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Imaging Anxiety

Neurochemistry in Anxiety Disorders Assessed by Positron Emission Tomography

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Abstract

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Anxiety disorders, including social anxiety disorder (SAD) and posttraumatic stress disorder (PTSD) are common and disabling conditions. Largely based on animal and pharmacological studies, both the serotonergic and substance P/neurokinin-1 (SP/NK1) systems have been implicated in their underlying pathology. However, only few neuroimaging studies have directly assessed these neurotransmitter systems in human sufferers of anxiety disorders, and none have addressed possible between-systems relationships.

The overall aim of this thesis was to study possible neurochemical alterations associated with anxiety disorders. To this end, three studies using positron emission tomography (PET) for in-vivo imaging of the brain serotonergic and SP/NK1 systems in patients with SAD and PTSD were conducted. The radiotracers [^{11}C]5-HTP, [^{11}C]DASB, and [^{11}C]GR205171 were used to index serotonin synthesis rate, serotonin transporter (SERT) availability, and NK1 receptor availability respectively.

In **Study I**, patients with SAD relative to controls exhibited enhanced serotonin synthesis rate and serotonin transporter availability. Serotonin synthesis rate in the amygdala was positively related to social anxiety symptom scores. **Study II** demonstrated increased NK1 receptor availability in the amygdala in patients with SAD relative to controls. In **Study III,** patients with PTSD showed elevated NK1 receptor availability in the amygdala as compared to controls. SERT availability in the amygdala was negatively related to PTSD symptom severity, a relationship that was moderated by NK1 receptor levels. The regional overlap between SERT and NK1 receptor expression was altered in patients with PTSD, with reduced overlap linked to more severe symptoms.

Collectively, the findings are consistent with the view that serotonin in the amygdala induces rather than reduces anxiety and links exaggerated anxiety to an overactive presynaptic serotonin system. In addition, the involvement of the SP/NK1 system in stress and anxiety, as suggested by animal studies, was demonstrated in two common human anxiety disorders. Finally, PTSD symptomatology is better accounted for by interactions between the serotonergic and SP/NK1 systems in the amygdala than by each system separately. In conclusion, this thesis supports that both the serotonergic and SP/NK1 systems in and of themselves, but also interactively, may be important contributors to anxiety symptomatology.

Keywords: Fear, Brain, Serotonin, Neurokinin, Substance P

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To Matilda, Vera, and Melker

List of Papers

This thesis is based on the following papers, which are referred to in the text by their Roman numerals.

I Frick, A., Ahs, F., Engman, J., Jonasson, M., Alaie, I., Björkstrand, J., Frans, Ö., Faria, V., Linnman, C., Appel, L., Wahlstedt, K., Lubberink, M., Fredrikson, M., Furmark, T. (2015). Serotonin synthesis and reuptake in social anxiety disorder: A positron emission tomography study. *JAMA Psychiatry*, *72*(*8*): 794-802

II Frick, A., Ahs, F., Linnman, C., Jonasson, M., Appel, L., Lubberink, M., Långström, B., Fredrikson, M., Furmark, T. (2015). Increased neurokinin-1 receptor availability in the amygdala in social anxiety disorder: A positron emission tomography study with [^{11}C]GR205171. *Translational Psychiatry*, *5*: e597

III Frick, A., Ahs, F., Michelgård Palmquist, Å., Pissiota, A., Wallenquist, U., Fernandez, M., Jonasson, M., Appel, L., Frans, Ö., Lubberink, M., Furmark, T., von Knorring, L., Fredrikson, M. (2015). Co-expression of serotonin transporters and neurokinin-1 receptors in posttraumatic stress disorder: A multi-tracer PET study. *Submitted for publication*

Contents

Abbreviations

5-HIAA	5-hydroxyindoleacetic acid
5-HT	5-hydroxytryptamine, serotonin
5-HTP	5-hydroxytryptophan
5-HTT	Serotonin transporter
5-HTTLPR	Serotonin transporter-linked polymorphic region
AADC	Amino acid decarboxylase
AC	Adenylyl cyclase
ACC	Anterior cingulate cortex
BLA	Basolateral amygdala
BNST	Bed nucleus of stria terminalis
BP	Binding potential
cAMP	Cyclic adenosine monophosphate
CAPS	Clinician-Administered PTSD Scale
CeA	Central amygdala
DAG	Diacylglycerol
DASB	3-amino-4-(2-dimethylaminomethylphenylsulfanyl)-benzonitrile
DSM	Diagnostic and Statistical Manual
DVR	Distribution volume ratio
FWE	Family-wise error
GABA	Gamma-aminobutyric acid
GAD	Generalized anxiety disorder
G-protein	Guanine nucleotide binding protein
GSAD	Generalized social anxiety disorder
HC	Healthy control
ICD	International Classification of Diseases
IP3	Inositol triphosphate
LSAS	Liebowitz Social Anxiety Scale
LSD	Lysergic acid diethylamide
MAO	Monoamine oxidase
MINI	Mini International Neuropsychiatric Interview
MNI	Montreal Neurological Institute
mRNA	Messenger ribonucleic acid
NK1	Neurokinin-1
NMDA	N-methyl-D-aspartate
OCD	Obsessive-compulsive disorder
pCPA	para-chlorophenylalanine

PD	Panic disorder
PET	Positron emission tomography
PKA	Protein kinase A
PKC	Protein kinase C
PLC	Phospholipid C
PPT-A	Preprotachykinin-A
PTSD	Posttraumatic stress disorder
ROI	Region of interest
SAD	Social anxiety disorder
SCID	Structured Clinical Interview for DSM
SERT	Serotonin transporter
SNP	Single nucleotide polymorphism
SP	Substance P
SPECT	Single photon emission computed tomography
SPM	Statistical parametric mapping
SPSQ	Social Phobia Screening Questionairre
SSRI	Selective serotonin reuptake inhibitor
TAC	Time-activity-curve
TACR1	Tachykinin receptor 1
TPH	Tryptophan hydroxylase
VMAT	Vesicular monoamine transporter
vmPFC	Ventromedial prefrontal cortex
VOI	Volume of interest

Introduction

Mental disorders affect a large proportion of the population, causing considerable distress for the individual and costs for society. Current pharmacological treatment options leave a substantial proportion of patients suffering, prompting research into the neural underpinnings of these common and debilitating conditions. More than 60 years ago, Woolley and Shaw (1954) suggested that alterations in the serotonergic system underlie mental disorders. This first proposal that altered neurotransmitter functioning was related to psychiatric illness has spurred enormous interest; for example the search-term "serotonin" currently yields 130,000 hits on PubMed. Following the discovery that effective antidepressants increase the tone of serotonin and other monoamines, Coppen (1967) proposed that lowered mood may be caused by deficiencies in the monoamine systems, paving the way for the serotonin deficiency theory of depression. In contrast to what was suggested for depressed mood, exaggerated anxiety was initially linked to an excess of serotonin (Iversen, 1984). Later studies found contradictory evidence, and the question of whether anxiety disorders are related to excessive or deficient serotonin levels in the brain has since been a matter of debate.

Substance P (SP), a peptide neurotransmitter, is implicated in the pathophysiology of anxiety disorders, even though it has not been studied to the same extent as serotonin. SP is the preferred endogenous ligand to the neurokinin-1 (NK1) receptor. Because the serotonergic and SP/NK1 systems are frequently co-expressed and interact in the brain, both systems are of interest when characterizing the neurobiological underpinnings of fear and anxiety.

Here, positron emission tomography was used to study the serotonergic and SP/NK1 systems in the living brain of sufferers of anxiety disorders. The results demonstrate that social anxiety disorder is associated with an overactive presynaptic serotonin system, that both social anxiety disorder and posttraumatic stress disorder are associated with increased NK1 receptor levels in the amygdala, and that posttraumatic stress disorder is characterized by altered co-expression between the serotonergic and SP/NK1 systems. Collectively, the findings are consistent with the views that serotonin induces rather than reduces anxiety, and that PTSD symptomatology is better accounted for by systems interactions than by each system separately. Thus, studies addressing joint contributions of neurochemical systems may be crucial to understanding, treating, and preventing these common and impairing conditions.

Background

Anxiety disorders

One of the most prevalent forms of suffering in modern society is exaggerated anxiety, which affects about one third of all individuals over their lifetime (Kessler et al., 2005; Kessler, Petukhova, Sampson, Zaslavsky, & Wittchen, 2012). Whereas fear is the response to imminent and proximal threat, anxiety can be conceptualized as anticipatory fear in response to potential and distant threat. Fear and anxiety share the same apprehensive mood including increased arousal and vigilance, and it has been suggested that fear is short-lived and rapidly attenuates when the threat is removed, whereas anxiety is a more long-lasting state of apprehension (Davis, Walker, Miles, & Grillon, 2009). Thus, anxiety can be seen as enduring fear without the presence of any imminent threat. Anticipatory identification of possible threat may have constituted an evolutionary advantage and is adaptive at normal levels to avoid harm, but can cause severe impairment if persistent and exaggerated. In the latter case, the individual may qualify for an anxiety disorder.

The focus of the present thesis is on two common anxiety disorders, social anxiety disorder (SAD, also known as social phobia) and posttraumatic stress disorder (PTSD). Although PTSD was recently moved from the Anxiety Disorders to the new category of Trauma- and Stressor-related Disorders in the 5th version of the Diagnostic and Statistical Manual (DSM-5; American Psychiatric Association, 2012), I will refer to PTSD as an anxiety disorder in the present thesis. The justification for this is three-fold. First, the participants with PTSD included in the thesis were recruited and diagnosed using the DSM-IV criteria, where PTSD was classified as an anxiety disorder (American Psychiatric Association, 2000). Second, PTSD is associated with similar exaggerated threat-related activity in the brain's fear circuitry as for example SAD and specific phobia (Etkin & Wager, 2007). Third, ICD-11, the upcoming version of the World Health Organization's International Classification of Diseases, will most probably define PTSD according to the more narrow criteria used in DSM-IV, namely avoidance of stimuli associated with the trauma, re-experiencing the trauma, and hyperarousal (Maercker et al., 2013). Furthermore, based on the association between external cues and fear, both SAD and PTSD, together with specific phobia, are situationally elicited anxiety disorders, meaning that the onset of fear and apprehension is triggered by outside events or memories. With that said, I do not want to

minimize the recent addition of posttraumatic changes in cognition and mood to the diagnostic criteria of PTSD in DSM-5. The next sections will give a description of SAD and PTSD in more detail.

Social anxiety disorder

Social anxiety disorder is characterized by fear of being negatively evaluated or scrutinized in social situations such as public speaking (American Psychiatric Association, 2000). The excessive concern about negative evaluation leads to marked anxiety in, or avoidance of, social situations. Untreated SAD is considered to be a chronic condition (Keller, 2006) associated with social and workplace impairment, individual suffering (Fehm, Pelissolo, Furmark, & Wittchen, 2005; M. B. Stein & Kean, 2000), and high societal cost (Acarturk et al., 2009; Olesen et al., 2012; Whiteford et al., 2013). It is the second most common anxiety disorder with a lifetime prevalence exceeding 10% (Furmark et al., 1999; Kessler et al., 2005, 2012). If exaggerated fear is present in most social situations, it is denoted as generalized SAD (American Psychiatric Association, 2000). SAD is often comorbid with other anxiety disorders, as well as mood and substance abuse disorders (Chartier, Walker, & Stein, 2003).

SAD typically develops in childhood or adolescence (M. B. Stein & Stein, 2008), but the etiology of the disorder is not fully understood. Based on the excessive fear of social situations, fear conditioning, pairing social situations with strong negative affect, provides an attractive etiological pathway. Moreover, risk factors for developing SAD include the temperament behavioral inhibition, characterized by strong fear of novel stimuli, particularly social stimuli (Biederman et al., 2001; Clauss & Blackford, 2012; Essex, Klein, Slattery, Goldsmith, & Kalin, 2010). However, only 50% of children with behavioral inhibition develop an anxiety disorder, prompting research into characterizing these high-risk children. This has led to the suggestion that the subgroup of children with behavioral inhibition that also display strong fear of ambiguous threat are at higher risk of developing anxiety disorders later, including SAD (Pine & Fox, 2015). Genetic contribution to SAD is in the order of 30-40% (Hettema, Neale, & Kendler, 2001), with probable gene × environment interactions (Hettema, Prescott, Myers, Neale, & Kendler, 2005).

Posttraumatic stress disorder

Following exposure to a traumatic event, the majority of individuals show transient symptoms of hyperarousal, intrusive memories and avoidance of stimuli associated with the event (Galea, Nandi, & Vlahov, 2005; Rothbaum, Foa, Riggs, Murdock, & Walsh, 1992). These symptoms of posttraumatic stress disorder (American Psychiatric Association, 2000) persist in around

20% of individuals exposed to trauma (Breslau, Davis, Andreski, & Peterson, 1991), causing great suffering and high societal costs (Breslau et al., 1991; Olesen et al., 2012; Pietrzak, Goldstein, Southwick, & Grant, 2012). PTSD is, similar to SAD, considered to be a chronic condition if not adequately treated (Perkonigg et al., 2005). The lifetime prevalence of the disorder is around 5-6% (Frans, Rimmö, Åberg, & Fredrikson, 2005; Kessler et al., 2012), but much higher in highly exposed groups (Galea et al., 2005) and in regions with high occurrence of potentially traumatic events such as post-conflict settings (de Jong et al., 2001). PTSD is often comorbid with other anxiety disorders, mood disorders, and substance abuse disorders (Brady, Killeen, Brewerton, & Lucerini, 2000).

Although exposure to a traumatic event is a necessary criterion for PTSD, not everyone who experiences trauma develops the disorder. Research into the risk factors predicting who will develop PTSD after a traumatic event has identified contribution by both environmental and genetic factors (Almli, Fani, Smith, & Ressler, 2014; Voisey, Young, Lawford, & Morris, 2014). One of the strongest predictors is multiple trauma exposures (Suliman et al., 2009). Situational risk factors have also been identified, such as degree of exposure to the traumatic event, where more intense and direct exposure increases the risk of subsequent PTSD (Galea et al., 2005). Genetic contributions include the lower-expressing short allele in the serotonin transporter-linked polymorphic region (5-HTTLPR; Gressier et al., 2013; Kimbrel et al., 2014). In addition, gene × environment interactions have been proposed (Liberzon et al., 2014). Moreover, PTSD is characterized by the pairing of previous neutral stimuli with fear responses, making fear conditioning a potential model for studying the etiology of the disorder (Mahan & Ressler, 2012; Peri, Ben-Shakhar, Orr, & Shalev, 2000; Pole, 2007). Because symptoms of PTSD, highly prevalent immediately following trauma, progressively attenuate for all but a subset of individuals, deficits in extinction of conditioned fear or inability to incorporate safety cues may be considered risk factors for PTSD. Indeed, individuals with PTSD exhibit deficits in recall of fear extinction (Milad et al., 2009).

Treatment of anxiety disorders

Because of the high individual and societal costs associated with anxiety disorders (Olesen et al., 2012), it is important to adequately treat and if possible prevent these disabling conditions. First-line treatment for anxiety disorders include both pharmacological and psychological options, for example selective serotonin reuptake inhibitors (SSRIs), serotonin-noradrenaline reuptake inhibitors, and cognitive-behavior therapy (Murrough, Yaqubi, Sayed, & Charney, 2015). However, response rates in clinical pharmacological trials are in the order of 50-60% (Blanco, Bragdon, Schneier, & Liebowitz, 2013; D. J. Stein, Ipser, & Seedat, 2006), indicating that a significant

proportion of patients with anxiety disorders do not respond to current treatment options. Proper understanding of the pathophysiology and etiological mechanisms, including neurobiological underpinnings, may therefore lead to novel treatment and preventive strategies.

Brain regions involved in anxiety disorders

Even though the neural underpinnings of anxiety disorders are still not fully understood, there is evidence that they include alterations in brain function (Brühl, Delsignore, Komossa, & Weidt, 2014; Etkin & Wager, 2007; Sartory et al., 2013; Shin & Liberzon, 2010), brain structure (Brühl et al., 2014; Frick, Howner, Fischer, Eskildsen, et al., 2013; Frick, Engman, et al., 2014; Kühn & Gallinat, 2013; van Tol et al., 2010), and brain neurochemistry (Durant, Christmas, & Nutt, 2010; Maron, Nutt, & Shlik, 2012). This section will describe the alterations in more detail, starting with an introduction to the brain regions involved.

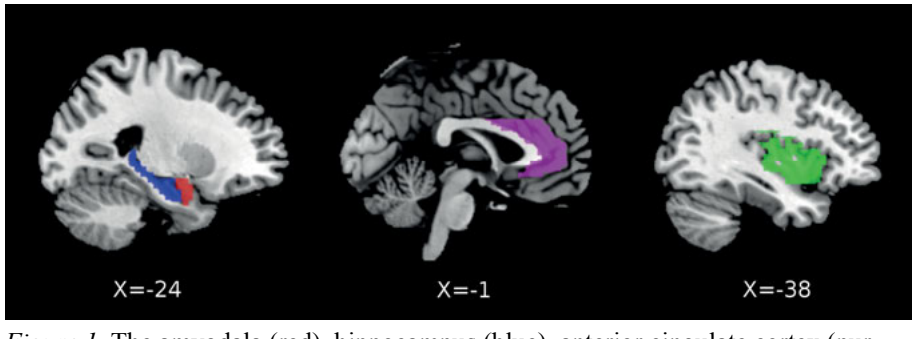

Figure 1. The amygdala (red), hippocampus (blue), anterior cingulate cortex (purple), and insular cortex (green) are part of the brain's fear circuitry. X indicates sagittal slice position in Montreal Neurological Institute standard space. Based on Shin and Liberzon (2010).

The brain fear circuitry

Anxiety disorders are characterized by excessive fear-responses, which has influenced the search for the neural underpinnings of these conditions. Shin and Liberzon (2010) have proposed overlapping neural substrates for fear, stress, and anxiety disorders. This so called fear circuitry includes the amygdala, medial prefrontal and anterior cingulate cortex (ACC), hippocampus, and insular cortex (see Figure 1). In addition, the hypothalamus, thalamus, periaqueductal gray, and brain stem nuclei play important parts in mediating fear-responses. The amygdala and dorsal parts of the ACC are considered to be involved in fear expression, whereas the rostral and ventral parts of the ACC and medial prefrontal cortex are involved in emotion regulation. The

ventromedial prefrontal cortex (vmPFC) is also involved in fear extinction (Milad & Quirk, 2002; Phelps, Delgado, Nearing, & LeDoux, 2004), supposedly playing a part in the creation of so called safety memories, where cues that signal the absence of threat override the fear memories. The hippocampus plays a pivotal role in contextual memories and context-dependent fear expression, while the insula monitors internal states and has been suggested to be involved in the experience of fear (Shin & Liberzon, 2010).

The amygdala

The main hub of the fear circuitry is believed to be the amygdala, located in the anterior medial temporal lobe and named after its almond-like shape (Davis, 1992; LeDoux, 2007). The amygdala is not a unitary region, but consists of several subnuclei (Amunts et al., 2005). Here I will use the division of amygdala into the basolateral (BLA) and the central parts (CeA) (Janak & Tye, 2015). Included in the amygdaloid complex are also the intercalated cells, groups of GABA-ergic neurons situated in between the basolateral and central regions of the amygdala (Royer, Martina, & Paré, 1999). Roughly, the BLA acts as the major input station of the amygdala, receiving afferents from various cortical and thalamic sensory regions, as well as the hippocampus. The BLA projects back to the cortex and hippocampus, acting as a reciprocal feedback system, and to the CeA, which is the major output station of the amygdala. The BLA also sends efferents to the striatum, (i.e. the caudate nucleus, putamen, and nucleus accumbens) involved in learned approach and avoidance behavior, and to the bed nucleus of stria terminalis (BNST) (Davis et al., 2009). The intercalated cells receive projections from the BLA and exert feedforward inhibition of the CeA. The CeA in turn projects to the hypothalamus, periaqueductal gray of the midbrain, and monoamine nuclei in the brain stem, which mediate the fear-responses at the autonomic, behavioral, and endocrine levels. The CeA has been suggested to be involved in the short-lived fear response, while the BNST has been related to the sustained fear-response associated with anxiety (Davis et al., 2009).

It should already at this stage be noted that the amygdala is involved in many functions, including threat detection, appetite, and sexual behavior. Indeed, it has been proposed that one of the tasks undertaken by the amygdala is to judge the valence of stimuli, thereby preparing the organism for action (Morrison & Salzman, 2010). Of relevance to the present thesis, damage to the amygdala results in impairments in fear-expression and fear conditioning (S. Brown & Schafer, 1888; Janak & Tye, 2015; Weiskrantz, 1956), suggesting an important role for the amygdala in anxiety disorders. Indeed, exaggerated neural activity in the fear-expressing amygdala and dorsal ACC of the brain fear circuitry is seen across anxiety disorders (Brühl et al., 2014; Etkin & Wager, 2007; Fonzo et al., 2015; Pitman et al., 2012; Sartory et al., 2013; Shin & Liberzon, 2010).

The amygdala receives afferents from the prefrontal cortex, including the rostral ACC and vmPFC that regulate amygdala activity (M. J. Kim et al., 2011). Consequently, neurocircuitry models of exaggerated anxiety have suggested that failure of the vmPFC to inhibit amygdala responses leads to threat bias, heightened fear responses, and emotional dysregulation (Frick, Howner, Fischer, Kristiansson, & Furmark, 2013; Milad & Quirk, 2002; Pitman et al., 2012; Quirk & Beer, 2006; Shin & Liberzon, 2010). Also other regions of the fear circuitry display altered activity in anxiety disorders, such as the hippocampus and insula, although the directions of the alterations are not as consistent as for the amygdala (Shin & Liberzon, 2010).

Structural changes in the fear circuitry have also been reported in both SAD and PTSD. In SAD, findings are inconsistent (Brühl et al., 2014; Frick, Gingnell, et al., 2014; Frick, Howner, Fischer, Eskildsen, et al., 2013), whereas in PTSD, reduced volume has been reported most consistently in the amygdala, hippocampus, vmPFC, and dorsal ACC (Kühn & Gallinat, 2013; O'Doherty, Chitty, Saddiqui, Bennett, & Lagopoulos, 2015; Pitman et al., 2012).

Thus, anxiety disorders are characterized by elevated fear expression, paralleled by increased neural reactivity to threat-related stimuli most reliably in the amygdala. Knowledge of the underlying wiring of the fear circuitry is fundamental to fully understanding how the brain processes threat-related stimuli, but the picture is nowhere near complete without proper knowledge regarding the neurotransmitters involved (Marder, 2012). This becomes especially important when considering that current pharmacological treatment options almost exclusively target neurotransmitter systems.

Neurotransmitter systems involved in anxiety disorders

As noted above, understanding the neurochemical modulation of the brain's fear circuitry may be pivotal to understanding the pathophysiology of anxiety disorders. A number of neurotransmitter systems have been implicated in anxiety disorders, including the two major neurotransmitters in the human brain, the mainly excitatory glutamate and inhibitory gamma-aminobutyric acid (GABA; Durant et al., 2010). For example, blocking the glutamatergic N-methyl-D-aspartate (NMDA) receptor has anxiolytic effects in animal models of anxiety. Evidence for the involvement of GABA in anxiety disorders comes partly from anxiolytic effects of barbiturates and benzodiazepines, both being agonists at the $GABA_A$ receptor. Inverse agonism of the $GABA_A$ receptor, on the other hand, increases anxiety. Furthermore, molecular imaging has revealed downregulated benzodiazepine binding sites in patients wih anxiety disorders (Fredrikson, Faria, & Furmark, 2014). Certainly, both glutamate and GABA are important contributors to the neurobiological underpinnings of anxiety disorders. However, the focus of the present

thesis is on the role of the two modulatory systems serotonin and substance P (SP), to which we now turn our attention.

Serotonin

Serotonin has been suggested to be one of the oldest signaling molecules, evolving perhaps as long as 2-3 billion years ago. It is present in almost all living organisms including animals, fungi, and plants (Azmitia, 2010; Peroutka & Howell, 1994). Serotonin was initially discovered and isolated by Vittorio Erspamer, who conducted studies on an extract of enterochromaffin that caused smooth muscle contraction (Erspamer & Asero, 1952; Vialli & Erspamer, 1937). However, Erspamer called the substance enteramine, and it was not until a decade later that the substance was named serotonin by Maurice Rapport and colleagues who isolated serotonin from serum during the search for vasoconstrictor substances involved in hypertension (Rapport, 1949; Rapport, Green, & Page, 1948). In fact, the name serotonin is a combination of the Latin word *serum* and the Greek *tonic*, from its identified function as a vasoconstrictor. It was later discovered that the active component of enteramine and serotonin were identical and characterized as 5-hydroxytryptamine (5-HT). Subsequent investigations found serotonin to be present in the brain (Amin, Crawford, & Gaddum, 1954; Twarog & Page, 1953), and although the majority of serotonin is synthesized in the gut, serotonin is perhaps best known as one of the major modulatory neurotransmitter systems in the central nervous system (Brodie & Shore, 1957). It is now recognized that brain serotonin is involved in numerous functions, including aggression, appetite, cognition, emesis, temperature regulation, nociception, stress response, mood, and anxiety (Berger, Gray, & Roth, 2009).

Metabolic and signaling pathways of serotonin

Serotonin does not pass the blood brain barrier, meaning that it needs to be synthesized in the human brain. Synthesis of central serotonin proceeds in two steps (Ruddick et al., 2006) (see Figure 2). Dietary tryptophan is passed through the blood brain barrier by the large neutral amino acid transporter and hydroxylates by tryptophan hydroxylase 2 (TPH2) to 5-hydroxytryptophan (5-HTP) in the serotonergic neuron (Walther et al., 2003). This hydroxylation is considered the rate-limiting step. 5-HTP is subsequently decarboxylated by aromatic L-amino acid decarboxylase (AADC) to serotonin and transported by vesicular monoamine transporter 2 (VMAT2) into vesicles for calcium-dependent exocytosis. After release, the action of serotonin is terminated predominantly through clearance of serotonin from the extracellular space by high-affinity presynaptic serotonin transporter proteins (SERT), effectively regulating serotonin signaling (Hoffman, Mezey, & Brownstein, 1991; Ramamoorthy et al., 1993). Synthesis and release of serotonin is regulated by inhibitory somatodendritic 5-HT_{1A} and

axonal 5-HT_{1B} autoreceptors (Boadle-Biber, 1993). Serotonin metabolism proceeds via the enzyme monoamine oxidase (MAO), preferentially the MAO-A isoenzyme, and aldehyde dehydrogenase to 5-hydroxyindoleacetic acid (5-HIAA), which is excreted primarily in urine (Charnay & Leger, 2010). MAO is present both in glia cells and at mitochondrial membranes inside monoaminergic neurons.

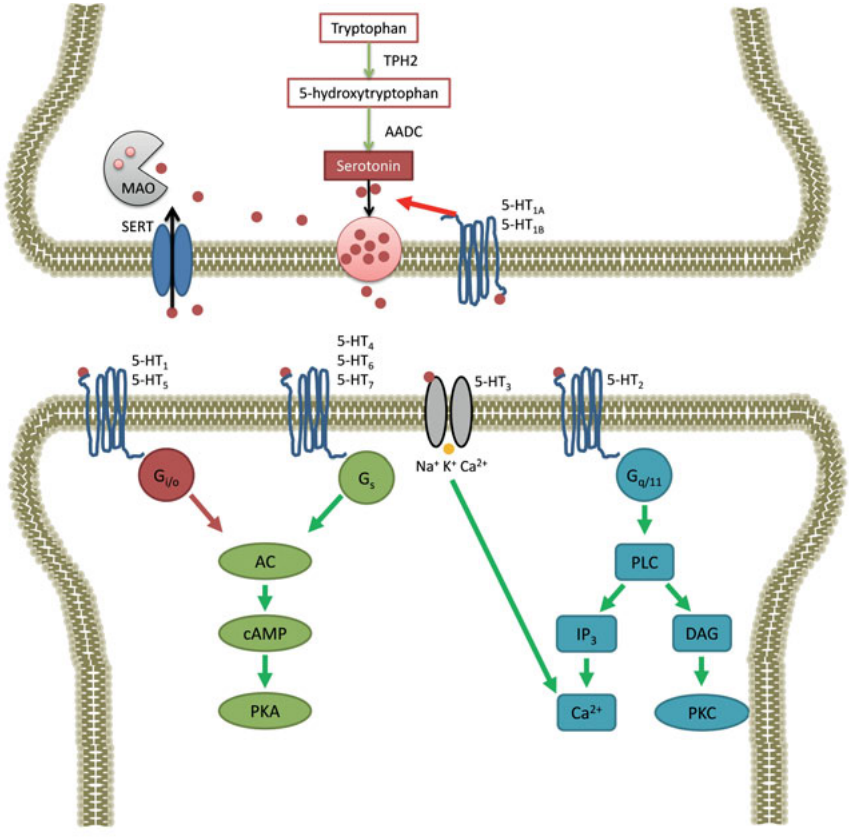

Figure 2. Simplified depiction of serotonin signaling pathways. Dietary tryptophan crosses the blood brain barrier and is hydroxylated by TPH2, the product 5-hydroxytryptophan is decarboxylated by AADC into serotonin and entered into vesicles for release into the extracellular space. Binding of serotonin to its receptors exerts different influences on the postynaptic neuron depending on receptor subtype. Biological outcomes are often mediated by second messenger systems. Green arrows indicate an increase in target activity, whereas red arrows indicate an inhibitory influence. After dissociation from the receptor, serotonin is transported back into the presynaptic neuron by SERT and degraded by MAO. Abbreviations: 5-HT: serotonin, AADC: amino acid decarboxylase, AC: adenylyl cyclase, cAMP: cyclic adenosine monophosphate, DAG: diacylglycerol, MAO: monoamine oxidase, G: G-protein, IP3: Inositol triphosphate, PKA: protein kinase A, PKC: protein kinase C, PLC: phospholipid C, SERT: serotonin transporter, TPH2: tryptophan hydroxylase.

Ascending projections from the approximately 300,000 serotonergic neurons in the rostral group of raphe nuclei, including the dorsal and median raphe nuclei (Charnay & Leger, 2010; Hornung, 2003) innervate in a defined and organized pattern the forebrain (Dahlström & Fuxe, 1964) (see Figure 3), where serotonin exerts its influence through binding to one of its receptors. Serotonin neurotransmission and modulation occurs both as hard-wired, synaptic transmission and as volume transmission. In the latter, serotonin diffuses from the release site and acts at longer ranges and affects multiple neurons (Bunin & Wightman, 1998). Various subtypes of serotonin receptors exist, and to date at least 14 subtypes have been cloned and characterized (Hoyer, Hannon, & Martin, 2002; Nichols & Nichols, 2008). All but the ionotropic 5-HT_3 receptor are G protein-coupled (guanine nucleotide binding proteins), and binding of serotonin to its receptors has differential effects depending on the type and localization of the receptor (Figure 2). As mentioned, the 5-HT_{1A} receptor has an inhibitory feedback function when expressed as an autoreceptor on the soma or dendrites of serotonergic neurons in the raphe nuclei, but it is also expressed as a heteroreceptor on non-serotonergic neurons where it exerts inhibitory influences. Indeed, 5-HT_{1A} is the main inhibitory serotonergic receptor in the brain. Conversely, the 5-HT_{2A} receptor is the primary central excitatory serotonin receptor.

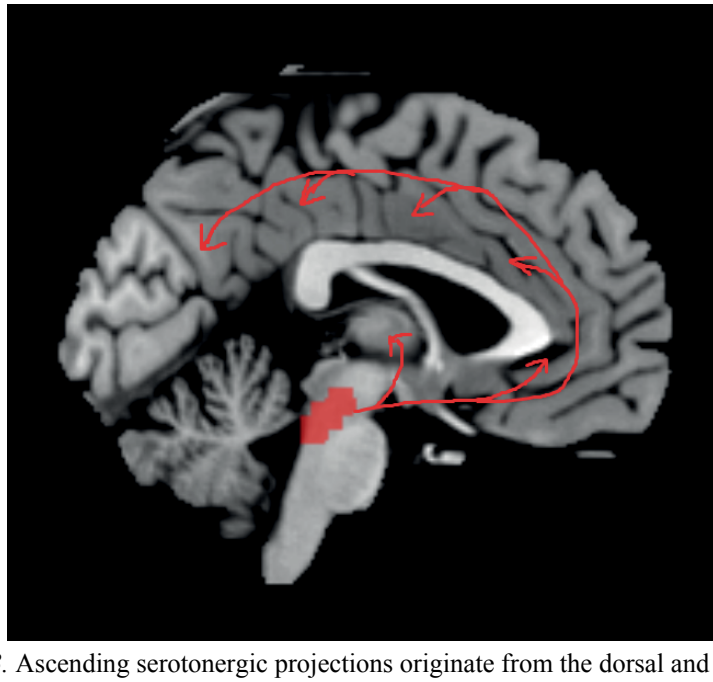

Figure 3. Ascending serotonergic projections originate from the dorsal and median raphe nuclei in the brain stem, here depicted in red, and innervate cortical and subcortical regions.

Serotonergic involvement in anxiety

In 1954, Woolley and Shaw (1954) in the USA and Gaddum in the UK independently proposed that serotonin could be involved in the mental disturbances caused by the mind-altering drug lysergic acid diethylamide (LSD; Green, 2008). Woolley and Shaw went on to suggest that mental disturbance may be caused by an imbalance in serotonin levels (Woolley & Shaw, 1957), be it excess or deficiency of serotonin. They proposed that normal serotonin levels caused continuous contraction and relaxation of oligodendroglia and that this rhythmic movement stirred the extravascular fluid and thereby circulated oxygen, food, and waste products necessary for normal brain function. Imbalance in serotonin levels was hypothesized to lead to mental disturbances by disrupted stirring and subsequent deficiencies in oxygen supply and waste removal. Although the proposed mechanism has been abandoned, the idea that alterations in neurochemistry could be related to psychiatric disorders has been suggested to be a starting point for modern neuropsychopharmacology (Nichols & Nichols, 2008).

Following Woolley and Shaw's proposal, two serendipitous findings strengthened the hypothesis of serotonergic involvement in psychiatric disorders. First, clinical trials revealed mood-elevating properties of the drug iproniazid used to treat tuberculosis. Subsequent studies demonstrated that iproniazid was a monoamine oxidase inhibitor, blocking the degradation of serotonin. Iproniazid thus decreases the metabolism of serotonin and increases serotonergic levels (Zeller & Sarkar, with the assistance of Renate M. Reinen, 1962). The second finding regarding the involvement of serotonin in psychiatric disorders was that reserpine, used to treat hypertension, reduces serotonin levels and leads in some cases to depressive mood (Achor, Hanson, & Gifford Jr., 1955; Dustan, Taylor, Corcoran, & Page, 1954). It is now known that reserpine acts as an antagonist at VMAT2, blocking the transport of serotonin and other monoamines into the vesicles used for exocytotic release of the neurotransmitter. These findings together with reports of the mood-elevating effects of tricyclic antidepressants that block the reuptake of monoamines (Marshall, Stirling, Tait, & Todrick, 1960) led to the monoamine deficiency theory of depression (Coppen, 1967). Subsequent pharmacological development led to the selective serotonin reuptake inhibitors (Carlsson, 1987; Carlsson & Wong, 1997) used today as first-line treatment for mood and anxiety disorders (Murrough et al., 2015).

Soon after the proposal of involvement in lowered mood, serotonin was suggested to be associated with anxiety (Griebel, 1995; Iversen, 1984). Initial animal studies reported that serotonin antagonists such as parachlorophenylalanine (pCPA; blocking conversion of tryptophan to 5-HTP, the first step in serotonin synthesis) had an anxiolytic-like effect (Rex & Fink, 2011; Robichaud & Sledge, 1969), whereas administration of the serotonin precursor 5-HTP prevented this effect (Geller & Blum, 1970). Based

on these and similar findings, the "classic" hypothesis of serotonin as anxiogenic was formulated (Iversen, 1984). This hypothesis has later been extended to state that increased serotonin release or hypersensitive postsynaptic serotonin receptors underlie the anxiogenic activity of serotonin (Durant et al., 2010; Maron et al., 2012). Supporting this "classic" hypothesis, both the serotonin releasing agent fenfluramine (Tancer et al., 1994) and serotonin agonists (Charney, Woods, Goodman, & Heninger, 1987; Kennett, Whitton, Shah, & Curzon, 1989) increase anxiety levels.

On the other hand, long-term blockage of serotonin reuptake by SSRIs increases extracellular serotonin levels in projection areas (Ceglia et al., 2004) and alleviates anxiety (Koen & Stein, 2011), suggesting that anxiety may be related to diminished levels of serotonin. Additional support for this notion comes from studies employing acute depletion of the serotonin precursor tryptophan. Such depletion leads to a dramatic drop in available serotonin and worsens anxiety following symptom provocation in patients with anxiety disorders successfully treated with SSRIs (Argyropoulos et al., 2004; Corchs, Nutt, Hood, & Bernik, 2009).

In an attempt to reconcile the conflicting reports of serotonin in anxiety, Deakin and Graeff (1991; Graeff, Guimarães, De Andrade, & Deakin, 1996) proposed that distinct subpopulations of serotonergic neurons modulated specific anxiety-related functions through differential projections to key components of the fear circuitry. Conditioned fear, they proposed, was associated with increased serotonergic neurotransmission in the amygdala. Innate fear, on the other hand, was inhibited by serotonergic projections to the periaqueductal gray, such that increased serotonergic firing inhibits innate panic- and escape-like behavior and physiological responses.

Genetic studies linking serotonin to anxiety

Further evidence for serotonergic involvement in anxiety disorders comes from genetic studies. T carriers relative to GG homozygotes of the rs4570625 (G-703T) single nucleotide polymorphism (SNP) in the putative promoter region of the TPH2 gene have increased amygdala reactivity to emotional stimuli (S. M. Brown et al., 2005; Canli, Congdon, Gutknecht, Constable, & Lesch, 2005; Furmark et al., 2009). In addition, a repeat length polymorphism exists in the promoter region of the SERT gene, the serotonin transporter-linked polymorphic region (5-HTTLPR), with a low-expressing short (s) and a high-expressing long (l) variant (Lesch et al., 1996). The low-expressing variant increases risk for anxiety disorders (Gressier et al., 2013; Lesch et al., 1996), facilitates fear conditioning (Garpenstrand, Annas, Ekblom, Oreland, & Fredrikson, 2001) and enhances amygdala reactivity (Furmark et al., 2009; Murphy et al., 2013), possibly through de-coupling of the regulatory connection from the rostral ACC to the amygdala (Pezawas et al., 2005). Also, genetic variants of the TPH2 and SERT possibly influence the outcome of SSRI treatment (M. B. Stein, Seedat, & Gelernter, 2006) and

pill placebo delivered under double-blind, randomized conditions (Furmark et al., 2008). Moreover, TPH2 knockout mice have marked reductions in serotonin formation (Gutknecht et al., 2008) and reduced anxiety-like behavior (Mosienko et al., 2012), whereas SERT knockout mice have increased extracellular serotonin levels, increased anxiety-like behavior and attenuated fear extinction recall (Holmes, Murphy, & Crawley, 2003; Wellman et al., 2007).

Serotonin, anxiety, and the amygdala

The amygdala is heavily innervated by serotonergic neurons, which modulate neural activity, including the response to threat (Fisher, Meltzer, Ziolko, Price, & Hariri, 2006). The most studied serotonergic receptors in the amygdala are the 5-HT_{1A}, 5-HT_2, and 5-HT_3 receptor subtypes, all of which are expressed throughout the subnuclei albeit in a subnuclei-dependent fashion (Asan, Steinke, & Lesch, 2013). The 5-HT_{1A} receptors are mainly expressed in the CeA, the 5-HT_{2A} on GABA-ergic interneurons and pyramidal neurons in the BLA, whereas the 5-HT_3 receptors are expressed almost exclusively on GABA-ergic neurons throughout the whole amygdala. There is evidence for anxiolytic effects of serotonin acting at amygdala 5-HT_{1A} receptors (Akimova, Lanzenberger, & Kasper, 2009), whereas serotonergic activation of 5-HT_{2C} receptors is anxiogenic (Q. Li, Luo, Jiang, & Wang, 2012; Salchner & Singewald, 2006).Thus, the anatomy of serotonergic innervation in the amygdala indicates that serotonin is involved in modulation of neural activity and possibly also anxiety. This has been corroborated by human neuroimaging studies demonstrating a negative relationship between SERT availability and threat-related activity in the amygdala (Rhodes et al., 2007). In accordance with these results, reduced amygdala SERT availability has been linked to facilitated fear conditioning in healthy subjects (Åhs, Frick, Furmark, & Fredrikson, 2015), a process associated with heightened amygdala activity (Furmark, Fischer, Wik, Larsson, & Fredrikson, 1997; LaBar, Gatenby, Gore, LeDoux, & Phelps, 1998). Furthermore, 10 days' administration of the SSRI escitalopram modulates amygdala activity to fearful faces, partly by strengthening the inhibitory connection from medial prefrontal cortex (Sladky et al., 2015).

Molecular neuroimaging findings of serotonin's role in anxiety

Molecular neuroimaging studies have associated anxiety disorders with altered in vivo serotonin transporter binding (Maron et al., 2012). The exact alterations, however, differ between disorders. In SAD, van der Wee et al. (2008) found increased thalamic SERT availability, whereas PTSD has been associated with decreased SERT availability in the amygdala (Murrough, Huang, et al., 2011). One common finding seen across many anxiety disorders is reduced 5-HT_{1A} receptor availability (Akimova et al., 2009; Fredrikson et al., 2014). Consistently, SAD has been associated with reduced 5-

HT_{1A} receptor binding in the raphe nuclei, amygdala, anterior cingulate cortex, and insular cortex (Lanzenberger et al., 2007). However, in patients with PTSD, reports of serotonin 5-HT_{1A} receptor availability are inconsistent with one study finding no change (Bonne et al., 2005) and another finding overall increase in all studied regions including the amygdala and the raphe nuclei (Sullivan et al., 2013). PTSD has further been associated with reduced availability of serotonin 5-HT_{1B} receptors in the amygdala, ACC, and caudate nucleus (Murrough, Czermak, et al., 2011). Thus, serotonergic alterations have been reported for both SAD and PTSD, but the directions of the alterations differ; SAD being associated with increased SERT and decreased 5-HT_{1A} availability, and PTSD with decreased SERT and increased 5-HT_{1A} binding.

Collectively, many lines of research provide evidence for serotonergic involvement in anxiety disorders, and suggest that neuromodulation of the fear circuitry of the brain may be an important aspect to take into consideration when probing the neural underpinnings of exaggerated anxiety. However, despite massive research into the specifics of serotonergic involvement in anxiety disorders in general, and SAD and PTSD specifically, it is still a matter of controversy as to whether exaggerated anxiety is associated with an excessive or deficient serotonergic system (Durant et al., 2010; Maron et al., 2012).

Substance P and neurokinin-1 receptors

SP is a neuropeptide expressed over large parts of the brain as well as in the periphery (Hökfelt, Kellerth, Nilsson, & Pernow, 1975; Ribeiro-da-Silva & Hökfelt, 2000; von Euler & Gaddum, 1931). The name SP comes from the powder preparation extracted from horse brain and intestine used by von Euler and Gaddum in the initial experiments of the contractile substance (Gaddum & Schild, 1934; von Euler & Gaddum, 1931). The amino acid structure of SP was identified by Chang et al. (1971), and SP was together with neurokinin A and neurokinin B characterized as belonging to the tachykinin family for their common ability to rapidly contract smooth muscle (Erspamer, 1981; Maggio, 1988). The precursor of SP is synthesized from the gene preprotachykinin-A (PPT-A) in the cell soma, followed by transport of the precursor propeptide to the axon terminals where it is cleaved into bioactive SP and made ready for release by large dense-core vesicles (Harrison & Geppetti, 2001; Steinhoff, Mentzer, Geppetti, Pothoulakis, & Bunnett, 2014) (see Figure 4). Secretion of SP and other neuropeptides from large dense-core vesicles requires enhanced or multiple stimulation of the neuron compared to release of serotonin and other monoamine neurotransmitters from small vesicles (Merighi, Salio, Ferrini, & Lossi, 2011).

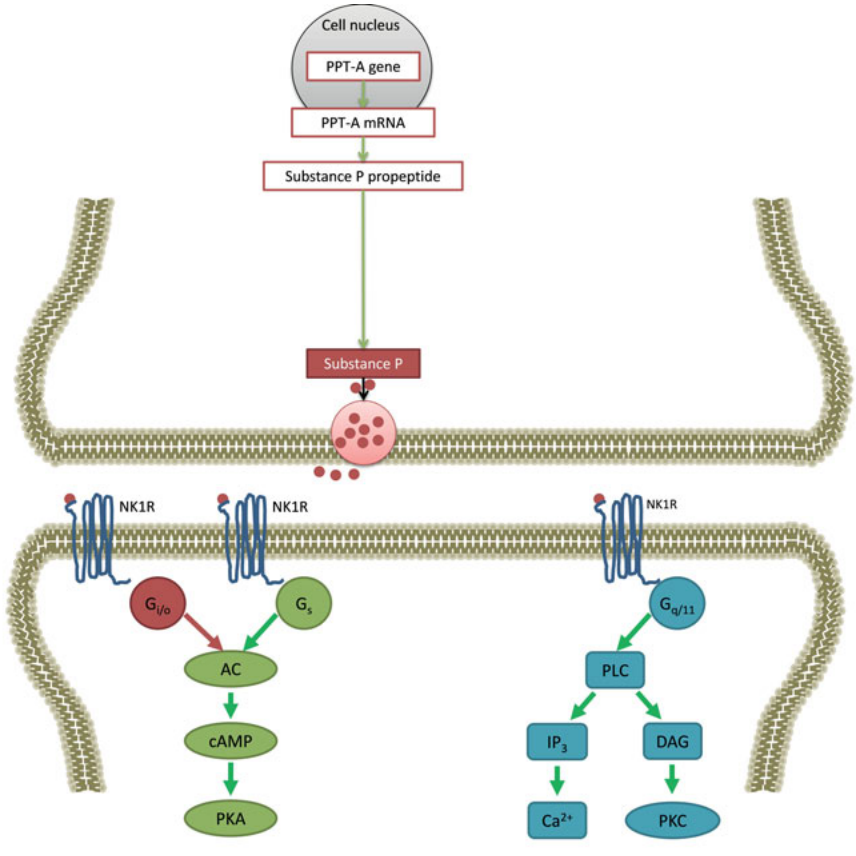

Figure 4. Simplified depiction of substance P signaling through NK1 receptors. Substance P originates from the PPT-A gene, which is transcribed to PPT-A mRNA and translated to substance P propeptide in the cell soma. Subsequent axonal transport of the propeptide and cleavage to substance P makes the neurotransmitter ready for release by large dense core vesicles. Substance P exerts its effects through binding to the G-protein coupled NK1 receptors. Biological effects are mediated by second messenger systems. Green arrows indicate an increase in target activity, whereas red arrows indicate an inhibitory influence. Abbreviations: cAMP: cyclic adenosine monophosphate, Ca: calcium, DAG: diacylglycerol, G: G-protein, IP3: Inositol triphosphate, mRNA: messenger ribonucleic acid, NK1R: neurokinin-1 receptor, PKA: protein kinase A, PKC: protein kinase C, PLC: phospholipid C, PPT-A: preprotachykinin A.

SP mainly exerts its actions in the brain through seven-transmembrane G-protein coupled neurokinin-1 (NK1) receptors, although it has some affinity also to the other tachykinin receptors, i.e. the neurokinin-2 and neurokinin-3 receptors (Ohkubo & Nakanishi, 1991). Thus, in this thesis, I will refer to the SP/NK1system, and by that mean the neurotransmitter system with SP as endogenous ligand and NK1 receptors as target. Following activation by SP and other agonist ligands, the NK1 receptor rapidly desensitizes. Receptor

complex endocytosis follows activation and the fate of the receptor differs depending on the stimulation conditions. Brief exposure of SP leads to rapid recycling of the receptor, whereas after sustained exposure, the receptor is degraded (Steinhoff et al., 2014).

The role of substance P and neurokinin-1 receptors in anxiety

There are converging lines of evidence supporting the involvement of the SP/NK1 system in anxiety disorders (Ebner & Singewald, 2006). First, brain regions included in the fear circuitry have dense expression of NK1 receptors (Ribeiro-da-Silva & Hökfelt, 2000). Moreover, animal and pharmacological intervention studies have shown that the SP/NK1 system in the amygdala modulates stress and anxiety (Ebner, Rupniak, Saria, & Singewald, 2004; Ebner & Singewald, 2006). Increased release of SP in the amygdala is induced by stress (Ebner et al., 2004). Consistently, in humans, exposure to phobic stimuli in patients with specific phobia increases stress-induced endogenous SP release (Michelgård et al., 2007). Also, patients with PTSD exhibit elevated cerebrospinal fluid SP concentrations that are further heightened by symptom provocation (Geracioti et al., 2006). Moreover, genetic variation in the TACR1 gene, encoding the NK1 receptor, has been associated with stress reactivity and receptor expression in the rat amygdala (Schank et al., 2013).

Furthermore, in animals, anxiogenic effects result from administration of SP into the amygdala (Bassi, de Carvalho, & Brandão, 2014; Ebner et al., 2004), whereas anxiety-like behavior is reduced by pharmacological blockage of the NK1 receptor (Ebner et al., 2004; Ebner & Singewald, 2006). Consistently, in patients with SAD, attenuated amygdala reactivity and reduced self-reported anxiety during public speaking were noted after 6 weeks' treatment with the selective NK1 receptor antagonist GR205161 (Furmark et al., 2005). However, a large-scale depression study (Keller et al., 2006) failed to replicate initial positive effects of NK1 receptor antagonists (Kramer et al., 1998, 2004) and treatment findings from clinical trials of NK1 receptor antagonists for psychiatric disorders are mixed (Keller et al., 2006; Kramer et al., 1998, 2004; Mathew et al., 2011; Michelson et al., 2013; Tauscher et al., 2010). Hence, findings both in animals and humans support that SP, acting through NK1 receptors in the amygdala, is anxiogenic. Nonetheless, mapping of NK1 receptors in anxiety disorders is largely lacking, with only one study assessing disease-related changes using molecular neuroimaging, in which Fujimura and colleagues reported on decreased NK1 receptor availability in patients with panic disorder (Fujimura et al., 2009).

Relationship between the serotonergic and SP/NK1 systems

Serotonin interacts with many other neurotransmitter systems, both in the raphe nuclei and in projection areas through serotonergic heteroreceptors and reciprocal regulatory connections between systems (Charnay & Leger, 2010). As has been briefly reviewed in the preceding pages, both the serotonergic and SP/NK1 systems are implicated in the pathophysiology of anxiety disorders. Moreover, the serotonin and SP/NK1 systems are frequently co-expressed (Sergeyev, Hökfelt, & Hurd, 1999), and there is evidence of crosstalk and interactions between the systems (Gobbi & Blier, 2005; Rojas et al., 2010; Santarelli et al., 2001; Shirayama, Mitsushio, Takashima, Ichikawa, & Takahashi, 1996; Valentino & Commons, 2005). In the amygdala, NK1 receptors are mainly co-expressed with 5-HT_{1A} receptors on GABA-ergic neurons (Hafizi, Serres, Pei, Totterdell, & Sharp, 2012) and animal studies have shown that blocking NK1 receptors increases firing in serotonergic neurons (Gobbi & Blier, 2005; Valentino & Commons, 2005) while blocking SERT reduces SP levels (Shirayama et al., 1996). Furthermore, NK1 receptor antagonist-facilitated active stress coping in rats is mediated by increased serotonergic binding to 5-HT_{1A} receptors (Ebner, Singewald, Whittle, Ferraguti, & Singewald, 2007). In addition, it is possible that the anxiolytic effect of SSRIs could be augmented by combining blockage of serotonin reuptake with NK1 receptor antagonism. Indeed, the combination exhibited increased antidepressant effect in an animal model of depression (Chenu, Guiard, Bourin, & Gardier, 2006). However, a clinical trial in patients with depression found no effect of adding the NK1 receptor antagonist aprepitant to the SSRI paroxetine (Ball et al., 2014). In summary, the relationship between the serotonergic and SP/NK1 systems may be of great importance for understanding the pathophysiology of exaggerated anxiety. However, such relationships have not been studied using molecular imaging in any anxiety disorder to date.

Positron emission tomography

Molecular neuroimaging techniques, such as positron emission tomography (PET), have made it possible to study neurochemistry in the living human brain (Farde, Hall, Ehrin, & Sedvall, 1986; Wong et al., 1984, 1986), including possible neurochemical alterations in patients with anxiety disorders (Fredrikson et al., 2014). In PET imaging, compounds are labeled with a short-lived positron emitting radionuclide, thereby creating a radiotracer. The most commonly used radionuclides for PET imaging of the human brain include ^{15}O (half-life:~2 min), ^{11}C (half-life:~20 min), and ^{18}F (half-life:~110 min). The half-life of the radionuclide determines scan length, and thereby the suitability to image different biological processes as well as the exposure

to radioactivity. A multitude of radiotracers exist for imaging of biological targets such as enzymes, receptors and transporters, and physiological and metabolic processes in vivo in humans, e.g. [^{11}C]raclopride for dopamine D_2-like receptors, [^{15}O]water for cerebral blood flow, and [^{18}F]fluorodeoxyglucose for glucose metabolism. For studies of receptors and transporters in the brain, high specific radioactivity (radioactivity/mass) of the radiotracer is essential, and as a rule of thumb no more than 5% of the target protein should be occupied by the tracer itself to fulfill the tracer concept (de Hevesy & Paneth, 1913).The administered amount of a radiotracer is thus very small, in the range of a few micrograms, which certifies that non measurable perturbation of the biological system occurs.

Basic principles of positron emission tomography

In PET imaging, the radiotracer is administered to the subject via intravenous injection, either as bolus or as bolus plus constant infusion, and binds to the biological target. During PET scanning, the radionuclide undergoes positron emission decay, emitting a positron that travels a short distance until it combines with an electron to form a short-lived composition called positronium. The positronium eventually gets annihilated, producing two 511 keV photons moving anti-parallel. These photons can be recorded in the PET scanner by the ring of detectors surrounding the scanned object. When two photons are registered simultaneously (i.e. within less than 10 nanoseconds) at approximately 180° from each other, it is assumed that the photons originate from the same annihilation event and the camera registers this as a coincidence event. During a PET scan, many coincidence events are registered, allowing computer algorithms to reconstruct the location and concentration of the radioactivity (i.e. the radiotracer) in three dimensions. Splitting the PET scan into a number of shorter time frames, so called dynamic PET scanning, allows for calculation of the location and concentration of the radioactivity over time. Thus, for each voxel in the brain, dynamic PET data consist of a series of radioactivity measurements reflecting radioactivity concentration over time in this location. Plotting the dynamic PET data for a voxel against time produces a time activity curve (TAC). Assuming that no radiolabeled metabolites of the radiotracer interfere, this information can be used to assess parameters of the biological target, for example the binding potential of the serotonin transporter to the radiotracer, reflecting serotonin transporter availability, in different regions of the brain.

Analysis of positron emission tomography data

Assessment of biological parameters such as binding potential can be performed by means of pharmacokinetic modeling, which assumes that the radiotracer is distributed in a number of compartments (i.e. in different states

or spaces). Within tissue, distribution of the radiotracer can be represented by a 3-tissue compartment model, where the radiotracer exists in specifically bound (bound to the target), nonspecifically bound (bound to non-target proteins), or free (unbound) states (see Figure 5a). The model is mathematically described by a set of differential equations. Because of the large number of parameters being estimated for the 3-tissue compartment model, there may be substantial error in the estimates. Therefore, if the free and non-specific bound compartments reach equilibrium quickly, which they often do, the non-specifically bound compartment can be discarded as it would be difficult to differentiate it from the free compartment. This results in a simplified model, reducing the number of estimates. Here, I have called the remaining free compartment the non-displaceable compartment, denoting that the radiotracer in the non-specifically bound and free compartments cannot be displaced by ligands acting at the same target as the radiotracer. This simplification describes the standard 2-tissue compartment model (see Figure 5b). If the radiotracer is irreversibly bound to the target during the time of the PET scan (i.e. if $k_4 = 0$), the model depicted in Figure 5c can be used.

Estimation of biological parameters of interest from dynamic PET data can also be accomplished by data-driven, graphical methods, which are not dependent on a specific model structure with a predefined number of tissue compartments. Logan (Logan et al., 1996) and Patlak (Patlak, Blasberg, & Fenstermacher, 1983) have described graphical methods to estimate parameters for reversible and irreversible binding of radiotracers respectively. Logan's method can be used to calculate the binding potential (BP_{ND}) (Innis et al., 2007; Logan et al., 1996), i.e. the ratio of the specifically bound radiotracer to non-displaceable radiotracer, reflecting receptor availability. Patlak plot estimates the influx parameter of the irreversibly bound radiotracer, which can be used to index for example receptor availability or enzyme activity. So far, the models described have used radiotracer concentration in arterial plasma as input function. Because of the invasive nature of arterial blood sampling, techniques substituting the arterial input function with the TAC of a reference region devoid of the biological target have been developed. Cerebellum is devoid of some of the important receptors and transporters of interest in neuroscience and is therefore often used as a reference region reflecting the non-displaceable fraction. Both Logan and Patlak methods can be modified to use reference region TAC instead of the plasma input function.

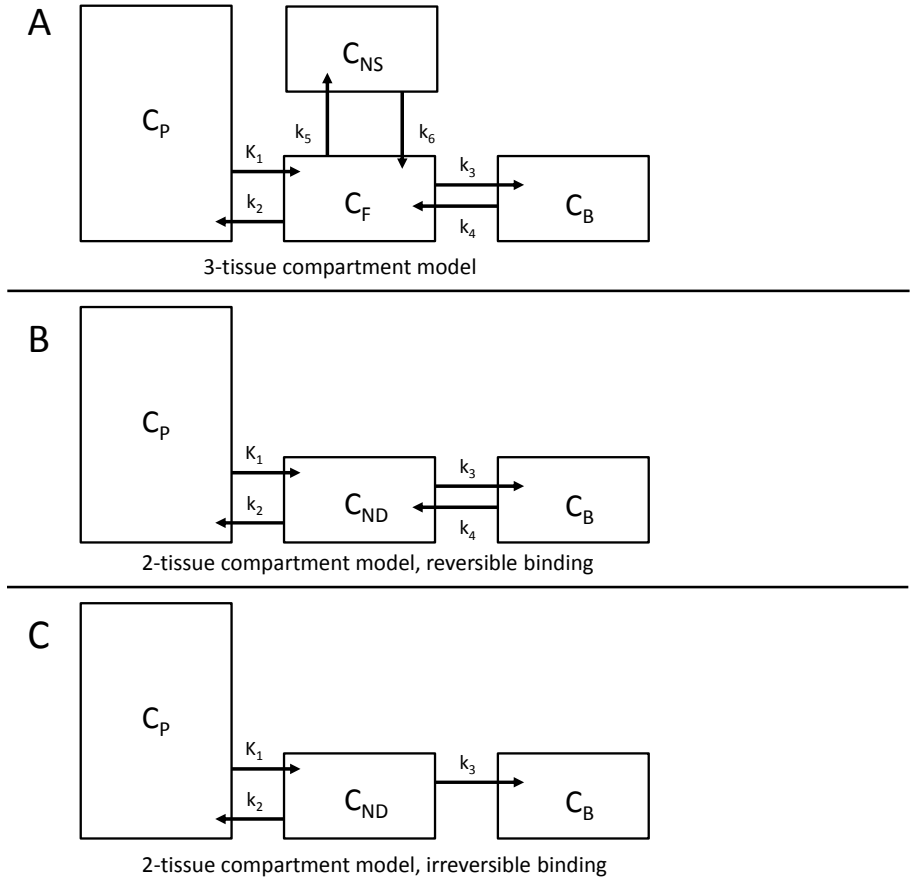

*Figure 5.*Compartment models used in PET analysis. (A) 3-tissue compartment model including plasma concentration of radiotracer (C_P), specifically bound to target (C_B), free (C_F), and non-specifically bound (C_{NS}) radiotracer. Rate constants include the influx from plasma to tissue (K_1), efflux from tissue to plasma (k_2), transfer between free and specifically bound (k_3 and k_4), and between free and non-specifically bound (k_5 and k_6) radiotracer. (B) 2-tissue compartment model where the free and non-specifically bound compartments have been reduced to one non-displaceable (ND) compartment. This model is useful if the radiotracer exhibits reversible binding to the target. (C) 2-tissue compartment model useful for irreversibly bound radiotracers, i.e. the efflux k_4 from C_B to C_{ND} is 0.

Aims

The general aim of this thesis was to study possible neurochemical alterations associated with anxiety disorders. Based on the previous literature, three questions were asked: (1) Is serotonin anxiogenic or anxiolytic?; (2) Is the SP/NK1 system involved in anxiety disorders?; (3) Is the relationship between the serotonergic and SP/NK1 systems altered in anxiety disorders? Three empirical studies contained in the thesis examined different aspects of these questions using PET for in-vivo imaging of the brain serotonergic and SP/NK1 systems in patients with SAD (Study I and Study II) and PTSD (Study III).

I The first study evaluated brain serotonin synthesis rate and SERT availability, major contributors to serotonin neurotransmission and indices of presynaptic serotonergic activity, in patients with SAD as compared to healthy controls and in relation to symptom severity.

II The second study characterized NK1 receptor availability, as a marker for the SP/NK1 system, in patients with SAD as compared to healthy controls and in relation to symptom severity.

III The third study assessed SERT and NK1 receptor availability, in order to study the serotonergic and SP/NK1 systems separately and their co-expression, in patients with PTSD as compared to healthy controls and in relation to symptom severity.

Methods

Participants

Participants' demographic and clinical characteristics can be found in Table 1. Patients with SAD in Study I and Study II, as well as healthy controls (HC), were recruited through newspaper advertising. Patients meeting the initial screening criteria for social phobia (i.e. SAD) from the Social Phobia Screening Questionnaire (SPSQ; Furmark et al., 1999) and those who did not fulfill any exclusion criteria were subsequently interviewed using the Mini International Neuropsychiatric Interview (MINI; Sheehan et al., 1998) and the Structured Clinical Interview for the DSM-IV (SCID-I; First, Gibbon, Spitzer, & Williams, 1998) to ascertain that they fulfilled the DSM-IV criteria for social phobia (i.e. SAD) (American Psychiatric Association, 2000) and to assess psychiatric comorbidity. All patients with SAD had a primary SAD diagnosis. Severity of social anxiety symptoms was evaluated with the Liebowitz Social Anxiety Scale (LSAS; Liebowitz, 1987). Finally, a medical examination was performed.

For Study III, patients with PTSD were recruited from the Department of Psychiatry and the Department of Obstetrics and Gynecology at Uppsala University Hospital. In addition to a clinical psychiatric evaluation using DSM-IV criteria for PTSD (American Psychiatric Association, 2000) a medical examination was performed. All patients with PTSD had a primary PTSD diagnosis. Symptom severity was evaluated with the Clinician-Administered PTSD Scale (CAPS; Blake et al., 1995), and the MINI (Sheehan et al., 1998) was used to assess psychiatric comorbidity.

Similar assessments were made for the HC participants. All subjects were appraised as healthy and none of the HCs fulfilled criteria for any current psychiatric disorder, as assessed with the MINI (Sheehan et al., 1998), nor did they have a lifetime history of such disorders.

The main exclusion criteria for all studies were any other major psychiatric (e.g. schizophrenia) or neurologic disorder, somatic disease, ongoing or discontinued (within 2 months) psychological treatment, treatment with psychotropic medication, chronic use of prescribed medication, current drug or alcohol abuse/dependency, previous PET-examination, pregnancy, or menopause.

Table 1. *Study and participant characteristics*

	Study I		Study II	Study III
Target	Serotonin synthesis rate	Serotonin transporter (SERT)	NK1 receptor	SERT and NK1 receptor
Radiotracer	[^{11}C]5-HTP	[^{11}C]DASB	[^{11}C]GR205171	[^{11}C]DASB and [^{11}C]GR205171
Patients	SAD	SAD	SAD	PTSD
Scanned	18	26	18	18
Analyzed[a]	18	26	17	16
Age mean (SD)	32.6 (8.2)	35.2 (10.7)	30.9 (7.3)	38.7 (13.0)
Sex (M/F)	9/9	14/12	8/9	8/8
Handedness (R/L)	18/0	24/2	17/0	16/0
GSAD	9 (50%)	16 (67%)	10 (59%)	-
LSAS	62.8 (12.6)	73.0 (25.5)	80.6 (20.6)	-
Symptom duration years	26.3 (9.8)	24.1 (11.3)	19.4 (9.4)	11.5 (8.8)
Trauma type				
Combat	-	-	-	8
Non-combat	-	-	-	8
CAPS	-	-	-	68.3 (16.7)
Recruitment	Advertisement	Advertisement	Advertisement	Clinic
Treatment history				
SSRI	3	4	4	0
Propranolol	1	0	3	0
Comorbidity				
Depression	0	1	0	12
GAD	0	4	5	3
OCD	0	1	0	3
Specific phobia	2	3	2	0
PD	0	1	0	4
Controls[b]				
Scanned	18	18	18	18
Analyzed[l]	17	17	17	16
Age mean (SD)	34.9 (9.4)	34.1 (9.4)	34.6 (9.8)	34.0 (9.7)
Sex (M/F)	8/9	9/8	8/9	8/8
Handedness (R/L)	17/0	17/0	17/0	16/0
Recruitment	Advertisement	Advertisement	Advertisement	Advertisement

Abbreviations: CAPS: Clinician-administered PTSD-scale, GAD: generalized anxiety disorder, GSAD: generalized SAD, LSAS: Liebowitz social anxiety scale, NK1: neurokinin-1, OCD: obsessive-compulsive disorder, PD: panic disorder, PTSD: posttraumatic stress disorder, SAD: social anxiety disorder, SERT: serotonin transporter.
a Due to technical problems, not all scanned individuals were analyzed.
b The same 18 controls were scanned with all three tracers.

Patients and controls did not differ with regard to age, sex distribution, or handedness in any study (*P*s > .26). The two SAD groups in Study I did not differ with respect to age, sex distribution, social anxiety severity, number of individuals with generalized SAD, previous use of psychotropic medication, or number of individuals with current psychiatric comorbidity (*P*s > .12).

Clinical instruments

Mini International Neuropsychiatric Interview

The MINI (Allgulander, Waern, Humble, Andersch, & Ågren, 2006; Sheehan et al., 1998) is a structured interview for the diagnosis of axis I disorders and antisocial personality disorder from DSM-IV. In the present thesis, MINI was used to assess psychiatric comorbidity.

Structured Clinical Interview for DSM-IV

In Study I and Study II, the social phobia questions from the SCID-I (First et al., 1998) were used to ascertain that all patients with SAD fulfilled DSM-IV criteria for SAD. Furthermore, for patients with SAD in Study I that underwent [^{11}C]5-HTP PET imaging and for patients with SAD in Study II, the anxiety questions from SCID-I were used to assess comorbid anxiety disorders.

Liebowitz Social Anxiety Scale

The LSAS (Liebowitz, 1987) is used as a measure of severity of social anxiety symptoms. For each of 24 social situations, the respondent rates on a scale 0-3 how much anxiety he/she feels in the situation and how often he/she avoids the situation. The total LSAS score ranges from 0 to 144, with higher scores indicating greater symptom severity. LSAS can be administered by the clinician or as a self-report version (Fresco et al., 2001), and the two versions show high reliability. In the present thesis, the clinician-administered version was used for patients with SAD that underwent [^{11}C]DASB PET imaging in Study I. For patients with SAD in Study I that underwent [^{11}C]5-HTP PET imaging and for patients with SAD in Study II, the self-report version was administered.

Clinician-Administered PTSD Scale

The CAPS (Blake et al., 1995) is used by clinicians to rate PTSD symptom severity. The version used in the present thesis is based on the DSM-IV criteria for PTSD and starts off by assessing the exposure to traumatic events.

The clinician then rates the frequency and intensity of symptoms from the three symptom clusters arousal, avoidance, and intrusion. This can be done for either the lifetime or the last week. Here, the assessment for the last week was used. Both frequency and intensity are scored on a 0-4 scale and subsequently summed to produce a severity score for each item. Item severity scores for each symptom cluster are then summed. The total CAPS score, a measure of overall PTSD symptom severity, is the sum of all symptom cluster scores and the measure used in Study III in the present thesis. The total CAPS score ranges from 0 to 136, with higher scores indicating greater symptom severity.

Positron emission tomography

Image acquisition

All PET imaging was performed on a 32-ring ECAT EXACT HR+ camera (Siemens/CTI, Knoxville, Tennessee) with acquisition of 63 contiguous planes of data with a slice thickness of 2.46 mm, resulting in a total axial field of view of 155 mm. In all studies, subjects fasted for three hours, and refrained from tobacco, alcohol, and caffeine for twelve hours, before PET-investigations, assessed by self-report. A venous catheter was inserted in the arm of the subject and used for tracer injection. For each PET investigation, the subjects were positioned supine in the scanner with the head gently fixated and a 10-minute transmission scan was performed using three retractable germanium (^{68}Ge) rotating line sources. All PET image acquisition was performed in the resting state.

5-HTP

In vivo assessment of serotonin synthesis rate can be accomplished by targeting the second enzymatic step of serotonin synthesis using PET and the tracer 5-hydroxy-L-[β -^{11}C]tryptophan ([^{11}C]5-HTP) as the marker (Hagberg et al., 2002; Lundquist et al., 2006; Visser et al., 2011). Metabolism of [^{11}C]5-HTP follows the normal serotonin pathway, it is converted by AADC to [^{11}C]5-HT and degraded by MAO to [^{11}C]5-HIAA. Elimination of [^{11}C]5-HIAA from the brain is negligible during the 60 minute scan duration (Lundquist et al., 2006) used in the present thesis. Accumulation of radioactivity from the tracer and its metabolites in the nerve terminals may thus serve as an index of serotonin synthesis rate (see Figure 6). In Study I, the tracer [^{11}C]5-HTP was injected as a rapid bolus whereupon the emission scanning started. Data were acquired in three-dimensional (3D) mode and consisted of 17 frames (5 × 60s, 3 × 120s, 3 × 180s, 4 × 300s, 2 × 600s) acquired during 60 minutes. In addition, a [^{15}O]water PET scan for spatial

normalization was made with administration of approximately 10 MBq/kg body weight and acquisition of three 30-second frames.

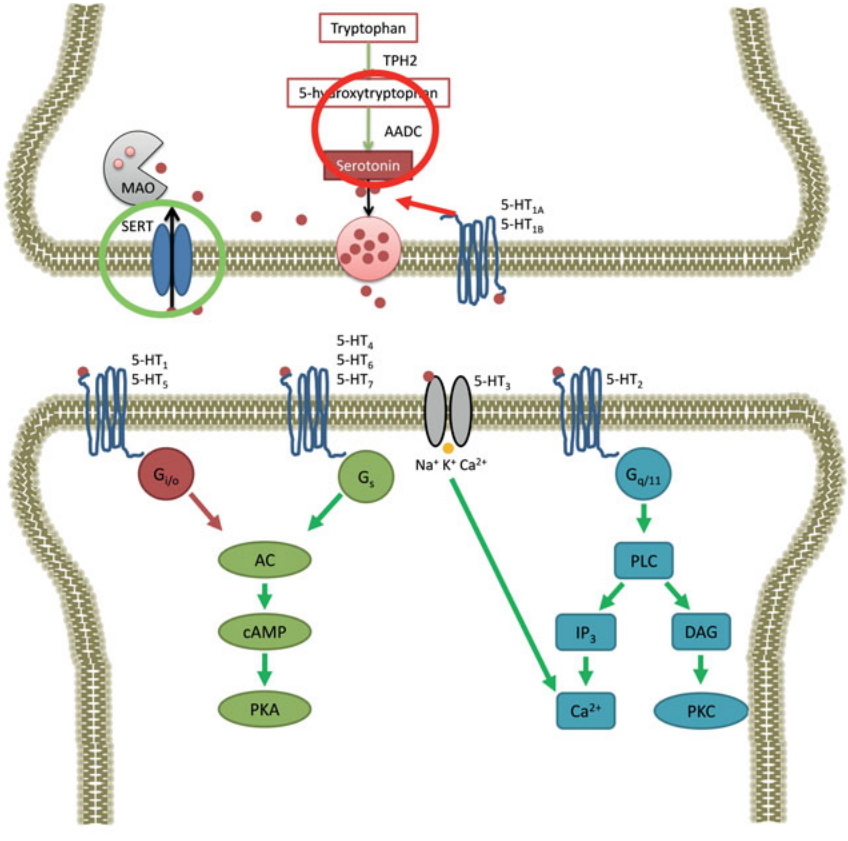

Figure 6. Serotonin synthesis, i.e. activity of the amino acid decarboxylase (AADC), is the target of [^{11}C]5-hydroxytryptohan positron emission tomography (PET) imaging and is here shown highlighted by the red circle in this simplified depiction of a serotonergic synapse. The green circle highlights the serotonin transporter (SERT), the target of [^{11}C]-3-amino-4-(2-dimethylaminomethylphenylsulfanyl)-benzonitrile ([^{11}C]DASB) PET imaging.

DASB

In Study I and Study III, SERT availability (see Figure 6) was studied using [^{11}C]-3-amino-4-(2-dimethylaminomethylphenylsulfanyl)-benzonitrile ([^{11}C]DASB) (Houle, Ginovart, Hussey, Meyer, & Wilson, 2000). [^{11}C]DASB is a highly selective ligand to the SERT. Test-retest studies have revealed around 5-10% variability in SERT availability (i.e. binding potential) (J. S. Kim, Ichise, Sangare, & Innis, 2006; Meyer et al., 2001). Coinciding with the start of the emission scan, a rapid bolus injection of [^{11}C]DASB was administered to the participant and 22 frames of data were acquired in

3D mode during 60 minutes (1 x 60s, 4 x 30s, 3 x 60s, 4 x 120s, 2 x 180s, 8 x 300s).

GR205171

For Study II and Study III, [^{11}C]GR205171 (Bergström et al., 2000) PET assessments were performed to assess the brain NK1 receptor availability (see Figure 7). [^{11}C]GR205171 is a highly selective, non-peptide ligand for the NK1 receptor. [^{11}C]GR205171 was injected intravenously as a fast bolus simultaneously with the start of the emission scan. Data were acquired in 3D mode and consisted of 17 frames (4 x 60s, 3 x 120s, 10 x 300s) with a total duration of 60 minutes. For Study II, an additional [^{15}O]water PET scan used for spatial normalization was acquired (3 frames x 30s) with administration of approximately 10 MBq/kg body weight.

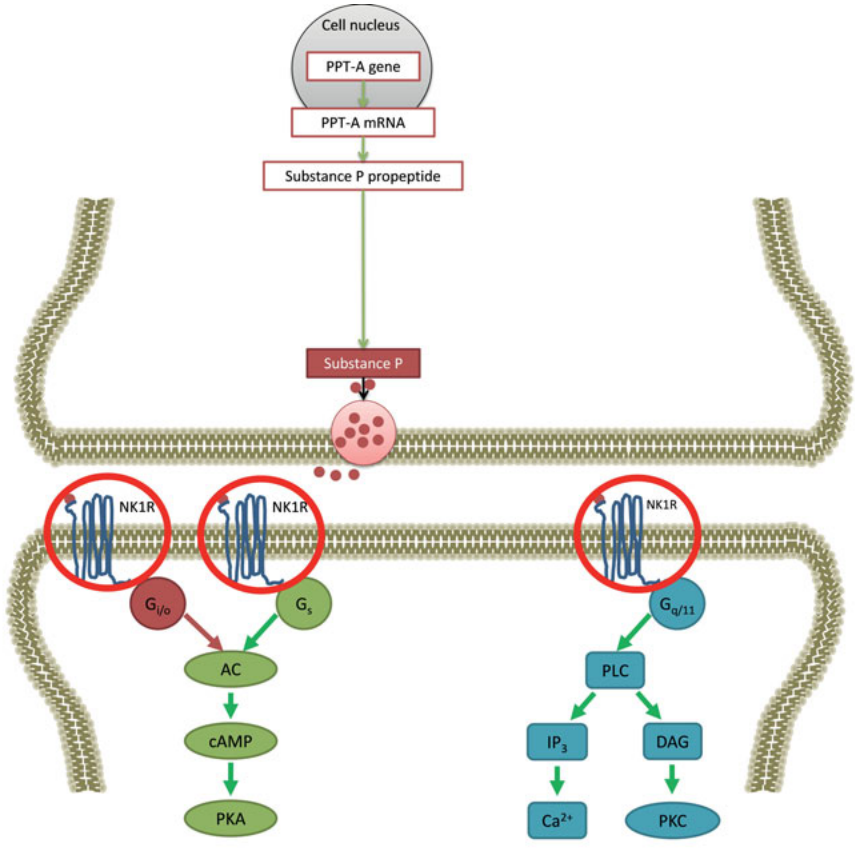

Figure 7. The red circles highlight the neurokinin-1 receptor (NK1R), the target of [^{11}C]GR205171 positron emission tomography imaging, in a simplified depiction of a substance P/neurokinin-1 synapse.

Image analysis

Dynamic images were reconstructed using ordered subset expectation maximization using 6 iterations and 8 subsets and a 4 mm Hanning post-filter, applying all appropriate corrections (e.g. attenuation correction using the transmission scan, and correction for scattered radiation). All PET tracer kinetic modeling was performed voxel-wise using graphical methods with cerebellum as reference region input. The cerebellum was chosen as the reference region for all tracers based on its lack of specific binding of [^{11}C]DASB and [^{11}C]GR205171, and minimal uptake of [^{11}C]5-HTP. It was defined using the PVElab software (Svarer et al., 2005), an observer independent approach for automatic generation of volumes of interest (VOIs). For [^{11}C]5-HTP in Study I and [^{11}C]GR205171 in Study II, the VOI template was applied to each participant's summed [^{15}O]water frames. For [^{11}C]DASB in Study I and [^{11}C]GR205171 in Study III the VOI template was applied to each participant's PET [^{11}C]DASB image summed over all 22 frames (Jonasson, 2014).

5-HTP

For [^{11}C]5-HTP, tracer binding is assumed to be irreversible during the scan period used in this thesis (60 minutes), and a modified reference Patlak method (Bergström et al., 1998; Hagberg et al., 2002; Patlak et al., 1983) correcting for binding of [^{11}C]5-HTP in cerebellum was performed on a time interval of 30-60 minutes (Hagberg et al., 2002; Lundquist et al., 2006) to estimate the influx parameter of the tracer, K_i (ml cm^{-3} min^{-1}), an index of serotonin synthesis rate.

DASB

[^{11}C]DASB binding potential (BP_{ND}) (Innis et al., 2007) images were calculated for each voxel as an index of SERT availability. Because [^{11}C]DASB binding is reversible, the reference Logan method (Logan et al., 1996) performed on a time interval of 30-60 minutes and BP_{ND} was estimated as the distribution volume ratio (DVR)-1 relative to cerebellum. Binding potential is proportional to the product between the number of targets (e.g. transporters), B_{max}, and the affinity of the tracer at the target, $1/K_d$, (Mintun, Raichle, Kilbourn, Wooten, & Welch, 1984).

GR205171

[^{11}C]GR205171 is assumed to be irreversible during the scan time and parametric images showing influx rate K_i (ml cm^{-3} min^{-1}) of [^{11}C]GR205171 for each voxel, i.e. an index of NK1 receptor availability, were calculated using a modified reference Patlak method (Bergström et al., 1998; Michelgård et al., 2007; Patlak et al., 1983) and the time interval of 30-60 minutes.

Preprocessing of parametric images

Before statistical analyses, all images were normalized to the Montreal Neurological Institute (MNI) standard space using the MATLAB toolbox Statistical Parametric Mapping 8 (SPM8; Wellcome Department of Cognitive Neurology, University College London, www.fil.ion.ucl.ac.uk). In Study I and Study II, the [^{11}C]5-HTP K_i, and [^{11}C]GR205171 K_i images were co-registered to the summation image of the 3 frames of [^{15}O]water for each subject, whereas the [^{11}C]DASB BP_{ND} in Study I, and both [^{11}C]DASB BP_{ND} and [^{11}C]GR205171 K_i images in Study III were co-registered to the summation image of all 22 [^{11}C]DASB frames for each subject. The summation images were then normalized to the PET template from SPM8, and the calculated transformation parameters applied to the parametric images, resulting in images normalized to the Montreal Neurological Institute (MNI) standard space. In Study I and Study II, voxels were isotropic $2 \times 2 \times 2$ mm^3 voxels and smoothing was performed using a 12 mm isotropic Gaussian kernel. In Study III, voxels were isotropic $4 \times 4 \times 4$ mm^3 and smoothed with an 8 mm isotropic Gaussian kernel in order to minimize computing demands and following Touminen et al. (2014).

Statistical analysis

Symptom ratings and participant characteristics in all studies were analyzed using R 3.1.0 (R Foundation for Statistical Computing, Vienna, Austria).

PET data were analyzed voxel-wise using both regions of interest (ROI) and whole brain approaches. Anatomical ROIs were chosen *a priori* based on earlier neuroimaging findings in SAD and PTSD. In Study II, these included nodes in the brain fear circuitry: the amygdala, hippocampus, insular cortex, and ACC (Shin & Liberzon, 2010). In Study I, regions rich in serotonin synthesis and reuptake as well as those found to differ between patients with SAD and controls with regard to SERT and 5-HT_{1A} availability (Lanzenberger et al., 2007; Savli et al., 2012; van der Wee et al., 2008) were added to the list of ROIs, thus comprising the amygdala, hippocampus, insular cortex, ACC, raphe nuclei, thalamus, caudate nucleus, and putamen. In Study III, the amygdala was chosen as the sole ROI, based on a previous report of reduced SERT availability in this region in patients with PTSD (Murrough, Huang, et al., 2011), and because it is the central hub in the fear circuitry (Davis, 1992; LeDoux, 2007) and demonstrated as being pivotal in a recent meta-analysis of functional activation studies in PTSD (Sartory et al., 2013). *A priori* ROIs were defined using the Automated Anatomical Labeling library from the Wake Forest University Pickatlas (Maldjian, Laurienti, Kraft, & Burdette, 2003), except for the raphe nuclei ROI that was defined from the PVElab software (Svarer et al., 2005). Additionally, whole-brain exploratory analyses were performed in all studies.

Statistical parametric mapping

Group differences in PET measures between patients and HC participants were examined using two-sample t-tests in SPM8. In Study I and Study II, the relationship between social anxiety symptom severity and serotonin synthesis rate, SERT availability, and NK1 receptor availability were assessed by entering parametric [^{11}C]5-HTP, [^{11}C]DASB and [^{11}C]GR205171 images into separate regression models with LSAS total score as predictor.

For Study I, the statistical threshold for significance was set at $P < .05$ family-wise error corrected (FWE) using random field theory for whole-brain analyses and with small volume correction within the ROIs. Study II applied the same statistical threshold for the ROIs, but a more liberal whole-brain statistical threshold of $P < .001$ in order to examine specificity of the ROI findings and to reveal additional affected areas.

In Study III, the statistical threshold for significance was set at combined height $P < .001$ and cluster extent of 640 mm^3 for whole-brain analyses of group differences. For the amygdala, results were thresholded with $P < .05$ and cluster extent of 640 mm^3 in order to balance type I and type II errors, as the amygdala is strongly implicated in PTSD pathophysiology (Murrough, Czermak, et al., 2011; Murrough, Huang, et al., 2011; Sartory et al., 2013; Shin & Liberzon, 2010).

Co-expression analyses

The correlation between [^{11}C]DASB BP_{ND} and [^{11}C]GR205171 K_i across individuals was determined for each voxel using Pearson's product-moment correlation coefficients. The correlation coefficients were calculated separately for the patients with PTSD and the HC participants and are here used as a measure of the co-expression of SERT and NK1 receptor availability in each voxel. The limited spatial resolution of PET imaging precludes conclusions regarding co-expressions between SERT and NK1 receptors at the cellular level. The analyses should therefore be thought of as co-existence of SERT and NK1 receptors in the same brain region, here defined by the voxel. The co-expression index ranged from -1 (lowest degree of co-expression) to +1 (highest degree of co-expression). Correlation coefficients were transformed using Fisher's r-to-z transformations in order to stabilize the variance of the estimate because the sampling distribution of correlation coefficients for $|r| > 0$ is not normal. The z-transformed correlation coefficients were used in subsequent voxel-wise within- and between-group comparisons.

Regression analyses within the PTSD group were performed using total CAPS score (i.e. symptom severity) as outcome and [^{11}C]DASB BP_{ND}, [^{11}C]GR205171 K_i and the interaction between them ([^{11}C]DASB BP_{ND} × [^{11}C]GR205171 K_i) as predictors. Age and sex were also entered as predictors to safeguard against potential confound from these variables. One pa-

tient did not complete the CAPS interview, leaving 15 patients with PTSD in the regression analyses. Both *a priori* defined amygdala ROI and voxel-wise whole-brain regression analyses were performed. For the amygdala ROI analyses, mean BP_{ND} and K_i values were extracted and four linear regression models tested. The first two models included only [^{11}C]DASB BP_{ND} and [^{11}C]GR205171 K_i respectively. In the third model, both [^{11}C]DASB BP_{ND} and [^{11}C]GR205171 K_i were entered. The fourth model added the interaction between [^{11}C]DASB BP_{ND} and [^{11}C]GR205171 K_i (both variables mean centered) to the third model. Positive interaction terms were taken to indicate that higher co-expression of SERT and NK1 receptors was related to greater PTSD symptom severity, while negative interaction terms were interpreted as a negative relationship between co-expression levels and PTSD symptom severity.

Only voxels showing specific binding were considered in the co-expression and regression analyses, i.e. where mean [^{11}C]DASB BP_{ND} and [^{11}C]GR205171 K_i exceeded 0.1 and 0.005 respectively. This resulted in approximately 10,000 voxels for DASB analyses and 25,000 voxels for GR205171 analyses.

Co-expression and regression analyses were performed with combined $P < .05$ and cluster extent of 640 mm^3, in order to balance type I and type II errors. Also, given the scarcity of in vivo measures of neurotransmitter systems in PTSD and the importance of finding effective treatments for this debilitating disorder, the relatively liberal statistical threshold was applied.

Ethical statement

The studies were approved by appropriate ethical review boards. The review boards have changed their names during the course of the studies and include the Uppsala University Medical Faculty Ethical Review Board, the regional Ethics Committee in Uppsala, the Regional Ethical Vetting Board, Uppsala, the Uppsala University Isotope Committee, the Radiation Hazard Ethics Committee at Uppsala University Hospital, and the Uppsala University Radiation Safety Committee. All study participants gave written informed consent before commencement of the study and were reimbursed for their participation.

Empirical studies

Study I

Serotonin synthesis and reuptake in social anxiety disorder: A positron emission tomography study

Background and aims

Genetic and pharmacological studies have suggested that SAD is associated with aberrations in serotonergic neurotransmission. However, only two studies have examined the serotonergic system in SAD directly using molecular imaging. Van der Wee et al. (2008) reported on increased SERT availability in the thalamus, and Lanzenberger and co-workers (2007) found reduced 5-HT_{1A} receptor binding in the raphe nuclei, amygdala, ACC, and insula. Here, PET imaging with [^{11}C]5-HTP and [^{11}C]DASB was used to evaluate brain serotonin synthesis rate and SERT availability respectively in patients with SAD and HC individuals. Based on earlier findings of reduced raphe nuclei 5-HT_{1A} autoreceptor density and enhanced SERT availability in the thalamus (Lanzenberger et al., 2007; van der Wee et al., 2008), it could be predicted that SAD is associated with increased serotonin synthesis rate and transporter binding, i.e. an overactive presynaptic serotonergic system. On the other hand, because chronic treatment with SSRIs increases extracellular serotonin availability (Ceglia et al., 2004) and alleviates SAD symptoms (Blanco et al., 2013), reduced serotonin synthesis rate is a possibility. Furthermore, because attenuated SERT binding has been reported in other anxiety disorders (Maron et al., 2004; Murrough, Huang, et al., 2011), and since genetically modified SERT knock-out mice show increased anxiety (Holmes et al., 2003), SAD could also be characterized by reduced SERT availability. Thus, while abnormalities in presynaptic serotonin functioning in SAD were predicted, no *a priori* hypotheses regarding the direction were formulated.

Results

Increased [^{11}C]5-HTP influx rate (K_i) was observed in the amygdala, raphe nuclei region, caudate nucleus, putamen, hippocampus, and anterior cingulate cortex of patients with SAD as compared to HC individuals, supporting enhanced serotonin synthesis rate (see Figure 8 and Table 2). Within the

SAD group, social anxiety symptom scores (LSAS) correlated positively with [^{11}C]5-HTP K_i in the right amygdala (MNI x, y, z: 24, 4, -16; $Z = 3.29$; 1056 mm^3, $P_{FWE} = 0.001$) (see Figure 9).

Table 2. *Statistical parametric mapping of increased serotonin synthesis rate ([^{11}C]5-HTP K_i) and serotonin transporter availability ([^{11}C]DASB binding potential, BP_{ND}) in social anxiety disorder (SAD) as compared to healthy controls (HC).*

	Z	P_{FWE}	Volume[a]	X	Y	Z[b]
SAD > HC						
Serotonin synthesis rate						
Brainstem corresponding to the Raphe Nuclei region	4.52	<.001	528	-4	-24	-22
Anterior Cingulate Cortex	3.87	.01	448	0	22	24
Putamen	3.67	.02	48	26	8	-10
	3.55	.03	32	18	14	-10
	3.49	.04	56	-30	-10	-8
Amygdala	3.64	<.001	104	24	6	-16
	3.53	<.001	176	-28	-8	-12
Caudate Nucleus	3.70	.02	48	14	14	-12
Hippocampus	3.64	.02	80	-30	-10	-12
Serotonin transporter						
Putamen	5.02	<.001	3936	28	-12	14
Thalamus	4.66	<.001	10440	-10	-12	8
Insula Cortex	4.32	.002	848	30	-18	18
Caudate Nucleus	3.88	.005	720	14	8	6
	3.24	.04	24	20	-14	20
Brainstem corresponding to the Raphe Nuclei region	2.73	.02	160	-2	-24	-12

a Volume in mm^3.
b Coordinates in Montreal Neurological Institute (MNI) standard space.

Increased SERT availability in SAD relative to HC individuals, as reflected by elevated [^{11}C]DASB $BP_{ND,}$ was evident in the amygdala, raphe nuclei region, caudate nucleus, putamen, thalamus, and insular cortex, see Figure 10 and Table 2. A whole-brain exploratory analysis likewise revealed augmented [^{11}C]DASB BP_{ND} in SAD compared to HC individuals in the right putamen (MNI x, y, z: 26, -12, 12; $Z = 5.11$; 2624 mm^3, $P_{FWE} = 0.003$) and left thalamus (MNI x, y, z: -10, -12, 8; $Z = 4.66$; 296 mm^3, $P_{FWE} = 0.02$) with no additional regional differences. Within the SAD group, there was a significant negative correlation between social anxiety symptom severity scores (LSAS) and [^{11}C]DASB BP_{ND} in the left dorsal ACC (MNI x, y, z: -10, 32, 26; Z = 3.44; 256 mm^3, $P_{FWE} = 0.02$) (see Figure 9).

Removing patients without lifetime psychiatric comorbidity or history of psychotropic medication produced the same pattern of results. Comparing patients with and without lifetime psychiatric comorbidity or history of psychotropic medication yielded no significant differences.

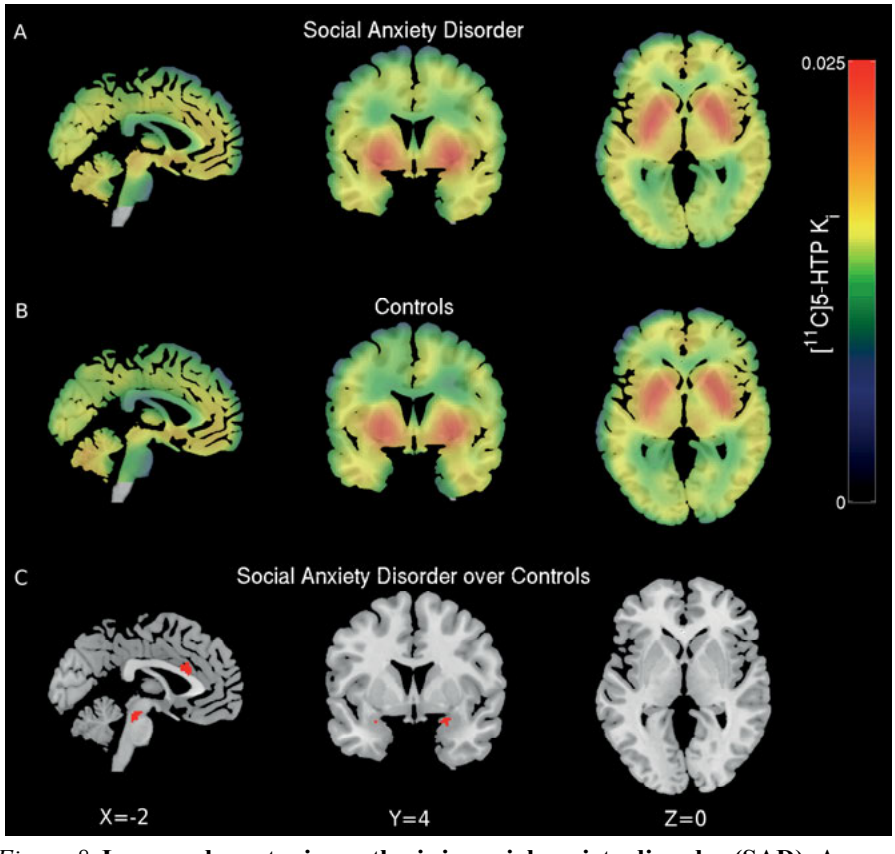

Figure 8. **Increased serotonin synthesis in social anxiety disorder (SAD).** Average serotonin synthesis rate ([^{11}C]5-HTP K_i) in **(A)** patients with SAD and **(B)** healthy controls, and **(C)** clusters of significantly increased [^{11}C]5-HTP K_i in SAD. Parametric images of serotonin synthesis rate overlaid on a standard MRI image, illustrating increased serotonin synthesis rate in the amygdala, brain stem corresponding to the raphe nuclei region, and anterior cingulate cortex. All rows depict slices at MNI coordinate (-2, 4, 0). The colorbar indicates [^{11}C]5-HTP K_i for the two top rows.

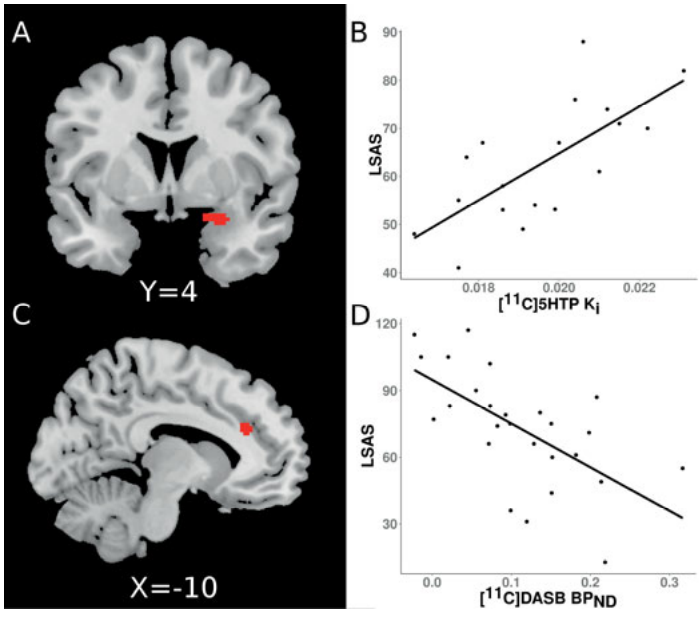

Figure 9. **Correlations between social anxiety symptom severity and serotonin synthesis rate and serotonin transporter availability. (A)** Significant cluster defining the anatomical extent of the positive relationship between serotonin synthesis rate in the amygdala (MNI coordinate 24, 4, -16), indexed by [^{11}C]5-HTP K_i, and social anxiety symptom severity, as measured by Liebowitz social anxiety scale (LSAS), in patients with social anxiety disorder. **(B)** [^{11}C]5-HTP K_i values (min^{-1}) plotted against LSAS for illustrative purposes. **(C)** Significant cluster defining the anatomical location of the negative relationship between serotonin transporter availability in the dorsal anterior cingulate cortex (MNI coordinate -10, 32, 26), indexed by [^{11}C]DASB binding potential, and social anxiety symptom severity, as measured by LSAS, in patients with social anxiety disorder. **(D)** [^{11}C]DASB BP_{ND} (arbitrary units) plotted against LSAS for illustrative purposes.

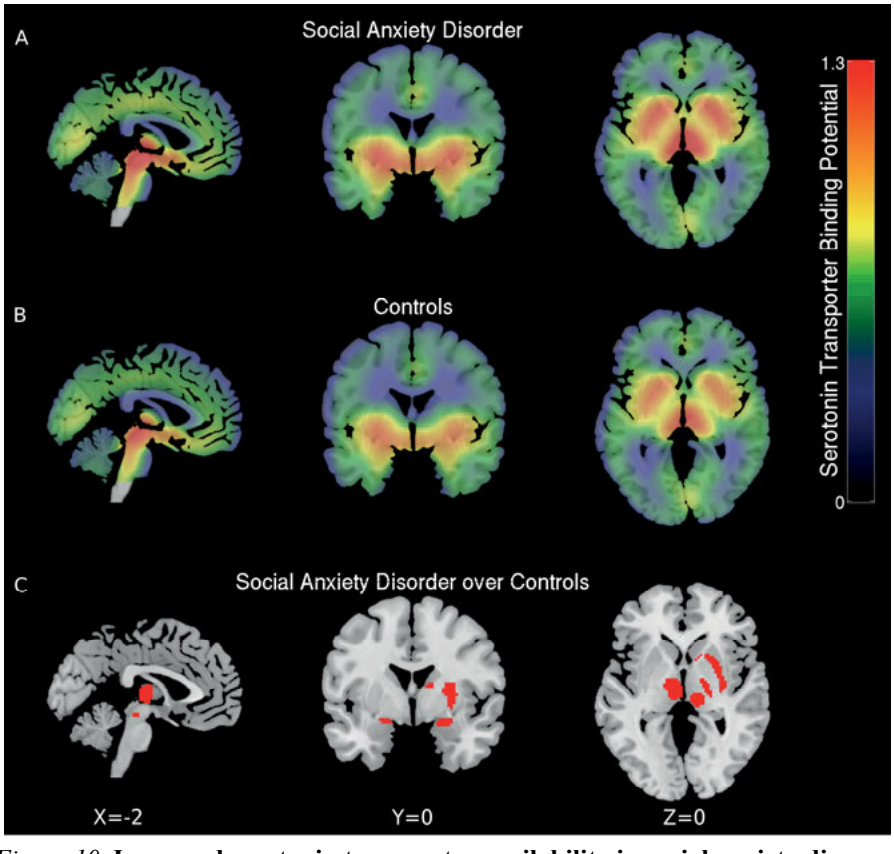

Figure 10. **Increased serotonin transporter availability in social anxiety disorder (SAD).** Average serotonin transporter availability indexed by [^{11}C]DASB BP_{ND} in **(A)** patients with SAD and **(B)** healthy controls, and **(C)** clusters of significantly increased [^{11}C]DASB BP_{ND} in SAD in the brain stem corresponding to the raphe nuclei region, caudate nucleus, putamen, thalamus, insula cortex, and amygdala. The amygdala is shown at $P < .05$ uncorrected for illustrative purposes. Parametric BP_{ND} images are overlaid on a standard MRI image. All rows depict slices at MNI coordinate (-2, 0, 0). The colorbar indicates [^{11}C]DASB BP_{ND} for the two top rows.

Discussion

Study I demonstrated increased serotonin synthesis and transporter availability, supporting an overactive presynaptic serotonin system, in patients with SAD relative to healthy controls. Correlations between severity of social anxiety symptoms and serotonergic measures in the fear-expressing brain regions amygdala and dorsal ACC further suggest region-specific anxiogenic effects of serotonin. These findings are widely consistent with previous imaging reports of anxiety conditions and support an anxiogenic effect of serotonin.

Study II

Increased neurokinin-1 receptor availability in the amygdala in social anxiety disorder: A positron emission tomography study with [^{11}C]GR205171

Background and aims

The SP/NK1 system is involved in stress and anxiety (Ebner & Singewald, 2006), but there is a lack of studies examining the SP/NK1 systems in vivo in the human brain. Given the implicated role of the SP/NK1 system in anxiety disorders and because treatment with NK1 receptor antagonists has shown promising initial results in SAD, including attenuation of amygdala activity concomitant with anxiety reductions during stressful public speaking (Furmark et al., 2005), it could be hypothesized that SAD is associated with altered NK1 receptor availability particularly in the amygdala. The aim of Study II was therefore to examine NK1 receptor availability in patients with SAD as compared to HC using PET and the highly selective NK1 receptor antagonist radiotracer [^{11}C]GR205171 (Bergström et al., 2000).

Results

Voxel-wise statistical parametric mapping analyses revealed that patients with SAD had higher NK1 receptor availability in the right amygdala (MNI x, y, z: 28, -2, -20; $Z = 3.79$, $P_{FWE} = 0.004$; 496 mm^3) relative to controls (see Figure 11). Extracting mean regional NK1 receptor availability from the right amygdala ROI indicated an 18.5% increase in patients with SAD (mean ± SD: 0.0128 ± 0.0017) relative to the HCs (mean ± SD: 0.0108 ± 0.0018) ($t(32) = 3.294$, $P = 0.002$). No other statistically significant clusters were found within the *a priori* ROIs (the amygdala, hippocampus, insular cortex, and ACC) or in exploratory whole-brain analyses. Nor were there any significant associations between social anxiety symptom severity and NK1 receptor availability, or between duration of SAD symptoms and NK1 receptor availability. After removal of patients with psychiatric comorbidity or history of psychotropic medication, the right amygdala uptake remained significantly different between patients and controls (MNI x, y, z: 28, -4, -20; $Z = 3.16$, $P_{FWE} = 0.03$; 104 mm^3). Patients without psychiatric comorbidity or history of psychotropic medication had higher NK1 receptor availability than patients with comorbidity or history of medication in the right amygdala (MNI x, y, z: 30, -2, -28; $Z = 3.12$, $P_{FWE} = 0.041$; 24 mm^3) and the ACC (MNI x, y, z: -10, 40, -2; $Z = 3.56$, $P_{FWE} = 0.046$; 8 mm^3).

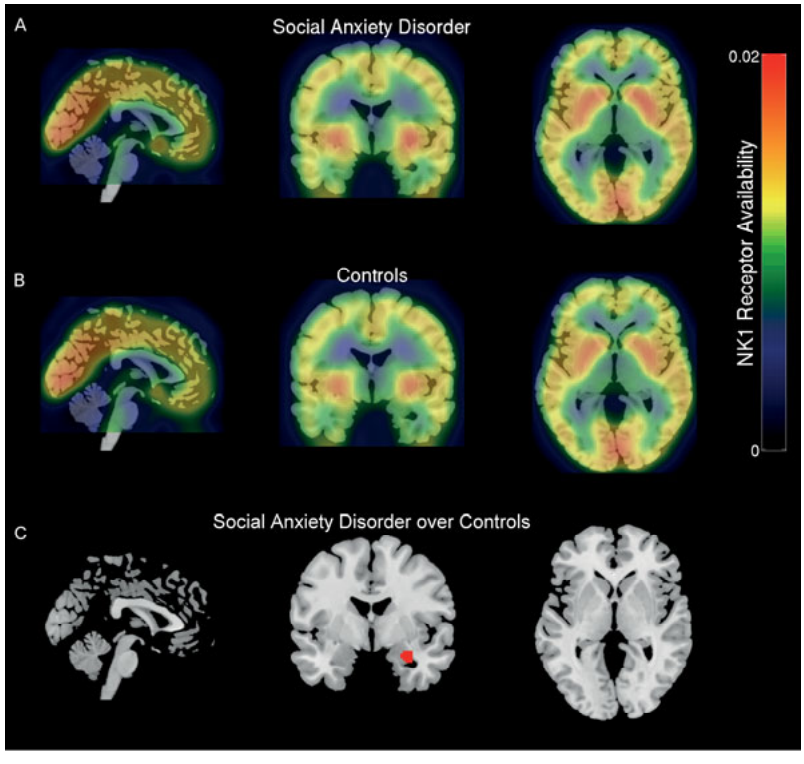

Figure 11. Parametric [^{11}C]GR205171 K_i images showing mean neurokinin-1 (NK1) receptor availability in patients with (A) social anxiety disorder and (B) healthy controls. The colorbar indicates [^{11}C]GR205171 K_i values. (C) Patients with social anxiety disorder showed increased NK1 receptor availability in the amygdala. Voxels within the amygdala were thresholded at $P < .05$, family-wise error corrected for multiple comparisons. Mean parametric images of [^{11}C]GR205171 K_i and the statistical maps from the group comparison were overlaid on standard MRI images. All rows depict slices at MNI coordinate (0, -2, 0). Reprinted by permission from Macmillan Publishers Ltd: Translational Psychiatry, copyright 2015.

Discussion

Study II showed that SAD is associated with increased NK1 receptor availability in the right amygdala, but no correlation between NK1 receptor levels and severity of social anxiety symptoms was detected. This finding supports involvement of the SP/NK1 system not only in animal models of stress and anxiety (Ebner & Singewald, 2006) but also in humans with anxiety disorders. Enhanced SP/NK1 neurotransmission may in part exaggerate fear-related amygdala activity and our results may thus help explain previous reports of enhanced amygdala responses to threat-related stimuli in anxiety disorders (Brühl et al., 2014; Etkin & Wager, 2007; Shin & Liberzon, 2010) and attenuated amygdala reactivity, with concomitant anxiety reduction, following NK1 antagonism in SAD (Furmark et al., 2005).

Study III

Co-expression of serotonin transporters and neurokinin-1 receptors in post-traumatic stress disorder: A multi-tracer PET study

Background and aims

There is evidence for serotonergic and SP/NK1 involvement in PTSD (Geracioti et al., 2006; Mathew et al., 2011; Murrough, Czermak, et al., 2011; Murrough, Huang, et al., 2011; Sullivan et al., 2013), and the two systems are frequently co-expressed (Sergeyev et al., 1999) and interact in the brain (Gobbi & Blier, 2005; Santarelli et al., 2001; Shirayama et al., 1996; Valentino & Commons, 2005), suggesting that interactions between these neurotransmitter systems may influence anxiety disorders such as PTSD. However, only a few studies have directly examined the serotonergic system, and the SP/NK1 system has not been assessed at all, using molecular neuroimaging in patients with PTSD. Murrough et al. (2011) recently reported that PTSD is associated with reduced SERT availability in the amygdala.

Using PET with carbon-11 labeled DASB (Houle et al., 2000) and GR205171 (Bergström et al., 2000), Study III aimed at examining SERT and NK1 receptor availability, both independently and in terms of their co-expression, in patients with PTSD and HC, as well as the relation to symptomatology.

Results

Voxel-wise analyses in the *a priori* defined ROI amygdala did not show any difference in SERT availability between patients with PTSD and controls, but exploratory whole-brain analyses revealed increased SERT availability in the precentral gyrus (MNI x, y, z: -26, -12, 62; $Z = 3.45$, $P < .001$, cluster = 768 mm^3) and posterior cingulate cortex (MNI x, y, z: -10, -40, 22; $Z = 3.39$, $P < .001$, cluster = 768 mm^3) (see Figure 12).

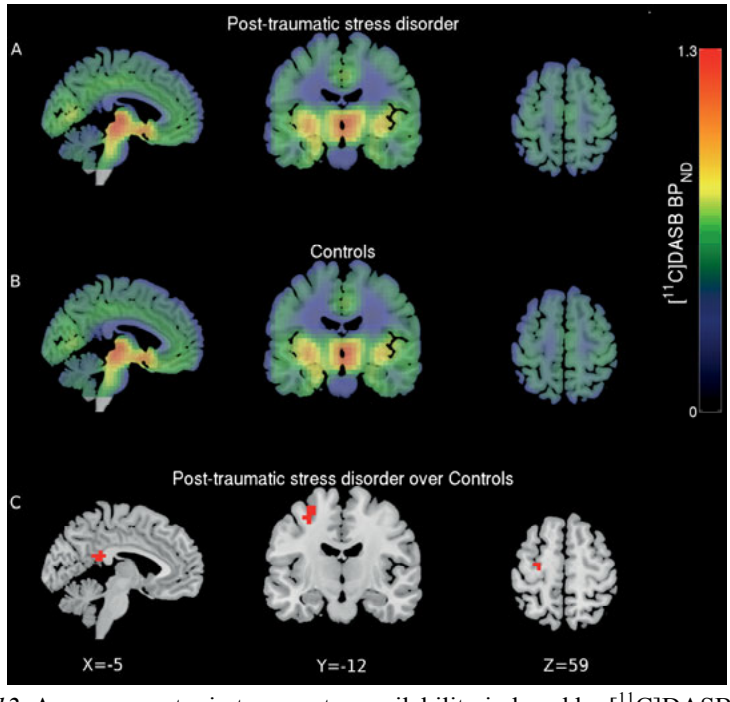

Figure 12. Average serotonin transporter availability indexed by [^{11}C]DASB BP_{ND} in **(A)** patients with posttraumatic stress disorder (PTSD) and **(B)** healthy controls, and **(C)** clusters of significantly increased [^{11}C]DASB BP_{ND} in patients with PTSD in the precentral gyrus and posterior cingulate cortex. All rows depict slices at MNI coordinate (-5, -12, 59). The colorbar indicates [^{11}C]DASB BP_{ND} for the two top rows.

Upregulated NK1 receptors were found in the amygdala only (MNI x, y, z: 26, 0, -18; $Z = 2.21$, $P = .011$, cluster = 832 mm^3) in the PTSD group (see Figure 13). A lower degree of co-expression of SERT and NK1 receptors in patients relative to controls was revealed in the putamen, thalamus, insula, and lateral orbitofrontal gyrus, and higher co-expression was found in the inferior temporal gyrus, cuneus, middle cingulate cortex, and media orbitofrontal gyrus (see Figure 14 and Table 3).

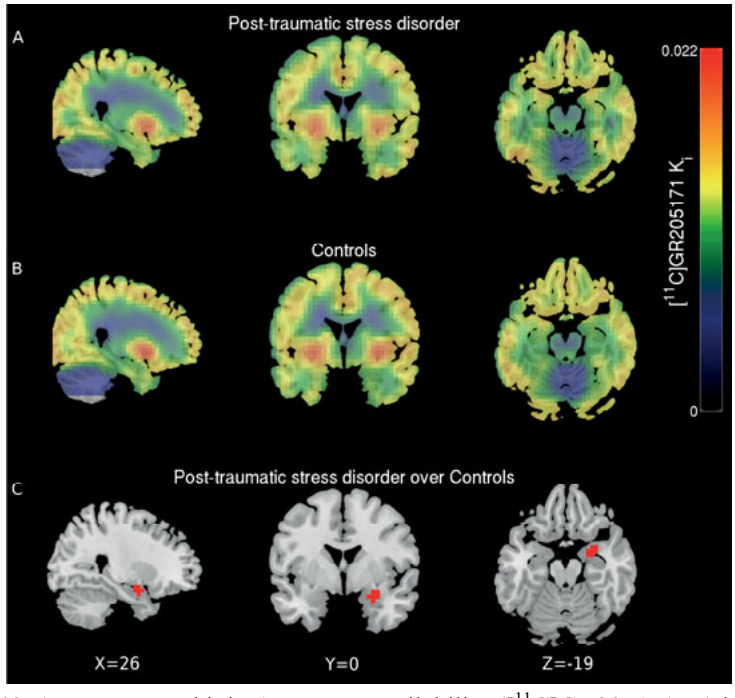

Figure 13. Average neurokinin-1 receptor availability ([^{11}C]GR205171 K_i) in **(A)** patients with PTSD and **(B)** healthy controls, and **(C)** cluster of significantly increased [^{11}C]GR205171 K_i in patients with PTSD in the amygdala. All rows depict slices at MNI coordinate (26, 0, -19). The colorbar indicates [^{11}C]GR205171 K_i for the two top rows.

Table 3. *Co-expression of serotonin transporters (SERT; [^{11}C]DASB binding potential, BP_{ND}) and neurokinin-1 receptors (NK1R; [^{11}C]GR205171 influx rate, K_i) in posttraumatic stress disorder (PTSD) as compared to healthy controls (HC).*

	HC r[a]	PTSD r[a]	Diff r[b]	P	Volume[c]	X	Y	Z[d]
HC>PTSD								
Putamen	.78	-.47	1.25	<.001	2048	26	8	-10
Thalamus	.73	-.43	1.16	.001	1048	-14	-20	6
Insula	.51	-.58	1.09	.004	640	-38	-20	-2
Lateral orbitofrontal gyrus	.75	-.24	0.99	.005	640	34	56	-2
Insula	.69	-.14	0.83	.020	704	-26	16	14
PTSD>HC								
Inferior temporal gyrus	-.70	.58	-1.28	<.001	704	46	-52	-22
Cuneus	-.62	.66	-1.28	<.001	3392	-6	-84	30
Middle cingulate cortex	-.29	.82	-1.11	<.001	896	10	8	34
Medial orbitofrontal gyrus	-.22	.82	-1.04	.001	832	6	56	-10

a Co-expression indexed by Pearson's product-moment correlation coefficient, *r*.
b Differences in Pearson's correlation coefficient between groups: r_{HC} - r_{PTSD}, as an index of difference in co-expression.
c Volume in mm^3.
d Coordinates in Montreal Neurological Institute (MNI) standard space.

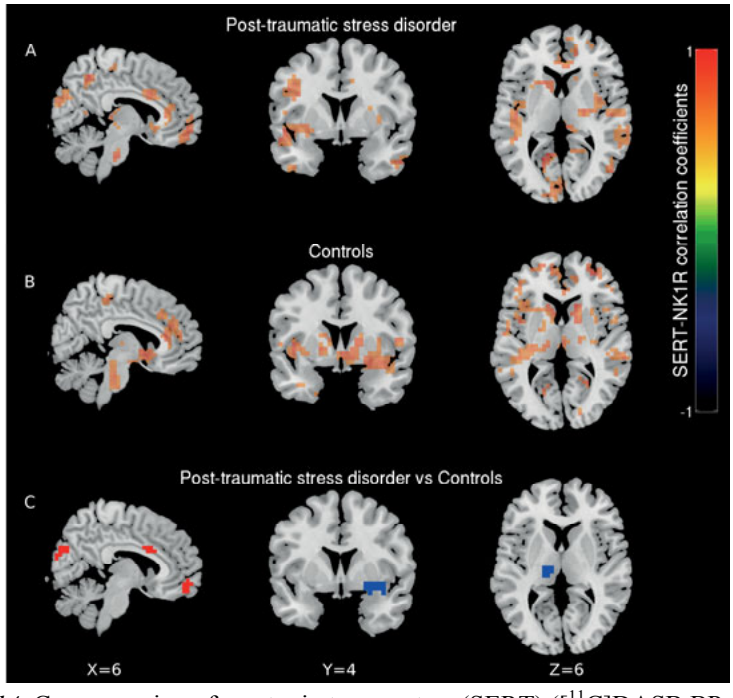

Figure 14. Co-expression of serotonin transporters (SERT) ([^{11}C]DASB BP_{ND}) and neurokinin-1 receptors (NK1R) ([^{11}C]GR205171 K_i) in **(A)** posttraumatic stress disorder and **(B)** healthy controls, and **(C)** clusters of significantly reduced (blue) and heightened (red) co-expressions in posttraumatic stress disorder. Parametric and statistical images overlaid on a standard MRI image. All rows depict slices at MNI coordinate (6, 4, 6). The colorbar indicates Pearson's product-moment correlation coefficients for correlations between [^{11}C]DASB BP_{ND} and [^{11}C]GR205171 K_i for the two top rows.

In *a priori* defined ROI analyses of the bilateral amygdala, mean [^{11}C]DASB BP_{ND} and [^{11}C]GR205171 K_i in the amygdala were extracted for each participant and entered into four separate linear regression models with CAPS score as outcome. In the first model, only [^{11}C]DASB BP_{ND} was entered in a simple linear regression model revealing significant negative contribution of SERT availability to symptom scores (β = -0.67, P = .016). Second, only [^{11}C]GR205171 K_i was entered, revealing no significant contribution of NK1 receptor availability to symptom scores (β = -0.32, P = .212). Third, both [^{11}C]DASB BP_{ND} and [^{11}C]GR205171 K_i were entered together into a multiple linear regression model. This model revealed a significant contribution of [^{11}C]DASB BP_{ND} (β = -0.64, P = .046), whereas the contribution of [^{11}C]GR205171 K_i remained non-significant (β = -0.08, P = .736). The fourth model added the interaction between [^{11}C]DASB BP_{ND} and [^{11}C]GR205171 K_i to the third model, significantly increasing the explained variance (adjusted R^2) in CAPS score from 53% to 75% ($F(2,9) = 9.67$, P = .013). Importantly, in this model, only the interaction term (β = -0.49, P =

.013), but not [^{11}C]DASB BP_{ND} (β = -0.31, *P* = .195) or [^{11}C]GR205171 K_i (β = -0.08, *P* = .656), significantly predicted symptom severity, suggesting that NK1 receptor availability moderated the inverse relationship between SERT availability and symptom severity (see Figure 15).

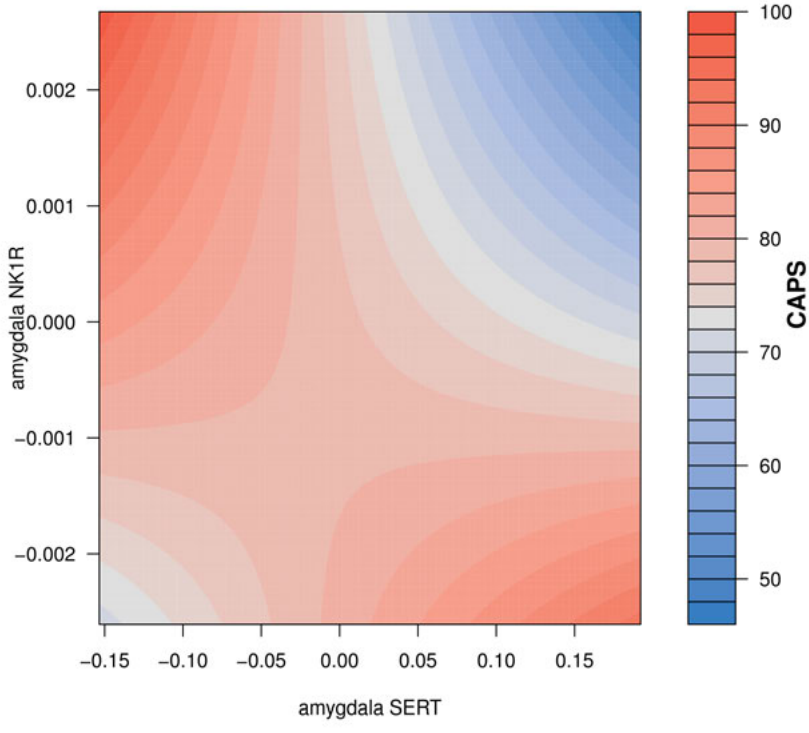

Figure 15. Interaction plot illustrating the relationship between neurotransmitter system co-expression in the amygdala and severity of PTSD symptoms as measured by CAPS. The amount of NK1 receptors modulated the relationship between SERT availability and symptom severity because low SERT levels were associated with relatively higher anxiety, irrespective of number of NK1 receptors, while high SERT availability was associated with relatively higher anxiety only when NK1 receptor levels were low. The scale on the right denotes CAPS score. Abbreviations: CAPS: Clinician-Administered PTSD Scale, NK1R: neurokinin-1 receptors, PTSD: post-traumatic stress disorder, SERT: serotonin transporter.

Whole-brain analyses revealed that in general both SERT and NK1 receptor availability as well as co-expression were negatively associated with degree of symptomatology in the PTSD group (see Figure 16 and Table 4), indicating that worse symptoms were related to a lower degree of co-expression between SERT and NK1 receptors.

Table 4. *Relationship between symptom severity (Clinician-Administered PTSD Scale; CAPS Total score) and the co-expression between neurotransmitter systems, serotonin transporter (SERT) availability ([^{11}C]DASB binding potential, BP_{ND}) and neurokinin-1 receptor (NK1R) availability ([^{11}C]GR205171 influx rate, K_i), in post-traumatic stress disorder (PTSD).*

	β[a]	P	Volume[b]	X	Y	Z[c]
Negative relationship with CAPS						
Middle frontal gyrus	-1.17	.004	832	34	40	30
Inferior frontal trigeminal gyrus	-1.11	.026	2304	46	24	22
Inferior occipital gyrus	-1.04	<.001	2880	-50	-64	-18
Inferior temporal gyrus	-1.04	.010	2304	46	0	-38
Middle temporal gyrus	-1.02	<.001	896	46	-68	6
Insula	-1.01	.049	4416	-26	16	-10
Parahippocampal gyrus	-0.92	.003	960	22	-12	-30
Lingual gyrus	-0.91	.012	1152	22	-56	-6
Anterior cingulate cortex	-0.88	.009	2944	-2	36	6
Precentral gyrus	-0.87	.022	1152	38	-8	54
Precuneus	-0.84	.002	1984	6	-52	22
Precuneus	-0.84	.019	1856	2	-64	30
Middle frontal gyrus	-0.80	.002	768	46	20	38
Orbitofrontal gyrus	-0.78	.008	4800	26	8	-18
Middle temporal gyrus	-0.77	<.001	2688	62	-44	2
Middle cingulate cortex	-0.74	.033	768	2	-4	34
Middle cingulate cortex	-0.74	.028	896	-10	-28	38
Superior temporal lobe	-0.72	.004	1472	50	4	-10
Middle frontal gyrus	-0.71	.039	896	34	52	10
Rolandic operculum	-0.54	.039	1024	-46	-8	14
Positive relationship with CAPS						
Fusiform gyrus	0.95	.011	1088	30	-36	-14
Inferior occipital gyrus	0.75	.017	640	26	-92	-6

CAPS: Clinician-Administered PTSD Scale.
a Regression coefficient.
b Volume in mm^3.
c Coordinates in Montreal Neurological Institute (MNI) standard space.

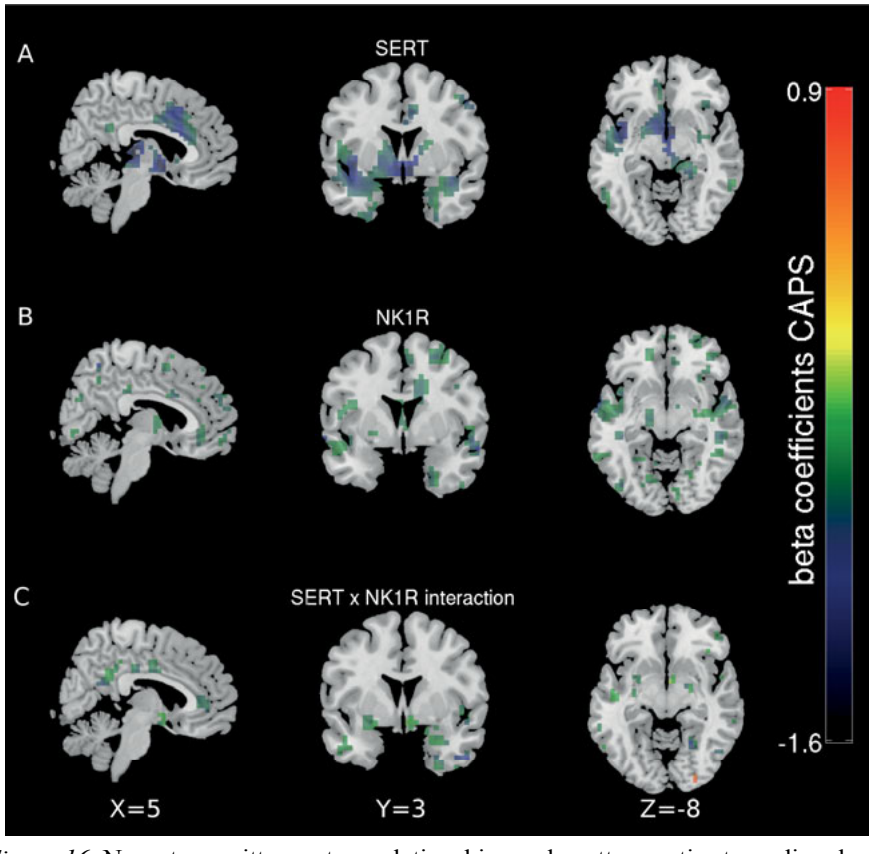

Figure 16. Neurotransmitter system relationships and posttraumatic stress disorder (PTSD) symptom severity, indexed by the Clinician-Administered PTSD Scale (CAPS). Beta weights for relationship between total CAPS score for separate simple regression models including **(A)** transporter (SERT) availability ([^{11}C]DASB BP_{ND}) and **(B)** neurokinin-1 receptor (NK1R) availability ([^{11}C]GR205171 K_i) respectively, and **(C)** for the interaction between SERT and NK1R availability in a multiple regression model including both SERT and NK1R availability and the interaction. Beta coefficient images overlaid on a standard MRI image. All rows depict slices at MNI coordinate (5, 3, -8). The colorbar indicates beta coefficients.

Discussion

Study III demonstrated that PTSD is associated with heightened SERT and NK1 receptor availability, as well as altered co-expression of SERT and NK1 receptors. In the amygdala, patients with PTSD had higher NK1 receptor, but not SERT, availability as compared to the controls. A negative relationship between SERT availability in the amygdala and PTSD symptom severity was moderated by NK1 receptor levels, such that a lower degree of co-expression was related to more severe PTSD symptoms. In general, reduced co-expression of SERT and NK1 receptors predicted more severe PTSD symptoms. Given that SP is a neuropeptide backup system for seroto-

nin during periods of strong stress, the findings support a disrupted role of SP in patients with PTSD. Studying multiple aspects of several neurotransmitter systems represents a new path in revealing the neurobiological underpinnings of anxiety disorders and may potentially serve to guide future therapeutics.

General summary

Table 5. *Summary of aims, findings, and conclusions from the studies in the thesis.*

	Study I	**Study II**	**Study III**
Aims	Investigate serotonin synthesis rate and serotonin transporter availability in patients with social anxiety disorder.	Examine the availability of neurokinin-1 receptors in patients with social anxiety disorder.	Study the availability of serotonin transporters and neurokinin-1 receptors separately as well as their co-expression in patients with posttraumatic stress disorder.
Main findings	Patients with social anxiety disorder had elevated serotonin synthesis rate and serotonin transporter availability. Social anxiety symptom severity was positively associated with serotonin synthesis rate in the amygdala and negatively related to serotonin transporter availability in the dorsal anterior cingulate cortex.	Patients with social anxiety disorder had increased neurokinin-1 receptor availability in the amygdala.	Patients with posttraumatic stress disorder had heightened serotonin transporter and neurokinin-1 levels, as well as altered co-expression between them. Lower levels of serotonin transporter, neurokinin-1 receptors, and co-expression predicted higher symptom scores. In the amygdala, the negative relationship between serotonin transporter availability and symptom severity was moderated by neurokinin-1 receptor levels.
Conclusions	Social anxiety disorder is characterized by an overactive presynaptic serotonin system. Serotonin in the amygdala and dorsal anterior cingulate cortex may be anxiogenic.	The substance P/neurokinin-1 system is involved in social anxiety disorder.	The results support that aberrant couplings between the serotonergic and substance P/neurokinin-1 systems contribute to the pathophysiology of posttraumatic stress disorder.

General discussion

The three studies in this thesis examined the serotonergic and SP/NK1 systems in SAD and PTSD. Based on the previous literature, three main questions were asked: (1) Is serotonin anxiogenic or anxiolytic?; (2) Is the SP/NK1 system involved in anxiety disorders?; (3) Is the relationship between the serotonergic and SP/NK1 systems altered in anxiety disorders? The findings support an anxiogenic effect of serotonin in the amygdala and links exaggerated anxiety to an overactive presynaptic serotonin system. In addition, the involvement of the SP/NK1 system in stress and anxiety, as suggested by animal studies, was demonstrated in two human anxiety disorders. Finally, the co-expression of SERT and NK1 receptors is altered in patients with PTSD, with reduced co-expression linked to more severe symptoms.

Anxiogenic effects of serotonin

It has been debated whether anxiety disorders are characterized by excess or deficit serotonin, or in other words, whether serotonin is anxiogenic or anxiolytic (Maron et al., 2012). Collectively, the findings from Study I and Study III support an anxiogenic effect of serotonin in the amygdala.

In Study I, SAD was associated with an elevated serotonin synthesis rate in the amygdala, and the serotonin synthesis rate in this region was positively related to the severity of social anxiety symptoms. Moreover, in the fear-expressing dorsal ACC, patients with SAD had increased serotonin synthesis, and SERT availability was negatively related to symptom severity. Given an inverse relationship between SERT availability and extracellular serotonin, these findings, together with the negative association between reduced amygdala SERT levels and greater PTSD symptom severity found in Study III, supports a positive relationship between extracellular serotonin in fear-expressing brain regions and anxiety symptoms. However, no assessments were made of serotonin synthesis in Study III, preventing strong conclusions regarding possible other changes in the presynaptic serotonergic system in patients with PTSD. Collectively, these findings are consistent with the notion that elevated extracellular serotonin levels are anxiogenic, either through increased synthesis or reduced reuptake, or possibly both. Further strengthening the conclusion that serotonin acts anxiogenic in the amygdala, and in agreement with animal studies (Carlsson & Lindqvist, 1978; Honig,

Jongsma, van der Hart, & Tecott, 2009; Stenfors, Yu, & Ross, 2001), we recently reported preliminary evidence that six weeks' treatment with the SSRI citalopram or the NK1 receptor antagonist GR205171 reduced serotonin synthesis in the amygdala as measured by [^{11}C]5-HTP PET imaging (Frick et al., 2015). The degree of synthesis reduction was positively related to symptom improvement. The findings from the present thesis are also consistent with a recent animal study from Näslund and colleagues (2015) that showed that anxiety-like behavior in rats was positively related to serotonin content in the amygdala as well as expression of the enzyme TPH2, involved in serotonin synthesis. Notably, high-anxiety rats also had increased expression of the SERT gene, consistent with increased SERT availability in SAD and PTSD. In conclusion, all these findings support the initial postulation of serotonin as anxiogenic in the "classic" hypothesis (Iversen, 1984), and are consistent with Deakin and Graeff's proposal that serotonin in the amygdala facilitates fear-response (Deakin & Graeff, 1991; Graeff et al., 1996). However, no support for the anxiolytic function of serotonin in the periaqueductal gray was found, as serotonin synthesis in patients with SAD was increased in all brain regions.

In Study I, both serotonin synthesis rate and transporter availability were elevated in the raphe nuclei, whereas a previous study reported decreased 5-HT_{1A} receptor levels in this region in patients with SAD relative to controls (Lanzenberger et al., 2007). The 5-HT_{1A} receptors have dual roles in the raphe nuclei, acting both as inhibitory autoreceptors located on the soma and dendrites of the serotonergic neurons, where they regulate synthesis and firing of the cell, and as heteroreceptors conveying inhibitory influences on postsynaptic neurons. A lack of this negative feedback may increase the extrasynaptic concentration of serotonin in the raphe and projection areas, as suggested in Study I. An alternative way of regulating the extrasynaptic serotonin levels is through reuptake by SERT. Increased SERT availability may thus compensate the proposed increase in extracellular serotonin. Without proper longitudinal studies, it is however not possible to discern order effects between the serotonergic changes noted in this thesis and previous studies.

The increased SERT availability in the thalamus reported by van der Wee et al. (2008) was replicated in Study I. Furthermore, we found that SAD was associated with increased SERT availability and serotonin synthesis rate in the striatum, comprised of the caudate nucleus and putamen. These findings support serotonergic underpinnings of previously proposed alterations in striatal-thalamic circuits (D. Li, Chokka, & Tibbo, 2001). Furthermore, the serotonergic alterations in the striatum may underlie changes in striatal activity and behavioral response to social cooperation (Sripada, Angstadt, Liberzon, McCabe, & Phan, 2013) and implicit learning (Sareen et al., 2007) as reported in patients with SAD.

It is noteworthy that all the participants in this thesis were adult, and the reported serotonergic changes may thus be consequences of the anxiety disorders. However, it is also possible that the serotonergic changes were present already during brain development, and thus constituted risk factors for anxiety disorders. Indeed, alterations in the serotonergic system during development may lead to changes in brain circuits and concomitant altered emotional processing (Booij, Tremblay, Szyf, & Benkelfat, 2015), possibly through serotonin's modulation of synaptic plasticity (Lesch & Waider, 2012). In accordance, alterations in neuroplasticity during development is associated with an early-life anxious temperament (Fox & Kalin, 2014), which in turn increases the risk for development of anxiety disorders including SAD (Clauss & Blackford, 2012; Fox & Kalin, 2014). Intriguingly, expression of the serotonin 5-HT_{2C} receptor, reported to be anxiogenic in adult life (Q. Li et al., 2012), is negatively related to anxious temperament in developing rhesus monkeys (Fox & Kalin, 2014). Hence, the question of whether serotonin is anxiogenic or anxiolytic becomes more complex when taking developmental aspects into consideration. The anxiogenic effects of serotonin in adult patients reported in this thesis should be contrasted with possible anxiolytic influences of serotonin and serotonergic receptors during brain formation and development.

Moreover, SAD (Brühl et al., 2014) is associated with exaggerated amygdala reactivity to disorder-relevant stimuli. Because both synthesis and reuptake contribute to the potential release of serotonin through their role in replenishment of the releasable pool of serotonin (Borue, Condron, & Venton, 2010), the increased capacity for serotonin release from combined elevated synthesis and reuptake, as found in Study I, may contribute to the increased amygdala reactivity reported for SAD (Brühl et al., 2014). I therefore propose that elevated serotonin levels may underlie not only anxiety symptoms as suggested above, but also increased amygdala reactivity in general.

The SP/NK1 system is involved in anxiety disorders

Evidence from animal studies indicates that the SP/NK1 system is involved in stress and anxiety reactions (Ebner & Singewald, 2006), but direct evidence from neuroimaging assessments of this system in patients with anxiety disorders has been largely lacking. The only previous study reported that panic disorder is associated with reduced NK1 receptor availability. In Study II and Study III, SAD and PTSD were associated with increased NK1 receptor levels in the right amygdala. Collectively, these findings support involvement of the SP/NK1 system in anxiety disorders, but the direction of the alterations may differentiate SAD and PTSD from panic disorder.

Binding of SP to NK1 receptors rapidly and transiently desensitizes SP response. The mechanisms include receptor internalization (Steinhoff et al.,

2014). This was utilized by Michelgård et al. (2007), who demonstrated that viewing images of phobic stimuli resulted in lower [^{11}C]GR205171 binding in the amygdala in individuals with specific phobia. Greater reduction in [^{11}C]GR205171 binding was related to a greater increase in state anxiety symptoms. These findings were interpreted as increased SP release to the emotional challenge, and support that SP acting through NK1 receptors in the amygdala underlie fear-responses (Bassi et al., 2014; Ebner et al., 2004; Maubach et al., 2001; Truitt, Johnson, Dietrich, Fitz, & Shekhar, 2009). Thus, increased resting-state NK1 receptor availability in SAD and PTSD, as found in the present thesis, may be associated with increased potential for SP neurotransmission and more severe responses to symptom provocation.

It should be noted that anxiolytic treatments targeting the SP/NK1 system have been mixed, and to date no SP/NK1 drugs have been approved for anxiety disorders. Initial positive clinical trials of NK1 antagonists (Furmark et al., 2005; Kramer et al., 1998, 2004) have been difficult to replicate in larger trials (Keller et al., 2006; Kwako et al., 2015; Tauscher et al., 2010). If SAD and PTSD are associated with increased NK1 receptor availability, why do NK1 receptor antagonists not reduce anxiety sufficiently? One potential answer to this intriguing and important question may be that the larger trials did not achieve the necessary receptor occupancy needed for effective treatment (Ratti et al., 2011). This is noteworthy, because patients with anxiety disorders have increased levels of NK1 receptors, and receptor occupancy studies in healthy controls may underestimate the doses needed to achieve adequate occupancy. Moreover, as reported by Furmark and colleagues (2005), in SAD, the NK1 antagonist GR205171 relative to placebo attenuated amygdala reactivity and subjective distress during a stressful public speaking task, but not general social anxiety symptoms as measured by LSAS. This suggests that NK1 blockage may be selectively effective for state-dependent high-stress symptoms, such as symptom provocation, not readily assessed in clinical trials. Consistently, in a trial of GR205171 for PTSD, only hyperarousal symptoms were attenuated (Mathew et al., 2011). Thus, NK1 receptors in the amygdala may selectively modulate anxiety and fear-responses in high-level stress states, which are perhaps not captured by the symptom scales ordinarily used in clinical trials if the patient does not encounter their feared objects.

Interestingly, in Study II and Study III, elevated NK1 receptor availability was found only in the right amygdala of both patient groups. It has been suggested that the right amygdala is involved in rapid detection of preferentially negative emotional stimuli, whereas the left amygdala plays a part in more elaborate evaluations (Dyck et al., 2011; Sergerie, Chochol, & Armony, 2008). The findings from this thesis thus suggest that the SP/NK1 system may be preferentially disrupted in the rapid and automatic evaluation of negatively valenced stimuli in anxiety disorders, which speculatively may translate to the more automatic processes in high-stress symptom provocation

paradigms and hyperarousal symptoms, as compared to symptom questionnaires that may require more complex cognitive elaboration involving the left amygdala. Thus, this may be part of an explanation why NK1 receptor antagonists have anxiolytic effect on symptom provocation paradigms but not on symptom questionairres, as proposed above. Of note, the positive relationship between serotonin synthesis and symptom severity was found only for the right amygdala, consistent with an anxiogenic effect of serotonin in the right amygdala in rats (Andersen & Teicher, 1999), and further suggesting specific right amygdala alterations in neurotransmitter systems in SAD. However, it should be noted that reports of lateralization of amygdala functions are mixed, and recent meta-analyses in both SAD (Brühl et al., 2014) and PTSD (Sartory et al., 2013) have failed to find any hemispheric differences in amygdala reactivity to emotional stimuli.

Hence, although more knowledge is needed regarding the specifics, the present thesis corroborates findings from animal studies that the SP/NK1 system is involved in anxiety disorders.

Altered co-expression between the serotonergic and SP/NK1 systems in anxiety disorders

As concluded in the previous sections, the serotonergic and SP/NK1 systems are involved in anxiety disorders. Because they are frequently co-localized (Hafizi et al., 2012; Sergeyev et al., 1999) and interact (Gobbi & Blier, 2005; Rojas et al., 2010; Santarelli et al., 2001; Shirayama et al., 1996; Valentino & Commons, 2005) in the brain, their co-expression may be important for elucidating the neurobiological underpinnings of exaggerated anxiety. Support for this was found in Study III, where altered co-expression of SERT and NK1 receptors was found in patients with PTSD, and reduced co-expression in various brain regions including the amygdala predicted more severe PTSD symptoms. Importantly, these co-expression analyses do not address the cellular level, but are indices of joint expression of SERT and NK1 receptors at the voxel level.

Interestingly, combining Study I and Study II showed that both serotonin synthesis and NK1 receptor availability were heightened in the amygdala of patients with SAD. In addition, NK1 receptors in the amygdala are co-expressed with serotonin 5-HT_{1A} receptors (Hafizi et al., 2012), and patients with SAD have a reduced number of 5-HT_{1A} receptors in this region (Lanzenberger et al., 2007). Together, these findings suggest possible interaction effects between the serotonergic and SP/NK1 systems on social anxiety. This could not be evaluated due to different patient samples in Study I and Study II. However, the present thesis shows that the co-expression between the serotonergic and SP/NK1 systems is altered in patients with PTSD, with lower co-expression predicting worse PTSD symptoms. In the amygdala, the

inverse relationship between SERT levels and PTSD symptom severity was moderated by NK1 receptor availability. These findings indicate that balanced co-expression of SERT and NK1 receptors may be vital for mental health and underscore the importance of taking interactions between neurochemical systems into account when assessing and treating the pathophysiology of anxiety disorders. Indeed, combining SSRIs with NK1 receptor antagonists has been tried for depression, but not found to be superior to SSRI monotherapy (Ball et al., 2014) despite promising results in an animal study (Chenu et al., 2006). To the best of my knowledge combined SSRI and NK1 receptor antagonists have not been reported for any anxiety disorder.

It is of note that the release of serotonin and SP into the extracellular space requires different degrees of neuronal stimulation due to serotonin and SP being stored in different types of vesicles. Serotonin is stored in small vesicles and SP in large dense-core vesicles (Merighi et al., 2011). Exocytotic release from both vesicle-types is mediated by calcium binding, but because the small vesicles are located closer to the voltage-gated Ca^{2+} ion channels that open when the neuron fires, serotonin is released into the extracellular space by fewer action potentials than SP. SP requires multiple action potentials for Ca^{2+} ions to reach the vesicle by diffusion, suggesting that SP is released only after enhanced stimulation. More stressful stimuli may translate to enhanced stimulation, suggesting that SP may act as a back-up system for serotonin in situations with intense or enduring aversive stimuli. This modulating role of the SP/NK1 system may be disrupted in patients with anxiety disorders such as SAD and PTSD.

Limitations

Sample sizes are generally small, but reasonable for PET studies. This may be something to consider especially when interpreting the findings from Study III, which should be regarded as tentative until replicated. Since Study III is the first study evaluating the co-expression between neurotransmitter systems in vivo in an anxiety disorder, liberal statistical thresholds were chosen to avoid type II errors. To mitigate small spurious findings, statistical level and extent criteria were combined. It should also be noted that the *a priori* amygdala ROI analyses were statistically significant, supporting the voxel-wise findings.

In addition, the limited spatial resolution of PET imaging prevented conclusions regarding co-expression of SERT and NK1 receptors at the cellular level. The co-expression results should therefore be thought of as co-existence of SERT and NK1 receptors in the same brain region, here defined by the voxel. Furthermore, neither SERT levels, nor serotonin synthesis rate, could be correlated with NK1 availability in SAD because of different samples in Study I and Study II. This precluded direct conclusions regarding the

co-expression and possible interactions between the serotonergic and SP/NK1 system in SAD.

The currently used measure of serotonin synthesis rate, [^{11}C]5-HTP K_i, may be partly confounded by the presence of AADC, catalyzing the conversion of 5-HTP to serotonin, also in other than serotonergic neurons, for example dopaminergic and noradrenergic neurons, and the influx of [^{11}C]5-HTP into those neurons could contribute to the tracer accumulation. However, some evidence of substrate-specific enzyme activity has emerged. For example, there is not a complete overlap between AADC activity for the substrates [^{11}C]5-HTP and [^{11}C]DOPA (3,4-dihydroxy-L-phenylalanine, the immediate precursor of dopamine) (Ågren et al., 1993). Furthermore, destruction of the serotonergic raphe system significantly reduces decarboxylation of 5-HTP (Korf, Venema, & Postema, 1974), indicating that 5-HTP is decarboxylated by serotonergic neurons. Thus, [^{11}C]5-HTP K_i could be regarded as an index of serotonin synthesis. In addition, a review concluded that [^{11}C]5-HTP is the most suitable PET radiotracer for measuring serotonin synthesis presently available (Visser et al., 2011).

Moreover, binding potential is proportional to the B_{max}/K_d, where B_{max} is the receptor density (i.e. the capacity for ligand-binding site interaction) and $1/K_d$ is the affinity of the ligand for the binding site (Mintun et al., 1984). This means that the findings of altered SERT availability in anxiety disorders cannot discriminate between changes in SERT density and changes in SERT affinity.

Furthermore, the studies were cross-sectional, meaning that predisposing risk factors cannot be separated from effects of having the disorder. Also, the lack of a non-PTSD trauma control group prevented us from discerning the effects of trauma exposure. Only SAD and PTSD were studied in this thesis and generalization to other anxiety disorders such as panic disorder and generalized anxiety disorder should be done with caution if at all.

Summary and directions for future research

This thesis provides support for alterations in the serotonergic and SP/NK1 systems in anxiety disorders. Specifically, the thesis supports an anxiogenic effect of serotonin in the amygdala and links anxiety to an overactive presynaptic serotonin system. The involvement of the SP/NK1 system in stress and anxiety, as suggested by animal studies, was confirmed in human anxiety disorders. Moreover, PTSD was associated with altered co-expression of serotonin transporters and NK1 receptors, with reduced co-expression being related to increased symptom severity.

Although this thesis answers some important questions regarding the neurochemical underpinnings of anxiety disorders, the findings give rise to even more questions. First, are the alterations in neuromodulatory systems seen in

SAD and PTSD causes or consequences of the disorder? Proper prospective longitudinal studies addressing this important question remain to be performed.

The proposed relationship between elevated extracellular serotonin levels and exaggerated anxiety could and should be tested by combining a symptom provocation paradigm in SAD and PTSD with PET measures of extracellular serotonin levels. Recently, two candidate radiotracers assessing changes in extracellular serotonin have been developed for use in humans (Finnema et al., 2010; Haahr et al., 2014; Nord, Finnema, Schain, Halldin, & Farde, 2013). Adding recent technological advancements in simultaneous acquisition of brain neurochemistry and brain function using combined PET and functional magnetic resonance imaging would make it possible to factor in brain function and thus explore the relations between serotonin and neural activity in the amygdala and anxiety.

On another note, SSRIs alleviate both anxiety and depression, but the mechanisms underlying the anxiolytic and antidepressant effects are still unknown. An intriguing question that remains to be solved is the relationship between anxiety and depressive disorders, given the high rate of comorbidity and the difference in presynaptic serotonergic functioning. SAD is associated with elevated SERT levels, whereas in major depressive disorder, SERT availability is downregulated (Gryglewski, Lanzenberger, Kranz, & Cumming, 2014). The solution might lie in the postsynaptic neuron, but very little is known about postsynaptic activity in anxiety disorders. Although an imbalance between postsynaptic serotonergic 5-HT_{1A} and 5-HT_{2C} receptors has been suggested in anxiety conditions (Q. Li et al., 2012), no studies have examined 5-HT_2 receptors of any type in vivo in humans with excessive anxiety. Tracers targeting the 5-HT_{2A} receptor exist (e.g. [^{18}F]altanserin), but there is still need for a suitable PET tracer for the 5-HT_{2C} receptor (Paterson, Kornum, Nutt, Pike, & Knudsen, 2013). Indeed, such a tracer could help test the hypotheses that excessive serotonergic stimulation of 5-HT_{2C} receptors is associated with anxiety (Q. Li et al., 2012) and that SSRIs exert some of their effect through 5-HT_{2C} receptors (Salchner & Singewald, 2006).

Furthermore, recent studies have suggested that the downregulated postsynaptic cAMP cascade in major depressive disorder is normalized by SSRI treatment (Fujita et al., 2012, 2014). It would be interesting to extend this to anxiety disorders, where one prediction would be that anxiety disorders are associated with an upregulated cAMP cascade that is downregulated with SSRI treatment. However, the radiotracer used in these PET studies, the phosphodiesterase-4 inhibitor rolipram labeled with carbon-11, is not selective for serotonergic signaling. As can be seen from this and the preceding example for 5-HT_{2C}, a crucial factor in studies of neurochemical functioning in health and disease is the development of suitable and specific radiotracers.

Another question in need of an answer is why NK1 receptor antagonists have not been successful in large randomized clinical trials of anxiety and

depression. As mentioned above, some suggestions have been formulated, including the lack of near-complete receptor occupancy (Ratti et al., 2011) and the focus on inadequate outcome measures. Another possibility is differential down-stream signaling in responders and nonresponders, including effects on other neurotransmitter systems. Future studies may provide more robust evidence if a multi-system approach is taken. Following the findings from Study III, it would be interesting to see the effect of pharmacological and psychological treatment on the couplings between the serotonin and SP/NK1 systems, for example by combining SSRI with NK1 receptor antagonist.

Staying with the multi-system approach, there are known interactions between the serotonergic and dopaminergic systems (MacGillivray, Reynolds, Sickand, Rosebush, & Mazurek, 2011) that would be interesting to explore further. Indeed, both serotonin and dopamine have been implicated in anxiety disorders (Maron et al., 2012; Schneier et al., 2000; van der Wee et al., 2008). Furthermore, there is evidence for changes in dopamine transporter levels following SSRI treatment (Kugaya et al., 2002), and serotonin is transported by dopamine transporters following blockage of SERT by SSRIs (Morelli et al., 2011), suggesting possible serotonin-dopamine interaction effects on anxiety and anxiolytic treatment.

Concluding remarks

In the present thesis, two abundant modulatory neurotransmitter systems, serotonin and SP/NK1, were examined in two common psychiatric anxiety disorders, SAD and PTSD. The main message is that both disorders are associated with alterations in both neurotransmitter systems, and that each system in and of itself, but also interactively, may be important contributors to anxiety symptomatology.

Mapping psychological functions to single neurotransmitter systems runs the risk of over-simplifying complex relationships, especially when considering the hundreds of neurotransmitter systems working together in the human brain. However, I believe that although we may not fully elucidate the intricate workings of the brain, molecular imaging together with converging methods may help us put together small pieces of the puzzle. If we can use this information to develop therapies and prevention strategies for debilitating disorders such as those studied in this thesis, I think we will have achieved a great deal.

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Andreas Frick

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