Serotonin Transporter (5-HTTLPR) Variation and Anterior Cingulate Cortex in Relation to Cognitive Load: An fMRI Study

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Abstract

Background: The serotonin transporter (5-HTTLPR) short allele has reduced transcriptional efficiency compared to the long allele, and individuals carrying the short allele tend to have increased risk for depression. Major Depressive Disorder is characterized by region-specific anterior cingulate cortex (ACC) dysfunction. However, whether this represents a trait marker of depressive mood or a vulnerability factor has not yet been addressed. A logical approach to answer this question is to study healthy individuals with or without genetic risk. The main aim of this study was to explore whether ACC activation is associated with 5-HTTLPR variants when cognitive load increases in healthy subjects. A secondary aim was to investigate behavioural data from the fMRI events and its potential association with functional activity within the anterior cingulate.

Methods: A total of 38 healthy female participants, screened for symptomatology related to anxiety and depression, were included in the fMRI study. Participants were genotyped according to the 5-HTTLPR triallelic model. Cognitive load was measured in an fMRI modified n-back procedure.

Results: The main finding was that dorsal ACC activation increased with the number of short alleles and cognitive load. The behavioural data showed significant group differences on 3-back accuracy, such that the short allele carriers made the most errors.

Conclusion: The increased dorsal ACC activation is interpreted as a cognitive compensation mechanism, whereby it is suggested that SS carriers find the task the most difficult. This may indicate an association between 5-HTTLPR variability and cognitive vulnerability.

Introduction

Mood disorders are a leading cause of disability and represent a significant mental health concern to individuals and to our society. The lifetime prevalence for Major Depressive Disorder (MDD) is as high as 13.5% - 21.2% (Kessler et al., 2005; Turner & Gil, 2002). Depression is one of the main disease burdens throughout the world and is associated with serious medical conditions and mortality across the lifespan (Lyche, Jonassen, Stiles, Ulleberg, & Landrø, 2011). Together with schizophrenia, depression is related to 60% of all suicides worldwide (World Health Organization, [WHO], 2009). MDD is associated with significant disability and poorer quality of life. Many patients have problems in fully participating in social and/or family life, and often have problems in meeting other expectations from society (Hammar & Årdal, 2009). Historically, MDD was seen as an episodic disorder but recent findings have indicated that developing a chronic course of the disease has been underestimated (Rush, 2001). Patients diagnosed with MDD show neurological abnormalities in emotion processing (Phillips, Drevets, Rauch, & Lane, 2003). MDD patients show a tendency to orient toward negative emotional stimuli and have difficulties disengaging attention from emotional stimuli that are congruent with depressive mood (Mathews, Ridgeway, & Williamson, 1996).

Serotonin has been found to be critical for the development of emotional circuitry in the brain (Pezawas et al., 2005). In particular, one allelic variant (short allele) of the serotonin transporter (5-HTTLPR) is associated with an increased vulnerability for developing depression within the context of negative life events (Caspi et al., 2003). The importance of serotonergic neurotransmission for the pathogenesis of depression is shown in pharmacological studies. Pharmacological manipulation of serotonin represents a common approach to treating depression (Murphy et al., 2003). Neuroimaging studies have also demonstrated abnormal brain structure and functioning in MDD patients associated with variations in the serotonergic circuitry (Malison et al., 1998; Pezawas et al., 2005; Phillips et al., 2003).

Although depression is traditionally seen as affective in nature, the last decade's research has shown that depression is also associated with disturbance in cognitive functioning (Hammar & Årdal, 2009). MDD patients often report attentional problems, and impaired ability to think and concentrate is one criterion for the MDD diagnosis (American Psychiatric Association [*DSM-IV-TR*], 2000). Several studies have reported attentional deficits in neutral (non emotional) tasks in MDD patients (Koetsier et al., 2002; Majer et al.,

2004; Porter, Bourke, & Gallagher, 2007). Depression is also associated with impairment in different cognitive domains, such as executive functions, attention, memory and psychomotor speed (Elliott, 1998; Harvey et al., 2004; Landrø, Stiles, & Sletvold, 2001; Porter, Gallagher, Thompson, & Young, 2003). Neuropsychological deficits in emotion processing among depressed patients have been associated with serotonin levels in the brain (Hariri et al., 2002). It has also been suggested that deficits in emotion processing in depressed patients could be associated with synaptic plasticity in serotonin neurons (Branchi, 2011), but the role of serotonin in cognition is still not clear.

An important approach to the study of emotion and cognition has been to consider the genetic, neural, neurochemical and neuropsychological substrates of mental illness. Cognitive neuroscience is a relatively new field that studies the neurobiological mechanisms that underly mental functioning, and which increasingly incorporates genetic data. Research that combines genetic and cognitive neuroscience data has the potential to elucidate the mechanisms that underlie human behaviour by looking at intermediate phenotype; variations in brain function. One of the aims of this hybrid approach is to relate variation in specific genes to variation in brain activity and psychological phenotypes, including cognitive, affective and social information processing (Green et al., 2008).

Emotion and Serotonin

Emotion processing model. The interaction between emotion and cognition has been studied using a model based on the theoretical framework of disturbances associated with MDD (Phillips, Ladouceur, & Drevets, 2008). Functional abnormalities in these neural systems may cause severe emotion dysregulation and vulnerability to develop psychiatric disorders. Studying deficits in emotion perception and behaviour may be important to understanding emotional processing in healthy individuals (Phillips et al., 2003).

Patients with MDD, compared with healthy controls, have shown increased activation in areas central for generating emotional behaviour, and in areas involved in the identification of emotional stimuli. Structures with increased activation in MDD patients include the amygdala, ventral striatum, thalamus, orbitofrontal cortex (OFC), anterior cingulate cortex (ACC), ventrolateral prefrontal cortex (VLPFC), and anterior insula. In addition, decreased activation in MDD patients has been found in dorsomedial prefrontal cortex (DMPFC) and dorsolateral prefrontal cortex (DLPFC), which are central emotion regulation (Phillips et al., 2003).

Philips' et al. (2008) model for emotion regulation consists of a medial prefrontal cortex (PFC) system and a lateral prefrontal cortex (PFC) system. The medial PFC system is involved in automatic aspects of emotion regulation. This feedforward system is made up of OFC, subgenual anterior cingulate gyrus (ACG), rostral ACG, and hippocampus. The DMPFC and dorsal ACG are involved in both automatic and voluntary emotion regulation. The second system, lateral PFC also described as a feedback system, consists of DLPFC and VLPFC. These structures subserve voluntary aspects of emotion regulation. The lateral and medial system work together and are both active in regulation of emotions and emotion perception. The two systems depend upon subcortical regions such as the amygdala, ventral striatum, and thalamus, which are involved in emotion perception and identification. This emotion regulation model indicates top-down processing in emotion regulation, where top-down emotion regulatory processes involve dorsal prefrontal cortical regions, and bottom-up processes involve the generating of emotion in subcortical, limbic neural regions (see Figure 1).

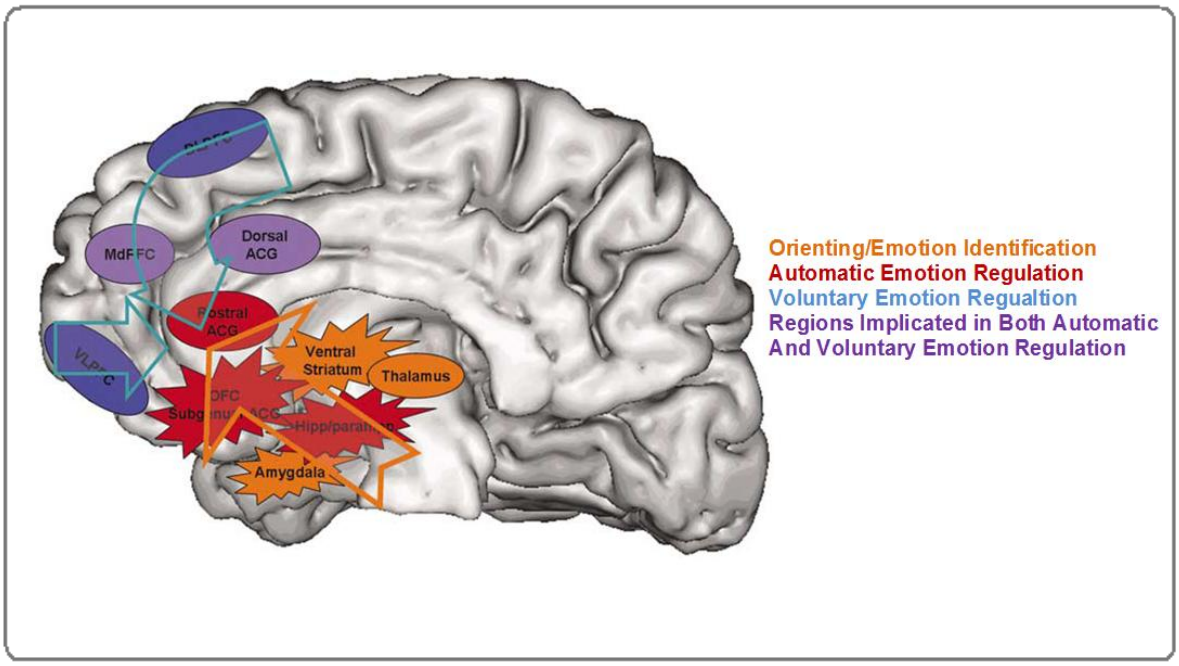

Figure 1. Emotion processing model with two major emotion regulating systems; (1) feedforward pathway (orange arrow): medial prefrontal cortical system, including the orbital frontal cortex, subgenual anterior cingulate gyrus, rostral anterior cingulate gyrus, hippocampus and parahippocampus, and dorsomedial prefrontal cortex, and (2) feedback pathway (green arrow): lateral prefrontal cortical system, including dorsolateral prefrontal cortex and ventrolateral prefrontal cortex. DLPFC: dorsolateral prefrontal cortex; DMPFC (MdPFC): dorsomedial prefrontal cortex; ACG: anterior cingulate gyrus; VLPFC: ventrolateral prefrontal cortex; OFC: orbito frontal cortex; hipp/parahip: hippocampus-parahippocampus region. Figure 1 is modified from Philips et al. (2008).

Anterior cingulate cortex function in emotion processing. The emotion processing model divides the ACC into separate subregions. ACC is often found to be divided into the following regions; dorsal ACC (BA 32) also called caudal ACC, perigenual ACC also known as rostral/ventral ACC, and subgenual ACC (BA 25). Subgenual ACC can also be further divided into posterior and anterior components (Bush, Luu, & Posner, 2000; Pezawas et al., 2005). The subgenual ACC and rostral ACC are involved in automatic emotion regulation, while the dorsal ACC is involved in both automatic and voluntary emotion regulation (Philips et al., 2008). The ACC is located in the human limbic system, and is known to be functionally divided between emotion and cognitive processing. Healthy participants performing cognitive tasks, show a relative deactivation in rostral regions of ACC. In contrast, when given significant emotion-related stimuli, participants show a relative deactivation in dorsal ACC (Bush et al., 2000; Pezawas et al., 2005; Ridderinkhof, Ullsperger, Crone, & Nieuwenhuis, 2004). During emotional-related tasks, participants also show an increased rostral ACC activation (Bush et al., 1998; Gruber, Rogowska, Holcomb, Soraci, & Yurgelun-Todd, 2002; Whalen et al., 1998).

A recent review of several neuroimaging studies demonstrates emotion and cognitive activation overlap in the dorsal ACC, and conceptualizes this region as anterior midcingulate cortex (aMCC). These anatomical studies reveal that the aMCC constitutes a hub involved in monitoring, generating and processing several distinct aspects of information. The aMCC has been linked to motor centres, functions responsible for expressing affect, and executing goal-directed behaviour. On this basis, it is claimed that the cingulate cortex is no longer strictly separated into cognitive and affective subregions (see Figure 2) (Shackman et al., 2011).

The serotonin system highly affects ACC, and subgenual ACC displays the highest density of 5-HTT terminals within the human cortex. In addition, serotonin affects several functions, and regulates synaptic plasticity and neural activity patterns in both serotonergic and non-serotonergic neurons (Gaspar, Cases, & Maroteaux, 2003). Abnormal or manipulated functioning in the serotonin system will alter the duration and intensity of 5-HT communication with its receptors and postsynaptic targets which are mainly located in limbic structures such as ACC. The 5-HTT function has both excitatory and inhibitory effects on the postsynaptic cell. Decreased 5-HTT gene function increases serotonin levels and leads to reduced receptor binding to receptor 5-H1A, but at the same time, increases binding in other 5-HTTLPR receptors (Borg et al., 2009; Lesch & Gutknecht, 2005).

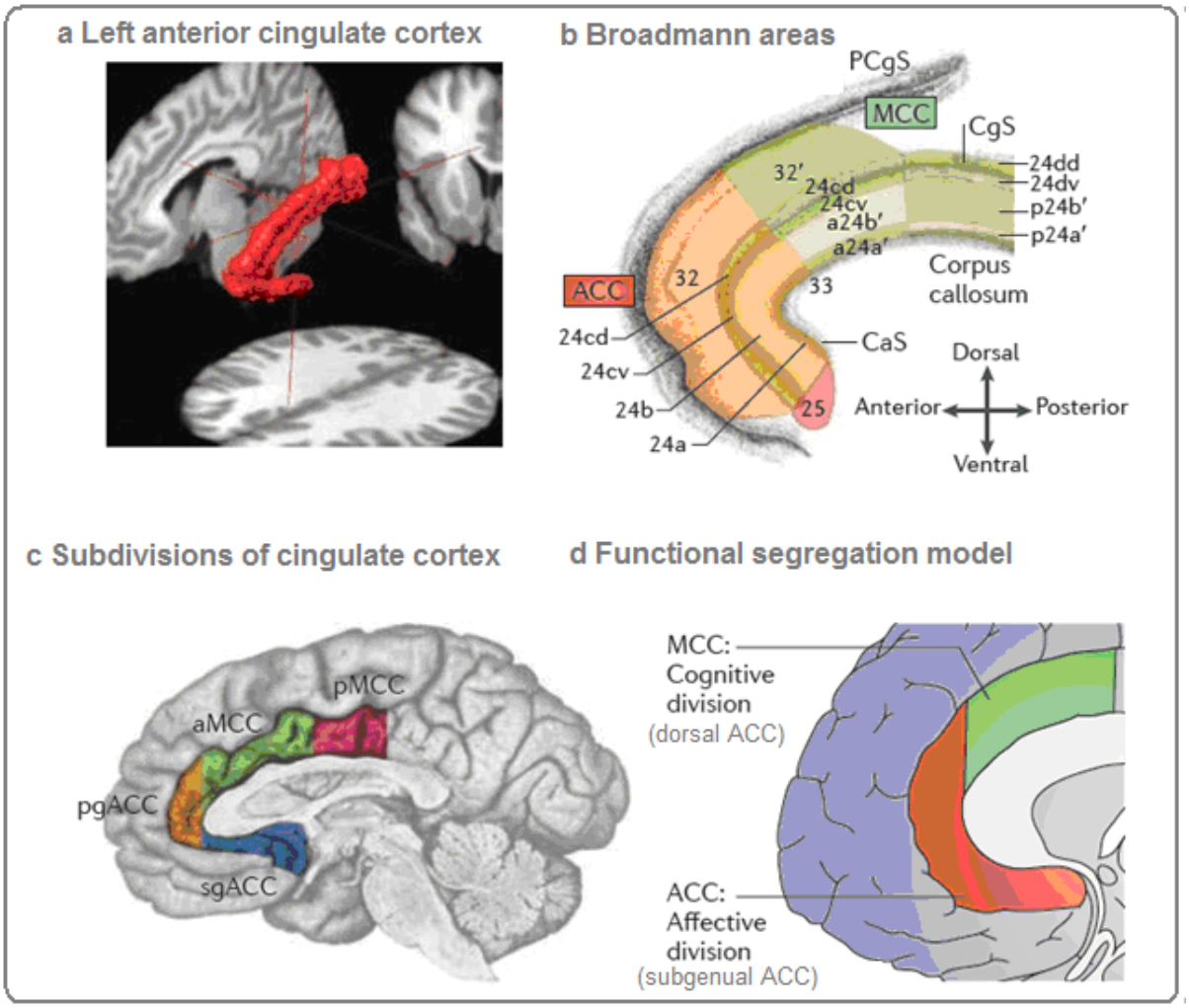

Figure 2. Functional subdivisions within the human anterior cingulate cortex; **a**: Three-dimensional rendering of the left anterior cingulate cortex (shown in red); **b:** Anterior cingulate subdivisions defined based on Broadmann areas (BA). The areas were defined on the basis of differences in microanatomy and neurotransmitter chemistry, hence differing somewhat from the classical descriptions of Brodmann and other pioneering neuroanatomists; **c:** Four major subdivisions of the cingulate cortex, a relatively new approach to how ACC could be divided, based on the article form Shackman et al. (2011). The supracallosal portion of the cingulate is designated the midcingulate cortex (MCC) and is divided into anterior (aMCC; shown in green) and posterior (pMCC; shown in magenta) subdivisions. The portion of the cingulate lying anterior and ventral to the corpus callosum is designated the anterior cingulate cortex (ACC) and is divided into pregenual (pgACC; shown in orange) and subgenual (sgACC; shown in cyan) subdivisions by the coronal plane at the anterior tip of the genu; **d:** The functional segregation model of Bush et al. (2000). On physiological and anatomical grounds, Bush et al. (2000) divide ACC into two functionally segregated regions: (1) rostroventral affective division (subgenual ACC, BA 25) and (2) dorsal ACC cognitive division (also referred to as aMCC, BA 32). PCgS: paracingulate sulcus; CaS: callosal sulcus; CgS: cingulate sulcus. Figure 2 is modified from Shackman et al. (2011).

Variability in the Serotonin Transporter Linked Region (5-HTTLPR)

Expression of the 5-HTT gene depends on the kinds of transcriptional factors that are connected to the gene in that particular case. The link between variations in 5-HTTLPR length and the 5-HTT function is studied by looking at the 5-HTTLPR genotype, 5-HTT gene transcription, and 5-HT reuptake. Transcriptional activity in the 5-HTT gene promoter is mediated by the presentation of short (S) or long (L) allelic variation in 5-HTTLPR. Cells homozygous for the long allele (LL) produce higher concentrations of the 5-HTT mRNA and have the highest expression of serotonin, compared to cells with one or two copies of the short variant. Cells homozygous for the short allele (SS) have the lowest expression of serotonin. The significant reduced 5-HTT mRNA and protein in SS carriers leads to slower reuptake and, consequently, to higher concentrations of serotonin in the synaptic gap. Research combining data technology, mathematics, biochemistry, and statistics has made it possible to detect both the quantity of the 5-HTT protein and the activity in this channel linked to 5-HTTLPR variation (Lesch & Gutknecht, 2005; Mortensen, Thomassen, Larsen, Whittemore, & Wiborg, 1999).

There has been some hesitance when defining genotypes. Until recently, the 5-HTTLPR genotypes have been considered to be functionally biallelic (Lesch et al., 1996). By using current technology; data technology, mathematics, biochemistry and statistics, two variants within the long variant allele were identified, L_A and L_G, suggesting a triallelic functional polymorphism. The L_G and S alleles have comparable levels of 5-HTT transporter expression and are both low expressing alleles compared to the L_A allele. Studies not using a triallelic model, and include L_G with LS and LL genotypes, may underestimate the effect of 5-HTTLPR polymorphism. It has also been suggested that 5-HTT genotypes incorporating L_G will better predict 5-HTT expression in the brain (Hu et al., 2006). However, there are contradicting results demonstrating that L_G could be associated with either long or short variants (Wendland, Martin, Kruse, Lesch, & Murphy, 2006).

Imaging Genetics Associated with 5-HTTLPR Variability

The majority of studies on 5-HTTLPR variation function have been conducted by non-invasive techniques. Several different techniques, such as functional neuroimaging procedures like ERP (event-related potentials), fMRI (functional magnetic resonance imaging), perfusion MR and PET (positron emission tomography) have influenced research on 5-HTTLPR allelic variation considerably.

The first study that explored genetic variation using neurophysiological imaging was based on an endophenotypic approach using ERP (Fallgatter, Jatzke, Bartsch, Hamelbeck, & Lesch, 1999). An endophenotype, such as a characteristic difference in brain function, is hypothesised to convey a simple genetic architecture, and may be more directly linked to genomic variation than highly variable behavioural phenotypes (Canli & Lesch, 2007). In the study, Falgatter et al., (1999) tested healthy individuals without symptomatology on cognitive response control (Go-No Go) task and the results showed an association between 5-HTTLPR genotype and prefrontal cortex-limbic excitability. They found that participants with one or two 5-HTT short variants showed higher prefrontal ERP activity than non carriers (carriers homozygous for the long variant). The increased response in the frontal cortex, especially in ACC, suggests a relationship between cognitive processing and the allelic variation in 5-HTTLPR function (Fallgatter et al., 2004; Fallgatter et al., 1999). Given the role of 5-HTT in emotion processing, these studies highlight the importance of studying gentics associated with complex brain functions.

Hariri et al. (2002) demonstrated higher functional amygdala activation in short allele carriers when given emotion-related tasks (potential fright-evoking stimuli), compared to neutral control tasks (Hariri et al., 2002). A follow-up study with larger sample size and similar design has show convergent results (Hariri et al., 2005; Heinz et al., 2005). In addition Hariri et al. (2002) also report a correlation between amygdala and VMPFC as a function of the 5-HTTLPR short allele. This association was specific to the presentation of negative images, relative to neutral images.

The 5-HTTLPR variability is also associated with significant differences in frontal cortical regions, anterior cingulate, and cerebellum. These significant differences in frontal activation indicate that the 5-HTTLPR transporter may have a much broader role in brain processes than previously assumed. The 5-HTTLPR variability may not only affect emotion processing, but also neural systems controlling cognitive processes (Canli et al., 2005).

Research on functional connectivity has shown that 5-HTTLPR variability also affect structural connections and functional interactions in the brain. An fMRI study by Pezawas et al. (2005) found that amygdala and ACC were significantly functionally connected and that healthy short variant carriers showed a decrease in connectivity between the structures. Furthermore, there were found two distinguished functionally divergent regions within ACC when studying amygdala connectivity; rostral ACC activity was positively correlated with the amygdala activity, and dorsal ACC activity was negatively correlated with amygdala activity.

Rostral ACC and dorsal ACC also showed strong positive connectivity with each other. The study suggests that the structures form a functional feedback loop with the amygdala. Short variant carriers in the same study also showed decreased grey matter volume in both amygdala and subgenual ACC, compared to the LL and LS groups. The results suggest a possible link between 5-HTTLPR variation and amygdala regulation when processing stimuli. The functional feedback loop between ACC and amygdala also points towards 5-HTTLPR-dependent variability in basic brain mechanisms, such as the interplay between cognitive and emotional processing (Pezawas et al., 2005).

A tonic activation model has also revealed 5-HTTLPR variability to be associated with differences in brain activation, proposing an alternative explanation to the amygdala activation. This theory by Canli et al. (2006) posits that presence of the short variant allele does not enhance amygdala reactivity to emotional stimuli, but rather enhances amygdala activation at rest. The perfusion data confirms that 5-HTTLPR short variant carriers show elevated levels of amygdala activation at rest, compared with carriers homozygous for the long variant. Furthermore, it has been demonstrated that increased amygdala activation found in short variant carriers when exposed to stimuli (negative versus neutral stimuli), is not driven by increased activation to negative stimuli. The observed increased amygdala activation is instead suggested to be driven by decreased activation to neutral stimuli, when compared with a fixation rest condition (Canli et al., 2006).

Research concerning the 5-HTTLPR transporter has been considerably elaborated on and widely published. A meta-analysis reanalysed data from fourteen studies using fMRI, PET and perfusion MR. The results showed that the association of the 5-HTTLPR polymorphism and amygdala account for up to 10% of phenotypic variance (Munafò, Brown, & Hariri, 2008).

Anterior Cingulate Functioning in Cognitive Processing

Together, these neuroimaging studies indicate that 5-HTTLPR allelic variation may not only influence emotional regulation processes, but also influence brain circuits involved in different types of monitoring, such as cognitive processing. The dorsal subdivision of ACC is involved in both automatic and voluntary emotion regulation (Philips et al., 2008). Dorsal ACC is also found to be active when solving cognitive tasks and is interconnected with lateral PFC, parietal cortex, and premotor and supplementary motor areas which together make up an attentional network. When tasks require a high level of mental effort, both dorsal ACC and areas of the lateral PFC operate together (Bush et al., 2000). The PFC is made up of

orbitofrontal and ventromedial areas, DLPFC and cingulate cortex. Together, these structures are involved in planning complex cognitive behaviour. Mental shifting, updating and inhibition of new information are key functions for efficient cognitive processing (Miyake et al., 2000). Due to its connections to other PFC structures, ACC is known to be involved in various functions such as modulating executive functions, monitoring competition, anticipation of cognitively demanding tasks, complex motor control, motivation, novelty, error detection and working memory (Bush et al., 2000).

It has recently been suggested that there is no separate subregion for emotional and cognitive processing within the ACC, although there are contradictory findings concerning this argument (Shackman et al., 2011). The importance of dorsal ACC in cognitive processing has also been demonstrated in a review exploring several functional studies that focus on posterior medial frontal cortex (pMFC) and lateral PFC, both structures involved in monitoring cognitive control such as performance adjustment, optimizing information processing and maintenance of flexible goal-directed behaviour. pMFC is interconnected with dorsal ACC (BA 32), ventral ACC (BA 24), and premotor cortices. The result showed that altogether, the most pronounced activation was located in dorsal ACC for all types of monitored events. These studies suggest that dorsal ACC is responsible for a unified performance monitoring function, which involves several cognitive functions (Ridderinkhof et al., 2004).

Anterior cingulate cortex and working memory. Several fMRI studies, including one meta-analysis have found increased dorsal ACC activation in healthy participants performing working memory tasks (Owen, McMillan, Laird, & Bullmore, 2005). Working memory is important for a wide range of executive functions and the dorsal ACC activation is described in relation to increased effort, complexity, and attention (Barrett, Tugade, & Engle, 2004). The n-back paradigm is often used in studies investigating the neural basis of working memory and cognitive load. In an n-back task, participants are instructed to monitor a series of stimuli and to respond whenever a stimulus is presented that is the same as the one presented n trials previously. The general cognitive load is predicted to increase as the number of n-backs increase. The task requires a continuous process of building, maintaining, updating, and releasing arbitrary bindings between items and temporal order of the stimuli positions (Schmiedek, Hildebrandt, Lovden, Lindenberger, & Wilhelm, 2009).

Owen et al. (2005) reported activation of several other cortical regions across fMRI studies using different variants of the n-back procedure. Regions active during the n-back

paradigms included the lateral- and medial premotor cortex, DLPFC, VLPFC, frontal poles and medial and lateral posterior parietal cortex. DLPFC has been suggested to play an essential role in improving task performance, by selecting appropriate high-level organizational chunks that serve to facilitate memory by reducing the overall cognitive load (Bor, Cumming, Scott, & Owen, 2004; Bor, Duncan, Wiseman, & Owen, 2003). The dorsal ACC is found to be highly interconnected with frontal structures. The mid ventrolateral frontal cortex has shown robust activation in tasks requiring selection, comparison, and judgment of stimuli held in short- and long term memory; in stimulus selection; during task switching; elaboration and encoding of information into episodic memory. Others have reported mid ventrolateral frontal activation in reversed learning (Cools, Clark, Owen, & Robbins, 2002). The combined results suggest that dorsal ACC, together with other prefrontal regions, is involved in modality-independent manner, with a variety of explicit task demands. An fMRI study exploring this n-back neural network in MDD patients, found greater lateral PFC activation and ACC activation in this group, compared to healthy subjects. The ACC activation was modulated by task complexity. This study indicates that depressed patients need greater activation within the same neural networks to maintain high level of cognitive control, compared to healthy individuals (Harvey et al., 2005).

Summary

The studies presented above confirm that serotonin plays a role in emotion-regulating networks, affecting brain function. In particular, the 5-HTTLPR short allele is associated with deficits in emotion processing and depressive vulnerability (Caspi et al., 2003). ACC is highly affected by the serotonin system and is involved in an emotion functional feedback loop. The dorsal subdivision of ACC is also found to be important for cognitive processing. Structural and functional deficits in dorsal ACC may lead to impairment in executive functions such as working memory (Phillips et al., 2003). Depression is also associated with disturbance in cognitive function (Landrø et al., 2001), and MDD patients show hyperactivity in dorsal ACC when performing working memory task, compared to healthy controls (Harvey et al., 2005). Further, neuroimaging studies indicate serotonin influence on cognitive processing. The question of how 5-HTTLPR variability influences cognitive circuits is still not answered. The field lacks studies that report whether 5-HTTLPR allelic variation affects cognitive processing in healthy individuals.

Objective

The main aim in this study was to explore 5-HTTLPR allelic variation in healthy subjects and how a polymorphic genetic region affects brain function. An fMRI study was conducted to examine 5-HTTLPR variability in association with anterior cingulate BOLD activation when cognitive load increased. Cognitive load was studied through an fMRI modified n-back task. A secondary aim was to explore performance in accuracy from the fMRI events and its potential association with 5-HTTLPR variability.

When studying genetics and functional activity in healthy individuals, the effects are expected to be rather small. Therefore, the current study did not predict high differences in BOLD activation based on 5-HTTLPR variability.

Methods and materials

Participants and Context

A total of 42 (mean age = 37.7, SD = 12.6) healthy Norwegian female participants were included in the study. Participants were recruited through newspaper ads and posters. Pre-criteria for inclusion in the fMRI study were females between 18 and 65 years, without organic brain disease, psychopharmacologic medication or alcohol/drug addiction. They were also required to be syndrome or symptom free in relation to anxiety and depression. Screening inventories included Structural Clinical Interview for DSM-IV, Axis I and II disorders (SCID I and SCID II), Beck Depression Inventory (BDI) cut off 9, and Beck Anxiety Inventory (BAI) cut off 11. The subscales Similarities (WAIS SI) cut off 4, and Picture Completion (WAIS PC) cut off 4, from WAIS-III were used as indication of potential group differences in underlying general cognitive ability, i.e. IQ. Education level was classified by means of the International Standard classification of Education (ISCED).

Prior to this study, all participants were screened for symptomatology, given background information interviews, based on the Diagnostic Interview for Genetic Studies (Nurnberger et al., 1994), had 3 to 4 hours of cognitive testing, and gave blood samples for genotyping. The SCID interviews were collected and recorded by trained test leaders, and a group of trained clinical psychologists reached consensus for inclusion based on these recordings. During the Informed Consent procedure, participants were asked if they were willing to be contacted for an imaging (fMRI) part of the study. In total, four participants were excluded from the study due to low quality of the functional images from the scanning

procedure, and one participant due to phobic anxiety of the scanning procedure. After exclusion, 38 participants were included in the analysis (See Table 1).

This study is part of a larger project at The Center for the Study of Human Cognition, UiO which applied to the Regional Ethics Committee and adheres to the Helsinki Convention. All data was collected and stored according to prescribed procedures fulfilling these standards.

Genotyping

The procedure for genotyping the triallelic 5-HTTLPR polymorphism located in the SLC6A4 gene, coding the serotonin transporter protein (HTT), has been performed essentially as described in detail elsewhere (Gelernter, Kranzler, & Cubells, 1997; Stein, Seedat, & Gelernter, 2006). Briefly, genomic DNA was amplified by polymerase chain reaction (PCR) on a real-time fluorescence LightCycler instrument in a final volume of 20 ul using LightCycler Faststart DNA SYBR Green kit (Roche cat no 12239264001) with specific primers (0.5 uM) (Gelernter et al., 1997) generating a long (L) 419 bp or a short (S) 375 bp PCR product depending on the presence of a 16 bp or a 14 bp sequence repeat, respectively, in the promoter region. Cycle conditions were the following: 10 min denaturation (95 ^{o}C), 45 cycles at 95 ^{o}C (10 s), 66 ^{o}C (10 s) and 72 ^{o}C (0 s). For the detection of an additional A/G single nucleotide polymorphism (SNP) that occurs within the L fragment (L allele), the PCR fragments were digested with 1 U MspI restriction enzyme (New England Biolabs, Beverly, Massachusetts) for 2 hour at 37 ^{o}C. The PCR fragments contain two obligatory MspI sites, whereas the A/G substitution creates an additional MspI site. Thus, a single PCR reaction and restriction digest followed by size fractioning on a gel provides classification of the S, L_A and L_G alleles. The triallelic classification was then reclassified into a diallelic model, based on the level of 5-HTT gene expression as follows: L_G/S, L_G/L_G and S/S participants were classified as S/S; L_A/S and L_A/L_G participants were classified as L/S; and L_A/L_A participants were classified as L/L (Hu et al., 2006; Neumeister et al., 2006). Participants were then divided into three 5-HTTLPR genotypes in a triallelic model; homozygote long allele (LL), heterozygote allele (LS) and homozygote short allele (SS).

A biobank was established at the Psychopharmacological Department at Diakonhjemmet Hospital, where the blood samples were implemented. The Clinical Chemical Department at Ullevål University Hospital conducted genotyping procedures using polymerase chain reaction (PCR). Extracted DNA and classification from Yale University

School of Medicine, used in Walderhaug's (2007) doctoral dissertation, were used as control samples.

fMRI Acquisition and Analyses

The paradigm was designed using E-prime 2.0 studio software. The stimuli were series of 2 times 12 upper case and lower case letters in 16 randomized blocks. Stimulus duration time was set to 300ms with fixation points at 1650ms between. Four types of stimulus sets were randomized, two types containing 2 n-backs and two types containing 4 n-backs, giving a total of 48 events and 144 non events in each run (64/128 in the 0-back condition). Each of the 16 series has a total duration of 23,4s. An 8000 ms resting condition was presented between series in the form of a centred exclamation point. Behavioural measures for group comparison were accuracy and reaction time. Outcome measures used in the fMRI analyses were onset time and duration in n-back series compared to the 8000ms resting conditions in a block related design.

The n-back paradigm is designed to measure cognitive load when performing working memory task. Task difficulty increases as the number of n-back increases. The 0-back, 1-back, 2-back and 3-back conditions were contrasted in a parametric model (see Figure 3).

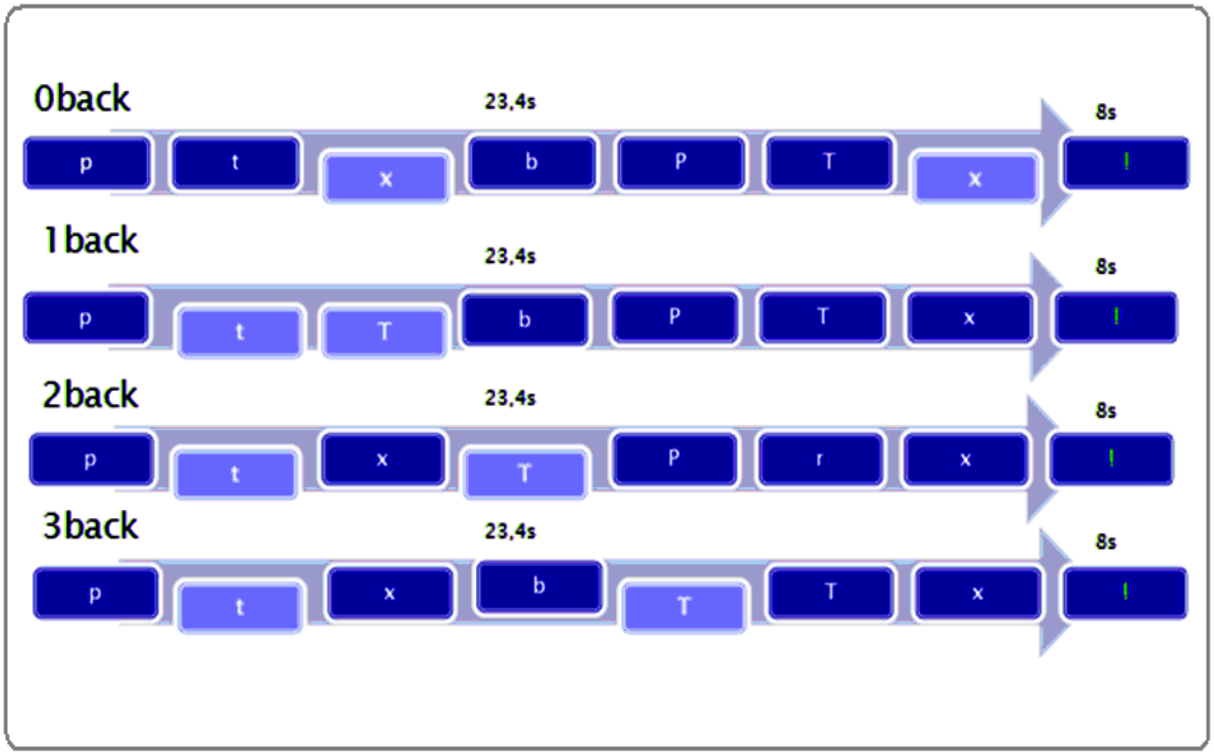

Figure 3. The n-back paradigm; Participants are asked to respond whenever a stimulus is presented that is the same as the one presented n trials previous (do not distinguish between lower case and upper case letters); 0-back: give response whenever x appears on the screen; 1-back: give response when stimulus is identical to the last stimulus presented before; 2-back: give response when stimulus is identical to the stimulus presented two trials before; 3-back: give response when stimulus is identical to the stimulus presented three trials before.

Imaging procedures. Blood- oxygen- level- dependant (BOLD) imaging data were acquired on a Philips Archieva 3T MR scanner (Rikshospitalet University Hospital, Norway) using gradient echo EPI 34 transverse 3mm slices (no gap), parallel to the AC- PC line. Repetition time (TR) = 2000 ms, slice echo time (TE) = 30 ms. Flip angle = 80%, field of view = 240 x 240 x 102 mm. The structural images were 170 T1 weighted sagittal slices.

fMRI analysis. fMRI data processing was carried out using FEAT (FMRI Expert Analysis Tool) version 5.98, part of FSL software (FMRIB's Software Library). The data was preprocessed using the following analysis; motion correction using MCFLIRT; slice-timing correction using Fourier-space time-series phase-shifting; spatial smoothing using a Gaussian kernel of FWHM 5mm; grand-mean intensity normalisation of the entire 4D dataset by a single multiplicative factor; highpass temporal filtering (Gaussian-weighted least-squares straight line fitting, with sigma=50.0s) (Jenkinson, Bannister, Brady, & Smith, 2002). Structural images for use as high resolution images in registration were manually and individually prepared based on region of interest (removing non brain regions such as neck, ears and nose), and then scull stripped using the FSL BET command. Registration to high resolution structural and/or standard space images was carried out using FLIRT (FMRIB's Linear Image Registration Tool). Registration in 6 or 7 degrees of freedom from standard to high resolution was individually applied based on the procedure that gave the most accurate registration. All registrations from the high resolution to standard MNI space allowed 12 degrees of freedom (Jenkinson et al., 2002; Jenkinson & Smith, 2001). Mid level analysis was conducted for within-subjects for each n-back variance estimated in first-level analyses.

Higher-level analysis was carried out using a mixed effect model to explore variation associated with the genotypes, by forcing the random effects variance to zero in FLAME (FMRIB's Local Analysis of Mixed Effects) (Beckmann, Jenkinson, & Smith, 2003; Woolrich, 2008; Woolrich, Behrens, Beckmann, Jenkinson, & Smith, 2004). Z-stat images were masked before thresholding, to constrain the search for activation to the anatomical ROI (ACC). The ACC mask was made in Harvard-Oxford Cortical Structural Atlas, Cingulate Gyrus, anterior division (Desikan et al., 2006; Hu et al., 2006). The ROI intensity threshold was set to 50-100 based on the probability atlas (Worsley, 2001). Z statistic images were thresholded using clusters determined by $Z > 1.0$ and a cluster significance threshold of $p < 0.05$ (Hanson, Rebecchi, Hanson, & Halchenko, 2007).

Statistical Analysis

Behavioural data analysis was completed in SPSS 18.0 (SPSS Inc., Chicago, Illinois). A one-way ANOVA between-group analysis of variance was conducted to spot potential group differences in age, BAI, BDI, ISCED, WAIS SI and WAIS PC.

Two way analysis of variance (ANOVA) was conducted to investigate potential differences in accuracy in n-back performance between the three genotypes. To rule out speed-accuracy effect as a confounding variable, the analysis was conducted with reaction time as covariate for each of the n-backs.

Results

Table 1

Group Descriptives

	Homo LL (n = 10)		Hetero LS (n = 15)		Homo SS (n = 13)	
	Mean (SD)	Range	Mean (SD)	Range	Mean (SD)	Range
Age	33.50 (13.22)	21-59	35.93 (10.51)	23-58	43.00 (13.42)	21-61
BAI	1.70 (1.34)	0-4	1.80 (3.08)	0-11	.69 (.75)	0-2
BDI	2.00 (2.45)	0-8	1.80 (2.51)	0-7	1.38 (2.47)	0-9
ISCED	4.50 (.85)	4-6	4.67 (.98)	3-6	5.23 (.73)	4-6
WAIS SI	11.10 (3.07)	7-17	10.60 (2.23)	5-16	11.46 (3.31)	4-17
WAIS PC	13.00 (2.36)	8-16	13.67 (4.16)	8-18	13.24 (3.30)	8-18

Table 1. Participants homozygous for long variant (LL), heterozygous (LS), and homozygous for short variant (SS), mean, standard deviation (SD) and range for descriptive variables: age, Beck Anxiety Inventory (BAI; cut off 11, max score 63), Beck Depression Inventory (BDI; cut off 9, max score 36), education level measured with International Standard Classification of Education (ISCED; max score 7), IQ measured with the Weschler Adult Intelligence Scale Similarities Test (WAIS SI; cut off 4), and Weschler Adult Intelligence Scale Picture Completion (WAIS PC; cut off 4).

Demographic Measures

The one-way ANOVA revealed no statistically significant differences between genotypes on age, BAI, BDI, ISCED, WAIS SI and WAIS PC.

Functional Analysis

The results showed significant increase in ACC activation with the amount of SS alleles when cognitive load increased (see Figure 5). The two strongest clusters activated with cluster index threshold at Z >1.0 and cluster significance threshold of $p < .05$, where a band of cluster in dorsal ACC (BA 32), and a smaller cluster in subgenual ACC (BA 25) (See Table 2).

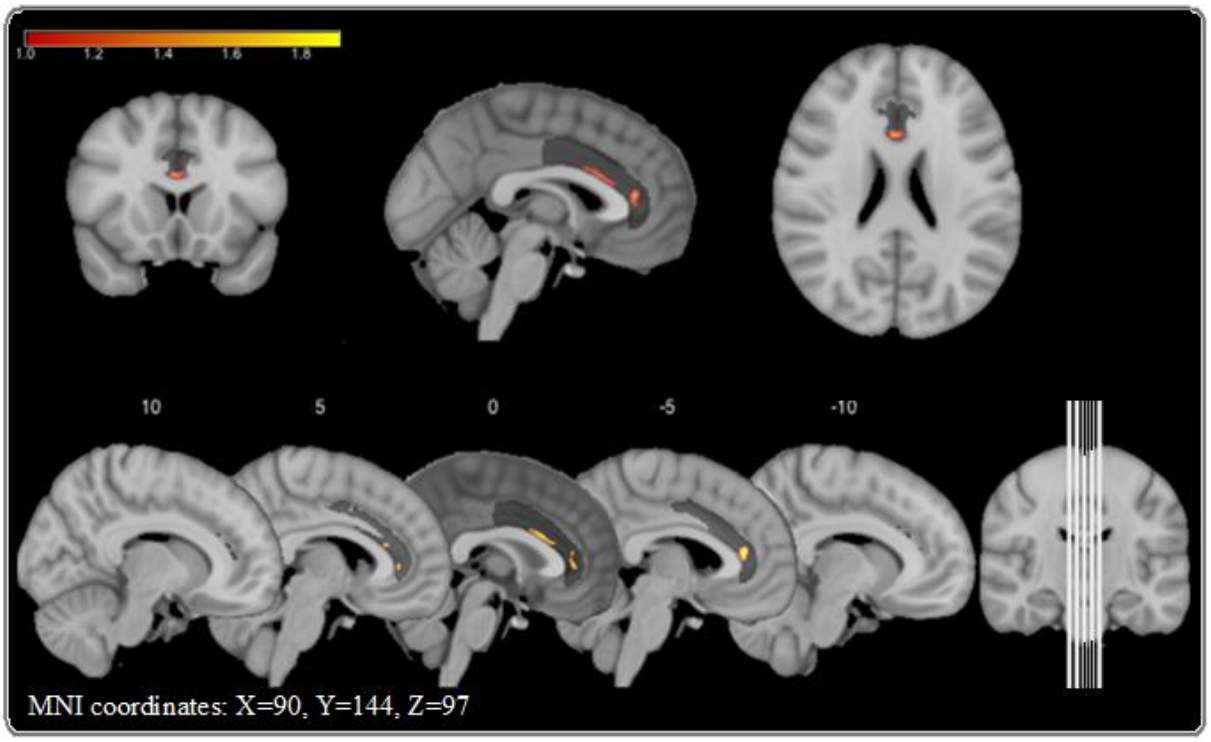

Figure 5. Coronal- sagittal- and transverse slices of standard template; ACC mask (shown in dark grey) with significant differences in activation during n-back paradigm when SS alleles and cognitive load increases, based on a parametric model. Areas of activation: dorsal ACC (BA 32) and subgenual ACC (BA 25) (shown in red and yellow).

Table 2

Cluster index

Cluster	Voxels	Z max	x	y	z	Label
5	1041	2.17	87	142	94	Cingulate Gyrus, Dorsal ACC (BA 32)
4	789	2.1	94	161	84	Cingulate Gyrus, Subgenual ACC (BA 25)
3	32	1.78	97	113	114	Posterior division, JLC, Precentral Gyrus
2	1	1	83	135	109	Paracingulate Gyrus, JLC
1	1	1.01	86	168	87	Paracingulate Gyrus

Note: ROI mask based on probability atlas; Harvard-Oxford Cortical Structural Atlas, Cingulate Gyrus, anterior division; JLC: Juxtapositonal Lobule Cortex.

N-back Performance

A two way ANOVA showed a statistically significant difference at $p < .01$ level between genogroups and 3-back accuracy: $F(2, 34) = 5.84$, $p = .007$ (parietal eta squared = .26). There were no significant difference in accuracy between genotype and 0-back, 1-back and 2-back (see Figure 4).

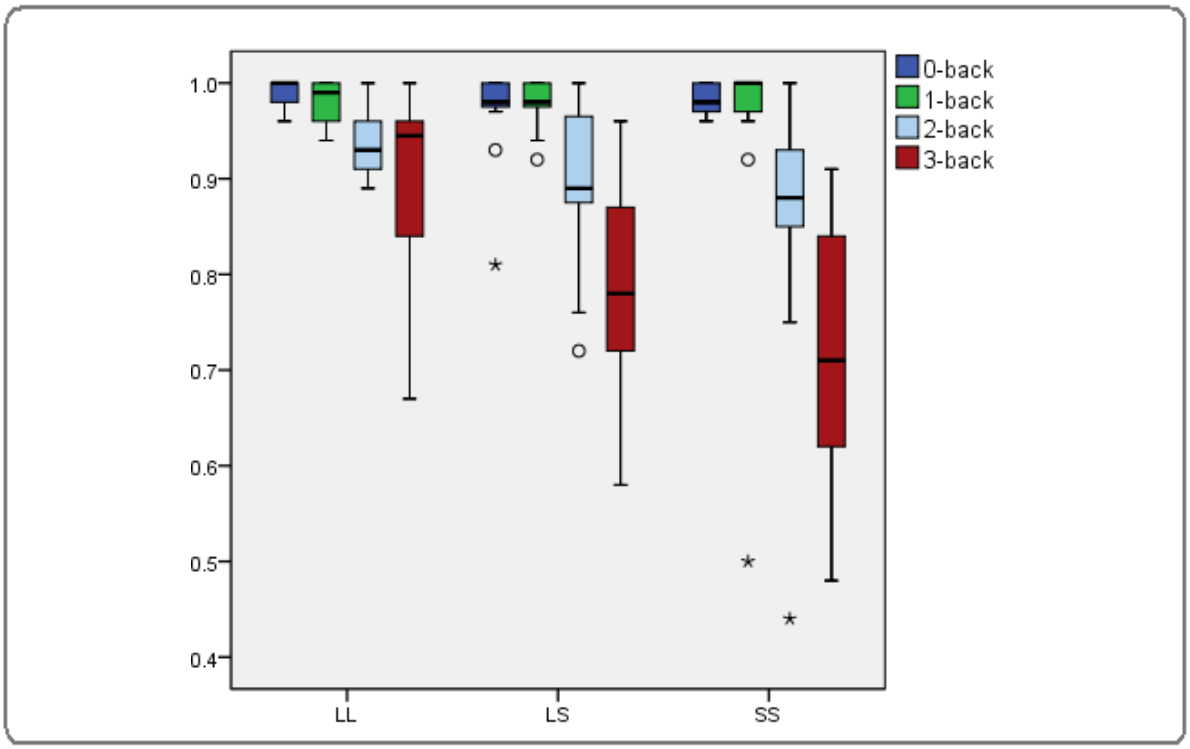

Figure 4. Percentage Accuracy from n-back events/100. There emerged a significant group difference in 3-back performance in accuracy, were accuracy decrease with increased number of SS alleles. However, there were no significant differences between the three groups on lower complexity tasks (0-back, 1-back and 2-back).

Discussion

The current study showed significant differences in dorsal ACC activation between the three 5-HTTLPR variants when cognitive load increased. Functional activation in dorsal ACC increased with the number of short alleles and cognitive load. The behavioural data showed a statistically significant difference in accuracy on the 3-back task. Short allele carriers showed lower accuracy compared to long allele carriers.

Activation in dorsal ACC in response to cognitive stimuli, is a frequent and common finding (Bush et al., 2000; Pezawas et al., 2005; Shackman et al., 2011). The current study indicates that 5-HTTLPR allelic variation modulates the amount of activation in dorsal ACC, and influences accuracy performance. The dorsal ACC is proposed to be involved in monitoring trade-off between speed and accuracy when responding to stimuli, which places

the cognitive system in a more cautious mode, as opposed to an impulsive response mode (Ridderinkhof et al., 2004). This is hypothesised to increase efficiency of information processing. Although the current study does not provide a statistical connection between performance and increased dorsal ACC activation, this study speculates that there is an association between increased functional activity and decreased accuracy associated with 5-HTTLPR variability. The BAI and BDI results showed that there were no significant differences between the three groups in symptomatology linked to anxiety and depression. In addition, there were no group differences on the WAIS SI and WAIS PC subtests, indicating that differences in working memory performance did not reflect variations in general cognitive abilities.

This study aimed to give a more profound understanding of how 5-HTTLPR variability modulates behaviour by studying cognitive processing in healthy individuals. Because the paradigm was strictly cognitive the study did not predict high activation in the region of interest (ACC) based on this working memory task. Instead, the study aimed to explore small differences in activation within ACC as a result of 5-HTTLPR variability. Therefore, the analysis was conducted with liberal thresholding ($Z>1.0$.) The highest activated cluster was found in dorsal ACC.

The increased dorsal ACC activation may be explained as a cognitive compensation mechanism, whereby the functional activation reflects cognitive control and the amount of intentional effort SS carriers used in the task. Dorsal ACC is claimed to be involved in performance monitoring and performance adjustment to achieve goals when solving cognitively demanding tasks (Pezawas et al., 2005; Ridderinkhof et al., 2004; Shackman et al., 2011). The increased dorsal ACC activation may indicate that SS carriers need more cognitive control monitoring because they find the task more difficult, compared to the LL and LS groups. The decrease in accuracy also supports this assumption, since SS carriers made the most errors. These results suggest a cognitive vulnerability associated with 5-HTTLPR variability.

Whether the increased dorsal ACC activation is the consequence of or the cause of decrease in accuracy is not clear. The current study suggests that when cognitive load increases, dorsal ACC is involved in the adaptive processes of reducing interfering stimuli, such as tonic overactive limbic structures. The dorsal ACC activation is predicted to be involved in down regulating amygdala, a structure highly involved in generating emotion. A tonic activation model reports higher amygdala activation at rest in 5-HTTLPR short variant

carriers, compared with carriers homozygous for the long variant (Canli et al., 2006). It is further reported that activation in dorsal ACC is negatively correlated with amygdala activation (Pezawas et al., 2005). When performing the n-back task it is crucial to down regulate emotional circuits to maintain a high level of cognitive control. The dynamic interplay is likely to create an activity gap between dorsal ACC and limbic regions (Pochon et al., 2002). The current study also found increased activation in subgenual ACC. Activation in subgenual ACC increased with the amount of SS alleles and cognitive load. Subgenual ACC is highly affected by the serotonin system and is found to be positively correlated with amygdala activation (Pezawas et al., 2005; Phillips et al., 2008). It is predicted that SS carriers in the current study also have higher amygdala activation at rest, compared with LS and LL groups. The increased subgenual ACC activation may therefore be a reflection of the high tonic amygdala activation in this group. Together, dorsal ACC, subgenual ACC and amygdala are involved in a functional feedback loop. The increased subgenual ACC activation may indicate a reduced capacity to decrease activation in limbic structures. Differences in accuracy performance could also be explained by overactive limbic structures in SS carriers, which could be interfering with cognitive monitoring structures. Under strong serotonin influence and 5-HTTLPR variability, it is likely that a reduced coupling translates into altered feedback regulation of amygdala activity. This altered connectivity between ACC and amygdala is also associated with vulnerability to depression (Elliott, Zahn, Deakin, & Anderson, 2011; Pezawas et al., 2005).

The adaptive control monitoring is effortful, and it may not be efficient to maintain high levels of control at all times. Therefore, the current study suggests that dorsal ACC is involved in determining the amount of cognitive control necessary for efficient task performance and communicating the need to recruit cognitive control from other brain regions. Performance adjustment is also associated with other frontal regions, and the literature agrees that dorsal ACC and lateral PFC operate together when cognitive load increases (Bush et al., 2000; Ridderinkhof et al., 2004). MDD patients show greater activation in lateral PFC and dorsal ACC as task difficulty increases, compared to healthy individuals (Harvey et al., 2005). The increased dorsal ACC activation in the current study may therefore be a result of recruiting functionally connected brain regions, such as DLPFC, which is critical for increasing task performance and controlled processing when cognitive load increases (Davidson et al., 2002). The results indicate that 5-HTTLPR serotonin transporter affects regions involved in cognitive processing, and that short allele carriers may need

greater activation within the same neural networks to maintain a high level of cognitive control, compared to long allele carriers.

A cognitive compensation mechanism is proposed as an adaptive process, wherein the increased dorsal ACC activation inhibits amygdala activation, monitors performance adjustment, and recruits other frontal structures to increase cognitive control and task performance. Even so, the behavioural measures in this study indicate that the SS carriers do not achieve the amount of cognitive control needed for efficient n-back performance, which is demonstrated by their low accuracy scores. The current study proposes several possible explanations for the cognitive vulnerability in SS carries. It is suggested that dorsal ACC does not succeed in down regulating emotion activation to the extent that tonic amygdala activation no longer interferes with performance. Another explanation could be that frontal brain structures, including dorsal ACC, are diminished by high serotonin levels, thereby affecting control monitoring. This leads to the possibility that increased dorsal ACC activation does not manage to compensate for the altered brain structures involved in cognitive processing.

How 5-HTTLPR variability may modulate cognitive processes could be explained through plasticity; modification of the functional properties of neurons and their networks according to experience. Serotonin metabolism has been widely associated with plasticity, and the level of plasticity is suggested to be influenced by the serotonin level (Azmitia, 1999; Gaspar et al., 2003; Gould, 1999; Mattson, Maudsley, & Martin, 2004; Wilson, Faber, & Haring, 1998). The current study hypothesised that SS alleles may be associated with cognitive vulnerability due to high serotonin levels in the brain. SS carriers show the lowest number of serotonin transporter proteins which lead to higher levels of extracellular serotonin (Schinka, Busch, & Robichaux-Keene, 2004). When serotonin levels are high, the level of neural plasticity is consequently high (Branchi, 2011). A high level of serotonin is associated with vulnerability to depression, and the current study proposes cognitive vulnerability associated with 5-HTTLPR variability.

The suggested pathway from genetics and deficits in information processing may be clarified by studies that explore the impact of stress on neural functioning. The 5-HTTLPR short allele has been especially identified as a genetic factor associated with an increased vulnerability for developing depressive disorder within the context of stress. The genetic predisposition accounts for 30–50% of the risk for depression, where social stress factors, such as early life adversity, are important interacting elements (Caspi & Moffitt, 2006; Hill et al., 2001). Overall, clinically depressed patients with the 5-HTTLPR short allele have severe

symptomatology, worse prognosis, and highest suicidality rates, compared with long allele carriers. They also show a lower effect of response to treatment with antidepressants (Caspi et al., 2003; Kendler, Kuhn, Vittum, Prescott, & Riley, 2005; Rausch et al., 2002; Uher & McGuffin, 2007).

The results from the current study are interpreted in light of the high serotonin level associated with SS alleles that allows plasticity to shift from one condition to the other; Plasticity increases the vulnerability in stressful contexts, but also enhances the capacity to recover when exposed to a supportive environment (Kaufman et al., 2004). It is therefore suggested that clinically healthy SS carriers also have a higher biological sensitivity to context compared with long allele carriers. The assumed increase in task difficulty and cognitive vulnerability in SS carriers reported in the current study, may also be explained in association with this biological sensitivity.

The current study has further strengthened the knowledge about 5-HTTLPR variability and its role in monitoring cognitive control, and has contributed to a more profound understanding of how genetic variation modulates behaviour. These findings suggest that 5-HTTLPR variability affects cognitive processing and not only sensory and emotion processing as previously suggested. The increased dorsal ACC activation could therefore be a result of a cognitive compensation mechanism, and this would indicate that SS carriers find the task the most difficult. These results are consistent with both behavioural and functional research on MDD patients (Harvey et al., 2005; Landrø et al., 2001), indicating cognitive vulnerability and deficits in executive processes associated with 5-HTTLPR variability. The current study further suggests that emotion related studies that demonstrate an influence on emotion processing by 5-HTTLPR variability, may actually reflect task difficulty. SS alleles are associated with an overactive amygdala, which interferes with processing of emotional stimuli (Hariri et al., 2002). This could make the task more demanding and result in an increase of cognitive load, which again affects performance.

Limitations

Although there are reasons for arguing low thresholding as a potential limitation, this study argued that the ROI analysis require corrected, but liberal thresholding ($Z>1.0$) to avoid Type 2 error. Concerning sample size, a higher N would increase the power of the study.

Future studies

While genetic studies have pointed to the innate biological vulnerability to stress leading to depression, research is still needed to further clarify the relation between serotonin

and cognitive vulnerability. This study indicates 5-HTTLPR variability associated with increased dorsal ACC activation as task difficulty increases. However, the interplay between dorsal ACC and other brain regions, such as when down regulating emotional structures and recruiting frontal brain regions, is still only speculative. For future research, diffusion tensor imaging (DTI) could be conducted to explore structural connectivity between dorsal ACC activation and medial – and lateral PFC, as cognitive load increases. Future studies should also consider a functional feedback loop between dorsal ACC and amygdala when given cognitive stimuli associated with cognitive load. This would give a better understanding of how cognitive load affects emotion regulation structures associated with 5-HTTLPR variability.

The 5-HTTLPR transporter may not have a consistent main effect on depression, but instead may be moderated through other variables, such as personality traits. There are still inconsistent findings between 5-HTTLPR variability and personality traits associated with anxiety and susceptibility for depression. Neuroticism is a personality trait with a strong genetic component, which may amplify the risk for depression in context with life stress. There has also been reported a tendency of increased neuroticism traits in healthy short allele carriers compared to long allele carriers (Kendler, Kuhn, & Prescott, 2004; Lesch et al., 1996; Schinka et al., 2004). Future studies may also benefit from including personality traits and by exploring, for example, whether high neuroticism scores linked to 5-HTTLPR variability are associated with functional activation in frontal brain regions and cognitive performance.

Studies operating with larger sample sizes have the potential to include several other genes involved in serotonin neurotransmission. Fitting associations between genes and phenotypes to molecular-genetic data can help to weed out spurious associations and to strengthen the link between cognition and molecular mechanisms that build and guide neural systems.

Conclusion

Research exploring cognitive load associated with 5-HTTLPR variability in healthy participants represents an advantageous contribution to a field that has, until now, mainly focused on emotional stimuli. The results demonstrated increased dorsal ACC activation with the number of short alleles and cognitive load. The behavioural data showed significant group differences in accuracy measures in the 3-back task, such that SS allele carriers made the most errors. The increased dorsal ACC activation is interpreted as a cognitive compensation

mechanism involved in down regulating tonic amygdala activation in order to filter out emotional distractions, and to recruit more activation from frontal regions, which are regions critical for performance adjustment and cognitive control. The current study suggests that SS carriers may find the task the most difficult. These findings may indicate biological sensitivity and cognitive vulnerability associated to 5-HTTLPR variability.

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