated administration (van Velzen *et al.*, 2014).

Given the beneficial effect of ARA290 on cell survival and tissue-protection and the absence of dangerous side effects, we investigated the potential antidepressant effects of ARA290 in a clinical trial in healthy volunteers (Cerit *et al.*, under review), using the neurocognitive model (Harmer *et al.*, 2009). This trial was modelled after the EPO studies and the effects of treatment were assessed one week after ARA290 administration. Some small effects were observed on attentional bias and on the BOLD response to emotional information, but not on the primary outcome measures. Furthermore, ARA290 elicited a larger BOLD response to fearful *vs* happy faces in the fusiform gyrus. This is one of the same regions that were sensitive to EPO administration (Miskowiak *et al.*, 2007a); however, this latter effect was not in the expected direction (Cerit *et al.*, under review).

Altered resting state-state connectivity is also suggested to contribute to the pathophysiology of MDD. Several pharmacological studies have investigated the effect of conventional antidepressant drugs on connectivity within the affective networks associated with MDD (Anand *et al.*, 2005; McCabe and Mishor, 2011; van Wingen *et al.*, 2014). These studies have mainly focussed on abnormalities in the cortico-limbic mood regulating circuit (MRC), the default-mode network (DMN) and the task-positive network (TPN) as these have been reported to be altered in depressed patients (see review by Wang *et al.*, 2012). Seven days of either citalopram, reboxetine or placebo was administered to healthy volunteers and a seed based connectivity analysis was conducted. Both citalopram and reboxetine reduced connectivity within the cortico-limbic network (McCabe and Mishor 2011). Two weeks of duloxetine administration in healthy volunteers resulted in reduced DMN and TPN connectivity (van Wingen *et al.*, 2014).

In order to gain a comprehensive understanding of the potential of ARA290 as an antidepressant, we investigated both its effects on cognitive and neural processing of emotional information (Cerit *et al.*, under review) and its effect on resting-state connectivity. This allows us to investigate whether the effects of ARA290 described in the previous report (Cerit *et al.*, under review) are task specific or rather a general effect of ARA290 on these regions. In summary, in the present study we examined the effect of ARA290 on resting state connectivity in the same healthy volunteers as in our previous report (Cerit *et al.*, under review). We employed a dual regression and a seed-based dual regression analysis using seed regions, known to be differentially activated in response to (emotional) stimuli following EPO and/or ARA290 administration compared to placebo (Miskowiak *et al.*, 2007a, 2007b, 2010; Cerit *et al.*, under review). Specifically, the following seeds were identified and compared over the groups: left and right hippocampus, left and right hippocampus-amygdala complex and bilateral fusiform gyrus. The following hypotheses were tested:

a) ARA290 affects connectivity in the brain (in relation to eight standard networks (Beckmann *et al.*, 2005), together covering around 80% of the brain) and specifically the default mode network (DMN) and the executive salience network, known to be involved in MDD.

b) ARA290 affects connectivity of the hippocampus, amygdala and/or the fusiform gyrus with other brain regions.

Methods

Participants

The data were acquired in the same participants as in a previous report, in which detailed information about the participants is outlined (Cerit *et al.*, under review). A total of 36 participants (18-35 years) were recruited and randomly assigned to either placebo or ARA290 condition. The groups did not differ in age, IQ, sex distribution and clinical characteristics (Cerit *et al.*, under review).

Design

We used a randomized, double-blind placebo-controlled, parallel-group design. Randomization was carried out in blocks of six, and was stratified for sex. The study was conducted at the LUMC and randomization was carried out by the LUMC pharmacy, Leiden. The study included two lab visits separated by 6 or 7 days. Participants came into the lab twice and were administered either placebo or ARA290 (2mg) on the first lab visit. During the second visit, which took place one week later, all participants underwent (functional) MRI (fMRI) scanning, both task-related (Cerit *et al.*, under review) and resting state fMRI scanning; both scans were part of one study and were acquired in one scanning session and in the same sample as reported in Cerit and colleagues (under review).

Image acquisition

Imaging data were acquired on a Philips 3.0-Tesla Achieva MRI scanner using a 32-channel SENSE head coil for radiofrequency transmission and reception (Philips Healthcare, Best, The Netherlands). RS-fMRI data were acquired using T2*-weighted gradient-echo echo-planar imaging with the following scan parameters: 200 whole-brain volumes; repetition time (TR) = 2.2 sec; echo time (TE)= 30 ms; flip angle= 80°; 38 axial slices scanned in ascending order; FOV = 220 x 220 mm; voxel size 2.75 x 2.75 x 2.75 mm, plus 10% interslice gap. For registration purposes and normalization to standard space, a high-resolution anatomical image (T1-weighted ultra-fast gradient-echo acquisition; TR=9.76 ms; TE= 4.59 ms; flip angle= 8°; 140 axial slices; FOV= 224 x 177.33 mm; in plane voxel resolution = 0.875 mm x 0.875 mm; slice thickness= 1.2 mm) and a high-resolution T2*- weighted gradient-echo EPI scan (TR= 2.2 sec; TE= 30 ms; flip angle=80°; 84 axial slices; FOV= 220 x 220 mm; in plane voxel resolution= 1.96 x 1.96 mm, slice thickness= 2 mm) were acquired for each participant. In accordance with the Leiden University Medical Center's policy, all anatomical MRI scans were screened by a radiologist to rule out incidental pathology.

Chapter 3

Data analysis

Before statistical analysis, all MRI scans were submitted to a visual quality control check to ensure that no gross artefacts were present in the data. Data analysis was performed with Functional Magnetic Resonance Imaging of the Brain Software Library (FSL version 5.0.1, Oxford, United Kingdom, www.fmrib.ox.ac.uk/fslSmith *et al.*, 2004). Anatomical locations were determined using the Harvard-Oxford cortical and subcortical structures atlas integrated in FSL.

Resting-State functional connectivity analysis

Pre-processing of RS-fMRI images was carried out using FEAT (FMRI Expert Analysis Tool) Version 5.98, part of FSL. The following processing steps were applied: motion correction (Jenkinson *et al.*, 2002), brain extraction (Smith, 2002), spatial smoothing using a Gaussian kernel with a full width at a half maximum (FWHM) of 6 mm, grand-mean intensity normalization of the entire 4D dataset by a single multiplicative factor and a high-pass temporal filtering equivalent to 100 sec. Time series statistical analysis was carried out with local autocorrelation correction (Woolrich *et al.*, 2001). After pre-processing, the functional images were registered to the corresponding high-resolution echo planar images, which were registered to the T1-weighted images, which in turn were registered to the 2 mm isotropic MNI-152 standard space images (T1 standard brain averaged over 152 subjects; Montreal Neurological Institute, Montreal, QC, Canada; Jenkinson and Smith, 2001; Jenkinson *et al.*, 2002). The registration parameters were combined to obtain the registration matrix from native space to MNI space and its inverse (from MNI space to native space).

The functional connectivity analysis was performed using the dual regression method of FSL, a technique that allows a voxel-wise comparison of resting-state functional connectivity (previously described in Filippini *et al.*, 2009). First, we performed a model-free analysis of eight standard resting state networks (Beckmann *et al.*, 2005), representing 80% of the total brain volume (Khalili-Mahani *et al.*, 2012). Previously, localized and drug-specific changes in functional brain connectivity have been shown using these networks in resting-state fMRI studies (Cole *et al.*, 2013; Khalili-Mahani *et al.*, 2012; Klumpers *et al.*, 2012; Niesters *et al.*, 2012). Secondly, we analysed the resting state connectivity changes induced by ARA290 in relation to two seeds, namely the amygdala-hippocampus complex (Miskowiak *et al.*, 2010), determined using the Harvard-Oxford cortical and subcortical structures atlas integrated in FSL, and the fusiform gyrus (functional seed, determined by the contrast: Fearful vs Happy in a previous report Cerit *et al., under review* and in line with Miskowiak *et al.*, 2007a).

In both analyses we used a dual regression analysis to create subject specific statistical maps: voxel-wise Z-scores of functional connectivity to each of the eight networks or to the seed regions. We accounted for non-specific and physiological variations by including nuisance variables, corresponding to fluctuations in the deep white matter and cerebrospinal fluid

(Birn, 2012). The statistical maps were then used for voxel-wise inference testing of the effect of ARA290 on functional connectivity with each of these networks or seed regions, using a General Linear Model (GLM) approach as implemented in FSL. Two statistical contrasts were made with regard to changes in functional connectivity between the two groups: ARA290>placebo and placebo>ARA290. Voxel-wise nonparametric permutation testing was performed using FSL-randomise (5000 permutations; Nichols and Holmes, 2001). All statistical maps were Family-Wise Error (FWE) corrected using p < 0.05, based on the Threshold-Free Cluster Enhancement (TFCE) statistic image (Smith and Nichols, 2009), applying a minimum cluster size of 80 mm^3.

Results

Voxel-wise comparison of each group of participants separately versus the eight predefined general resting-state networks (Beckmann *et al.*, 2005), using dual regression, revealed that the eight networks were significantly present in both groups of participants (Fig. 1). Figure 1 seems to show some differences between the two groups, for instance the contribution of the cingulate cortex to the auditory and somatosensory system (network c in Fig.1) in the placebo group is not significantly present in the ARA290 group. However, when directly comparing the two groups of participants, no significant differences in functional connectivity in any of the eight networks were found (p<0.05, threshold-free cluster enhancement corrected). Thus, the apparent difference between the groups in cingulate cortex contribution to network c (Fig. 1) did not reach significance (it was present in the uncorrected data p<0.05). Similarly, when comparing the two groups of participants, we did not find significant differences in functional connectivity in relation to the seed regions, anywhere in the brain.
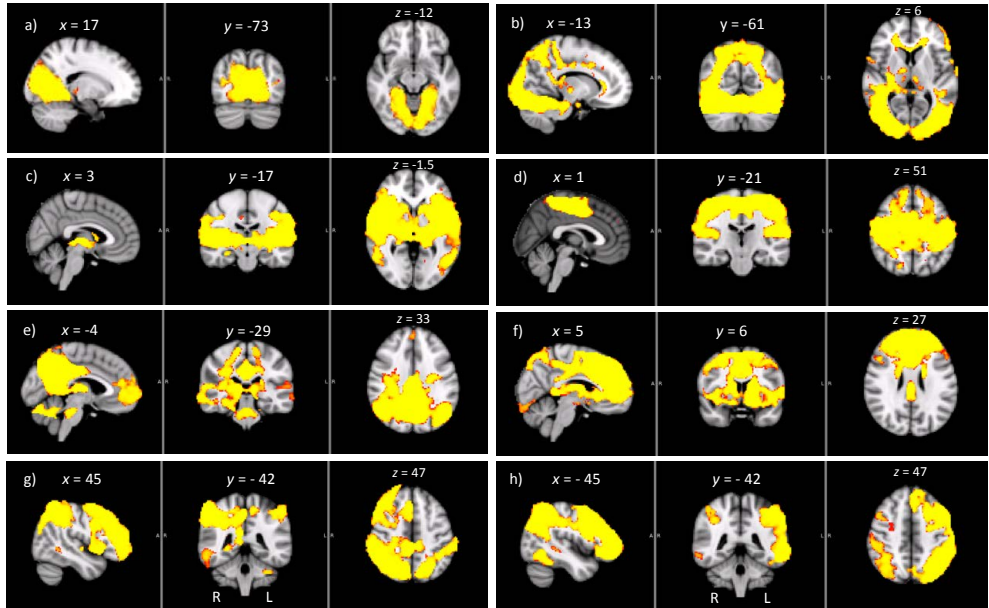
Placebo
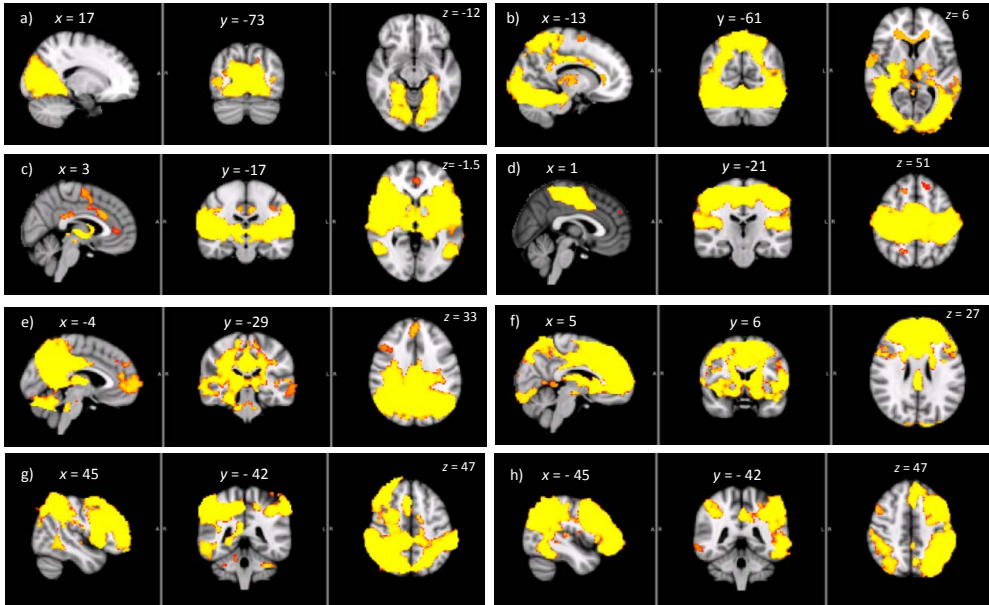
ARA 290

Figure 1. Statistical connectivity maps (P < 0.05, threshold-free cluster enhancement corrected) of the resting-state network connectivity in relation to eight template networks (Beckmann *et al.*, 2005): medial and lateral visual systems (networks a and b respectively), auditory and somatosensory system (network c), sensorimotor system (network d), the default mode network (network e), executive salience network (network f) and visual-spatial and working memory networks (networks g and h), separately for the placebo-group (at the top) and for the ARA290-group (at the bottom).

Chapter 3

Discussion

The present study examined the effect of ARA290, an EPO analogue, on resting state connectivity in healthy volunteers using a randomized, double-blind, placebo-controlled, parallel group design. We did not find evidence that ARA290 affects functional connectivity. Specifically, ARA290 did not affect functional connectivity with brain regions that showed effects in the EPO studies, nor with the networks typically affected by antidepressants. Our results indicate that the differential BOLD response of the bilateral fusiform gyrus in response to fearful *vs* happy faces after ARA290 administration (Cerit *et al.*, under review), is due to task specific activation rather than a general function of the fusiform gyrus as part of a coherent network. The other seeds included the hippocampus and hippocampus-amygdala complex and were based on ROIs which were found to be differentially activated after EPO administration. Although ARA290 did not have an effect on the hippocampus (or amygdala) during task related BOLD response (Cerit *et al.*, under review), these seeds were of interest since ARA290 was expected to elicit similar effects as EPO (Miskowiak *et al.*, 2007b; 2010). Overall, our findings indicate that the currently used dosage of ARA290 does not seem to exert antidepressant-like effects on resting state connectivity.

A strength of our study is that we had a relatively large sample size and power. Furthermore, participants were matched for age, sex and IQ. We conducted a model-free analysis of eight standard resting state networks (Beckmann *et al.*, 2005) which encompasses approx. 80% of the total brain volume (Khalili-Mahani *et al.*, 2012) and, therefore, ensures an thoroughgoing assessment of the possible effects of ARA290 on functional connectivity. However, this study has some limitations which need to be addressed.

We did not apply retrospective correction on our data in order to exclude the noise originating from physiological confounders (e.g., heartbeat, breathing related chest movement, respiration rates). Since physiological noise influences fluctuations in the MRI signal and as a consequence the estimates of functional connectivity (Birn, 2012), it is conceivable that a possible effect of ARA290 has been drown out by physiological noise.

The lack of effect may have been due to several reasons which may need attention in future studies investigating the effect of ARA290 on resting state connectivity. Since this is the first study assessing the antidepressant properties of ARA290 in humans, selecting the right dose and treatment duration was based on previous studies with pain patients. A single dose of 2 mg might have been too low to exert an antidepressant-like effect in healthy participants and/or the effect of ARA290 may have lasted shorter than one week. We might not have been able to examine the effect of ARA290 with the highest effective dose and the time point on which ARA290 is the most effective. Administration of this dose (2 mg i.v.) to somatic patients (i.e., neuropathic pain patients) raised no safety concerns (Heij *et al.*, 2012; Niesters *et al.*, 2013).

Within this context some pharmacological differences should also be mentioned. EPO was administered as 40 000 IU (equal to 336 micrograms) and has a half-life of approx. 5 hours (Miskowiak *et al.*, 2007a; Eckardt *et al.*, 1989; McMahon *et al.*, 1990) whereas, ARA290 was administered as 2000 micrograms to healthy volunteers and has a plasma half-life of 2 minutes (Niesters *et al.*, 2013). Once the IRR receptor is activated by ARA290 it initiates multiple signalling pathways resulting in tissue protective and anti-inflammatory actions. It is possible though that these effects are not enduring as the effects of EPO, since ARA290 and EPO differ in half-life and the exact signalling pathways they activate.

The antidepressant effects of six week sertraline treatment in MDD patients increased corticolimbic connectivity compared to baseline (at which the connectivity was reduced) (Anand *et al.*, 2005), therefore, it is conceivable that the effect of ARA290 may not have been detected due to its assessment in healthy volunteers i.e., in order to detect an antidepressant effect on connectivity associated with affective networks one may need to asses these specific effects in a patient group. On the other hand, administration of conventional antidepressant drug for a period of one to two weeks in healthy volunteers did elicit an difference between intervention and placebo groups as reduced connectivity in healthy volunteers was reported (McCabe and Mishor 2011; van Wingen *et al.*, 2014). Similar to pharmacological resting-state studies carried out with antidepressant drugs (i.e. including daily administration for at least one week), future resting-state studies with ARA290 may also benefit from repetitive administration (Anand *et al.*, 2005; McCabe and Mishor, 2011; van Wingen *et al.*, 2014) and from a baseline assessment in order to exclude intersubject variability (Anand *et al.*, 2005).

The fMRI study with ARA290 administration in the same healthy population yielded modest to small effects on neural and behavioural measures of emotional processing which was measured by means of the neurophysiological model of drug action (Cerit *et al.*, under review). The finding that ARA290 does not affect functional connectivity in the same healthy population does strengthen the possibility that the effect of ARA290 on task related activity was not affected by randomization failure. Although the neuropsychological model of drug action is validated and assesses depression related emotional processes both at neural as well as behavioural level, the emerging view is that instead of different brain regions activated in response to stimuli, alterations in specific neural circuits may underlie depressive symptoms (Wang *et al.*, 2012). Our finding of ARA290 not eliciting an effect on depression related networks may be interpreted as ARA290 lacking the strength to affect functional connectivity in a way that could contribute to observable/detectable antidepressant effects. Nonetheless, a future resting-state study would benefit from designs used in previous pharmacological resting state studies including baseline measurements (Anand *et al.*, 2005), administration of treatment for at least one week and a crossover design (van Wingen *et al.*, 2014). This may provide us with a better understanding of the effects of ARA290 on functional connectivity in healthy volunteers.

References

Anand A, Li Y, Wang Y, Wu J, Gao S, Bukhari L, Mathews VP, Kalnin A, Lowe MJ (2005) Antidepressant Effect on Connectivity of the Mood-Regulating Circuit: An fMRI Study. *Neuropsychopharmacology* 30: 1334-1344.

Beckmann CF, Deluca M, Devlin JT, Smith SM (2005) Investigations into resting-state connectivity using independent component analysis. *Phil Trans R Soc B Biol Sci* 360: 1001–1013.

Birn RM (2012) The role of physiological noise in resting-state functional connectivity. *NeuroImage* 62: 864-870.

Brines ML, Ghezzi P, Keenan S, Agnello D, de Lanerolle NC, Cerami C, Itri LM, Cerami A (2000) Erythropoietin crosses the blood–brain barrier to protect against experimental brain injury. *Proc Natl Acad Sci USA* 97: 10526-10531.

Brines M, Patel NSA, Villa P, Brines C, Mennini T, De Paola M, Erbayraktar Z, Erbayraktar S, Sepodes S, Thiemermann C, Ghezzi P, Yamin M, Hand CC, Xie Q, Coleman T, Cerami A (2008) Nonerythropoietic, tissue-protective peptides derived from the tertiary structure of erythropoietin. *Proc Natl Acad Sci USA* 105: 10925-10930.

Brines M, Cerami A (2012) The Receptor That Tames the Innate Immune Response. *Mol Med* 18: 486–496.

Cerit H, Veer IM, Dahan A, Niesters M, Harmer CJ, Miskowiak KW, Rombouts S, Van der Does W (2014) Testing the Antidepressant Properties of the Peptide ARA290 in a Human Neuropsychological Model of Drug Action. Manuscript sumbitted for publication.

Cole DM, Beckmann CF, Oei NY, Both S, van Gerven JM, Rombouts SA (2013) Differential and distributed effects of dopamine neuromodulations on resting-state network connectivity. *Neuroimage* 78: 59-67.

Eckardt KU, Boutellier U, Kurtz A, Schopen M, Koller EA, Bauer C (1989) Rate of erythropoietin formation in humans in response to acute hypobaric hypoxia. *J Appl Physiol* 66: 1785-1788.

Ehrenreich H, Hinze-Selch D, Stawicki S, Aust C, Knolle-Veentjer S, Wilms S, Heinz G, Erdag S, Jahn H, Degner D, Ritzen M, Mohr A, Wagner M, Schneider U, Bohn M, Huber M, Czernik A, Pollmächer T, Maier W, Sirén AL, Klosterkötter J, Falkai P, Rüther E, Aldenhoff JB, Krampe H (2007a) Improvement of cognitive functions in chronic schizophrenic patients by recombinant human erythropoietin. *Mol Psychiatry* 12: 206-220.

Ehrenreich H, Fischer B, Norra C, Schellenberger F, Stender N, Stiefel M, Siren AL, Paulus W, Nave KA, Gold R, Bartels C (2007b) Exploring recombinant human erythropoietin in chronic progressive multiple sclerosis. *Brain* 130: 2577-2588.

Filippini N, MacIntosh BJ, Hough MG, Goodwin GM, Frisoni GB, Smith SM, Matthews PM, Beckmann CF, Mackay CE (2009) Distinct patterns of brain activity in young carriers of the APOE-epsilon4 allele. *Proc Natl Acad Sci USA* 106:7209–7214.

Gonzalez FF, Abel R, Almli CR, Mu D, Wendland M, Ferriero DM (2009) Erythropoietin sustains cognitive function and brain volume after neonatal stroke. *Dev Neurosci* 31: 403-411.

Harmer CJ, Goodwin GM, Cowen PJ (2009) Why do antidepressants take so long to work? A cognitive neuropsychological model of antidepressant drug action. *Br J Psychiatry* 195: 102-108.

Heij L, Niesters M, Swartjes M, Hoitsma E, Drent M, Dunne A, Grutters JC, Vogels O, Brines M, Cerami A, Dahan A (2012) Safety and efficacy of ARA 290 in sarcoidosis patients with symptoms of small fiber neuropathy: A randomized, double-blind pilot study. *Mol Med* 18: 1430-1436.

Chapter 3

Jenkinson M, Smith S (2001) A global optimisation method for robust affine registration of brain images. *Med Image Anal* 5: 143-156.

Jenkinson M, Bannister P, Brady M, Smith S (2002) Improved optimization for the robust and accurate linear registration and motion correction of brain images. *Neuroimage* 17: 825-841.

Khalili-Mahani N, Zoethout RM, Beckmann CF, Baerends E, De Kam ML, Soeter RP, Dahan A, van Buchem MA, van Gerven JM, Rombouts SA (2012) Effects of morphine and alcohol on functional brain connectivity during ''resting state'': a placebo-controlled crossover study in healthy young men. *Hum Brain Mapp* 33: 1003–1018.

Klumpers LE, Cole DM, Khalili-Mahani N, Soeter RP, Te Beek ET, Rombouts SA, van Gerven JM (2012) Manipulating brain connectivity with δ^9-tetrahydrocannabinol: a pharmacological resting state FMRI study. *Neuroimage* 63: 1701-1711.

McCabe C, Mishor Z (2011) Antidepressant medications reduce subcortical–cortical resting-state functional connectivity in healthy volunteers. *Neuroimage* 57: 1317-1323.

McMahon FG, Vargas R, Ryan M, Jain AK, Abels RI, Perry B, Smith IL (1990) Pharmacokinetics and effects of recombinant human erythropoietin after intravenous and subcutaneous injections in healthy volunteers. *Blood* 76: 1718-1722.

Miskowiak K, O'Sullivan U, Harmer CJ (2007a) Erythropoietin reduces neural and cognitive processing of fear in human models of antidepressant drug action. *Biol Psychiatry* 62: 1244-1250.

Miskowiak K, O'Sullivan U, Harmer CJ (2007b) Erythropoietin enhances hippocampal response during memory retrieval in humans. *J of Neurosci* 27: 2788-2792.

Miskowiak K, Favaron E, Hafizi S, Inkster B, Goodwin G, Cowen P, Harmer CJ (2010) Erythropoietin modulates neural and cognitive processing of emotional information in biomarker models of antidepressant drug action in depressed patients. *Psychopharmacology (Berl)* 210: 419-428.

Nichols TE, Holmes AP (2001) Nonparametric permutation tests for functional neuroimaging: a primer with examples. *Hum Brain Mapp* 15: 1 - 25.

Niesters M, Khalili-Mahani N, Martini C, Aarts L, van Gerven J, van Buchem MA, Dahan A, Rombouts S (2012) Effect of subanesthetic ketamine on intrinsic functional brain connectivity: a placebo-controlled functional magnetic resonance imaging study in healthy male volunteers. *Anesthesiology* 4: 868-877.

Niesters M, Swartjes M, Heij L, Brines M, Cerami A, Dunne A, Hoitsma E, Dahan A (2013) The erythropoietin analog ARA 290 for treatment of sarcoidosis-induced chronic neuropathic pain. *Expert Opin on Orphan Drugs* 1: 77-87.

Shingo T, Sorokan ST, Shimazaki T, Weiss S (2001) Erythropoietin regulates the in vitro and in vivo production of neuronal progenitors by mammalian forebrain neural stem cells. *J Neurosci* 21: 9733-9743.

Smith SM (2002) Fast robust automated brain extraction. *Hum Brain Mapp* 17: 143- 155.

Smith SM, Jenkinson M, Woolrich MW, Beckmann CF, Behrens TE, Johansen-Berg H, Bannister PR, De Luca M, Drobnjak I, Flitney DE, Niazy RK, Saunders J, Vickers J Zhang Y, De Stefano N, Brady JM, Matthews PM (2004) Advances in functional and structural MR image analysis and implementation as FSL. *Neuroimage* 23: 208–219.

Smith SM, Nichols TE. 2009 Threshold-free cluster enhancement: addressing problems of smoothing, threshold dependence and localisation in cluster inference. *Neuroimage* 44: 83–98.

Van Velzen M, Heij L, Niesters M, Cerami A, Dunne A, Dahan A, Brines M (2014) ARA 290 for treatment of small fiber neuropathy in sarcoidosis. *Expert Opin on Investig Drugs* 23: 541-550.

Wang L, Hermens DF, Hickie IB, Lagopoulos J (2012) A systematic review of resting-state functional-MRI studies in major depression. *J of Affect Disord* 142: 6-12.

van Wingen GA, Tendolkar I, Urner M, van Marle HJ, Denys D, Verkes RJ, Fernández G (2014) Short-term antidepressant administration reduces default mode and task-positive network connectivity in healthy individuals during rest. *Neuroimage* 88: 47-53.

Woolrich MW, Ripley BD, Brady M, Smith SM (2001) Temporal autocorrelation in univariate linear modeling of FMRI data. *NeuroImage* 14: 1370–1386.

Chapter 3

Chapter 4

The Effect of Tryptophan on the Cortisol Response to Social Stress Is Modulated by the 5-HTTLPR genotype

H Cerit, LAW Jans, AJW Van der Does

Abstract

Objective: The S'/S' (S/S, S/Lg and Lg/Lg) variant of the serotonin (5-HT) transporter gene linked polymorphic region (5-HTTLPR) is associated with less efficient neurotransmission and may be more reactive to 5-HT manipulations. We tested the effects of l-tryptophan supplements on the cortisol response induced by a social stressor in S'/S' and L'/L' (La/La) carriers.

Methods: In a double-blind parallel design, 25 S'/S' carriers and 21 L'/L' carriers were randomized to take l-tryptophan (2.8 g/d) or placebo supplements for six days. At day 7 participants were exposed to the Trier Social Stress Test. Salivary cortisol and subjective mood states were monitored before, during and after the stress procedure.

Results: S'/S' carriers who took l-tryptophan supplements had a significantly lower cortisol response to stress than S'/S' carriers who took placebo. Tryptophan had no effect on cortisol in L'/L' carriers and no effect on subjective mood states in either genotype group.

Conclusion: Tryptophan attenuates the cortisol response to acute social stress depending on 5-HTTLPR genotype. S'/S' carriers may indeed be more reactive to 5-HT manipulations.

Introduction

The serotonin (5-hydroxytryptamine; 5-HT) neurotransmission system and the hypothalamic-pituitary-adrenal axis (HPA axis) have complex interrelationships (Porter *et al.*, 2004). Both systems have important roles in the response to stress and are implicated in depression. In the present study, we examined the effect of the 5-HT precursor l-tryptophan (TRP) on reactivity of the HPA axis, specifically investigating whether this effect is modulated by genetic variation in the 5-HT system.

The serotonin transporter (5-HTT) membrane protein is essential in regulating the concentration of 5-HT in the synaptic cleft. The 5-HTT is encoded by the SLC6A4 gene, and transcriptional activity of this gene is regulated by the 5-HTT-linked polymorphic region (5-HTTLPR). Two major variants of the 5-HTTLPR exist, with functional significance in that the short (S) allele of the 5-HTTLPR is associated with less transcriptional efficiency and less 5-HTT expression than is the long (L) allele (Lesch *et al.*, 1996). A functional A/G polymorphism (rs25531) has been found within the L allele (Nakamura *et al.*, 2000) as well, indicating that the 5- HTTLPR is functionally triallelic (S / Lg / La). The Lg variant is associated with reduced 5-HTT transcriptional efficiency, comparable to the S allele (Hu *et al.*, 2006; Praschak-Rieder *et al.*, 2007; Reimold *et al.*, 2006). The reduced efficiency 5-HTT variants (S and Lg) are referred to as S', the La variant as L'. Biallelic classifications are indicated as S and L.

Several studies have found that differences in 5-HTTLPR genotype are associated with differing HPA axis reactivity in healthy individuals. Healthy girls (aged between 9 and 14 years) who were homozygous for the S allele showed an increase in cortisol response during and following exposure to a laboratory stress task, whereas girls carrying at least one copy of the L allele had a slight decrease in cortisol response (Gotlib *et al.*, 2008). This differential pattern of cortisol response was independent from history of depression in participants' mothers. Way and Taylor (2010) exposed healthy young adults to a similar social stress task and found that individuals homozygous for the S allele had a significantly stronger cortisol response 40 minutes after onset of the stressor. The individuals homozygous for the L allele had the least response, with the heterozygous (S/L) falling in between.

Wüst *et al.* (2009), however, did not observe an effect of 5-HTTLPR on the cortisol response to a social stress task. This study had a large sample size (N = 216) and grouped individuals based on triallelic classification. The non-replication may have been caused by the fact that all 126 female participants used oral contraceptives, which dampen the cortisol response to stress (Kirschbaum *et al.*, 1999). Another recent study reported no effect of 5-HTTLPR genotype or neuroticism on the cortisol response to social stress in 94 college students (Verschoor & Markus, 2011). Mueller *et al.* (2011) even reported a **larger** cortisol response to social stress in both younger (18-31 years) and older (54-68 years) adults who were homozygous for the L' allele than individuals carrying at least one S' allele. This effect was not observed in children (8-12 years). In the younger adults (N = 106), genotype interacted with early (during the first five years of life) stressful life events: L'/L' individuals had a much higher cortisol response to

stress than S' carriers in the absence of early stressful life events. This pattern was reversed for people who had experienced three or more early life events. In a study with new-borns, S'/S' individuals had a larger cortisol response to a pain stimulus (heel prick) (Mueller *et al.*, 2010). Finally, male S'/S' carriers with a history of stressful life events had a larger cortisol response 35 minutes after the onset of the social stressor than male S'/S' carriers without such history. The cortisol response in S'/S' carriers with a history of stressful life events was also higher than that of male S'/L' and L'/L' carriers (with and without life events) (Alexander *et al.*, 2009).

The central theme of these reports demonstrates that 5-HT function and 5-HTTLPR genotype affect HPA axis reactivity to social stress; however, the direction of this interaction has yet to be conclusively established. Age as well as both type and time of occurrence of stressful life events have been suggested to play a role in the effect of gene-environment interactions on cortisol response (Mueller *et al.*, 2011).

The relationship between the 5-HT system and HPA axis reactivity has also been investigated by manipulating tryptophan (TRP) levels in stress-vulnerable populations, as defined by family or personal history of depression or by questionnaire scores indicating vulnerability, e.g. high neuroticism. Experimental interventions that temporarily increase TRP include: carbohydrate rich/protein poor (CR/PP) meal (Markus *et al.*, 1998; Markus *et al.*, 2000b), tryptophan-rich egg protein hydrolysate (EPH) (Markus *et al.*, 2010), carbohydrate rich drink (Markus, 2007), whey protein α-lactalbumin (α-lac) (Markus *et al.*, 2000a; Merens *et al.*, 2005; Booij *et al.*, 2006; Nesic & Duka, 2008) and tryptophan-rich hydrolyzed protein (HP) (Firk & Markus, 2009). Stress responses were induced with the cold pressor test (CPT), arithmetic stress task (under noise stimulation) or public speaking. Stress-vulnerable populations were expected to benefit from increasing TRP availability, as expressed in improved mood and lower cortisol responses to stress. The above-mentioned interventions and stressors revealed no consistent pattern of effects. The lack of a consistent effect may have been due to methodological limitations, including: a relatively small impact of the interventions on tryptophan concentrations, less than optimal timing of the cortisol measurements, or the nature of the stressor. Non-social stressors (e.g. cold pressor) are less reliable in eliciting a cortisol response than are social stressors (e.g. public speaking) (Dickerson & Kemeny, 2004).

A recent study combined a TRP manipulation and stress exposure with measurement of 5-HTTLPR genotype (Markus & Firk, 2009). Sixteen healthy S'/S' and 14 L'/L' carriers were exposed to stress one hour after a single dose tryptophan (0.8 g) and after taking placebo in a cross-over design. The stress procedure consisted of a combination of backward counting tasks and cold-pressor exposures at unpredictable intervals. TRP improved mood and reduced backward counting errors in S'/S' participants but not in L'/L' participants, and no group differences were found on the cortisol response to stress. Remarkably, cortisol concentrations were lower at post-stress than at pre-stress regardless of intervention or genotype. The number of cortisol measurements was limited to single pre- and post-

stress samplings. The authors concluded that TRP challenge improves mood and stress performance in S'/S' participants (Markus & Firk, 2009). 'Stress performance' referred to the number of errors in the backward counting task that was part of the stressor. Under placebo conditions, S'/S' participants made more mistakes than L'/L' participants, which seems to have been corrected by TRP.

Given the methodological issues in previous TRP loading studies, we examined the effect of six days of TRP supplementation on reactivity of the HPA axis to a social stressor, specifically investigating whether its role is modulated by 5-HTTLPR genotype. We used a parallel design, exposing every participant to the stressor only once. Furthermore, we monitored physiological data for 95 minutes at six time points pre- and post-stress. Possible confounders of the stress response were avoided by excluding users of oral contraceptives and by testing female participants during the luteal phase of their menstrual cycle. We tested the following hypotheses: 1) S'/S' carriers have larger cortisol responses to social stress than L'/L' carriers; 2) the increased cortisol response to stress of S'/S' carriers will be reduced by tryptophan.

Chapter 4

Methods and Materials

Participants

Participants were selected from a pool of 581 genotyped individuals who had been recruited at various sites at Leiden University and through local advertisements. Participants were non-smokers and were included if all four grandparents were West-European. For the present study, the age range was 18 to 35 years and Body Mass Index was between 19 and 29 kg/m²). Exclusion criteria were a current diagnosis of depression or post-traumatic stress disorder, a lifetime history of psychosis, and use of medication, including oral contraceptives. The presence of anxiety disorders was also assessed, but this was not an exclusion criterion. The following two genotype groups S'/S' (S/S, S/Lg and Lg/Lg variants) and L'/L' (La/La variant) were invited to participate in the present study. Participants received a reward of € 40. The research was approved by the Ethics Committee of the Leiden University Medical Center in The Netherlands.

Genotyping

Genetic Assessment. DNA was obtained using the Oragene Self-Collection Kit – DISC format (DNA Genotek Inc, Ottawa, ON, Canada); 200 µl of saliva was collected in lysis buffer (100 mM NaCl, 10 mM EDTA, 10 mM Tris pH 8, 0.1 mg/ml proteinase K and 0.5% w/v SDS) until further processing. Genomic DNA was isolated from the samples using the Chemagic buccal swab kit on a Chemagen Module I workstation (Chemagen Biopolymer-Technologie AG, Baesweiler, Germany). DNA concentrations were quantified by OD260 measurement and by agarose gel electrophoresis. The average yield was approximately 4 mg of genomic DNA per sample.

Polymerase chain reaction amplification

The region of interest from the 5-HTT gene was amplified by triplex PCR using the following primers: a FAM-labeled primer HTTLPR-FWFAM 5'-TCCTCCGCTTTGGCGCCTCTTCC-3', and a reverse primer HTTLPR-RV 5'-TGGGGGTTGCAGGGGAGATCCTG-3'. Typical PCR reactions contained between 10 and 100 ng genomic DNA template, 10 pmol of forward and reverse primer. PCR was carried out in the presence of 5% DMSO with 0.5U of BioThermAB polymerase (GeneCraft, Munster, Germany) in a total volume of 30 µl using the following cycling conditions: initial denaturation step of 5 min at 95°C, followed by 40 cycles of 30 sec 96°C, 30 sec 61°C, 60 sec 72°C and a final extension step of 10 min 72°C. After PCR 5 µl of the sample was subjected to restriction digestion with the enzyme HpaII in a total volume of 20 µl. Restriction enzyme mix was incubated with DNA for 3 hours at 37°C.

Analysis of PCR products

One µl of PCR product before and after restriction digestion was mixed with LIZ-500 size standard and formamide and run in two separate lanes on an AB 3100 genetic analyser set up for genotyping with 50 cm capillaries. Results were analysed using Genescan software version 3.7 (Applied Biosystems, Carlsbad, CA, USA), and alleles were scored visually according to the following scheme: Uncut: S, 469 bp; L, 512 bp. Cut: Sg, 402 + 67 bp; Lg, 402 + 110 bp.

Instruments

<u>Diagnosis</u>. The Mini International Neuropsychiatric Interview (M.I.N.I.) was administered (Sheehan *et al.*, 1997; Van Vliet *et al.*, 2000) to assess psychiatric diagnoses.

<u>Psychiatric symptoms and mood states</u>. Three self-report questionnaires were used. Current symptoms of anxiety and depression were measured using the hospital anxiety and depression scale (HADS; Zigmond & Snaith, 1983; Spinhoven *et al.*, 1997). The HADS is a 14-item self-report scale which assesses past-week severity of anxiety and depressive symptoms. Positive and negative affect was assessed by means of the Positive and Negative Affectivity Scales (PANAS), a 20-item questionnaire (Watson *et al.*, 1988). The state version (today) was used. The mood states Sadness, Annoyance, Tension and Anxiety were assessed using single-item Mood States Scales (MSS) with scores ranging from 0 (not at all) to 10 (extremely).

Stress Induction

The Trier Social Stress Test (TSST; Kirschbaum *et al.*, 1993) is a combination of a public speaking task in front of an unresponsive audience and a mental arithmetic task at high speed and with public correction of every mistake. During an initial resting period of 50 minutes, baseline saliva cortisol samples were obtained at time points t_{15} and t_{50}. Subsequently, participants were informed that they were about to give a speech in front of an audience consisting of three persons, and that they would also complete another task that this audience would announce to them (i.e. the mental arithmetic task). The TSST protocol further prescribes that participants were informed that their speech would be videotaped and evaluated by skilled psychologists with regard to content and performance. During the verbal instructions the experimenter briefly showed the room with the audience waiting for him/her. Subsequently, participants were placed in a quiet room and given six minutes to prepare themselves. They were instructed that it was not allowed to keep any notes during their speech. At the end of this anticipation period, another saliva sample was taken and the participant entered the room in order to deliver the speech and complete the arithmetic task. At the end of the arithmetic task, the experimenter entered the room and took another saliva sample in front of the audience. Subsequently, the participant was guided to a quiet room and was allowed to rest and read magazines for 45 minutes. Saliva samples were collected at six points in time (with t_0 representing the time of arrival): rest 1 (t_{15}), rest 2 (t_{50}), after anticipation (t_{56}), after

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speech and arithmetic task (t_{65}) and twice during the recovery period (t_{90} and t_{110}). During saliva sampling participants rated their mood states on the MSS.

Salivary cortisol assessment

Salivary cortisol was assessed using Salicaps (IBL International, Germany). Saliva samples were stored at -20°C until assayed at the laboratory of biopsychology at the University of Dresden, Germany. Free cortisol concentrations in saliva were measured using a commercially available "Luminescence Immunoassay for the in-vitro-diagnostic quantitative determination of cortisol in human saliva and serum" (IBL, Hamburg, Germany). The intra and interassay coefficients of variance for cortisol was below 8%.

Design and procedure

This study was a randomized double-blind placebo-controlled trial with stratification for genetic variation of the 5-HTTLPR genotype. Participants were randomly allocated to receive 7 capsules containing either TRP (total dose of 2.8 g/day) or placebo (cellulose microcrystalline) for a period of six days. The dosage and duration were based on previous studies that had shown social-behavioural effects of TRP administration (3 g/day) after a period of 15 days (Aan het Rot *et al.*, 2006) and cognitive effects after a single dose of 0.8g TRP (Markus & Firk, 2009). The experimental procedure included two visits to the laboratory, pre- and post-TRP.

First visit to laboratory.

Upon arrival at the laboratory, participants provided written informed consent for the study. Following the M.I.N.I. interview, participants performed experimental tasks and filled out questionnaires on a computer. At the end of the first visit, participants were provided with 42 capsules that contained 400 mg tryptophan or placebo (PLC). Oral and written instructions were provided to the participants regarding the timing of administration of capsules and lifestyle restrictions during the next six days and on the day of the second lab visit.

Tryptophan supplementation

Participants started to take the capsules the day after their first lab visit. They were instructed to take two capsules in the morning, two in the afternoon (before meals) and three in the evening (before 23.00h). Participants received a diary in which they were asked to write down the exact time of intake and number of capsules. Compliance was not measured through blood sample analyses, however participants were led to believe that compliance would be assessed at post-intervention through a saliva sample.

Lifestyle instructions included: no smoking, no use of dietary supplements and vitamins and consumption of alcohol limited to 3 units/day. Participants were also instructed to refrain from alcohol and caffeine-containing consumptions and avoid high carbohydrate meals on the day of their second visit. Further instructions for the day of the second visit included: no eating and drinking one hour before arriving at the laboratory (except water), and no physical exercise at least two hours before arrival. Female participants were tested in the luteal phase of their menstrual cycle. All test sessions started in the afternoon between noon and 5pm.

Second visit to laboratory

Upon arrival at the lab participants handed in their diary regarding the intake of capsules. In addition, they were asked to fill out a debriefing questionnaire regarding compliance to the instructions during the previous six days. They were also interviewed about their compliance to the instructions for the second lab visit. Next, participants were asked to perform a number of tests and to fill out questionnaires on a computer. Finally, participants performed the TSST. After completion of the TSST procedure participants were fully debriefed and paid.

Chapter 4

Results

Sample characteristics

For the total sample of n = 581, genotype frequencies were as follows: SS, 16,9%; SLg, 4,8%; LgLg, 0,7%; LaLg, 8,6%; SLa, 43%; LaLa, 26%. Participants were divided on the basis of the triallelic classification (Lg alleles were collapsed with S variants into three genotype groups: S'/S' (n = 130); L'/S' (n = 300); L'/L' (n = 151). Genotype frequencies were consistent with Hardy–Weinberg Equilibrium ($\chi2$ (1) = 0.67; p = 0.41). We contacted 92 S'/S' carriers and 92 L'/L' carriers by email. Sixty-four S'/S' and 66 L'/L' carriers expressed interest in the study. After screening for in- and exclusion criteria we included 26 S'/S' and 22 L'/L' participants. In each group, one participant dropped out before day 2. Analyses were conducted on 25 S'/S' and 21 L'/L' carriers.

The first two participants (one S'/S' and one L'/L' carrier) received tryptophan single blind. Since the TSST panel was double-blind for these participants and no observer ratings were collected, we kept these participants in the analyses. The demographic details of both groups are shown in Table 1. Groups did not differ significantly on assessed demographic characteristics. One participant in the PLC group (S'/S' carrier) had a current diagnosis of panic disorder, and one participant in the TRP group (S'/S' carrier) had a specific phobia (needles). Both participants were not taking any medication.

Table 1. Demographic characteristics for both genotype groups.

	S'/S' (S/S, S/Lg, Lg/Lg)	L'/L' (La/La)
Female	11	11
Male	14	10
Age (M±SD)	20.4 ± 3.5	20.3 ± 2.5
BMI (M±SD)	19.4 ± 3.0	19.2 ± 1.7

Note: Mean ± Standard Deviation. Abbreviation: BMI, Body Mass Index.

Compliance

According to self-report, approximately 98% of the capsules were taken according to instructions. The minimum percentage of capsules taken by a participant was 69%. Three participants had taken two capsules in the morning before the second lab visit. All these participants were retained.

Effects of TRP on psychiatric symptoms

In order to analyse the effects of intervention and genotype on psychiatric symptoms, separate RM-GLMs for each of the questionnaire scores (HADS Anxiety, HADS Depression, PANAS-S Pos and PANAS-S Neg) were conducted with Time as within subject factor, and Intervention (TRP *vs.* PLC) and Genotype (S'/S' *vs.* L'/L') as between subject factors. No significant effects were found for intervention or genotype (Table 2).

Table 2. Symptom scores for each genotype and intervention group assessed pre- and post-intervention.

		Anxiety	Depression	Negative Affect	Positive Affect
S'/S' TRP	Pre	3.6 ± 2.0	1.9 ± 1.2	12.1 ± 4.3	30.1 ± 6.7
	Post	3.8 ± 2.4	2.8 ± 2.8	13.3 ± 3.3	32.1 ± 5.2
S'/S' PLC	Pre	4.8 ± 3.3	1.4 ± 1.6	12.8 ± 2.8	32.6 ± 4.0
	Post	3.7 ± 3.7	1.3 ± 1.5	12.3 ± 2.3	34.6 ± 4.9
L'/L' TRP	Pre	3.8 ± 2.4	1.4 ± 1.4	12.2 ± 2.7	30.3 ± 8.5
	Post	4.2 ± 3.3	1.5 ± 1.4	11.9 ± 2.1	28.5 ± 8.9
L'/L' PLC	Pre	3.9 ± 3.8	2.4 ± 2.6	13.1 ± 2.4	29. 5 ± 5.9
	Post	3.7 ± 2.2	2.5 ± 2.3	15.4 ± 7.0	31.9 ± 8.2

Note: Mean ± Standard Deviation. Abbreviations: TRP, tryptophan; PLC, placebo.

Cortisol Response to TSST

The cortisol data were not normally distributed. Log^{10}-transformations were successful in normalizing the distributions. Analyses of transformed data are reported, but the figures represent untransformed data. RM-GLMs were conducted in the S'/S' and L'/L' groups separately on cortisol concentrations, with Time (the six cortisol measurements) as within subjects factor and Intervention (TRP *vs.* PLC) as a between subjects factor. Gender was included as covariate. Greenhouse-Geisser statistics are reported. In the S'/S' group, the main effect of Time was significant ($F(1.50, 33.07) = 6.72$, $p = 0.007$, $\eta p^2 = 0.234$) and the Time x Intervention interaction was borderline significant ($F(1.50, 33.07) = 3.55$, $p = 0.052$, $\eta p^2 = 0.139$). In the L'/L' group, the main effect of Time was a trend ($F(1.76, 31.70) = 3.09$, $p = 0.065$, $\eta p^2 = 0.146$) and the interaction was non-significant. Figure 1 displays the cortisol concentrations over time by intervention and genotype.
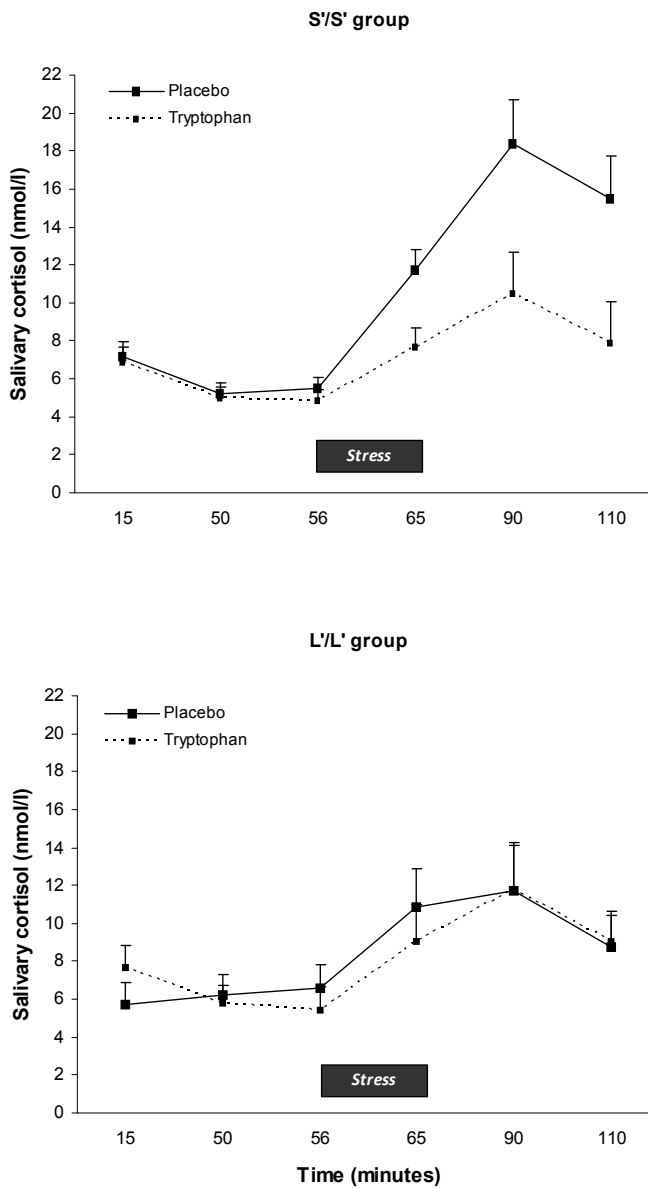
Figure 1. Salivary cortisol (nmol/l) before, during and after exposure to social stress in S'/S' group (Figure 1A) and in L'/L' group (Figure 1B).

To further probe the Time x Intervention interaction effect within the S'/S' group, Independent-samples t-tests were conducted, comparing cortisol concentrations at each time point between TRP- and placebo-treated individuals. Significant differences were observed at t_{65}, t_{90} and t_{110}. Within the S'/S' group, the TRP-treated participants differed significantly from

PLC-treated participants at t_{65} [$t(19)$= -2.73, p= 0.012, d = 1.11]. Significant differences were also observed at t_{90} [$t(23)$=-2.27, p= 0.033, d= 0.92] and at t_{110} [$t(23)$=-2.29, p= 0.031 d=0.92].

Subjective mood response to TSST

A separate analysis was conducted for each scale of the MSS questionnaire. RM-GLMs with Time as within subjects factor, Intervention and Genotype as between subjects factors and Gender as covariate revealed a significant main effect of Time for the Tension scale (F(2.78, 113.95) = 32.76, p < 0.01, ηp^2= 0.444), Anxiety scale (F(1.77, 72.59) = 11.23, p < 0.01, ηp^2= 0.215) and Annoyance scale (F(2.37, 97.24) = 4.46, p = 0.01, ηp^2= 0.098). Tension, Anxiety and Annoyance scores varied between 0 and 5; after reaching a peak at t_{56} (after anticipation stress) and t_{65} (after speech and arithmetic task), the scores normalized to 0-1 at t_{90} and t_{110} (rest) in all intervention and genotype groups. The ratings of sadness remained between 0 and 1 at all time points (F(2.16, 88.72) = 0.41, p = 0.68, ηp^2= 0.010) (Figure 2).

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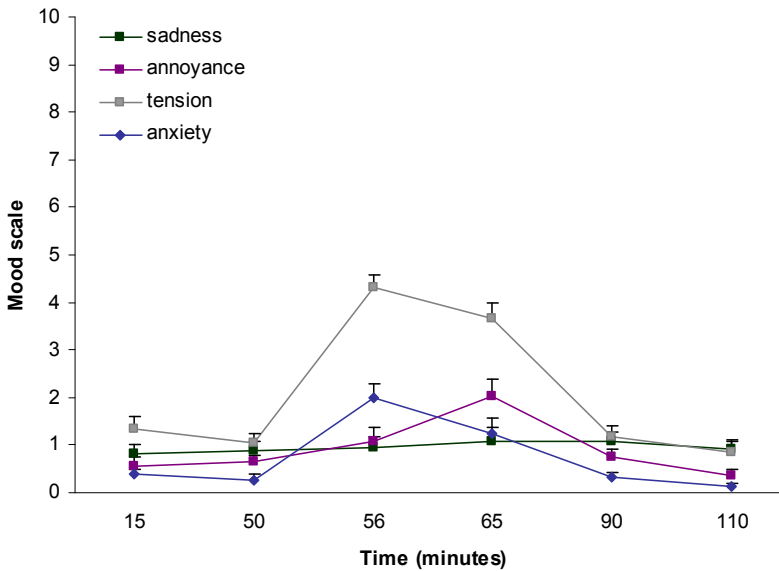
Figure 2. Mood state scores before, during and after exposure to social stress across genotype and intervention group.

Discussion

Six days of TRP supplements attenuated the cortisol response to stress in S'/S' carriers of the serotonin transporter gene. Following social stress, significantly lower cortisol concentrations were found in S'/S' carriers treated with TRP than in S'/S' carriers treated with placebo. TRP had no effect in L'/L' carriers, whose cortisol curves were comparable to TRP-treated S'/S' carriers. These effects of TRP on cortisol response to stress were observed in the absence of any effects on anxiety, depressive symptoms, or affect. Furthermore, neither genotype nor intervention had an effect on the subjective mood response to the TSST.

Previous studies have found differential HPA axis activity depending on 5-HTTLPR genotype (Gotlib *et al.*, 2008; Alexander *et al.*, 2009; Wüst *et al.*, 2009; Way & Taylor, 2010, Mueller *et al.*, 2010, Mueller *et al.*, 2011), but the effects of TRP supplements on this association had not been investigated. Only one study has investigated the effects of TRP (single dose, 0.8g) in S'/S' *vs.* L'/L' individuals (Markus & Firk, 2009), and found no effect on the cortisol response to stress in either genotype group. Notably, in this prior study the stressor failed to produce a rise in cortisol. While the lack of a cortisol response to stress in control conditions negated the opportunity to observe a dampening of this effect, a small improvement of mood was noted after 0.8 g TRP in S'/S' carriers but not in L'/L' carriers.

In the present study, we found no effect of TRP on anxiety and depression symptoms or mood states during exposure to public speaking stress in either L'/L' or S'/S' carriers. While the present findings seem to conflict with those of Markus & Firk (2009), the latter utilized pooled mood states (pre- and post-intervention) and also employed a crossover design. This means that their participants were exposed to the same stressor twice. Anticipation stress may have been different during the second administration. Earlier studies, in which genotype had not been assessed, found only small and rather inconsistent effects of tryptophan loading on mood and stress response (Markus *et al.*, 1998; Markus *et al.*, 2000a; Markus *et al.*, 2000b; Merens *et al.*, 2005; Markus, 2007; Nesic & Duka, 2008; Firk & Markus, 2009; Markus *et al.*, 2010). In contrast to the aforementioned studies, we pre-selected our participants based on genotype, used a longer intervention period of six days, and carefully selected participants to eliminate potential confounders as much as possible (e.g. use of contraceptives; smoking).

Although the complex relationship between HPA axis reactivity and 5-HTTLPR genotype remains to be further elucidated, several studies have indicated that neurobiological responses to negative or threatening stimuli are mediated by 5-HTTLPR genotype. Healthy S carriers had greater amygdala reactivity to negative facial expressions (e.g., fearful and angry) than L/L carriers in a face matching task (Hariri *et al.*, 2002; Hariri *et al.*, 2005). If 5-HTTLPR affects amygdala response to negative environmental stimuli in general, this may in turn also mediate HPA axis reactivity (Way and Taylor, 2009). At the molecular level the serotonin transporter promoter polymorphism alters SLC6A4 transcription efficiency and the level of

serotonin transporter function (Lesch *et al.*, 1996; Greenberg *et al.*, 1999). It remains unclear exactly how the 5-HTT polymorphism alters neurochemical processes between the pre- and post- synaptic cells. 5-HTTLPR genotype-related alterations found so far include: differential levels of extracellular 5-HT concentrations, difference in 5-HT clearance, changes in tissue 5-HT concentrations, and 5-HT synthesis and turnover in the brain (as reviewed by Murphy & Lesch, 2008). Since the short variant is associated with reduced uptake or clearance of 5-HT as compared with the long variant, it is also associated with higher concentrations of 5-HT in the synaptic cleft. Consequently, presynaptic 5HT1A autoreceptors may be overstimulated in S carriers by the increased 5-HT concentrations. The negative feedback mechanism of these autoreceptors causes a decrease in 5-HT synthesis in the presynaptic cell, reducing the amount of 5-HT in the synapse (Hoyer *et al.*, 1994). Subchronic administration of TRP might compensate this process and result in an attenuated cortisol response to social stress.

In summary, the 5-HTT polymorphism changes the dynamic between post- and pre- synaptic cell and this may also influence peripheral adrenomedullary and hypothalamo-pituitary responses (Murphy & Lesch 2008). As the precise neurochemical differences between S' and L' carriers remain unclear, the underlying mechanisms of our findings remain speculative.

Limitations and future directions

Limitations of our study include the relatively small sample size and the fact that we checked compliance only by self-report and not by measuring TRP concentrations in plasma before and after intervention. Self-reported compliance was excellent. To optimize compliance, participants kept a diary of the exact time of intake of capsules and had been led to believe that compliance would be checked through a saliva sample.

TRP supplementation of six days (2.8 g/day) may be used in further studies as an experimental intervention to increase TRP levels in 5-HT vulnerable populations (i.e. serotonergic genotypes associated with stress vulnerability). In this study we have shown that a group with a specific genotype seems to benefit more from TRP supplements, as TRP attenuated the cortisol response to stress. Future studies may investigate whether the same effects can also be reached with lower dosages of TRP supplementation. Another essential question that needs to be answered is the maximum duration of the intervention. The daily requirement of tryptophan for humans is between 3 and 5 mg/kg per day (World Health Organization, 2002). We administered 2800 mg/day, which is almost ten times this daily requirement. Although high tryptophan intake has no serious adverse effects in animals and humans (Garlick, 2004), the mechanism and safety of long-term and high-dose TRP supplementation needs further investigation (Le Floc'h *et al.*, 2010). For instance, cognitive dysfunctions have been observed after intravenous TRP infusion in healthy first-degree relatives of patients with bipolar disorder (Sobczak *et al.*, 2003). In 'quarrelsome' individuals, however, a high dose of tryptophan (3 g/d) for 15 days decreased quarrelsome behaviours and increased agreeableness (Aan het Rot *et al.*, 2006). Future studies may further investigate

how the effects of TRP supplements on the cognitive, physiological and behavioural levels depend on serotonergic genotypes. Research on TRP supplementation in individuals with serotonergic genotypes conferring vulnerability to stress might give new insights in personalized treatment of mood disorders.

References

Aan het Rot, M., Moskowitz, D.S., Pinard, G., Young, S.N., 2006. Social behaviour and mood in everyday life: the effects of tryptophan in quarrelsome individuals. *J. Psychiatry Neurosci*. 31: 253-262.

Alexander, N., Kuepper, Y., Schmitz, A., Osinsky, R., Kozyra, E., Hennig, J., 2009. Gene-environment interactions predict cortisol responses after acute stress: Implications for the etiology of depression. *Psychoneuroendocrinology* 34: 1294-1303.

Booij, L., Merens, W., Markus, R., Van der Does, A.J.W., 2006. Diet rich in α-lactalbumin improves memory in unmedicated recovered depressed patients and matched controls. *J. of Psychopharm*. 20(4): p. 526-535.

Dickerson, S.S., Kemeny, M.E., 2004. Acute stressors and cortisol responses: A theoretical integration and synthesis of laboratory research. *Psychol. Bull.* 130: 355-391.

Firk, C., Markus, C.R., 2009. Mood and cortisol responses following tryptophan-rich hydrolyzed protein and acute stress in healthy subjects with high and low cognitive reactivity to depression. *Clin. Nutr.* 28: 266-271.

Garlick, P.J., 2004. The nature of human hazards associated with excessive intake of amino acids. *J. Nutr.* 134: 1633S-1639S.

Greenberg, B.D., Tolliver, T.J., Huang S.J., Li Q., Bengel D., Murphy, D.L., 1999. Genetic variation in the serotonin transporter promoter region affects serotonin uptake in human blood platelets. *Am J Med Genet* 88:83–87.

Gotlib, I.H., Joormann, J., Minor, K.L., Hallmayer, J., 2008. HPA Axis Reactivity: A Mechanism Underlying the Associations Among 5-HTTLPR, Stress, and Depression. *Biol. Psychiatry* 63: 847-851.

Hariri, A.R., Mattay, V.S., Tessitore A., Kolachana, B., Fera, F., Goldman, D., Egan, M.F., Weinberger, D.R., 2002. Serotonin Transporter Genetic Variation and the Response of the Human Amygdala. *Science* 297: 400–403.

Hariri, A.R., Drabant, E.M., Munoz, K.E., Kolachana, B.S., Mattay, V.S., Egan, M.F., Weinberger, D.R., 2005. A Susceptibility Gene for Affective Disorders and the Response of the Human Amygdala. *Arch Gen Psychiatry* 62:146-152.

Hoyer, D., Clarke, D.E., Fozard, J.R., Hartig, P.R., Martin, G.R., Mylecharane, E.J., Saxena, P.R., Humphrey, P.P.A., 1994. International Union of Pharmacology classification of receptors for 5-hydroxytryptamine (Serotonin). *Pharmacol Rev* 46: 157-203.

Hu, X.Z., Lipsky, R.H., Zhu, G., Akhtar, L.A., Taubman, J., Greenberg, B.D., *et al.*, 2006. Serotonin transporter promoter gain-offunction genotypes are linked to obsessive—compulsive disorder. *Am. J. Hum. Genet.* 78, 815—826.

Kirschbaum, C., Pirke, K.M., Hellhammer, D.H., 1993. The "Trier Social Stress Test". *Neuropsychobiology* 28: 76-81.

Kirschbaum, C., Kudielka, B.M., Gaab, J., Schommer, N.C., Hellhammer, D.H., 1999. Impact of gender, menstrual cycle phase, and oral contraceptives on the activity of the hypothalamus-pituitary-adrenal axis. *Psychosom. Med.* 61: 154-162.

Le Floc'h, N., Otten, W., Merlot, E., 2010. Tryptophan metabolism, from nutrition to potential therapeutic applications. *Amin. Ac.* 1-11.

Lesch, K.P., Bengel, D., Heils, A., Sabol, S.Z., Greenberg, B.D., Petri, S., Benjamin, J., Müller, C.R., Hamer, D.H., Murphy, D.L., 1996. Association of Anxiety-Related Traits with a Polymorphism in the Serotonin Transporter Gene Regulatory Region. *Science* 274: 1527-1531.

Markus, C.R., Panhuysen, G., Tuiten, A., Koppeschaar, H., Fekkes, D., Peters, M.L., 1998. Does carbohydrate-rich, protein-poor food prevent a deterioration of mood and cognitive performance of stress-prone subjects when subjected to a stressful task? *Appetite* 31: 49-65.

Markus, C.R., Olivier, B., Panhuysen, G.E.M., Van der Gugten, J., Alles, M.S., Tuiten A., Westenberg, H.G.M., Fekkes, D., Koppenschaar, H.F., De Haan, E.E.H.F., 2000a. The bovine protein α-lactalbumin increases the plasma ratio of tryptophan to the other large neutral amino acids, and in vulnerable subjects raises brain serotonin activity, reduces cortisol concentration, and improves mood under stress. *Am. J. Clin. Nutr.* 71: 1536-1544.

Markus, C.R., Panhuysen, G., Tuiten, A., Koppeschaar, H., 2000b. Effects of food on cortisol and mood in vulnerable subjects under controllable and uncontrollable stress. *Physiol. Beh.* 70: 333-342.

Markus, C.R., 2007. Effects of carbohydrates on brain tryptophan availability and stress performance. *Biol. Psychol.* 76: 83-90.

Markus, C.R., Firk, C., 2009. Differential effects of tri-allelic 5-HTTLPR polymorphisms in healthy subjects on mood and stress performance after tryptophan challenge. *Neuropsychopharmacology* 34: 2667-2674.

Markus, C.R., Verschoor, E., Firk, C., Kloek, J., Gerhardt, C.C., 2010. Effect of tryptophan-rich egg protein hydrolysate on brain tryptophan availability, stress and performance. *Clin. Nutr.* 29: 610-616.

Merens, W., Booij, L., Markus, R., Zitman, F.G., Onkenhout, W., Van der Does, A.J.W., 2005. The effects of a diet enriched with α-lactalbumin on mood and cortisol response in unmedicated recovered depressed subjects and controls. *Br. J. Nutr.* 94: 415-422.

Mueller ,A., Brocke, B., Fries, E., Lesch, K.P., Kirschbaum, C., 2010. The role of the serotonin transporter polymorphism for the endocrine stress response in newborns. *Psychoneuroendocrinology* 35: 289-296.

Mueller , A., Armbruster, D., Moser, D.A., Canli, T., Lesch, K.P., Brocke, B. and Kirschbaum, C., 2011. Interaction of serotonin transporter gene-linked polymorphic region and stressful life events predicts cortisol stress response. *Neuropsychopharmacology* 36: 1332-1339.

Murphy, D.L., Lesch, K.P., 2008. Targeting the murine serotonin transporter: insights into human neurobiology. Nat. Rev. *Neuroscience* 9, 85-96.

Nakamura, M., Ueno, S., Sano, A., Tanabe, H., 2000. The human serotonin transporter gene linked polymorphism (5-HTTLPR) shows ten novel allelic variants. *Mol. Psychiatry* 5, 32—38.

Nesic, J., Duka, T., 2008. Effects of stress on emotional reactivity in hostile heavy social drinkers following dietary tryptophan enhancement. *Alcohol and Alcoholism* 43: 151-162.

Porter, R., Gallagher, P., Watson, S., Young, A., 2004. Corticosteroid-serotonin interactions in depression: a review of the human evidence. *Psychopharmacol.* 173: 1-17.

Praschak-Rieder, N., Kennedy, J., Wilson, A.A., Hussey, D., Boovariwala, A., Willeit, M., *et al.*, 2007. Novel 5-HTTLPR allele associates with higher serotonin transporter binding in putamen: a [(11)C] DASB positron emission tomography study. *Biol. Psychiatry* 62, 231—327.

Reimold, M., Smolka, M.N., Schumann, G., Zimmer, A., Wrase, J., Mann, K., *et al.*, 2006. Midbrain serotonin transporter binding potential measured with [11C]DASB is affected by serotonin transporter genotype. *J. Neural. Transm.* 114, 635—639.

Sheehan, D.V., Lecrubier, Y., Harnett Sheehan, K., Janavs, J., Weiller, E., Keskiner, A., Schinka, J., Knapp, E., Sheehan, M.F., Dunbar, G.C., 1997. The validity of the Mini International Neuropsychiatric Interview (MINI) according to the SCID-P and its reliability. *Eur. Psychiatry* 12: 232-241.

Sobczak, S., Honig, A., Schmitt, J.A.J., Riedel, W.J., 2003. Pronounced cognitive deficits following an intravenous L-tryptophan challenge in first-degree relatives of bipolar patients compared to healthy controls. *Neuropsychopharmacology* 28: 711–719.

Spinhoven, P., Ormel, J., Sloekers, P.P., Kempen, G.I., Speckens, A.E., Van Hemert, A.M., 1997. A validation study of the Hospital Anxiety and Depression Scale (HADS) in different groups of Dutch subjects. *Psychol. Med.* 27: 363–370.

Van Vliet, I.M., Leroy, H., Van Megen, H.J.M., 2000. De MINI-Internationaal Neuropsychiatrisch Interview. Leiden, NL: Leiden University Medical Center.

Verschoor, E., Markus, C.R.,2011. Effects of acute psychosocial stress exposure on endocrine and affective reactivity in college students differing in the 5-HTTLPR genotype and trait neuroticism. Stress. 14(4):407-19.

Watson, D., Clark, L.A., Tellegen, A., 1988. Development and validation of brief measures of positive and negative affect: The PANAS scales. *J. Pers. Soc. Psychol.* 54: 1063-1070.

Way, B.M., Taylor, S.E., 2010. The serotonin transporter promoter polymorphism is associated with cortisol response to psychosocial stress. *Biol. Psychiatry* 67: 487-492.

World Health Organization (WHO), 2002. Protein and amino acid requirements in human nutrition: report of a joint FAO/WHO/UNU expert consultation (WHO technical report series; no. 935). Geneva: WHO.

Wüst, S., Kumsta, R., Treutlein, J., Frank, J., Entringer, S., Schulze, T.G., Rietschel , M. 2009. Sex-specific association between the 5-HTT gene-linked polymorphic region and basal cortisol secretion. *Psychoneuroendocrinology* 34: 972-982.

Zigmond, A.S., Snaith, R.P., 1983. The hospital anxiety and depression scale. *Acta Psychiatr. Scand.* 67: 361–370.

Chapter 4

Chapter 5

The Effect of 5-HTTLPR Genotype and
Tryptophan Supplementation on the Response
to Unfairness in Healthy Volunteers

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Abstract

Experimental manipulation of the availability of serotonin (5-HT) has been shown to increase or decrease the rejection rates of unfair offers in the Ultimatum Game (UG). The effect of 5-HT manipulations on UG performance may also be moderated by genotypic variation, in particular the 5HT-transporter-linked polymorphic region (5-HTTLPR). The aim of the present study was to investigate the effect of 5-HTTLPR genotype, tryptophan (TRP) supplementation and their interaction on performance on the UG in healthy individuals. The UG was completed by 26 S'/S' and 21 L'/L' carriers of the 5-HTTLPR, before and after a 6-day intervention of TRP (2.8 g/day) or placebo. We also measured impulsivity with a response inhibition (Go-Stop) task and with a questionnaire. 5-HTTLPR genotype did not affect the rejection rate to unfair offers. TRP supplementation also had no effect, but there was a non-significant tendency in the opposite direction as expected: the TRP-group had higher rejection rates of very unfair offers than the placebo group. Neither genotype nor intervention had an effect on impulsivity. A limitation of this study is that no blood samples were taken. The lack of effects cannot be explained by low statistical power and/or weakness of the intervention since the effect went in the opposite direction as expected. Our findings are not consistent with earlier studies with other 5-HT manipulations.

Introduction

Serotonin (5-HT) is involved in the modulation of many aspects of social cognition and behaviour. Genotypic variation, in particular regarding the serotonin transporter gene (SLC6A4) affects social cognition (Canli & Lesch, 2007), as well as 5-HT manipulations. For instance, experimental depletion of tryptophan (TRP), a precursor of 5-HT, leads to reduced cooperative behaviour in a Prisoners Dilemma paradigm (Wood *et al.*, 2006). TRP depleted healthy volunteers judged couples as less intimate and less romantic (Bilderbeck *et al.*, 2011). TRP depletion also increased reaction times for happy but not sad faces in an affective go/no go task (Murphy *et al.*, 2002) and increased rapid- response impulsivity in healthy volunteers (Walderhaug *et al.*, 2002). Serotonin also plays a role in the processing of facial expressions and social interactions. For example, acute tryptophan depletion (ATD) decreased the recognition of fearful facial expressions in healthy females (Harmer *et al.*, 2003) and TRP supplementation for 15 days decreased quarrelsomeness in quarrelsome men and women and altered the perception of others in the quarrelsome males (Aan het Rot *et al.*, 2006). These studies show that lowering serotonin availability is associated with disruptive social behaviour, whereas increasing serotonin availability is associated with pro-social perception and behaviour.

Serotonergic manipulations also affect performance in the Ultimatum Game (UG) (Crockett *et al.*, 2008; Crockett *et al.*, 2010). In the UG, the participant (responder) is exposed to offers to split a sum of money from other individuals. The responder can either accept the offer (in which the money is divided accordingly) or reject the offer (in which case both players receive nothing). Rationally, the responder should accept every offer regardless of its fairness to earn the most. However, very unfair offers (20% of the total) have a 50% chance of being rejected (Güth, Schmittberger & Schwartze 1982; Bolton & Zwick, 1995), which indicates that emotion plays an important role in making those decisions. Acute tryptophan depletion was associated with a higher rejection rate (approximately 81%) of very unfair offers (18-22% of the stake) than PLC (appr. 65%) in healthy individuals (Crockett *et al.*, 2008). This effect was independent of the size of the offer and ATD had no effect on self-reported mood or on response inhibition. Conversely, a single dose of a selective serotonin reuptake inhibitor (SSRI), citolapram (30 mg) was associated with a lower rejection rate (appr. 34%) of unfair offers than PLC (appr. 48%) and atomoxetine (60 mg) a selective selective nor epinephrine reuptake inhibitor (NRI), (appr. 50%) in 30 healthy participants (Crockett *et al.*, 2010). This time the effect was restricted to moderately unfair offers (27-33% of the stake). Citalopram did not alter self-reported mood. In another study, healthy students who rejected an unfair offer had lower platelet serotonin content than participants who accepted the offer (Emanuele *et al.*, 2008). Finally, a PET study in 20 healthy males showed that individuals with low levels of 5-HT transporter binding in the dorsal raphe nucleus were more likely to reject unfair offers (Takahashi *et al.*, 2012). In summary, UG behaviour seems to be under serotonergic influence and the effects are consistent across studies. The direction of the effect on the UG

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is consistent with the direction of the 5-HT manipulation (Crocket *et al.*, 2008; Crocket *et al.*, 2010).

Although 5-HTTLPR genotype is linked to social cognition (Canli & Lesch, 2007; Antypa *et al.*, 2011), no studies have been conducted in order to investigate whether 5-HTTLPR moderates the effect of 5-HT manipulations on UG performance. In the present study we investigated whether performance on the UG is affected by 5-HTTLPR genotype and by an increase of 5-HT availability through tryptophan (TRP) supplementation. Specifically, short and long allele carriers of the 5-HTTLPR completed the UG prior to and after a 6-day intervention of TRP (2.8 g/day) or placebo. In a previous report on this study, we have shown that TRP normalized the cortisol response to social stress in S'/S' carriers only (Cerit *et al.*, 2013). Based on the less efficient serotonin neurotransmission in S'/S' carriers, the regulation of the reaction to unfairness was expected to be diminished in this group. We hypothesized that we would find higher rejection rates in S'/S' carriers than in L'/L' carriers and that this difference would be reduced after 6-day TRP supplementation. Response inhibition (Go-Stop task) and self-reported impulsivity (BIS-II) were measured as secondary outcomes. Based on Crockett *et al.* (2008) we expected a selective effect on the ultimatum game.

Methods and Materials

Participant pool

The participants of this study are the same as reported in Cerit et al. (2013). Participants were selected from a pool of 581 genotyped individuals who were non-smokers and whose grandparents were all West-European. The age range was 18 to 35 years and Body Mass Index was between 19 and 29 kg/m². Exclusion criteria were a current diagnosis of depression or post-traumatic stress disorder, a lifetime history of psychosis, and use of medication, including oral contraceptives. Genotype frequencies were as follows: SS, 16,9%; SLg, 4,8%; LgLg, 0,7%; LaLg, 8,6%; SLa, 43%; LaLa, 26%. Participants were divided on the basis of the triallelic classification (Lg alleles were collapsed with S variants into three genotype groups: S'/S' (n = 130); L'/S' (n = 300); L'/L' (n = 151). Genotype frequencies were consistent with Hardy–Weinberg Equilibrium ($\chi2$ (1) = 0.67, p = 0.41).

We invited only participants with two low-expressing alleles (S'/S': S/S, S/Lg and Lg/Lg variants) or two high-expressing alleles (L'/L': La/La). Written informed consent was obtained before data collection. The research was approved by the Medical Ethics Committee of Leiden University Medical Centre in The Netherlands. Participants received € 40 upon completing the study.

Genetic Assessment

DNA was obtained using the Oragene Self-Collection Kit – DISC format (DNA Genotek Inc, Ottawa, ON, Canada); 200 µl of saliva was collected in lysis buffer (100 mM NaCl, 10 mM EDTA, 10 mM Tris pH 8, 0.1 mg/ml proteinase K and 0.5% w/v SDS) until further processing. Genomic DNA was isolated from the samples using the Chemagic buccal swab kit on a Chemagen Module I workstation (Chemagen Biopolymer-Technologie AG, Baesweiler, Germany). DNA concentrations were quantified by OD260 measurement and by agarose gel electrophoresis. The average yield was approximately 4 µg of genomic DNA per sample.

Polymerase chain reaction amplification

The region of interest from the 5-HTT gene was amplified by triplex PCR using the following primers: a FAM-labeled primer HTTLPR-FWFAM 5'-TCCTCCGCTTTGGCGCCTCTTCC-3', and a reverse primer HTTLPR-RV 5'-TGGGGGTTGCAGGGGAGATCCTG-3'. Typical PCR reactions contained between 10 and 100 ng genomic DNA template, 10 pmol of forward and reverse primer. PCR was carried out in the presence of 5% DMSO with 0.5U of BioThermAB polymerase (GeneCraft, Munster, Germany) in a total volume of 30 ml using the following cycling conditions: initial denaturation step of 5 min at 95°C, followed by 40 cycles of 30 sec 96°C, 30 sec 61°C, 60 sec 72°C and a final extension step of 10 min 72°C. After PCR 5 µl of the

sample was subjected to restriction digestion with the enzyme HpaII in a total volume of 20 µl. Restriction enzyme mix was incubated with DNA for 3 hours at 37°C.

Analysis of PCR products

One µL of PCR product before and after restriction digestion was mixed with LIZ-500 size standard and formamide and run in two separate lanes on an AB 3100 genetic analyser set up for genotyping with 50 cm capillaries. Results were analysed using Genescan software version 3.7 (Applied Biosystems, Carlsbad, CA, USA), and alleles were scored visually according to the following scheme: Uncut: S, 469 bp; L, 512 bp. Cut: Sg, 402 + 67 bp; Lg, 402 + 110 bp.

Instruments

Diagnosis. The Mini International Neuropsychiatric Interview (M.I.N.I.) was administered (Sheehan *et al.*, 1997; Van Vliet *et al.*, 2000) to assess psychiatric diagnoses.

Ultimatum Game. The ultimatum game consists of three different conditions in which participants are exposed to fair (45% of stake), unfair (32% of stake) and most (i.e. very) unfair (21% of stake) offers from a "proposer", who had split a sum of money given by the experimenter. If the participant accepts the offer, both the proposer and participant will receive the money as promised in the offer. In case of a rejection by the participant neither one will receive money. An example of a highly unfair offer could be € 2 for the participant and € 8 for the proposer. Obviously, the most beneficial strategy economically for the participant is to accept each offer regardless of its fairness level. Next to social reward (fairness), monetary reward (offer size) was also manipulated as described in Crockett *et al.* (2008). The value of the stake was either low (between 1 and 7 euro) or high (between 8 and 33 euro).

After 10 practice trials, participants were asked to respond to 48 offers (16 per fairness level). With each offer a photograph of a new proposer, the amount of the stake, and the amount of the offer was shown. The 48 photographs were counterbalanced for gender (24 male and 24 female proposers). All 48 offers were presented in a random order at both sessions. The participants were told that they would receive a percentage of the total amount that they had gained after having completed both sessions. In reality, there were no actual proposers and all participants received the same propositions. To increase credibility, the participants were first asked to split 24 sums of money (on paper) and had their photograph taken to be used in future experiments.

Impulsivity. Self-reported impulsivity was assessed with the 30-item Barratt Impulsiveness Scale (BIS-II, state version; Patton *et al.*, 1995). The Go-Stop test is a stop-signal task and

measures response inhibition aspects of impulsivity (Dougherty 2005; Dougherty *et al.*, 2010). In this task a series of 5-digit numbers are displayed for 500 msec with a 1,500 msec inter-stimulus interval. The 5-digit numbers appear in series, and some of these numbers are identical to the immediately preceding 5-digit number. Participants are instructed to respond to these matching numbers (Go Signal). Some of these matching numbers are first presented in black and then suddenly turn red. This is a Stop Signal cue, and the participants are instructed to withhold responding to any matching numbers that turn red. The timing of these stop signals varied across the testing session (e.g. 50, 150, 250 and 350 msec). The two dependent measures of interest were: 1) correct responses and 2) response inhibition failures. The primary dependent measure is the Go-Stop Ratio, which is the ratio of these two measures. The Go-Stop Ratio has been validated as a measure of the ability to inhibit an already initiated response, and data from the 150 msec stop delay typically provides the best group discrimination (Dougherty *et al.*, 2010).

Design and procedure

This study was a randomized double-blind placebo-controlled experimental study with stratification for genetic variation of the 5-HTTLPR genotype. Participants were randomly allocated to receive 7 capsules containing either 400 mg TRP (total dose of 2.8 g/day) or placebo (cellulose microcrystalline) for a period of six days. The dosage and duration were based on previous studies that had shown social-behavioural effects of TRP administration (3 g/day) after a period of 15 days (Aan het Rot *et al.*, 2006) and cognitive effects after a single dose of 0.8g TRP (Markus & Firk, 2009). The experimental procedure included two visits to the laboratory on the days before and after the intervention (Day 0 and 7).

First visit to laboratory (Day 0)

Upon arrival at the laboratory, participants provided written informed consent. Following the M.I.N.I. interview, participants filled out the BIS-II, and completed the ultimatum game and Go-Stop test, respectively (as part of a larger test battery). At the end of the first visit, participants were provided with 42 capsules that contained 400 mg tryptophan or placebo (PLC). Oral and written instructions were provided to the participants regarding the timing of administration of capsules and lifestyle restrictions during the next six days and on the day of the second lab visit.

Tryptophan supplementation

Participants started to take the capsules the day after their first lab visit. They were instructed to take two capsules in the morning, two in the afternoon (before meals) and three in the evening (before 23.00h). Participants received a diary in which they were asked to write down the exact time of intake and number of capsules. Compliance was not measured through

blood sample analyses, however, participants were led to believe that compliance would be assessed at post-intervention through a saliva sample. Lifestyle instructions included: no smoking, no use of dietary supplements and vitamins and consumption of alcohol limited to three units/day. Participants were also instructed to refrain from alcohol and caffeine-containing consumptions and avoid high carbohydrate meals on the day of their second visit. Further instructions for the day of the second visit included: no eating and drinking one hour before arriving at the laboratory (except water), and no physical exercise at least two hours before arrival. Female participants were tested in the luteal phase of their menstrual cycle. All test sessions started in the afternoon between noon and 5pm.

Second visit to laboratory (Day 7)

Upon arrival at the lab, participants handed in their diary regarding the intake of capsules. In addition, they were asked to fill out a debriefing questionnaire regarding compliance to the instructions during the previous six days. They were also interviewed about their compliance to the instructions for the second lab visit. Next, participants were asked to complete the BIS-II questionnaire, the Ultimatum Game and Go-Stop test in fixed order.

Results

Sample characteristics

We contacted 92 S'/S' carriers and 92 L'/L' carriers from our participant pool (N=581) by email. Sixty-four S'/S' and 66 L'/L' carriers expressed interest in the study. After screening for in- and exclusion criteria we included 26 S'/S' and 22 L'/L' participants. In L'/L' group, one participant dropped out before the second visit to laboratory. Analyses were conducted on 26 S'/S' and 21 L'/L' carriers.

The first two participants (one S'/S' and one L'/L' carrier) who received tryptophan single blind were kept in the analysis. The demographic details of both groups are shown in Table 1. Groups did not differ significantly on demographic characteristics. One participant in the PLC group (S'/S' carrier) had a current diagnosis of panic disorder, and one participant in the TRP group (S'/S' carrier) had a specific phobia (for needles). Both participants were not taking any medication.

Table 1. Demographic Characteristics of the Tri-Allelic S'/S' and L'/L' 5-HTTLPR Genotype groups

	S'/S' (N = 26)	L'/L' (N = 21)
Age (M±SD)	20.4 ± 3.4	20.3 ± 2.5
Females/males	12/14	11/10

*Note: S'/S' includes: S/S, S/Lg, Lg/Lg and L'/L' includes: La/La; M, Mean; SD, Standard Deviation.

Compliance

According to self-report, approximately 98% of the capsules were taken according to instructions. The minimum percentage of capsules taken by a participant was 69%. Three participants had taken two capsules in the morning of the second lab visit. All these participants were retained.

Ultimatum Game

Genotype Effect on ultimatum game on Day 0. A Repeated Measures ANOVA (RM-ANOVA) with Offer size (low and high) and Fairness level (fair, unfair and most unfair) as within subjects factors and Genotype (S'/S' and L'/L') as between subjects factor on the rejection rates on Day 0 (pre-intervention) was conducted. This analysis revealed the expected main effects of Offer size (F (1.00, 45.00) = 5.66, p = 0.022, ηp^2= 0.112) and Fairness level (F (1.66, 74.60) =

100.49, $p < 0.001$, $\eta p^2 = 0.691$). The interaction of Offer size and Fairness was also significant (F (1.833, 82.49) =, $p = 0.039$, $\eta p^2 = 0.072$). No main or interaction effects involving genotype were found.

Genotype and intervention effects on ultimatum game on Day 0 and 7. A 2x2x3 RM-ANOVA with Time (pre/post intervention), Offer size and Fairness level as within subjects factors and Genotype and Intervention (TRP/PLC) as between subjects factors again revealed the expected main effects of Offer size and Fairness level (F (1.54, 66.26) = 112.44, $p < 0.001$, $\eta p^2 = 0.723$), but no main effects of Genotype, Time or Intervention. However, three-way interactions among Time x Offer size x Genotype (F (1.00, 43.00) = 6.22, $p = 0.017$, $\eta p^2 = 0.126$) and Time x Offer Size x Intervention (F (1.00, 43.00) = 8.12, $p = 0.007$, $\eta p^2 = 0.159$) were found.

Separate RM-ANOVAs with Time as within subject factor and Intervention as between subject factor were conducted on the rejection rates of high unfair, high most unfair, low unfair and low most unfair offers. None of these analyses revealed a main effect of time or intervention. However, in the analysis of the High Most Unfair offers, a significant Time x Intervention was found (F (1.00, 45.00) = 4.249, $p = 0.045$, $\eta p^2 = 0.086$) (Figure 1). The interaction was borderline significant ($p = 0.057$) when genotype was included as a between subjects factor. We conducted post-hoc Independent-Sample T-Test on day 7, and Paired-Sample T-Tests within the TRP and PLC groups separately. None of these analyses revealed a significant difference (all p-values around 0.15).

Figure 1. Rejection of Most Unfair Offers with High offer size following TRP +
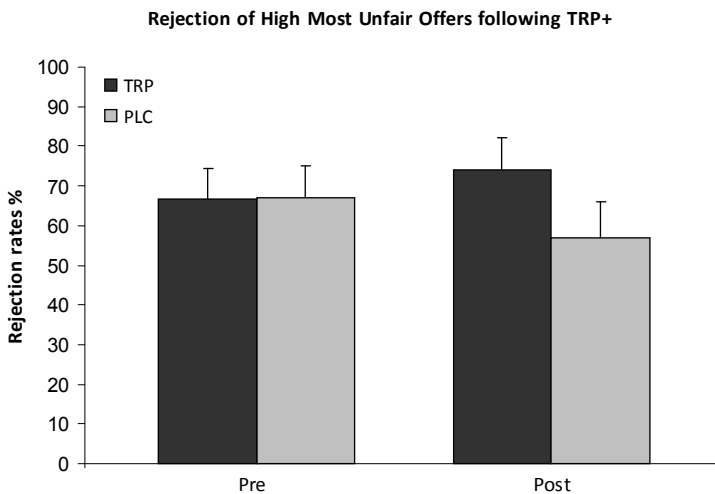
Note: Rejection rates of "most unfair offers" with high offer sizes pre and post intervention (TRP; Tryptophan, PLC; Placebo). Error bars represent Standard Error (SE)

Self-reported Impulsivity

Genotype Effect on impulsivity on Day 0. In order to assess effects of 5-HTTLPR genotype on impulsivity a RM-ANOVA with the Attentional, Motor and Non-planning scales of the BIS-II measured at Day 0 (pre-intervention), as within subjects factors and Genotype (S'/S' and L'/L') as between subjects factor on the scores of the three scales revealed a main effect of scale ($F_{(1.96, 88.09)} = 144.54$, $p < 0.001$, $\eta p^2 = 0.763$). Impulsivity did not interact with Genotype ($F_{(1.96, 88.09)} = 0.445$, $p = 0.638$, $\eta p^2 = 0.010$).

Genotype or intervention effects on impulsivity on Day 0 and 7. Effect of 5-HTTLPR genotype and intervention were assessed by conducting a RM-ANOVA with Time (pre/post intervention), and the three BIS-II scales as within subjects factors. Genotype and Intervention (TRP/PLC) were between subjects factors. No main effect of Time ($F_{(1.00, 43.00)} = 0.166$, $p = 0.686$, $\eta p^2 = 0.004$) was found. A main effect of Impulsivity ($F_{(1.98, 84.92)} = 183.34$, $p < 0.001$, $\eta p^2 = 0.810$) and Time x Impulsivity was found ($F_{(1.68, 72.17)} = 4.829$, $p = 0.015$, $\eta p^2 = 0.101$).

Time x Impulsivity x Genotype was not significant ($F_{(1.68, 72.17)} = 2.483$, $p = 0.100$, $\eta p^2 = 0.055$). Time x Impulsivity x Intervention was also not found ($F_{(1.68, 72.17)} = 1.688$, $p = 0.196$, $\eta p^2 = 0.038$). The scores on Attentional, Motor and Non-planning scales of the BIS-II are shown in Table 2.

Go-Stop Task

Genotype Effect on Response Inhibition on Day 0. RM-ANOVA with two impulsivity outcomes of the Go-Stop paradigm (Response Inhibition Failure and Correct Detections) as within subjects factors and Genotype (S'/S' and L'/L') as between subject factor on Day 0 (pre-intervention), revealed a main effect of impulsivity outcome ($F_{(1.00, 43.00)} = 1200.20$, $p < 0.001$, $\eta p^2 = 0.96$) interaction, but no effect of Genotype on the two impulsivity outcomes were found.

A separate One-Way ANOVA was conducted for the Go-Stop Ratio outcome with Go-Stop Ratio on Day 0 as dependent variable and Genotype as factor. The genotype groups did not differ on the Go-Stop Ratio outcome ($F_{(1, 45)} = 1.415$, $p = 0.241$).

Genotype and intervention effects on Response Inhibition on Day 0 and 7. RM-ANOVA with Time (pre/post intervention), and the two impulsivity outcomes of the Go-Stop paradigm (Response Inhibition Failure and Correct Detections) as within subjects factors, and Genotype and Intervention (TRP/PLC) as between subjects factors revealed a significant main effect of Impulsivity outcome, but no main effect on Time ($F_{(1.00, 43.00)} = 0.037$, $p = 0.848$, $\eta p^2 = 0.001$). No effect of genotype or Intervention on impulsivity outcomes on Day 0 and Day 7 were found (Table 3).

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A separate RM-ANOVA with Time (pre/post intervention), and the Go-Stop Ratio outcome as within subjects factors, and Genotype and Intervention (TRP/PLC) as between subjects factors revealed a main effect of Time (F (1.00, 43.00) = 11.89, p = 0.001, ηp^2= 0.217), but no interaction effects.

Table 2. Self- reported Impulsivity. BIS-II scores on the 2cnd order factors Attentional, Motor and Non-Planning with Intervention (TRP and PLC) and Genotype (S'/S' and L'/L')*, (Mean ± SD)

			Pre (M ± SD)	Post (M ± SD)
Attentional	TRP	S'/S'	16.3 ± 2.4	15.9 ± 2.6
		L'/L'	14.4 ± 2.5	14.6 ± 2.6
	PLC	S'/S'	15.3 ± 4.0	13.9 ± 3.5
		L'/L'	15.1 ± 3.9	14.7 ± 3.2
Motor	TRP	S'/S'	22.1 ± 3.8	23.1 ± 3.3
		L'/L'	20.8 ± 2.4	21.1 ± 2.3
	PLC	S'/S'	20.0 ± 2.6	21.5 ± 3.4
		L'/L'	21.0 ± 3.7	21.5 ± 4.1
Non-planning	TRP	S'/S'	27.0 ± 3.9	25.3 ± 3.6
		L'/L'	26.2 ± 3.3	25.6 ± 3.4
	PLC	S'/S'	25.0 ± 3.4	24.2 ± 3.7
		L'/L'	23.3 ± 4.0	23.9 ± 4.7

(M, Mean; SD, Standard Deviation; TRP, Tryptophan; PLC, Placebo)
*Note: S'/S' includes: S/S, S/Lg, Lg/Lg and L'/L' includes: La/La

Table 3. Go-Stop Impulsivity Measures at 150 ms, (Mean ± SD)

	Pre (M ± SD)	Post (M ± SD)
TRP		
Correct detections	67.28 (12.03)	63.36 (23.01)
Response Inhibition Failure	0.37 (0.20)	0.33 (0.20)
Go-Stop Ratio	0.48 (0.25)	0.36 (0.21)
PLC		
Correct detections	63.18 (13.62)	65.91 (14.51)
Response Inhibition Failure	0.42 (0.20)	0.36 (0.23)
Go-Stop Ratio	0.53 (0.23)	0.40 (0.28)

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Discussion

Genotypic variation of the 5-HTTLPR did not influence the response of healthy participants to unfair offers. Six days of tryptophan supplementation seemed to influence response to very unfair offers, however, this effect did not reach statistical significance. We also found no effects of genotype, intervention or their interactions on impulsivity as measured by the Go-Stop Task or by self-report.

Our findings are not consistent with other studies in which the ultimatum game was performed by healthy participants following a serotonergic manipulation. A single dose of citalopram reduced the rejection rate of unfair offers compared to placebo condition in healthy volunteers (Crockett *et al.*, 2010), whereas tryptophan depletion had an opposite effect (Crocket *et al.*, 2008). In another study, low platelet serotonin was found to be associated with heightened rejection of unfair offers (Emanuele *et al.*, 2008). Our non-significant findings were in the opposite direction as these three studies, which suggest that our findings were not due to low statistical power. Insufficient strength of our intervention is also unlikely, since we did observe an attenuated cortisol response to social stress in S'/S' carriers, as reported previously (Cerit *et al.*, 2013).

It is conceivable that the ultimatum game may be less sensitive when administered more than once. This cannot explain the lack of effect of genotypic variation, but might explain the findings regarding tryptophan supplements. Participants may have applied different strategies during the two experimental sessions. However, others (e.g., Crockett *et al.*, 2008) have also administered the UG more than once.

Our participants did not take TRP on the day of testing. Therefore, one might argue that the opposite trend in our findings is due to the acute withdrawal of tryptophan that caused a relative depletion. Since we did not take blood samples, we cannot be sure about the tryptophan concentrations at the time of testing. This relative depletion hypothesis seems quite unlikely, considering that we found a reduced cortisol response to social stress in the same participants, which is theoretically consistent with supplementation (Cerit *et al.*, 2013).

We have no information on the diet of the participants during the 6-day lasting TRP intervention, neither do we have information on the type of meal that participants have consumed on day 7. We did not take blood samples to measure peripheral parameters (e.g. TRP/LNAA ratios) which could provide us with 1) an indication of a possible interaction of nutrients with the intervention during the study and/or 2) an indication of the effect of the intervention on central serotonin levels. However, the aim of the current study was not to measure the acute effect of TRP loading on central serotonin levels and its consequent effect on behaviour. The perspective of the current study is rather based on the idea that increasing serotonin availability leads to a shift from negative towards positive information

processing (Harmer *et al.*, 2009) and alters social behaviour and perception of others in a positive manner (Aan het Rot *et al.*, 2006).

The effects of 5-HT manipulations on UG behaviour may be mediated by more complex underlying processes than we had presumed. For example, the personality trait "agreeableness" was found to be a mediating factor between 5-HTT binding and UG behaviour in healthy men: higher scores on "straightforwardness" and "trust" (two subscales of "agreeableness") were correlated with lower 5-HTT binding and higher rejection rates of unfair offers (Takahashi *et al.*, 2012). Further studies are required to unravel the underlying processes between 5-HT neurotransmission and behavioural responses to unfairness.

Acknowledgements

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References

Aan het Rot, M., Moskowitz, D.S., Pinard, G., & Young S.N. (2006). Social behaviour and mood in everyday life: the effects of tryptophan in quarrelsome individuals. *Journal of Psychiatry and Neuroscience 31*(4), 253–262.

Antypa, N., Cerit, H., Kruijt, A.W., Verhoeven, F.E.A., & Van der Does, A.J.W. (2011). Relationships among 5-HTT genotype, life events and gender in the recognition of facial emotions. *Neuroscience* 172, 303-313. doi: 10.1016/j.neuroscience.2010.10.042

Bilderbeck, A.C., McCabe, C., Wakeley, J., McGlone, F., Harris, T., Cowen, P.J., & Rogers, R.D. (2011). Serotonergic Activity Influences the Cognitive Appraisal of Close Intimate Relationships in Healthy Adults. *Biological Psychiatry 69*(8), 720-725. doi: 10.1016/j.biopsych.2010.12.038

Bolton, G.E., & Zwick, R. (1995). Anonymity versus punishment in ultimatum bargaining. *Games and Economic Behavior 10*(1), 95–121. doi: 10.1006/game.1995.1026,

Canli, T., & Lesch, K.P. (2007). Long story short: the serotonin transporter in emotion regulation and social cognition. *Nature Neuroscience 10*(9), 1103–1109. doi: 10.1038/nn1964

Cerit, H., Jans, L.A., & Van der Does, A.J.W. (2013). The effect of tryptophan on the cortisol response to social stress is modulated by the 5- HTTLPR. *Psychoneuroendocrinology 38*(2), 201-208. doi: 10.1016/j.psyneuen.2012.05.016

Crockett, M.J., Clark, L., Tabibnia, G., Lieberman, M.D., & Robbins, T.W. (2008). Serotonin Modulates Behavioral Reactions to Unfairness. *Science 320*(5884), 1739-1739. doi: 10.1126/science.1155577

Crockett, M.J., Clark, L., Hauser, M.D., & Robbins, T.W. (2010). Serotonin selectively influences moral judgment and behavior through effects on harm aversion. *Proceedings of the National Academy of Sciences 107*(40), 17433-17438. doi: 10.1073/pnas.1009396107

Dougherty, D., Mathias, C., Marsh, D., & Jagar, A. (2005). Laboratory behavioral measures of impulsivity. *Behavior Research Methods 37*(1), 82-90. doi: 10.3758/bf03206401

Dougherty, D.M., Richard, D.M., James, L.M., & Mathias, C.W. (2010). Effects of Acute Tryptophan Depletion on Three Different Types of Behavioral Impulsivity. *International Journal of Tryptophan Research 3*, 99-111. doi: 10.4137/ijtr.s4317

Emanuele, E., Brondino, N., Bertona, M., Re, S., & Geroldi, D. (2008). Relationship between platelet serotonin content and rejections of unfair offers in the ultimatum game. *Neuroscience Letters 437*(2), 158-161. doi: 10.1016/j.neulet.2008.04.006

Güth, W., Schmittberger, R., & Schwartze, B. (1982). An experimental analysis of ultimatum bargaining. *Journal of Economic Behaviour & Organization 3*(4), 367-388. doi: 10.1016/0167-2681(82)90011-7

Harmer, C.J., Rogers, R.D., Tunbridge, E., Cowen, P.J., & Goodwin, G.M. (2003). Tryptophan depletion decreases the recognition of fear in female volunteers. *Psychopharmacology 167*(4), 411-417. doi: 10.1007/s00213-003-1401-6

Harmer, C. J., Goodwin, G. M., & Cowen, P. J. (2009). Why do antidepressants take so long to work? A cognitive neuropsychological model of antidepressant drug action. *The British Journal of Psychiatry, 195*(2), 102-108. doi: 10.1192/bjp.bp.108.051193

Markus, C.R., & Firk, C. (2009). Differential effects of tri-allelic 5- HTTLPR polymorphisms in healthy subjects on mood and stress performance after tryptophan challenge. *Neuropsychopharmacology 34*(13), 2667-2674. doi: 10.1038/npp.2009.92

Murphy, F.C., Smith, K.A., Cowen, P.J., Robbins, T.W., & Sahakian, B.J. (2002). The effects of tryptophan depletion on cognitive and affective processing in healthy volunteers. *Psychopharmacology 163*(1), 42-53. doi: 10.1007/s00213-002-1128-9

Patton, J.H., Stanford, M.S., & Barratt, E.S. (1995). Factor structure of the Barratt impulsiveness scale. *Journal of Clinical Psychology 51*(6), 768-74.

Sheehan, D.V., Lecrubier, Y., Harnett Sheehan, K., Janavs, J., Weiller, E., Keskiner, A., … & Dunbar, G.C. (1997). The validity of the Mini International Neuropsychiatric Interview (MINI) according to the SCID-P and its reliability. *European Psychiatry 12*(5), 232-241. doi: 10.1016/S0924-9338(97)83297-X

Takahashi, H., Takano, H., Camerer, C.F., Ideno, T., Okubo, S., Matsui, H., … & Suhara T. (2012). Honesty mediates the relationship between serotonin and reaction to unfairness. *Proceedings of the National Academy of Sciences 109*(11), 4281-4284. doi: 10.1073/pnas.1118687109

Van Vliet, I.M., Leroy, H., & Van Megen, H.J.M. (2000). De MINI-Internationaal Neuropsychiatrisch Interview. Leiden, Netherlands: Leiden University Medical Center.

Walderhaug, E., Lunde, H., Nordvik, J.E., Landrø, N.I., Refsum, H., & Magnusson, A. (2002). Lowering of serotonin by rapid tryptophan depletion increases impulsiveness in normal individuals. *Psychopharmacology 164*(4), 385-391. doi: 10.1007/s00213-002-1238-4

Wood, R.M., Rilling, J.K., Sanfey, A.G., Bhagwagar, Z., & Rogers, R.D. (2006). Effects of Tryptophan Depletion on the Performance of an Iterated Prisoner's Dilemma Game in Healthy Adults. *Neuropsychopharmacology 31*(5), 1075-1084. doi: 10.1038/sj.npp.1300932

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Chapter 6

General Discussion

General Discussion

Aim of the dissertation

The aim of this dissertation was to investigate the antidepressant properties of two compounds: a novel compound, ARA290, and a well-known compound, L-tryptophan.

ARA290 is a relatively new compound and studies on its effects on cognition and behaviour in healthy individuals are sparse. We used a neuropsychological model of drug action (Harmer *et al.*, 2009) to test its possible antidepressant effects in healthy volunteers. As summarized in the introduction, this model has been validated with registered antidepressants and is based on the finding that single dose or short term (1 week) administration of antidepressants in healthy volunteers results in a shift towards a more positive bias in various domains of emotional information processing such as attention, memory and recognition of facial expressions (Harmer *et al.*, 2011). This early shift in emotional information processing may also be reflected in physiological (startle response) and neural changes (BOLD response) in healthy volunteers (Harmer *et al.*, 2011). In sum, the validated neuropsychological model of drug action includes behavioural, psychological and neural measures that can be applied in order to detect early antidepressant-like effects of new compounds in healthy volunteers. It is suggested that this model provides an early indication of how promising/efficient a new compound may eventually be in clinical populations (Harmer *et al.*, 2009; Harmer *et al.*, 2011). In addition to the primary outcomes of the neuropsychological model of drug action, which tap into emotional information processing, we also included resting-state fMRI as a measure in order to investigate the effects of ARA290 on resting-state connectivity.

Next to a new compound ARA290, we also investigated the antidepressant properties of a better-known compound, L-tryptophan which has been investigated as an add-on compound to conventional antidepressants (Coppen *et al.*, 1963; 1972). Since the effect of tryptophan manipulations on social-emotional information processing in healthy populations is relatively well known (Moskowitz *et al.*, 2001; Aan het Rot *et al.*, 2006; 2010), we aimed to further investigate the antidepressant effects of L-tryptophan by testing its effects in a healthy population possessing a genetic marker (serotonin transporter polymorphism) that is linked to depression vulnerability.

In order to assess the potential therapeutic effects of L-tryptophan, a selection of measures were applied. These measures include comparable behavioural and physiological vulnerability markers as those included in the neuropsychological model of drug action. The selection was based on recent studies on the effects of serotonin manipulations on cognition. Specifically, social-emotional decision making (behavioural) and stress or HPA-axis reactivity (physiological) were investigated.

The assessment of neuropsychological effects of interventions in healthy individuals has the advantage that they do not have a current disorder or a history of psychiatric disorders and that

their cognitive performances are not 'contaminated' by earlier pharmacological treatment. The idea is that vulnerability markers of depression are also present to varying degrees in healthy individuals who do not have a history of depression. The neuropsychological effects of ARA290 and tryptophan in healthy individuals were assessed by comparing placebo and intervention groups who had not had earlier treatment.

1a. Does ARA290, a novel pharmacological compound, have antidepressant effects?

Although a single dose of ARA290 (i.v. 2 mg) in healthy individuals did show an effect on some measures of the neuropsychological model of drug action, the effects were small and not all of these were in the expected direction of an antidepressant effect. Specifically, ARA290-treated individuals had smaller neural responses to happy faces in the fusiform gyrus, but no neural differences during the memory task. Furthermore, ARA-290 was not associated with changes in the functional connectivity of the brain. At the behavioural level, ARA290 had no effect on the recognition of emotions in facial expressions. ARA290 did have an effect on the categorization of self-referential words as ARA290 was associated with faster categorization of positive *vs* negative words, but not with a better memory for positive words. Furthermore, ARA290 was associated with a higher positive attentional bias score than placebo. No effects of ARA290 were observed on measures of mood and psychiatric symptoms. Taken together, these findings suggest that a single administration of ARA290 elicited the expected shift from positive to a negative bias on two measures of the emotional test battery. These were not the primary outcome measures, however – these outcome measures are a part of the emotional test battery and are based on previous effects of registered antidepressants or EPO. Furthermore, since we had no baseline assessments, it cannot be excluded that the effects represent pre-existing group differences.

1b. Effects of ARA290 - Limitations

Developed as an analogue of EPO, ARA290 is a peptide consisting of 11-amino acids which acts on the Innate Repair Receptor (IRR). The IRR consist of a β-common receptor (βCR) subunit (CD131) coupled to an EPO receptor (EPOR) and its activation initiates signalling pathways that mediate tissue protection, without initiating hematopoietic effects (Review, Brines and Cerami, 2012). Considering a) the tissue protective and anti-inflammatory role of ARA290 in animals and humans (Pulman *et al.*, 2013; Dahan *et al.*, 2013), b) the beneficial effects on cognitive performance in chronic schizophrenic patients (Ehrenreich *et al.*, 2007b) and c) the antidepressant-like effects of EPO in healthy individuals (Miskowiak *et al.*, 2007a; 2007b), we sought to answer the question of whether ARA290 exerts similar antidepressant-like effects as EPO does. Since EPO was tested with the neuropsychological model of drug action, we applied the same model and experimental design (incl. frequency of ARA290 administration and timing of testing).

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The absence of distinct antidepressant-like effects in our study may be due to differences in pharmacokinetics between EPO and ARA290. In humans, EPO has an elimination half-life of approximately 5 hours (Eckardt *et al.*, 1989; McMahon *et al.*, 1990), whereas ARA290 has an elimination half-life of approximately 2 minutes following i.v. administration (Niesters *et al.*, 2013). Despite the short elimination half-life, ARA290 is suggested to pass rapidly into the CNS and elicit durable effects due to the activation of IRR which regulates the innate tissue-protective response in various stages over a period from hours to days (Brines *et al.*, 2008a; Brines and Cerami, 2012). Consistent with this, a phase 1 study in patients suffering from sarcoidosis or diabetes-induced neuropathy showed that ARA290 (i.v. 2 mg) administration for 3 days (spread over one week) reduced pain scores; an effect that lasted for 3 days following the last treatment (Niesters *et al.*, 2013). Furthermore, first phase human studies with ARA290 indicated modest improvements in cognitive measures six hours after a single dose (Investigators brochure ARA290, 2009). Since first phase studies in humans did not raise safety concerns with the dose of 2 mg i.v. administration, we chose to administer the same dosage in our study. The time interval of measuring the antidepressant effects of ARA290 was chosen based on the studies investigating the antidepressant effects of EPO by means of the neuropsychological model of drug action (i.e., 6-7 days post administration). It is, therefore, conceivable that we have missed some effects of ARA290 on emotional information processing and cognition in general due to 1) possibly incorrect timing of measurement and 2) possibly ineffective dose of ARA290. An explanation for the lack of distinct antidepressant-like effects may be that either the timing of measurements should have been shorter after a single dose of administration (e.g., 2-3 days post administration) and/or that the dose of ARA290 administration should have been higher or that repeated administration (e.g., three administrations of 2 mg spread over a week) is needed in order to produce detectable effects at cognitive level.

Besides the differences in pharmacokinetics between EPO and ARA290, some limitations of the neuropsychological model also need to be addressed. The behavioural measures included in the test battery tap into different aspects of emotional information processing such as attention, memory and recognition of emotional facial expressions. Most of the stimuli used in these behavioural measures have an emotional content and are subject to habituation as well as learning effects. The majority of the tests included in the battery of emotional information processing can therefore be administered only once. Due to this restriction we chose not to take baseline measurements prior to the pharmacological intervention. In order to compensate for the lack of baseline measurements we increased the number of participants in our study (*N*=36). This is higher than the fMRI studies on EPO in healthy participants (*N*= 24) (Miskowiak *et al.*, 2007a), that also had no baseline assessments. Baseline measures would of course have provided objective measures of inter-subject variability and differences in response between groups prior to intervention.

Another possible point of criticism relates to the design of the cognitive challenges during fMRI scanning, which are a part of the neuropsychological model. To facilitate comparison

with prior research, the fMRI tasks in our study were presented in a block-design. Therefore, it is conceivable that we did not observe distinct differences in hippocampal function between groups during the picture recognition task as a block-design limits the possibility to analyse responses to individual trials. Presentation of trials in an event-related design would have made it possible to investigate the changes in hemodynamic response during correct and incorrect recognition of pictures. In a block-design, however, it is not possible to distinguish between correct *vs*. incorrect recognition. Consequently, changes in BOLD response associated with correct and incorrect recognition could not be modelled in the data.

Overall, the effects of ARA290 on the neural response during task performance are task specific rather than a general effect of ARA290 on these regions, as ARA290 was not associated with changes in the functional connectivity of the brain.

1c. Effects of ARA290 – Underlying biological mechanism

Although ARA290 seems to elicit changes in a few domains of emotional information processing, overall the results indicate that a single administration of ARA290 is not sufficient to initiate the explicit shift towards a more positive bias in emotional information processing as had been hypothesized based on the neuropsychological model we have applied. Possible limitations of our studies are discussed in ***paragraph 1b***, however, the question that needs to be answered at this point is a) whether it is worthwhile to continue to study ARA290 as a therapeutic compound for depression, and if so b) which steps are needed in order to further investigate the potential of ARA290 as antidepressant drug.

Our findings with a single administration of ARA290 in healthy individuals does not seem particularly promising as an antidepressant compound, however, when taking into consideration the positive findings reported in clinical trials with patients suffering from peripheral neuropathy together with the cellular pathways activated by ARA290, the antidepressant-like effects of ARA290 may need to be explored with a different approach.

ARA290 activates the IRR, a receptor which is locally up-regulated following tissue damage (Brines and Cerami, 2008b). In the brain, activation of the IRR leads to the activation of the STAT pathway (Fu *et al.*, 2010; Brines and Cerami, 2012) which in turn results in the up-regulation of survival signals and the inhibition of inflammation-induced apoptosis (Brines and Cerami, 2012). Furthermore, activation of the STAT3 and STAT5 pathways in human neural stem cells facilitate the differentiation of neural stem cells and outgrowth of neurites (Fu *et al.*, 2010). In rodents, ARA290 supresses the inflammation response in spinal microglia following nerve injury providing evidence for central anti-inflammatory actions of ARA290 (Swartjes *et al.*, 2014). Also, phase 1 and 2 clinical trials in neuropathy patients indicate beneficial effects of ARA290 on pain symptoms (Niesters *et al.*, 2013; van Velzen *et al.*, 2014) which are suggested to be mediated by the anti-inflammatory and tissue restorative actions initiated by ARA290 (van Velzen *et al.*,2014). Given these aforementioned findings, it

is conceivable that antidepressant-like effects of ARA290 occur and/or are only detectable in the "appropriate" biological environment, i.e. in individuals with increased levels of inflammatory biomarkers (i.e. elevated levels of pro-inflammatory cytokines). Inflammation has been proposed as one of the biological mechanisms underlying depression and has been recognized as a biological factor increasing the risk to develop MDD (as reviewed by Dantzer *et al.*, 2008; Rosenblat *et al.*, 2014), it is therefore possible that ARA290 exerts antidepressant-like effects in a subgroup of individuals who are vulnerable to develop depression due to the presence of high inflammation bio-markers. Besides the limitations discussed in *section 1b* concerning proper timing of assessment following ARA290 administration, future studies would also benefit from examining the effect of ARA290 on depressive symptoms (e.g., fatigue, sleep disturbances, changes in appetite, mood and cognition) in biologically vulnerable populations (i.e., individuals who are at risk to develop MDD due to conditions of high inflammation).

2a. Does L-tryptophan, a dietary compound, have antidepressant effects?

The effect of prolonged (six days) intake of TRP on HPA-axis reactivity (cortisol response) and social decision making (response to unfairness) was examined in a sample selected on genotype (i.e., S'/S' and L'/L' carriers of the 5-HTTLPR genotype). Our results indicate that TRP supplementation lowers the cortisol response to social stress in S'/S' carriers of the serotonin transporter gene while it has no effect on the cortisol response in L'/L' carriers. TRP supplementation for six days had no effect on mood or on symptoms of anxiety and depression in either of the genotype groups. The same study indicates that TRP supplementation does not influence the behavioural response to unfairness in the ultimatum game (UG). A non-significant difference was found in the opposite direction as expected: the tryptophan-group had higher rejection rates of very unfair offers than the placebo group. Also, 5-HTTLPR genotype did not influence the behavioural response to unfairness.

2b. Effects of L-tryptophan – Limitations

The beneficial effects of tryptophan on stress response in healthy volunteers appears to be specific for a certain genotype, i.e., the S'/S' variant of the 5-HTTLPR. Tryptophan did not have an effect on social decision making in either (L'/L' or S'/S') variant of the 5-HTTLPR genotype.

The response to unfairness in the ultimatum game in healthy subjects appears to be under serotonergic control as acute tryptophan depletion was associated with a higher rejection rate of very unfair offers (Crockett *et al.*, 2008), whereas a single dose of the selective serotonin reuptake inhibitor (SSRI) citalopram (30 mg) was associated with a lower rejection rate of unfair offers (Crockett *et al.*, 2010). Our study showed that tryptophan supplementation for six days did not modulate the response to unfairness in the Ultimatum game. At day 7 the

TRP group seemed to reject very unfair offers more often if the stakes were relatively high, which is in the opposite direction as expected based on the findings of Crockett *et al.* (2010). However, the effect was a non-significant in our study. S'/S' carriers of the 5-HTTPLR were expected to reject more offers (regardless the of level of unfairness) prior to treatment and were expected to benefit from the TRP supplementation in order to make more utilitarian decisions by lower rejection rates of unfair offers. However, 5-HTTPLR genotype did not influence the response to unfair offers.

In our study we did not draw blood samples pre- and post TRP supplementation to prevent possible interference with the cortisol response to the stress induction (i.e. Trier Social Stress Task). Although plasma TRP is an indirect measure of central serotonin availability, the lack of TRP plasma ratios pre- and post TRP supplementation complicates the interpretation of the null findings. Several possibilities could have been excluded if we would have measured plasma TRP levels. The last TRP intake was on the evening of the 6th day and the UG was performed on day 7. This may have turned our intended TRP supplementation into a relative depletion of TRP compared to the six days of supplementation prior to the day of testing. Oral TRP administration 50 mg/kg (3.5 g for 70 kg – mixed in milk) to humans showed that TRP concentration in blood reaches a peak approx. after 6 hours and returns to its baseline value after 12 hours, whereas, in the cerebrospinal fluid the peak is reached earlier (between six and ten hours) and returns to baseline after approx. 18 hours (Eccleston *et al.*, 1970). This early study of Eccleston *et al.* (1970) was carried out with a small sample size and needs to be interpreted with caution. However, even if a relative depletion was caused by the interruption of TRP supplementation at the day of testing, this may have pushed TRP levels back to baseline but would not cause the sizeable drop in TRP concentrations that is reached with an ATD procedure (i.e., approx. 70-90% drop from baseline, see review Van der Does, 2001). In addition, the effect we expected from prolonged TRP supplementation on ultimatum game behaviour was based on the idea of the cognitive neuropsychological model of Harmer *et al.* (2009) which hypothesizes that the neuro-adaptive effects of antidepressant agents, together with social interactions occur in parallel and reshape emotional biases which lead to a shift from negative to positive biases. In line with this hypothesis, TRP supplementation was expected to lead to a positive switch in which participants would be able to overrule their negative emotion towards unfair offers and would be able to make utilitarian decisions. Furthermore, at the same day of testing (i.e., day 7) we still observed an effect of TRP supplementation on the cortisol response to social stress in S'/S' carriers. Perhaps, in contrast to HPA-axis reactivity, social decision making and social cognition in general is more dependent on absolute TRP levels (i.e. acute increase or decrease in TRP availability). The studies that have shown the effect of serotonin availability on social decision making were interventions in which the effect of manipulating serotonin availability (either by means of SSRI's, TRP loading or depletion) was measured quickly after (approx. 1-2 hours) a single administration (Crockett *et al.*, 2008; 2010; Colzato *et al.*, 2013).

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Another possible methodological limitation may be the absence of dietary instructions on the day of testing itself (i.e., day 7). We asked participants to refrain from food intake 1 hour before arrival to the laboratory, however, more specific instructions (e.g. restricting protein rich food) regarding their food intake before arrival (i.e., for breakfast or lunch) to the laboratory may have been beneficial in order to control the possible drop in TRP availability to the brain due to competing amino acids.

Although we did find an effect of TRP supplementation on the cortisol response in S'/S' carriers, one could argue that our study was underpowered to detect genetic effects on social decision making as genetic studies require larger sample sizes (Review, Burmeister *et al.*,2008). Also, our parallel design, in combination with the number of participants may not have yielded enough power to replicate the pharmacological effects on the UG as Crockett *et al.* (2008) and Crockett *et al.* (2010) has found with a cross-over design, which has a larger statistical power.

Overall, our study may have benefitted from biological measures related to TRP metabolism. Although plasma TRP/LNAA ratio is a peripheral and indirect measure of TRP availability, plasma measures pre- and post-intervention a) would have given more insight in the possible explanation underlying the lack of behavioural effects on the UG following a 6-day TRP supplementation and b) would have revealed possible non-compliance, although the latter being very unlikely given the findings on the physiological level (Cerit *et al.* 2013).

2c. Effects of L-tryptophan – Underlying biological mechanism

The effects of tryptophan are rather well known as most of the early studies have investigated its additive antidepressant effects in combination with conventional antidepressant drugs or with electroconvulsive therapy (Coppen *et al.*, 1963; 1967; 1972; D'Elia *et al.*, 1977a,b; Møller *et al.*, 1980). Although research on the antidepressant properties of tryptophan has diminished due to the introduction of SSRIs (Parker and Brotchie, 2011), the effects of increasing serotonin availability on emotional information processing and mood are relatively well documented (Booij *et al.*, 2006; aan het Rot *et al.*, 2006). Therefore, in contrast to our ARA290 study - in which we directly applied the neuropsychological model of drug action in order to examine its antidepressant effects for the first time in healthy individuals - in the tryptophan study we included alternative measures in order to examine the beneficial effects of tryptophan in healthy individuals. Social decision making and HPA-axis reactivity were of special interest as both measures have been shown to be sensitive to serotonergic manipulations either induced by experimental interventions (TRP loading or depletion) or serotonergic antidepressants (Crocket *et al.*, 2008; 2010; Markus and Firk, 2009).

Tryptophan supplementation lowered the cortisol response to acute stress in S'/S'-carriers. The involvement of the serotonin system in the control of the HPA axis during an acute stress response is complex and remains a subject of study in both humans and animals (Markus

et al., 2000; Lepage *et al.*, 2002). The generally accepted view is that the serotonergic system is not an unitary system and has a stimulating as well as an inhibiting role in the regulation of a stress response (McAllister – Williams *et al.*, 1998; Markus *et al.*, 2000). The stimulatory effect of serotonin on the HPA-axis activity is mediated through 5HT1A and 5HT2a and 5-HT2c receptors in the hypothalamic paraventricular nucleus and the pituitary (McAllister – Williams *et al.*, 1998; Lowry, 2002), while the inhibiting effect of serotonin is mediated through 5-HT1A receptors in the hippocampus where serotonin exerts negative feedback control over the HPA axis (McAllister – Williams *et al.*, 1998; Markus *et al.*, 2000). Although the exact mechanism of the serotonergic control over the HPA axis is unclear, an imbalance/disturbance in the interaction between the two systems results in an inadequate biological response to stressful situations making it difficult to cope with stress (Markus *et al.*, 2000). Sufficient serotonergic activity during stressful situations is thought to be essential for an adequate biological response and stress adaptation (Markus *et al.*, 2000). Based on this premise Markus *et al.*, (2000) hypothesized that stress-prone individuals, defined as having high neuroticism scores, are subject to a constant lack of serotonin availability due to chronic stress exposure, and consequently, possess a hypersensitive serotonergic system. Stress-prone individuals with a hypersensitive serotonergic system are therefore expected to benefit from a diet that increases the TRP/LNAA ratios, and presumably central serotonin. Such diet would also result in a normalized HPA-axis activity/stress response, as indexed by a reduced cortisol response when exposed to acute stress (Markus *et al.*, 2000). The authors suggested two possible mechanisms of how increased serotonergic activity in stress-prone individuals may be involved in the control of the HPA-axis (i.e. reduced cortisol response): 1) Increasing serotonin availability may act on the serotonergic innervations of the hippocampus by the median raphe nucleus. It is known that serotonin in the hippocampus exerts negative feedback control over the HPA axis (Jacobson 1991; Markus 2000). Hippocampi with higher serotonergic sensitivity, as assumed to be the case in stress-prone individuals, may exert a stronger negative feedback on the HPA axis following increased TRP availability. As a result of this, a reduction of cortisol release will follow due to more pronounced inhibiting action of serotonin. 2) Increasing serotonin availability may lead to enhanced activation of higher cortical structures such as the prefrontal cortex, thereby increasing their control over limbic adreno-cortical system (Markus *et al.*, 2000).

Although these proposed underlying mechanisms remain speculative, the short variant of the 5-HTTLPR is associated with less serotonin uptake activity, leaving more serotonin in the synaptic cleft compared to the long variant. The 5HT1A auto-receptor is an important regulator in the serotonergic system and continuous stimulation of the 5HT1A receptor by 5HT in s/s carriers may decrease 5-HT synthesis and release resulting in shortage of 5-HT availability, similarly as has been shown for the mechanism of action of antidepressants (Blier and de Montigny, 1998). Assuming that the short allele carriers have a hypersensitive serotonergic system due to chronic shortage of serotonin availability (i.e. serotonergic activity is reduced by the inhibitory feedback of 5HT1A receptors), sub chronic administration of TRP

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may have normalized the reduced serotonergic activity. The normalization of serotonergic activity, in either the serotonergic innervations of the hippocampus or enhanced higher cortical control over limbic adrenocortical structures, may have resulted in normalization of cortisol response in S'/S' carriers of the 5-HTTTLPR genotype.

The underlying neural mechanism of the sensitivity to acute stress in S'/S' carriers of the 5-HTTLPR has been investigated by applying unpredictable electric shocks in healthy women during fMRI scanning (Drabant *et al.*, 2012). During anticipation to acute stress, S'/S' carriers exhibited an enhanced activation in the amygdala, hippocampus, anterior insula, thalamus, pulvinar, caudate, precuneus, anterior cingulate cortex, and the medial prefrontal cortex (mPFC) compared to L'-allele carriers (Drabant *et al.*, 2012). Although no increase of central serotonin availability was induced and no direct or indirect measures of HPA-axis were included, this study showed that brain structures involved in the processing of threat are more active during acute stress in S'/S' carriers, possibly due to a lack of (serotonergic) control of higher cortical structures over these limbic subcortical structures.

While a large meta- analysis on the effect of 5-HTTLPR on antidepressant efficacy concluded that 5-HTTLPR does not predict antidepressant response in MDD patients (Taylor *et al.*, 2010), another meta- analysis found that in Caucasians (but not in Asians), the L allele was associated with higher probability of response and remission when treated with SSRIs (Porcelli *et al.*, 2012). The finding that L allele carriers are more responsive to SSRIs, seems to contradict with our finding that tryptophan supplementation did not have an effect on the cortisol response during stress in L-allele carriers, while it did attenuate the cortisol response in S-allele carriers. Our study was conducted in a Caucasian population and although both tryptophan and SSRIs influence serotonergic neurotransmission, they are different interventions. SSRIs act on the serotonin transporter in order to block the re-uptake of serotonin from the synaptic cleft, whereas tryptophan is a precursor of serotonin. Besides its role in the brain, tryptophan metabolism occurs in other tissues, and it contributes to various bodily functions including immune response and intestinal functions (Le Floc'h *et al.*, 2011). Furthermore, the aforementioned meta-analyses have a focus on treatment response in patients, whereas our study has focussed specifically on the HPA axis reactivity as indexed by cortisol response to acute social stress in healthy individuals. These differences between the two interventions (antidepressant drugs and tryptophan) and the different focus of the meta-analysis (i.e. reduction of symptoms in patients), make it difficult to extrapolate the findings regarding 5-HTTLPR and SSRI efficacy to our study conducted with six days of tryptophan supplementation. In general our findings support the notion that variation of 5-HTTLPR genotype is associated with altered reactivity to serotonergic manipulations.

3. General Conclusion

The cognitive neuropsychological model has been proposed as a complementary tool to predict the efficacy of antidepressant compounds before the stage of large scale and

expensive RCTs (Harmer *et al.*, 2009; 2011). The effects of ARA290 were small and not all of these were in the expected direction of an antidepressant-like effect. Future studies may benefit from repeated ARA290 administration over time, and/or a shorter period between administration of ARA290 and testing. Furthermore, regardless of the model applied, it is essential to take into account the "biological environment" in which the compound is expected to exert an effect. In the future ARA290, as an anti-inflammatory compound, may be administered to healthy but depression vulnerable individuals as determined by high inflammation biomarkers. The beneficial effects of ARA290 may be more distinct when tapped into a specific (biological) vulnerability factor (i.e. inflammatory conditions).

Tryptophan lowered the cortisol response to stress only in S'/S' carriers of the 5-HTTLPR genotype while it did not affect the L'/L', which indicates a beneficial, although not necessarily an antidepressant effect, of tryptophan in a specific genetically vulnerable group. The effect of tryptophan on social decision making was not modulated by 5-HTTLPR genotype. In the future the modulation of social decision making by 5-HTTLPR genotype may be investigated by means of acute interventions (i.e. acute increase in TRP availability) rather than prolonged interventions (i.e. six days).

The potential of (human) experimental medicine models in predicting antidepressant drug efficacy may be improved by a) defining system-specific biological vulnerability factors implicated in the aetiology of depression together with the selection of the population based on these biological vulnerability factors, and by b) targeting biomarkers specific to the defective process implemented in depression.

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References

Aan het Rot M, Coupland N, Boivin DB, Benkelfat C, Young SN (2010). Recognizing emotions in faces: effects of acute tryptophan depletion and bright light. *J Psychopharmacol* 24: 1447-1454.

Aan het Rot M, Moskowitz DS, Pinard G, Young SN (2006). Social behaviour and mood in everyday life: the effects of tryptophan in quarrelsome individuals. *J of Psychiatry Neurosci* 31: 253-262.

Blier P, de Montigny C (1998). Possible serotonergic mechanisms underlying the antidepressant and anti-obsessive–compulsive disorder responses. *Biol Psychiatry* 44: 313-323.

Booij L, Merens W, Markus CR, Van der Does AJ (2006). Diet rich in α-lactalbumin improves memory in unmedicated recovered depressed patients and matched controls. *J Psychopharmacol* 20: 526-535.

Brines M, Patel NS, Villa P, Brines C, Mennini T, De Paola M *et al* (2008a). Nonerythropoietic, tissue-protective peptides derived from the tertiary structure of erythropoietin. *Proc Natl Acad Sci USA* 105: 10925-10930.

Brines M, Cerami A (2008b). Erythropoietin-mediated tissue protection: reducing collateral damage from the primary injury response. *J Intern Med* 264: 405-432.

Brines M, Cerami A (2012). The receptor that tames the innate immune response. *Mol Med* 18: 486-496.

Burmeister M, McInnis MG, Zollner S (2008). Psychiatric genetics: progress amid controversy. *Nat Rev Genet* 9: 527-540.

Cerit H, Jans LA, Van der Does W (2013). The effect of tryptophan on the cortisol response to social stress is modulated by the 5-HTTLPR genotype. *Psychoneuroendocrinology* 38: 201-208.

Colzato LS, Steenbergen L, de Kwaadsteniet EW, Sellaro R, Liepelt R, Hommel B (2013). Tryptophan Promotes Interpersonal Trust. *Psychol Sci* 24: 2575-2577.

Coppen A, Shaw DM, Farrell JP (1963). Potentiation of the antidepressive effect of a monoamine-oxidase inhibitor by tryptophan. *Lancet* 1: 79-81.

Coppen A, Shaw DM, Herzberg B, Maggs R (1967). Tryptophan in the treatment of depression. *Lancet* 290: 1178-1180.

Coppen A, Whybrow PC, Noguera R, Maggs R, Prange AJ Jr (1972). The comparative antidepressant value of L-tryptophan and imipramine with and without attempted potentiation by liothyronine. *Arch Gen Psychiatry* 26: 234-241.

Crockett MJ, Clark L, Hauser MD, Robbins TW (2010). Serotonin selectively influences moral judgment and behavior through effects on harm aversion. *Proc Natl Acad Sci USA* 107: 17433-17438.

Crockett MJ, Clark L, Tabibnia G, Lieberman MD, Robbins TW (2008). Serotonin modulates behavioral reactions to unfairness. *Science* 320: 1739-1739.

Dahan A, Dunne A, Swartjes M, Proto PL, Heij L, Vogels O *et al* (2013). ARA 290 improves symptoms in patients with sarcoidosis-associated small nerve fiber loss and increases corneal nerve fiber density. *Mol Med* 19: 334-345.

Dantzer R, O'Connor JC, Freund GG, Johnson RW, Kelley KW (2008). From inflammation to sickness and depression: when the immune system subjugates the brain. *Nat Rev Neurosci* 9: 46-56.

D'Elia G, Lehmann J, Raotma H (1977a). Evaluation of the combination of tryptophan and ECT in the treatment of depression. I. Clinical analysis. *Acta Psychiatr Scand* 56: 303-318.

D'Elia G, Lehmann J, Raotma H (1977b). Evaluation of the combination of tryptophan and ECT in the treatment of depression. II. Biochemical analysis. *Acta Psychiatr Scand* 56: 319-334.

Drabant EM, Ramel W, Edge MD, Hyde LW, Kuo JR, Goldi PR *et al* (2012). Neural mechanisms underlying 5-HTTLPR-related sensitivity to acute stress. *Am J Psychiatry* 169: 397-405.

Eccleston D, Ashcroft GW, Crawford TB (1970). Effect of tryptophan administration on 5HIAA in cerebrospinal fluid in man. *J Neurol Neurosurg Psychiatry* 33: 269-272.

Eckardt KU, Boutellier U, Kurtz A, Schopen M, Koller EA, Bauer C (1989). Rate of erythropoietin formation in humans in response to acute hypobaric hypoxia. *J Appl Physiol* 66: 1785-1788.

Ehrenreich H, Hinze-Selch D, Stawicki S, Aust C, Knolle-Veentjer S, Wilm S *et al* (2007b). Improvement of cognitive functions in chronic schizophrenic patients by recombinant human erythropoietin. *Mol Psychiatry* 12: 206-220.

Fu ZQ, Shao QL, Shen JL, Zhang YJ, Zhao XX, Yao L (2010). Effect of carbamylated erythropoietin on major histocompatibility complex expression and neural differentiation of human neural stem cells. *J Neuroimmunol* 221: 15-24.

Harmer CJ, Cowen PJ, Goodwin GM (2011). Efficacy markers in depression. *J of Psychopharmacol* 25: 1148-1158.

Harmer CJ, Goodwin GM, Cowen PJ (2009). Why do antidepressants take so long to work? A cognitive neuropsychological model of antidepressant drug action. *Br J Psychiatry* 195: 102-108.

Investigator's Brochure (2009): Compound Research Number: ARA 290. Edition Nr: 05

Jacobson L, Sapolsky R (1991). The role of the hippocampus in feedback regulation of the hypothalamic-pituitary-adrenocortical axis. *Endocr Rev* 12: 118–34.

Le Floc'h N, Otten W, Merlot E (2011). Tryptophan metabolism, from nutrition to potential therapeutic applications. *Amino Acids* 41: 1195-1205.

Lepage O, Tottmar O, Winberg S (2002). Elevated dietary intake of L-tryptophan counteracts the stress-induced elevation of plasma cortisol in rainbow trout (Oncorhynchus mykiss). *J Exp Biol* 205: 3679-3687.

Lowry CA (2002). Functional Subsets of Serotonergic Neurones: Implications for Control of the Hypothalamic-Pituitary-Adrenal Axis. *J Neuroendocrinol* 14: 911-923.

Markus CR, Firk C (2009). Differential Effects of Tri-Allelic 5-HTTLPR Polymorphisms in Healthy Subjects on Mood and Stress Performance After Tryptophan Challenge. *Neuropsychopharmacology* 34: 2667-2674.

Markus R, Panhuysen G, Tuiten A, Koppeschaar H (2000). Effects of food on cortisol and mood in vulnerable subjects under controllable and uncontrollable stress. *Physiol Behav* 70: 333-342.

McAllister-Williams RH, Ferrier IN, Young AH (1998). Mood and neuropsychological function in depression: the role of corticosteroids and serotonin. *Psychol Med* 28: 573-584.

McMahon FG, Vargas R, Ryan M, Jain AK, Abels RI, Perry B *et al* (1990). Pharmacokinetics and effects of recombinant human erythropoietin after intravenous and subcutaneous injections in healthy volunteers. *Blood* 76: 1718-1722.

Miskowiak K, O'Sullivan U, Harmer CJ (2007a). Erythropoietin Reduces Neural and Cognitive Processing of Fear in Human Models of Antidepressant Drug Action. *Biol Psychiatry* 62: 1244-1250.

Miskowiak K, O'Sullivan U, Harmer CJ (2007b). Erythropoietin enhances hippocampal response during memory retrieval in humans. *J Neurosci* 27: 2788-2792.

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Møller SE, Kirk L, Honore P (1980). Relationship between plasma ratio of tryptophan to competing amino acids and the response to L-tryptophan treatment in endogenously depressed patients. *J Affective Disord* 2: 47-59.

Moskowitz DS, Pinard G, Zuroff DC, Annable L, Young SN (2001). The Effect of Tryptophan on Social Interaction in Everyday Life: A Placebo-Controlled Study. *Neuropsychopharmacology* 25: 277-289.

Niesters M, Swartjes M, Heij L, Brines M, Cerami A, Dunne A *et al* (2013). The erythropoietin analog ARA 290 for treatment of sarcoidosis-induced chronic neuropathic pain. *Expert Opin Orphan Drugs* 1: 77-87.

Parker G, Brotchie H (2011). Mood effects of the amino acids tryptophan and tyrosine: 'Food for Thought' III. *Acta Psychiatr Scand* 124: 417-426.

Porcelli S, Fabbri C, Serretti A (2012). Meta-analysis of serotonin transporter gene promoter polymorphism (5-HTTLPR) association with antidepressant efficacy. *Eur Neuropsychopharmacol* 22: 239-258.

Pulman KG, Smith M, Mengozzi M, Ghezzi P, Dilley A (2013). The erythropoietin-derived peptide ARA290 reverses mechanical allodynia in the neuritis model. *Neuroscience* 233: 174-183.

Rosenblat JD, Cha DS, Mansur RB, McIntyre RS (2014). Inflamed moods: A review of the interactions between inflammation and mood disorders. *Prog Neuropsychopharmacol Biol Psychiatry* 53: 23-34.

Swartjes M, van Velzen M, Niesters M, Aarts L, Brines M, Dunne A *et al* (2014). ARA 290, a peptide derived from the tertiary structure of erythropoietin, produces long-term relief of neuropathic pain coupled with suppression of the spinal microglia response. *Mol Pain* 10: 13.

Taylor MJ, Sen S, Bhagwagar Z (2010). Antidepressant response and the serotonin transporter gene-linked polymorphic region. *Biol Psychiatry* 68: 536-543.

Van der Does AJ (2001). The effects of tryptophan depletion on mood and psychiatric symptoms. *J Affec Disord* 64: 107-119.

Van Velzen M, Heij L, Niesters M, Cerami A, Dunne A, Dahan A *et al* (2014). ARA 290 for treatment of small fiber neuropathy in sarcoidosis. *Expert Opin Investig Drugs* 23: 541-550.

Chapter 6

Appendix

Samenvatting

Achtergrond

Het testen van de effectiviteit van farmaca is een langdurig proces dat uit verschillende fases bestaat. Deze fases omvatten proefdieronderzoek, onderzoek in kleine aantallen vrijwilligers en uiteindelijk onderzoek bij patiënten voor wie het farmacon bedoeld is. Het sluitstuk van dit proces vormt het testen van het geneesmiddel in grootschalige gerandomiseerde gecontroleerde trials (randomized clinical trials; RCTs). Hierin worden de effecten van het middel vergeleken met placebo of met een reeds bekend middel. Het afronden van het hele proces duurt vaak meer dan tien jaar en het komt geregeld voor dat pas in de laatste fase (bij de RCTs) de effectiviteit onvoldoende blijkt. Er is dus behoefte aan gevoeligere indicatoren voor effectiviteit, die al in de eerdere fasen van het testen van farmaca duidelijker aanwijzingen geven over welke middelen het meest veelbelovend zijn om in grootschalige RCTs te testen.

In het geval van antidepressiva laten de RCTs zien dat ook de reeds geregistreerde middelen matig effectief zijn. Een RCT met de serotonine heropname remmer (SSRI) sertaline in chronische depressie patiënten resulteerde, na een behandeling van 12 weken, in een respons rate van 22% (d.w.z. minimaal 50% reductie in symptomen) en een remissie rate van 36% (d.w.z. reductie van symptomen tot beneden de diagnose drempel) (Keller *et al*., 1998). Een ander voorbeeld is een observationele studie waarbij behandeling met citalopram resulteerde in een respons rate van 47% en een remissie rate van 28-33% na 8 weken (Trivedi *et al*., 2006). Dit betekent dat ongeveer 50% van de patiënten met een chronische depressie niet reageren op antidepressiva (Keller *et al*., 1998; Trivedi *et al*., 2006). Meta-analyses van gerandomiseerde gecontroleerde trials wijzen uit dat antidepressiva de symptomen van depressie verbeteren, maar dat het verschil met placebo klein is. Dit verschil is alleen van klinisch belang in patiënten met een ernstige depressie (bij wie placebo minder effectief is) (Khan *et al*., 2002; Kirsch *et al*. 2008; Fournier *et al*., 2010).

Samenvattend, er is een noodzaak voor het ontwikkelen van nieuwe en effectievere antidepressiva voor de behandeling van depressie. Zoals hierboven vermeld, loopt het proces van het ontwikkelen van nieuwe medicatie vaak stuk in de laatste fase, die van grootschalige en dure gerandomiseerde klinische trials. Dan blijkt een medicijn dat aanvankelijk veelbelovend leek – in diermodellen of open trials met kleine aantallen proefpersonen – toch niet voldoende effectief te zijn (Harmer *et al*., 2011). Hierdoor is er behoefte aan experimentele modellen, die (vóór de fase van grootschalige klinische trials) als preklinische instrumenten ingezet kunnen worden om de effectiviteit van nieuwe medicijnen beter te kunnen onderzoeken en een mogelijke potentie beter te kunnen voorspellen. Hiertoe zijn er verschillende experimentele modellen beschreven die relevant zijn voor medicijnontwikkeling voor psychiatrische stoornissen (Dawson *et al*., 2011). Een van deze

modellen is het cognitieve neuropsychologische model van antidepressiva (Harmer *et al.*, 2009; 2011).

In dit proefschrift worden studies gepresenteerd waarbij gebruik is gemaakt van dit cognitieve neuropsychologische model. Een studie (Hoofdstuk 1 en 2) had als doel om de antidepressieve werking van een potentieel nieuw antidepressivum te onderzoeken, de neuropeptide ARA290. De tweede studie (Hoofdstuk 3 en 4) betrof een bestaand voedingssupplement (tryptofaan, precursor van serotonine). Tryptofaan (TRP) is verkrijgbaar zonder recept en word reeds veelvuldig ingenomen als voedingssupplement (bijv. om beter te kunnen slapen). In tegenstelling tot ARA290, is veel meer bekend over het effect van TRP op de cognitie welke een gerichtere toepassing van het neuropsychologisch model toeliet. Het effect van ARA290 is onderzocht met behulp van een standaard cognitieve testbatterij. Het effect van tryptofaan is onderzocht in een genetische kwetsbare groep waarin de fysiologische respons op sociale stress is gemeten en een aangepaste cognitieve testbatterij, waaronder een test voor sociale cognitie.

Beide studies vonden plaats in een gezonde populatie, omdat kwetsbaarheidmarkers van depressie (zoals die gezien worden in depressie patiënten die in remissie verkeren) ook gemeten kunnen worden met farmacologische interventies in gezonde populaties (Harmer *et al.*, 2003; Harmer *et al.*, 2008; Arnone *et al.*, 2009; Murphy *et al.*, 2009; Rawlings *et al.*, 2010). Een bijkomend voordeel is ook dat gezonde deelnemers niet lijden aan een huidige vorm en ook geen geschiedenis hebben van een psychiatrische stoornis waardoor de respons op de interventie niet gecontamineerd is door eerder farmacologische behandeling(en). Deze eigenschappen maken een gezonde populatie geschikt voor het onderzoeken van de effecten van een nieuwe interventie in een experimenteel psychologische setting.

Cognitieve neuropsychologische model van antidepressiva

Bestaande antidepressiva hebben soms 6-12 weken nodig om een klinisch effect te laten zien in patiënten met een depressie, maar blijken direct effect te hebben op de verwerking van emotionele informatie - zelfs na eenmalige toediening in gezonde vrijwilligers (Harmer *et al.*, 2003; Harmer *et al.*, 2008; Arnone *et al.*, 2009; Murphy *et al.*, 2009; Rawlings *et al.*, 2010). Het neuropsychologisch model beschrijft dat deze vroege meetbare effecten een gevolg zijn van de directe biologische veranderingen in verschillende processen op moleculair niveau, die gelijk na inname van antidepressiva plaatsvinden (Figuur 1). De initiatie van deze directe effecten (zoals bijvoorbeeld de toename van serotonine in de synaptische spleet) leiden op hun beurt weer tot een cascade aan neuro-adaptieve processen, maar ook tot een overgang van een voornamelijk negatieve bias naar een (meer) positieve bias in de verwerking van emotionele informatie (Review Harmer *et al.*, 2009). Volgens het neuropsychologisch model vindt er een interactie plaats tussen geïnduceerde neuro-adaptieve processen en veranderingen in de verwerking van sociaal-emotionele stimuli uit de omgeving, welke na een bepaalde tijd resulteert in stemmingsverbetering. De vroege verandering van een negatieve

naar een meer positieve verwerking van sociaal-emotionele stimuli is tevens geassocieerd met neurale veranderingen in subcorticale en corticale circuits die gedetecteerd kunnen worden door middel van MRI-technieken (Review, Harmer *et al.*, 2009).
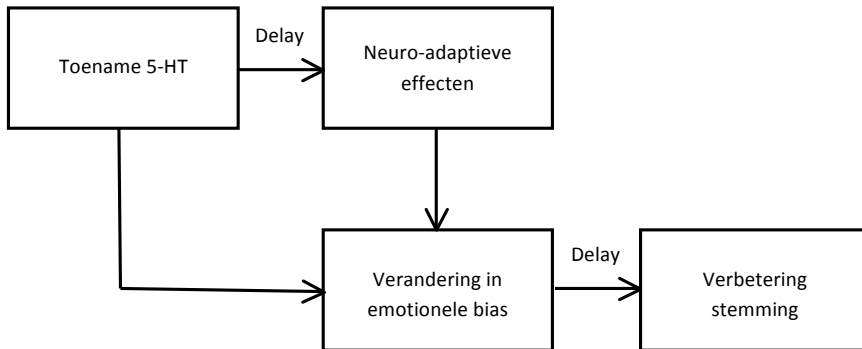
Figuur 1. Onderliggende mechanisme van de vertraagde klinische effect van antidepressiva als voorgesteld door het neuropsychologisch model (Harmer *et al.*, 2009).

Het cognitieve neuropsychologische model bestaat uit een reeks aan cognitieve en neurale maten die het doel hebben om de vroege antidepressieve effecten te meten in gezonde vrijwilligers en hierdoor een betere schatting te maken van de potentie van een antidepressieve interventie. De testen (ofwel taken) waarvan in dit model gebruik wordt gemaakt, richten zich op het oppikken van de verschuiving van een negatieve bias naar een meer positieve bias in de verschillende processen die een rol spelen in het verwerken van emotionele informatie zoals bijv. aandacht en geheugen (Review Harmer *et al.*, 2009).

Naast de bestaande gedrags- en neurale maten van dit model zijn in het onderzoek, dat in deze dissertatie is beschreven, maten meegenomen die specifiek gericht zijn op het mechanisme van de beoogde interventie (nl. tryptofaan). Een van de meest bestudeerde genetische variaties die gerelateerd is aan depressie, is een allelvariatie in de promotor regio van de serotonine transporter gen: het serotonine transporter polymorfisme (5-HTTLPR) (Heils *et al.*, 1996). Afhankelijk van de combinatie van de allelen (kort of lang) in de promotor regio van het serotonine transporter gen worden er meer of minder serotonine transporters tot expressie gebracht in de membranen van de presynaptische neuronen, wat op zijn beurt een invloed heeft op de serotonine neurotransmissie (Canli & Lesch, 2007). Naar de relatie tussen 5-HTTLPR en stress respons is veel onderzoek gedaan vanwege de a) rol van het serotonerge systeem in depressie b) rol van de hypothalamus-hypofyse-bijnier-as (HHB-as; Engels: HPA-axis) reactiviteit in depressie en c) de interactie tussen het serotonerge systeem en de HPA-axis in het reguleren van de neuro-endocriene responsen. Meerdere studies hebben laten zien dat de HPA-axis reactiviteit verschilt afhankelijk van de variant van het serotonine transporter polymorfisme (Gotlib *et al.*, 2008; Way and Taylor, 2010). Een meta-

analyse laat zien dat er een associatie bestaat tussen het 5-HTTLPR genotype en HPA-axis reactiviteit in respons op stress, waarbij de homozygoot kortere variant (met een minder efficiënte serotonine neurotransmissie) een hogere cortisol respons laat zien (Miller *et al*., 2012). Behalve HPA-axis reactiviteit is het 5-HTTLPR genotype ook geassocieerd met een variatie in verwerking van emotionele informatie (Perez-Edgar *et al*., 2010; Antypa *et al*., 2011; Koizumi *et al.*, 2013). In deze dissertatie zijn 5-HTTLPR genotype samen met HPA-axis reactiviteit en sociale-emotionele informatie verwerking geïncludeerd als maten die specifiek gericht zijn op de beoogde interventie: tryptofaan.

Samenvatting van de bevindingen

Wij onderzochten de antidepressieve effecten van ARA290 (*i.v.* 2 mg) op gedrags- en neurale (functionele MRI) maten gerelateerd aan de verwerking van emotionele informatie in gezonde proefpersonen (N= 36) (**Hoofdstuk 2**). Deze placebo gecontroleerde studie werd dubbelblind en gerandomiseerd (in parallel groep design) uitgevoerd. ARA290 groep liet (t.o.v. placebo groep) een verlaagde neurale (BOLD) respons zien in de bilaterale gyrus fusiformis tijdens het verwerken van blije gezichten, terwijl het verwerken van angstige gezichten een verhoogde neurale activiteit liet zien in de rechter gyrus fusiformis. Op de gedragsmaten leek de ARA290 groep (t.o.v. placebo groep) gezichtsexpressies van blijdschap en walging minder goed te herkennen. Hoewel ARA290 niet geassocieerd is met een beter geheugen voor positieve woorden, vonden we wel een associatie van ARA290 met snellere categorisatie van positieve *vs.* negatieve woorden. De ARA290 groep liet (t.o.v. placebo) tevens een verhoogde aandacht voor emotioneel positieve plaatjes zien. We vonden geen effecten van ARA290 op stemming of affectieve symptomen. In dezelfde groep hebben we het effect van ARA290 op resting-state activiteit onderzocht (**Hoofdstuk 3**). Een week na eenmalige toediening heeft ARA290 geen effect op a) de connectiviteit van de acht standaard netwerken (beschreven zijn door Beckmann *et al.,* 2005) en b) de vooraf gedefinieerde seed regio's (hippocampus/amygdala en gyrus fusiformis).

Uit Hoofdstuk 2 blijkt dat ARA290 enige aspecten van het verwerken van emotionele informatie moduleert, echter, de richting en de sterkte van de effecten ondersteunen niet een eenduidig antidepressief effect. Aangezien ARA290 geen invloed heeft op depressie gerelateerde netwerken, kan worden geconcludeerd dat de cognitieve effecten van ARA290, zoals beschreven in Hoofdstuk 2, niet gemedieerd worden door een effect op functionele connectiviteit.

Toekomstig onderzoek zou het effect van hogere doseringen en kortere tijdsinterval op functionele connectiviteit moeten uitwijzen. Vanwege de anti-inflammatoire eigenschap van ARA290, is het mogelijk dat effecten van ARA290 gemedieerd worden door anti-inflammatoire acties binnen het centrale zenuwstelsel. Toekomstig onderzoek zou zich daarom kunnen richten op het onderzoeken van de cognitieve effecten van ARA290 in populaties waar sprake is van inflammatie in het centrale zenuwstelsel. Aangezien het ontwikkelen van nieuwe

antidepressiva met klinisch relevante effecten mede bemoeilijkt wordt door heterogeniteit van depressie, zou onderzoek naar de cognitieve effecten van ARA290 in een subgroep van depressie patiënten (waarin de aanwezigheid van hoge inflammatie bio-markers een belangrijke rol spelen) meer licht kunnen werpen op de effectiviteit en het onderliggende antidepressieve mechanisme van ARA290.

In **Hoofdstuk 4** onderzochten we de effecten van tryptofaan inname gedurende zes dagen (2.8 g/d) op HPA-axis reactiviteit in gezonde proefpersonen (N=46) die dragers zijn van het homozygoot korte (S'/S') of homozygoot lange (L'/L') variant van de serotonine transporter polymorfisme (5-HTTLPR). Deze placebo gecontroleerde studie werd dubbelblind en gerandomiseerd (in parallel groep design) uitgevoerd. Zes dagen na tryptofaan of placebo inname werd in alle proefpersonen stress geïnduceerd middels een sociale stressor (Trier Social Stress Taak) en cortisol gemeten als een maat voor HPA-axis reactiviteit. TRP verlaagt de cortisol respons na blootstelling aan sociale stress in de S'/S' groep, terwijl TRP geen effect heeft op de cortisol respons in de L'/L'groep. Tryptofaan lijkt de acute stress respons in een genetisch kwetsbare groep te normaliseren, hetgeen kan duiden op een antidepressieve werking. Hoewel tryptofaan suppletie een effect heeft op fysiologie, lijkt het geen effect te hebben op socio-emotionele informatieverwerking welke is gemeten met de Ultimatum Game (**Hoofdstuk 5**). Deze taak vereist het vermogen om een initiële emotionele reactie op een oneerlijk bod van de tegenspeler te overwinnen ten behoeve van eigen baat. Hoewel de TRP groep neigde naar meer weigering van onrechtvaardige aanbiedingen van de tegenspellers was deze bevinding niet significant. Ook blijkt 5-HTTLPR genotype geen rol te spelen.

Conclusie

In deze dissertatie is het cognitieve neuropsychologische model toegepast als preklinisch instrument om de effectiviteit van mogelijke antidepressieve middelen te kunnen voorspellen. ARA290 heeft effect op enkele domeinen van emotionele informatie verwerking, echter, de richting en de sterkte van de effecten ondersteunen niet een eenduidig antidepressief effect. Tryptofaan lijkt de cortisol respons in een genetisch kwetsbare groep te normaliseren, hetgeen kan duiden op een antidepressieve werking. De potentie van preklinische experimentele modellen t.b.v. het voorspellen van effectiviteit van (nieuwe) antidepressieve middelen zouden verbeterd kunnen worden door het zorgvuldig definiëren van biologische en systeem-specifieke kwetsbaarheidsfactoren gerelateerd aan de etiologie van depressie.

Appendix

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Hilal Cerit, Leiden

Biography

Hilal Cerit (06-12-1981) was born in Zaandam, The Netherlands. She obtained her HAVO diploma at CVO Pascal College in 2000. From 2001 to 2005 she studied Biomedical engineering at HU University of Applied Sciences in Utrecht, Netherlands. After obtaining her Bachelor's degree she continued her education at the VU University in Amsterdam where she completed the Research Master Neurosciences (MSc) in 2008. From February till November 2007 she conducted research for her Master thesis under supervision of Prof. dr. Robert Rogers at the Department of Psychiatry at the University of Oxford, United Kingdom. In 2009 she started her PhD work under the supervision of Prof. dr. Willem van der Does at the Department of Clinical, Health and Neuropsychology at Leiden University, The Netherlands.

Appendix

Publication list

- **H Cerit**, LAW Jans, AJW Van der Does (2013) The effect of tryptophan on the cortisol response to social stress is modulated by the 5- HTTLPR genotype. *Psychoneuroendocrinology* 38, 201 - 208.

- N Antypa, **H Cerit**, AW Kruijt, FEA Verhoeven, AJW Van der Does (2011) Relationships among 5-HTT genotype, life events and gender in the recognition of facial emotions. *Neuroscience* 172, 303-313.

- FEA Verhoeven, L Booij, AW Kruijt, **H Cerit**, N Antypa, AJW Van der Does (2012) The effects of MAOA genotype, childhood trauma and sex on trait and state-dependent aggression. *Brain and behavior* 2, 806-813.

- **H Cerit**, IM Veer, A Dahan, M Niesters, CJ Harmer, KW Miskowiak, SARB Rombouts and AJW Van der Does. Testing the antidepressant properties of the peptide ARA290 in a human neuropsychological model of drug action. *Under review.*

- **H. Cerit**, RJ Schuur, AJW Van der Does. The effect of 5-HTTLPR genotype and tryptophan supplementation on the response to unfairness in healthy volunteers. *Under review.*

- **H. Cerit**, E Ghariq, KG Ramlakhan, IM Veer, SARB Rombouts, AJW Van der Does and M de Rover. The Effects of ARA290, an Erythropoietin Analogue, on Resting State Networks Associated with Depression: a randomized placebo-controlled trial. *In preparation.*

Appendix