On the anxiogenic influence of serotonin

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2015

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Printed by Ale Tryckteam AB, Bohus, Sweden

ISBN: 978-91-628-9457-3
hdl.handle.net/2077/38386
ISBN: 978-91-628-9458-0 (electronic version)

Till mamma och pappa

On the anxiogenic influence of serotonin

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ABSTRACT

Despite over half a century of research on the role of serotonin in modulating anxiety, no consensus exists as to if serotonin should be regarded as acting mainly anxiety-dampening or anxiety-enhancing. This question is the focus of this thesis, with special emphasis on the role of serotonin in upholding differences in anxiety between and within sexes, and on the issue why some but not others report enhanced anxiety when exposed to selective serotonin reuptake inhibitors (SSRIs).

In paper I, we investigate the impact of serotonin elevation and depletion on inter-individual differences in anxiety-like behaviour of male Wistar rats as measured using an animal model of anxiety, the elevated plus maze (EPM). We also investigate biochemical correlates of temperament, mainly through gene expression analyses using real-time quantitative PCR (rt-qPCR). Briefly, these experiments indicate that more "anxious" rats display a gene expression profile suggesting a higher capacity for serotonin production, and are more prone to display enhanced such behaviour when acutely exposed to an SSRI and also that differences in baseline temperament are abolished by serotonin depletion.

In paper II, we investigate the possible role of three serotonin receptor subtypes in mediating the anxiogenic effect of acute SSRI administration (as studied in paper I) and find evidence for SSRI-induced acute anxiogenesis being dependent on 5-HT6 signalling.

In paper III, we show that the oft-reported difference in anxiety-like behaviour between the sexes is serotonin-dependent. Further underlining the importance of sex steroid vs. serotonin interactions for EPM behaviour, the results in paper IV suggest that castration of male rats abolishes inter-individual differences in EPM behaviour and that the anxiogenic effect of this treatment in non-anxious rats is reversed by serotonin depletion.

In paper V, we employ rt-qPCR to explore the effects of short-term administration of a serotonin synthesis inhibitor and an SSRI, respectively, on the expression of serotonin-related genes in six brain areas, the aim being to shed light on to what extent a measurable change in gene expression is a common adaptive response to changes in extracellular serotonin levels. While many genes were unaffected, some were markedly influenced.

In paper VI, we perform a post hoc analysis of patient level-data from a large number of placebo-controlled depression trials, the aim being to investigate the prevalence of enhanced anxiety following initiation of treatment (as commonly seen in patients with panic disorder). We note such reactions to be rare in this patient

population, and also find no support for a suicide-provoking effect of these substances, but, in contrast, reduced rating of suicidal ideation in SSRI-treated subjects already after one week of treatment.

In summary, our studies suggest that i) SSRI-induced enhanced anxiety, in both animals and humans, is confined to subjects with high baseline anxiety, ii) that enhanced anxiety in animals is associated with indices of enhanced serotonergic activity, and iii) that inter-individual differences in anxiety are abolished by serotonin depletion. The importance of interactions between sex steroids and serotonin in this context gained support by the observation that sex differences in EPM behaviour were abolished by serotonin depletion, and that castration-induced anxiety in non-anxious males (unlike the effects on aggression and sexual behaviour of such treatment) could be reversed by serotonin depletion.

Keywords: Serotonin, anxiety, SSRI
ISBN: 978-91-628-9457-3

LIST OF PAPERS

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

I. **Jakob Näslund**, Erik Studer, Robert Petterson, Melker Hagsäter, Staffan Nilsson, Hans Nissbrandt, Elias Eriksson. 2015. Differences in anxiety-like behaviour within a batch of Wistar rats are associated with differences in serotonergic transmission, enhanced by acute SSRI administration and abolished by serotonin depletion. *Int J Neuropsychopharmacol*, Advance online publication, doi: 10.1093/ijnp/pyv018

II. **Jakob Näslund**, Erik Studer, Elin Johansson, Fredrik Stenfors, Jaroslav Eriksson, Elias Eriksson. 2015. The anxiety-enhancing effect of acute SSRI administration is antagonised by a 5-HT6 receptor antagonist. *Submitted.*

III. **Jakob Näslund**, Erik Studer, Karin Nilsson, Lars Westberg, Elias Eriksson. 2013. Serotonin depletion counteracts sex differences in anxiety-related behaviour in rat. *Psychopharmacology*, 230:29-35

IV. **Jakob Näslund**, Erik Studer, Elias Eriksson. 2015. Effects of gonadectomy and serotonin depletion on inter-individual differences in anxiety-like behaviour in Wistar rats. *Submitted.*

V. **Jakob Näslund**, Erik Studer, Staffan Nilsson, Elias Eriksson. 2015. Effects of an acute increase or reduction in extracellular levels of serotonin on the expression of serotonin-related genes. *Submitted.*

VI. **Jakob Näslund**, Johan Fredrik Emilsson, Staffan Nilsson, Fredrik Hieronymus, Elias Eriksson. 2015. Do SSRIs cause an initial increase in suicidal ideation and anxiety in depressed patients participating in placebo-controlled trials? *Manuscript.*

CONTENT

ABBREVIATIONS

5-HT	5-hydroxytryptamine; serotonin
ADHD	Attention deficit hyperactivity disorder
ANOVA	Analysis of variance
ANCOVA	Analysis of co-variance
DSM	Diagnostic and Statistical Manual [of Mental Disorders]
EMA	European Medicines Agency
EPM	Elevated plus-maze
FDA	Food and Drug Administration (U.S.)
HAMD	Hamilton Depression Rating Scale (also HDRS)
ICD	International Classification of Diseases (WHO)
MDD	Major depressive disorder
OCD	Obsessive-compulsive disorder
p-CPA	*para*-Chlorophenylalanine
PMDD	Premenstrual dysphoric disorder
PTSD	Post-traumatic stress disorder
rt-qPCR	Real-time quantitative polymerase chain reaction
SERT	Serotonin transporter
SRI	Serotonin reuptake inhibitor (i.e. antidepressants exerting a strong influence on serotonin reuptake, including SSRIs and clomipramine)
SSRI	Selective serotonin reuptake inhibitor
TPH2	Tryptophan hydroxylase 2

INTRODUCTION

Serotonin has in the popular mind become somewhat of the archetypical feel-good chemical; countless books, web pages and articles in newspapers and magazines discuss or advertise various ways of increasing brain serotonin levels as a way to improve mood. Likewise, anxiety and depression often are referred to as states in which there is a lack of serotonin in the brain and that successful treatment of them works by raising serotonin levels.

The wide dissemination of this idea can to some extent be traced to how antidepressants, especially of the SSRI class, were initially marketed. Unfortunately this explanation, while appealing, is almost certainly a matter of gross over-simplification and possibly even incorrect, and it has never reflected any scientific consensus; indeed there is no nor has there ever been anything approaching a consensus regarding the mode of action of these drugs beyond their immediate pharmacological targets. While not uncommon in medicine, the mode of action of paracetamol not being fully understood despite over a century of use, the lack of knowledge regarding the mechanism of action of the SSRIs is a humbling and slightly embarrassing state of affairs considering their wide use.

From a clinical perspective, the SSRIs are, on the other hand, a success story; they have been used to successfully treat hundreds of millions of people over the course of less than four decades and are by many (though not all) seen as one of the main reasons for the dramatic decrease in suicides seen in the Western world during recent decades. Apart from being first line of treatment for depression, they have also come to revolutionise the treatment of a number of other psychiatric disorders, such as obsessive-compulsive disorders, panic disorder, social phobia (social anxiety disorder) and premenstrual dysphoric disorder.

Nevertheless they are no panacea; a large proportion of patients suffering from depression and anxiety disorders do not respond, or respond inadequately. Thus, there is an obvious need for improved strategies to combat these conditions. However, development of new drugs would be much facilitated by a greater understanding of how current antidepressants work.

The uncertainty of the mode of action of SSRIs is mirrored by a likewise considerable uncertainty about the role of serotonin in regulating normal as well as pathologically altered mood, fear and anxiety. Even though much evidence points to disturbances in

the serotonin system as important biological factors behind depression and anxiety disorders, the exact nature of these tentative imbalances remain contentious issues.

As implied by the title as well as this introduction, the role of serotonin in regulating anxiety is the focus of this thesis. More specifically, it can be described as an attempt to address the question if serotonin should be regarded as acting mainly dampening or enhancing on anxiety, with a special focus on its role in maintaining differences in anxiety between and within sexes, as well as on the issue why some but not all report enhanced anxiety when exposed to SSRIs. These are the issues we investigate in the first four papers, using behavioural and biochemical methods. Prompted by our use of assessment of the expression of serotonin-related genes in some of these papers, the project described in paper V had the purpose of shedding further light on to what extent measurable changes in gene expression is a common mechanism for the brain to maintain homeostasis in situations of short-term changes in extracellular serotonin levels. .

The sixth paper is a subject-level meta-analysis (or mega-analysis) of patient data from all studies of major depressive disorder MDD included in the development program for three of the major SSRIs. As a clinical counterpart to paper I, we here wanted to investigate the prevalence of an acute anxiogenic effect of SSRIs, similar to that often seen in panic disorder patients, and variously named "activation" or "jitteriness syndrome", in patients with depression rather than an anxiety condition. At the same time, we also assessed the risk for an initial increase in suicidal ideation in SSRI-treated depressed patients.

MOOD AND ANXIETY DISORDERS

INTRODUCTION

Mood disorders (or affective disorders) are psychiatric disorders in which a change in mood is the most prominent feature. Unipolar depression, bipolar depression, mania and dysthymia all belong to this group. Anxiety disorders are psychiatric conditions dominated by anxiety, such as panic disorder, generalized anxiety disorder, obsessive-compulsive disorder, social anxiety disorder (social phobia), posttraumatic stress disorder (PTSD) and simple phobias. It should however be noted that anxiety is a frequent symptom also in many other psychiatric disorders, including depression and psychosis.

Lifetime prevalence for major depression in the longitudinal Lundby study where the population of two Swedish parishes were studied for 50 years was 22.5% for men and 30.7% for women in the 1972-1997 period[1], similar to estimates for the Netherlands and Australia[2] but somewhat higher than figures from studies in the US and New Zeeland[3,4]. Anxiety disorders have a lifetime prevalence of approximately 40% in females and 20% in males with the most common single disorders, social anxiety disorder and specific phobia both having a prevalence of about 10%[3,4].

Diagnosing psychiatric illness

Mood and anxiety disorders, as well as other psychiatric conditions, are diagnosed by way of a synthesis of information (both verbal and non-verbal) obtained in a clinical interview, and sometimes supplemented with information from relatives and caregivers, with focus not only on the present situation but also on heredity for psychiatric disease and factors in childhood and adult life that could have predisposed for, or precipitated, mental illness. Laboratory tests and medical imaging may be useful as to eliminating other causes (e.g. neurological or endocrinological) of the observed symptoms, but provide as yet (with a few exceptions) no aid in establishing a diagnosis. In principle, the diagnosis is up to the clinician's global impression, although many choose to rely heavily on various rating scales and diagnostic manuals and may indeed be expected to do so by regulators and employers.

The DSM system

The dominating classification system for psychiatric disorders is the Diagnostic and Statistical Manual of Mental Disorders (DSM). Produced by the American Psychiatric Association, and being the result of years of work of numerous experienced clinicians and researchers, the DSM has been revised four times since its first edition in 1952, hence reflecting the development of the understanding of psychiatric illness during the decades since then.

Central to the DSM are the naming of criteria for the various conditions, a number of which must be fulfilled for a diagnosis to be made. These manuals do not presume to give absolute definitions of psychiatric disease but rather to aid in establishing diagnoses, ensuring inter-rater reliability and to provide a common language for clinicians and researchers. Over-reliance can be problematic; as the (relatively small) discrepancies between DSM versions, and between DSM-5 and the alternative nomenclature ICD-10 demonstrate, such a manual can be no "Bible" of psychiatry, as the DSM has often been labelled, but should rather be seen as a consensus document whose categories aim to encompass the majority of cases.

Furthermore, most diagnoses are not valid in the sense that they are clearly separated from each other or from normality[5], something which is often stated in the manuals themselves: "there is no assumption that each category of mental disorder is a completely discrete entity with absolute boundaries dividing it from other mental disorders or from no mental disorder" (DSM-IV, p. xxii), a fact that is not always sufficiently considered in preclinical studies, but should be, e.g., in the context of animal models of these disorders.

ANXIETY DISORDERS

The Indo-European verb root of words such as Ancient Greek "ἄγχω", Latin "ango" (the source, by way of French, of the English word "anxiety"), German "Angst" and Swedish "ångest" has been reconstructed to have had a meaning of "to narrow" or "to strangle", with cognates in many daughter languages denoting intense psychic pain - *anxiety*.

The word as used in English signifies an apprehension over future events and choices and is distinct from the response to an acutely threatening situation: that is, anxiety is

distinct from fear. Anxiety can be evoked by e.g., presentation of new individuals and situations, or in the context of a situation that is or can be perceived as a test of one's ability. It can also have deeply existential connotations related to the anxiety elicited by the realisation of not only mortality but also of the myriad of choices, large and small, of everyday life; Kierkegaard speaks of the "dizziness of freedom". In a psychiatric context, anxiety can both be one of several features of a disorder (as in depression), and sometimes, as is the case for the anxiety disorders, the dominating symptom.

In the DSM-5, the anxiety disorders include generalised anxiety disorder, panic disorder, social anxiety disorder (formerly social phobia) and a few others, such as agoraphobia and specific phobias. Entities related to the anxiety disorders and earlier included among them are obsessive-compulsive disorders and trauma and stress-related disorders (of which post-traumatic stress disorder, PTSD, is the most prominent).

Generalised anxiety disorder is characterised by excessive and hard-to-control worrying relating to a number of domains such as work, relationships and health and persisting for more than six months. This may often be associated with symptoms such as irritability, difficulty to concentrate, muscle tension, sleep disturbances, increased fatigability and restlessness.

Panic disorder is characterised by recurrent panic attacks; intense spells of fear accompanied by symptoms such as palpitations, tachycardia, shortness of breath, chest pain, trembling, nausea, feelings of choking, paresthesias and fear of dying. These attacks may lead to a persistent fear of experiencing additional attacks, resulting in maladaptive changes in behaviour related to the attacks such as avoiding situations eliciting them or where they would be dangerous (e.g. driving) or perceived as socially embarrassing (e.g. public transportation, cinema, lecture halls, parties).

Social anxiety disorder entails a disproportionate and impairing apprehension in the face of one or several social situations where the individual is exposed to possible scrutiny by others and fears that he or she will act in a way that will provoke negative reactions and rejection from others. These situations almost always provoke intense fear or anxiety and are either avoided or endured with great anguish.

Post-traumatic stress disorder, either classified as an anxiety disorder or as a separate entity, may develop after exposure to a traumatic event. It entails very vivid recollections of the event in the form of e.g. dreams or intrusive thoughts. Exposure

to stimuli reminiscent of the situation may elicit considerable distress and the patient is likely to avoid situations that may remind him or her of the event. Sleep disturbances are common and the patient often experiences a state of hyper-arousal with exaggerated fear responses.

DEPRESSION

One of the earliest descriptions in world literature of depression can be found in Euripides' *Orestes* where the protagonist exhibits loss of appetite, lack of motivation, great exhaustion, excess sleeping and feelings of helplessness and futility. This is what the author's contemporary Hippocrates labels *melancholia*; an excess of cold black bile, associated from Aristotle and on not only with sadness and rumination but, in its milder forms, also with genius and profundity. This association of depression with artists, philosophers and statesmen is a recurrent theme in Western thought; the black dog of Churchill and others thus has been romanticised far more than any other mental illness. Nonetheless, depression is a debilitating condition with considerable direct mortality through suicide[6,7] and indirect through increased risk for a number of other conditions, such as cardiovascular disease[8], diabetes[9] and cancer[10].

The term depression in modern psychiatric parlance signifies a state, much like that of Orestes, characterised by lowered mood, diminished ability to feel joy, tendency towards rumination, feelings of guilt and thoughts of death. It is a characteristic of several closely related psychiatric conditions such as major depressive disorder and dysthymia. Some patients experience not only depressive episodes but also spells of mania or hypomania, a condition labelled bipolar disorder, as contrasted with unipolar disorder where only depression is present.

The HAMD-17

The Hamilton Depression Rating Scale, originally published in 1960[11], is a rating scale for depression, designed not to diagnose depression but to provide a reasonably objective instrument for measuring its depth and also to assess the effects of treatment. In its most common form it comprises 17 questions, or items, that can give a score of 0-2 or 0-4 with a total added score sum presumed to give a measure of depth of illness.

The scale quickly became very popular and came to be used as the golden standard in clinical trials. The Hamilton scale is however multi-dimensional, that is, it includes

different clusters of items that measure things that do not co-vary with other clusters. In case some symptoms, or clusters of symptoms, are absent already at baseline in many subjects, including these items and clusters in a scale may enhance the variability and hence reduce the sensitivity of the instrument. Likewise, when using a multi-dimensional rating instrument, improvements with respect to one important domain may be masked by lack of improvement in another less significant domain; with respect to the HAMD-17, this problem is further enhanced by the fact that some side effects of pharmacological treatment may be inaccurately picked up by the scale as symptom aggravation.

These weaknesses were pointed out early on[12] and several uni-dimensional variants have been proposed[13], but almost all of the large antidepressant studies of the 80ies and 90ies nevertheless used the HAMD-17 as primary measure of treatment efficacy. On the basis of meta-analyses of drug company-sponsored studies, some debaters have suggested that antidepressants in fact do not work[14,15]; recent reports however suggest that this conclusion may be a consequence of the use of an insensitive measure of improvement rather than an actual lack of efficacy of the tested drugs[16].

SEROTONIN

INTRODUCTION

In 1935, the 26-year-old Italian pharmacologist and chemist Vittorio Erspamer isolated a substance from rabbit enterochromaffin cells capable of making intestinal tissue contract[17]. He would two years later demonstrate that this substance was an amine, previously unknown to science, and name it enteramine[18].

In 1948 a group of American biochemists working at the Cleveland Clinic discovered a substance that was present in blood and capable of inducing vasoconstriction[19]. It was subsequently named *serotonin*. Four years later serotonin and enteramine were shown to be the same substance[20] and a year later, in 1953, it was shown to be present in the brain[21].

Figure 1: Vittorio Erspamer at work in his laboratory in Rome, circa 1990. Public domain

NEUROBIOLOGY OF SEROTONIN

A monoamine of considerable age

Serotonin, or 5-hydroxytryptamine (5-HT), belongs to a class of compounds called monoamines that are defined as such by having an amino group bound to an aromatic ring by a two-carbon chain. In the metazoa, that is the animals, serotonin is synthesised from the essential amino acid *l*-tryptophan by way of two steps (Figure 2).

Serotonin is present in a number of organisms throughout all three domains of life. In metazoans, serotonin acts as a mediator of behaviour related to feeding, reproduction and dispersion with enzymes as well as several receptors being homologous[22,23], indicating that the general role as well as the basics of the molecular machinery of serotonin has been conserved throughout the animal kingdom for the better part of a billion years.

The molecular machinery of serotonin

The enzyme defining the serotonergic neurons is tryptophan hydroxylase (TPH) which catalyses the rate-limiting step of serotonin synthesis, i.e. the conversion of the essential amino acid tryptophan to 5-hydroxytryptophan. TPH exists in two isoforms, one of which (TPH2) is exclusively expressed in the brain[24]. The next step, the conversion from 5-HTP to serotonin, is catalysed by amino acid decarboxylase (also known as dopa-decarboxylase; AADC/DDC).

In anticipation of release, serotonin is protected by degradation by being stored in vesicles into which it is transported through the action of vesicular monoamine transporter 2 (VMAT2). Reuptake from the synaptic cleft back into the presynaptic neuron is mediated by the serotonin transporter (SERT). The degradation of the transmitter is accomplished through two steps, the first generally catalysed by monoamine oxidase A (MAOA) and the second by aldehyde dehydrogenase (ADH).

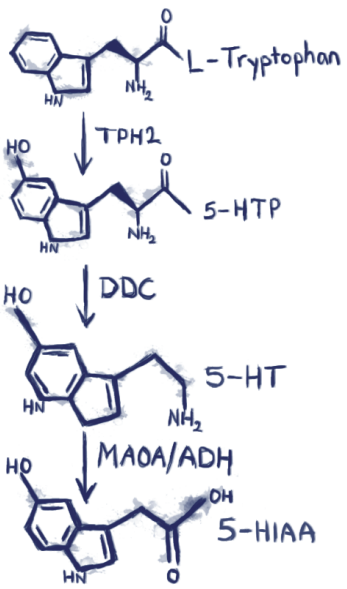

Figure 2: Serotonin metabolism

Serotonin exerts its actions through a number of receptors. Apart from the 5-HT3 receptor, which is a ligand-gated ion channel, all are G-protein coupled receptors. The 5-HT1 subfamily comprises five (in murids four) G_i-coupled receptors which not only serve as post-synaptic receptors but also as autoreceptors, the 5-HT1A receptor being situated in the somatodendritic region and exerting a negative feed-back influence on the firing of serotonergic neurons, and the 5-HT1B and 5-HT1D receptors being situated at serotonergic nerve terminals and exerting a local inhibitory influence on serotonin synthesis and release. All other serotonin receptors are post-synaptic, i.e, situated at non-serotonergic neurons and mediating an influence of serotonin upon these.

The raphe nuclei

The serotonergic cell bodies of the vertebrate brain reside in several distinct nuclei arranged symmetrically along the midline, from the medulla oblongata to the midbrain. The general organisation of this system is phylogenetically conservative, with the general organisation in cyclostomes being similar to that of jawed vertebrates[25] and only small differences existing among mammals[26]. A caudal group of nuclei projects mainly to the brainstem itself and the spinal cord while a rostral group projects mainly to the forebrain. The division into a rostral and a caudal group displays an approximate overlap with gene expression profiles[27]

The rostral group contains about 85% of all serotonergic neurons in the brain and comprises three nuclei, the dorsal and median raphe nuclei (the former being considerably larger than the latter) as well as the small caudal linear nucleus that to a large extent shares projections with the dorsal raphe nucleus. The projections from the dorsal and median raphe nuclei largely overlap, although important differences in innervation exist. Structurally the axons of the dorsal raphe nucleus are characterised by small varicosities that in general do not form classical synapses but instead signal through volume transmission while the neurons originating in the median raphe nucleus to a greater extent form true synapses[28].

SEROTONIN IN ANXIETY AND MOOD DISORDERS

Introduction

Serotonin has numerous roles in vertebrate physiology, being a regulator of, e.g., platelet and vascular function, bone growth and gastrointestinal motility. However, its effects in the CNS are by far the most investigated.

Initial suspicion of a role for serotonin in psychiatric disorders arose from the observation that LSD and other serotonergic agents were able to profoundly affect mental states in humans[29]. The nature of the effects of these drugs rather suggested a role in the pathophysiology of schizophrenia, but indications of importance for depressive illness came with the discovery that the monamine-depleting plant alkaloid reserpine was able to induce depression in a subset of patients receiving this drug as an antihypertensive agent[30]. Important was also the observation that the antitubercular agent iproniazid, earlier having been found to act as an inhibitor of monoamine oxidase[31], could improve mood in tubercular[32] as well as non-tubercular patients[33].

The discovery by Roland Kuhn in 1956 that the tricyclic agent imipramine exerts antidepressant effects[34], and the subsequent finding by Julius Axelrod that it inhibits the reuptake of noradrenaline[35], further strengthened the monoamine hypothesis of depression, most early workers however focussing on the catecholamines rather than on serotonin. Although Arvid Carlsson and co-workers had pointed out that imipramine and other tricyclic antidepressants may block also serotonin reuptake in 1968[36], and serotonin hypotheses of depression had been formulated, e.g., by Lapin and Oxenkrug in 1970[37], it was not until the introduction of selective serotonin reuptake inhibitors, the first of which was developed by Arvid Carlsson in collaboration with Astra, i.e. during the 80s, that serotonin came in focus for the hypotheses aiming to explain the aetiology of affective disorders.

Serotonin and anxiety

Serotonin being a factor of importance for the pathophysiology or anxiety disorders is strongly implicated by the excellent therapeutic effect of drugs such as clomipramine and the selective serotonin reuptake inhibitors (SSRIs) in panic disorder and several

other anxiety disorders [38-40] as well as by various genetic findings [41,42]. How serotonin influences proneness for anxiety however is far from clear. For example, while some claim pathological anxiety to be associated with too much serotonergic output[43,44] and some have suggested it to be the result of a shortage of serotonin there are also those claiming that serotonin may both enhance and reduce anxiety[45].

In animal models, acute administration of SSRIs [46,47] or the serotonin releaser fenfluramine [48] enhances anxiety-like behaviour, as do optogenetic activation of raphe neurons [49], while depletion of brain serotonin in pharmacological [50] as well as genetic knock-out models [51] attenuates it. Similarly, acute SSRI or fenfluramine administration may elicit heightened anxiety in humans, especially in patients suffering from panic disorder [52,53].

These findings could be taken to indicate a generally anxiogenic role of serotonin in rodents and men; acute elevation elicits anxiety while depletion attenuates it. There are however some problems with such an assumption. Serotonin depletion in humans tends to have mild and transient effects on mood[54], but if anything a worsening of symptoms of anxiety [55] and depression is seen[56,57], although a direct comparison with animal studies may be difficult as the method used in humans, dietary tryptophan depletion, is considerably less effective at lowering brain serotonin levels than methods available to researchers working with animal models [58,59]. Also, non-serotonergic effects of tryptophan depletion should not be excluded, since only a small fraction of the tryptophan normally ingested is converted to serotonin.

Genetic studies are also hard to interpret in terms of *increased* or *decreased* serotonergic transmission as developmental effects of the studied gene variant may induce various compensatory mechanisms. Nevertheless, it may be noted that the 5-HTTLPR polymorphism, which leads to a decreased serotonin transporter (SERT) expression, and hence presumably (although this is difficult to confirm in humans) to enhanced extracellular levels of serotonin, has usually been associated with heightened anxiety[60,61].

SEROTONIN AND ANDROGENS

Male gonadal hormones, or androgens, are steroid hormones that include, among others, testosterone, dihydrotestosterone and androstenedione. They are produced in the testes of the male as well as in the adrenal cortex of both sexes and signal through

the nuclear androgen receptor, as well as through the estrogen receptors if first converted to estrogens by the enzyme aromatase. They are crucial for establishing the male phenotype and in maintaining normal male behaviour and sexual function. As it happens, behavioural domains where androgens have a profound effect are also to a great extent modulated by serotonin.

There exist important differences between the sexes in relation to serotonergic signalling. Several studies have demonstrated lower levels of brain serotonin in males[62-64], a difference seemingly related to gonadal and hormonal status[64-66].

To a considerable extent serotonin and androgens exercise opposite effects. Both in respect to sexual and aggressive behaviour, exogenous androgens generally exert an enhancing[67-69] and castration a dampening[70] influence, while the situation for serotonin is the opposite; enhancing serotonergic output dampens[71-74] while serotonin depletion promotes[75-79] both sexual activity and aggression.

Also with respect to the regulation of anxiety-like behaviour, as assessed, e.g., using the elevated plus-maze (EPM), there appears to exist an antagonism. Acute serotonin elevation is known to exert an anxiogenic[47,48,80] and serotonin depletion an anxiolytic influence[51,81]. Regarding androgens, castration has been reported to be anxiogenic[82] and exogenous androgens to exert the opposite effect[83].

How serotonin and androgens interact is somewhat unclear. It could be so that behavioural effects of androgens on aspects of behaviour that are dampened by serotonin are partly mediated by a hormone-induced reduction in serotonergic transmission. However, it is also possible that androgens promote and serotonin dampens aggression and sexual activity by parallel paths, a notion that gains support from studies indicating that serotonin depletion does not restore sexual behaviour[84,85] or aggression[86] after gonadectomy, thus implying that while serotonin effects are dependent of presence of androgens, androgens act independently of serotonin.

SELECTIVE SEROTONIN REUPTAKE INHIBITORS

History and indications

The first SSRI to reach the market was zimelidine, developed by Astra Hässle in collaboration with the Swedish pharmacologist and later Nobel prize winner Arvid Carlsson. However, after a successful introduction it became apparent that zimelidine could elicit a cumbersome side-effect; about 1 in 10 000 patients developed a Guillain-Barré-like syndrome. It was for this reason withdrawn from the market.

Meanwhile, the American pharmaceutical company Eli Lily had been working on its own SSRI: fluoxetine, better known as Prozac. Approved for marketing in 1987 and introduced to the American market in 1988, it came to not only revolutionise treatment of depression and anxiety disorders but also to have a profound effect on how psychiatric disorders, the mind and modern pharmacology are perceived by the public.

SSRIs are the most prescribed class of antidepressants and are used not only for the treatment of depressive disorders like major depression and dysthymia, and for the prevention of depression relapse, but also for a number of other conditions such as panic disorder, GAD, OCD, PTSD, social phobia, PMDD and bulimia nervosa.

Mechanism of action

SSRIs inhibit the serotonin transporter, a protein primarily localised at the cell membrane of serotonergic neurons that is responsible for eliminating serotonin from both the synaptic and extra-synaptic space. This is an immediate effect of SSRIs that leads to increased extracellular availability of serotonin[87]; the therapeutic effects of SSRIs are however in general not immediate, with the time to onset of the clinical effect usually being reported as 2-4 weeks and the time to maximal effect being much longer. Important exceptions are PMDD, where a therapeutic effect is evident within a few days[88], as well the various side-effects, who also generally display a rapid onset.

Numerous theories have been postulated to explain the considerable latency between initiation of treatment and clinical effect. According to one of these, the SSRIs elicit a 5HT1A-mediated feed-back inhibition of the serotonergic nerve activity that prevents any significant increase in extracellular levels of serotonin until the somatodendritic autoreceptors have been down-regulated[89,90] and according to another the SSRIs exert their effect by slowly down-regulating post-synaptic receptors[91,92].

Other theories have focussed on an influence of SSRIs on neurotrophins such as BDNF[93], often in conjunction with the assumption that SSRIs promote neoneurogenesis[94], and it has also been suggested that they modulate inflammatory components of alleged importance for the pathogenesis of depression[95]. In addition, it has been suggested that the delayed effect is related to a cognitive process where a negative emotional bias, known to quickly respond to administration of SSRIs[96,97], initiates gradual psychological processes favouring remission[98].

Side effects and the "jitteriness syndrome"

SSRIs have a generally mild side effect profile when compared to the old tricyclic antidepressants and are much less toxic at accidental or deliberate overdoses[99]. Common side effects include sleep disturbances and gastrointestial side effects[100] as well as disturbances of sexual function such as anorgasmia or prolonged time to orgasm[100,101]. Regarding the latter, SSRIs have been used to treat premature ejaculation and a short-acting SSRI with this specific indication, dapoxetine has been developed[102].

Though serotonin reuptake inhibition is effective in reducing anxiety, it is not uncommon for patients to experience an initial worsening of anxiety symptoms, sometimes referred to as the "jitteriness" syndrome, when initiating treatment with an SSRI. First primarily seen in patients with panic disorder medicating with a tricyclic antidepressant that is a stronger inhibitor of serotonin reuptake than the other tricyclic antidepressants, i.e. clomipramine[103,104], this phenomenon has later been reported also in patients on SSRIs and in patients with depression or anxiety disorders other than panic disorder.

Opinion differs with respect to which symptoms that should be included in this anxiogenic response to antidepressants, but a public health advisory released by the FDA in 2004[105] names insomnia, irritation, agitation, anxiety, panic attacks, akathisia (that is, restlessness and a feeling of not being able to remain still), impulsivity and

induction of mania/hypomania; a recent systematic review however found little support for the inclusion of this last item[106]. The reported incidence varies greatly, with a range of 4-44%[106], and it is usually claimed that this syndrome abates within a week or two.

This syndrome has some parallels in pre-clinical models as well as in findings in studies in experimental psychology using healthy volunteers. Thus, acute SSRI administration has been shown to increase neuroticism[107] fear responses[108], recognition of fearful faces[109] as well as amygdalar reactivity[110]. In animals, acute SSRIs (or similarly acting agents such as fenfluramine) reliably increase anxiety-like behaviour in various animal tests of anxiety [46-48].

SSRIs and suicide

Considerable attention has been given to a possible association between SSRI treatment and a transient increased risk of suicide, with the first reports of intense suicidal ideation in patients receiving fluoxetine published in the early nineties[111,112].

The literature on this important matter is far from unanimous, with several reports of increased suicidal ideation during the first weeks of treatment[113-115] as well as of non-fatal (but not fatal) suicide attempts[116]. Most large systematic reviews and meta-analyses however report no increased risk of suicidal behaviour in adult patients receiving SSRIs[117-119].

Akathisia has been proposed to explain a possible early suicidogenic effect of SSRIs in some patients[113,120]. This response is most commonly seen as a side effect of neuroleptic treatment, but has been recognised as a side effect also of SRIs[121]. Hansen, reviewing the literature on drug-induced akathisia in 2001, however found little support for an association between suicidality and SSRI-induced akathisia, noting the latter phenomenon generally to be reported as milder than the corresponding side effect induced by neuroleptics[122]. A more recent meta-analysis did however find support for such a connection, finding patients medicating with SSRIs and experiencing increased suicidal ideation to be more likely to report akathisia[123].

In 2004, the FDA issued a warning of increased suicidal ideation (but not of attempts or completed suicides) in children and adolescents initiating treatment with SSRIs[105], with the EMA and other regulatory bodies following suit. This led to a considerable reduction in prescriptions not only in these groups but also in young adults[124].

Subsequent analyses indicate an increase of completed suicides among children and adolescents (with an increase of no less than 49% in the Netherlands) in the years following the FDA warning[124,125], hence suggesting a negative net effect of the warning.

Important to note is that the overall incidence of suicide in the Western world has decreased dramatically over the last 40 years. It is evident that many factors such as, e.g. a change in societal attitudes to psychiatric illness can be expected to influence suicide rate, but it has often been pointed out that this decrease coincides with the marked increase in the prescription of antidepressants that was the result of the introduction of the SSRIs[126].

A reduction in suicide rates thus has coincided with the introduction of modern antidepressants in several countries and subscription rates often display a negative correlation with suicide rates[127-131], although some have failed to find such an association[132,133]. Needless to say, association in time is of course not evidence for causation and prescription rates could be a proxy, e.g., of improved availability of psychiatric care. Nevertheless, the above-mentioned correlation of decreased SSRI prescription and increased suicidality following the FDA warning is a strong indication of a causal role of SSRIs in the reduction of suicides seen in recent decades.

ANIMAL BEHAVIOUR

TEMPERAMENT

Why do individuals vary in behaviour?

The realisation that animals, just as humans, vary in temperament is likely at least as old as the first human attempts to domesticate other species. Scientific study of these differences is however considerably younger, and has mostly been undertaken by ethologists and ecologists.

Temperament, or personality, denotes inter-individual differences in behaviour that are stable over time and across situations. Similar to other phenotypic variation, variation in behaviour can be expected in any non-stable environment; a strategy that, e.g., emphasises boldness, competition and growth may be favoured in times of abundance and/or low predation pressure while a more cautious and conservative strategy can be expected to have higher fitness when the conditions are the opposite[134,135]. As populations encounter varying conditions across range as well as time, variation in temperament can be expected to be maintained.

Variation in behavioural strategies within a population is not restricted to 'higher' animals; differences in temperament or personality exist in e.g. arthropods such as spiders[136] and molluscs such as cephalopods[137] and gastropods[138] and the ecological mechanisms underlying the emergence and maintenance of such differences are similar irrespective of species.

Temperament in neurobiological research

To use inter-individual variation in neurobiological and psychopharmacological research is not a new concept; there has for example been considerable research done into how individual variation in novelty-seeking influences a tendency to develop an addiction to dopaminergic drugs[139,140].

A related approach is the creation of specific lines of animals characterised by e.g. increased aggression. A good example would be the High Anxiety Behaviour/Low Anxiety Behaviour (HAB/LAB) strains where rats have been bred on the basis of EPM behaviour to produce strains representing two extremes of anxiety-like

behaviour[141]. Considerable work has been done using these strains on, for example, the neuroendocrine basis of anxiety[142], the interaction of genetic and environmental factors[143], and the interplay between anxiety-like behaviour and aggression[144].

SEX DIFFERENCES IN BEHAVIOUR

In the vast majority of organisms with sexual reproduction, reproductive strategies differ between the sexes; indeed, almost by definition, sexes are different reproductive strategies[145]. A dimorphism in reproduction-related physiology and anatomy also extends to behaviour, the kind and degree of dimorphism being dependent on the life histories of the individual species. Sexual dimorphism in behaviour is commonplace throughout the vertebrate subphylum; as life histories differ between species and sexes, so do traits such as aggression[146] and novelty seeking[147,148].

In humans, a consistent dimorphism exists regarding personality traits related to depression and anxiety, and also with respect to the disorders themselves, with women generally displaying higher levels of neuroticism, regardless of culture[149], as well as a higher prevalence of affective disorders[1-4].

That biological factors play a central role in mediating sex differences in behaviour also in humans is largely uncontroversial from a scientific point of view, considering the high degree of heredity of these traits and disorders[150-152]. Of note is also that the higher prevalence of affective disorders in women emerges at menarche[153-155], hinting at an important role for sex hormones in maintaining certain behavioural differences.

In the rat, females make more frequent foraging trips[156] and display lower levels of neophobia[147,157] as well as higher activity levels[158,159]. In the context of animal models of human psychiatric disease, males generally register as displaying higher anxiety-like behaviour in tests such as the EPM[160,161]. Though different from the situation in humans, this is in line with what could be expected in organisms with short life spans and little or no bi-parental care; the female will need to forage to a greater extent than the male due to the higher reproductive costs of reproduction[162].

ANIMAL MODELS IN PSYCHIATRIC RESEARCH

Problems and strategies

Attempting to model psychiatric conditions in animals is problematic from several aspects. Psychiatric diagnoses are, as mentioned, made on the basis of structured clinical interviews and on what the patient and/or family of the patient report. There exist few laboratory tests to aid in diagnosis and thus few biomarkers or suchlike to look for in a putative animal model.

All models hence must be based on tentative similarities between human and animal behaviour. Usually, the only validation available is through pharmacology; animals in a putative model of anxiety should respond with increased anxiety-like behaviour when treated with, e.g., benzodiazepines but with increased anxiety if given, e.g., an inverse GABA-A agonist, in analogy with the situation in humans. Likewise, drugs not influencing anxiety in humans should also be inactive in the suggested model.

Furthermore, some of the disorders are to some extent of an existential character. For example, concepts such as worth and worthlessness, guilt and mortality may be central to someone afflicted by depression. It is highly unlikely that laboratory animals such as rats, that appear to be devoid of any sense of self, would ruminate over this kind of issues; what is likely modelled in them are instead more basal fear and stress responses.

Models of anxiety are usually based on the presentation of something assumed to be aversive to the species being used in the model; for rodents such stimuli could be open spaces, light, height, predator scent or sudden noises. Stimuli can thus either be very specific to the species or more general.

Related is the concept of endophenotypes, that is discrete and possibly more phylogenetically basal aspects of a complex human phenotype that can be present also in, e.g., rodents[163]. An example would be the startle response; patients with higher trait anxiety[164] or anxiety disorders[165] tend to respond (by e.g. flinching or blinking) more strongly to a sudden frightening stimulus such as a loud noise than others, and this response can often be attenuated by anxiolytic substances such as

benzodiazepines[166] and chronic SSRIs[167]. While often used in clinical research, this test has a well-validated equivalent in rodents[168,169].

Often, but not always, the stimulus is presented in the context of a choice in which something desirable (e.g. food or exploration of a new area) necessitates exposure to the aversive stimulus, creating a conflict. According to some authors, such conflicts might be similar to the ones allegedly underlying anxiety in humans, as once suggested by Sigmund Freud. Also for one not sharing the belief that psychiatric disorders are caused by unconscious conflict, it may however make sense to expose an animal for a choice, hence testing the willingness of the animal to expose itself for possible danger, as a measure of its proneness for fear or anxiety.

Figure 3: The elevated plus-maze.

An example: the elevated plus-maze

The model we chose to use to separate animals according to presumed anxiety level is the EPM. This paradigm exploits a natural tendency of rats and mice to avoid open spaces and bright light to create a conflict in which this aversion counteracts a drive to

explore new areas. Substances such as benzodiazepines with anxiolytic action in humans increase the propensity of animals to enter the aversive open arms[170] of the maze while anxiogenic substances[171,172] or stressors, such as predator odour[173], does the opposite. EPM is one of the most used behavioural tests in behavioural neuroscience, with over 6000 hits on PubMed in early May 2015, and it has been adapted for use in several other species, ranging from other murids such as gerbils[174] to crayfish[175].

PAPERS I-VI: AIMS, OVERVIEW AND RESULTS

AIMS

The aims of this thesis were to answer the following questions:

1. Numerous findings suggest an important role for brain serotonin in establishing inter-individual differences in anxiety-related traits in humans. Is there a relationship between anxiety-like behaviour in animals on the one hand and biochemical indices of serotonergic function on the other, and can such associations give an indication as to whether more "anxious" animals are characterised by *higher* or *lower* central serotonergic activity?

2. To what extent are inter-individual differences between rats with respect to anxiety-like behaviour dependent on brain serotonergic transmission, i.e. can such differences be abolished by serotonin depletion? To what extent can baseline anxiety-like behaviour predict an anxiogenic response to acute serotonin reuptake inhibition? And to what extent are inter-individual differences between rats with respect to anxiety-like behaviour influenced by subchronic administration of an SSRI?

3. Which receptor subtypes mediate the acute anxiogenic-like effect of SSRIs observed in anxious animals when assessed using the EPM?

4. There exists a considerable sexual dimorphism in animals as well as humans in regards to anxiety and anxiety-like behaviour, respectively. To what extent are such differences dependent on intact serotonergic neurotransmission?

5. Like serotonin, androgens may influence anxiety, castration of males reported to have an anxiogenic-like effect and exogenous androgens doing the opposite. Is the influence of androgens in the EPM paradigm to some extent mediated by an interaction with serotonergic neurotransmission?

6. How responsive are the degrees of transcription of genes of importance for serotonergic function to sub-acute elevation or reduction of the extra-cellular

levels of serotonin? What brain areas are most affected by such sub-acute perturbations of serotonergic output?

7. To what extent do SSRIs exacerbate anxiety in patients with depression during the first two weeks after initiation of treatment as captured by the relevant items of the HAMD-17?

8. To what extent do SSRIs exacerbate or induce suicidal ideation in patients with depression as measured using the suicidality item of the HAMD-17?

STUDY OVERVIEW AND RESULTS

1. Temperament and serotonergic neurotransmission (paper I)

Several animal studies suggest variation in the levels of serotonergic enzymes and transporters, such as TPH2 and SERT, to influence anxiety-like behaviour[51] and to be sensitive to various anxiogenic interventions[176]. Numerous genetic studies in human also support such a connection[177], but it remains an open question if such associations suggest serotonin to exert mainly an anxiety-enhancing or an anxiety-reducing influence.

We therefore chose to investigate the possible relationship between inter-individual variation in anxiety-like behaviour in male Wistar rats and inter-individual variation in brain serotonergic activity, and, more specifically to what extent EPM behaviour correlates with the expression of a number of serotonin-related genes in the region where the serotonergic cell bodies reside, i.e. the midbrain raphe. Animals were first pre-tested in the EPM to identify the 1/3 of the animals most or least prone to spend time on the open arms, the former tentatively non-"anxious" animals named “high open” (HO) and the latter tentatively "anxious" animals named “low open” (LO). The intermediate 1/3 was excluded from further analysis. Assessment of gene expression was done by way of rt-qPCR, of protein levels by way of western blot and of amine levels by way of HPLC.

We observed significantly higher expression of TPH2 in LO rats – a finding confirmed using Western blot – and also higher expression of a number of other genes expressed by serotonergic neurons and important for the functioning of the

serotonin system. We also observed higher levels of serotonin in LO animals in the amygdala.

2. Effects of acute serotonin elevation and depletion on temperament (paper I)

Acute administration of SRIs or the serotonin releasing agent fenfluramine[104,106,178,179] often causes a marked, paradoxical increase in anxiety in patients with anxiety disorders, such as panic disorder, and in subjects with anxiety-related personality traits[107,180]. This response is generally absent or mild in subjects with other diagnoses (see paper VI) or healthy volunteers when exposed to these drugs.

We wanted to investigate whether this holds true also in rats; i.e. if animals assayed as more "anxious" in the EPM display a higher tendency to respond with heightened anxiety upon acute SSRI administration than those with lower “anxiety” at baseline. We were also interested in how long-term treatment with another SSRI would impact the inter-individual variation in EPM behaviour observed at baseline, as well as the acute response to an SSRI injection. To this end, animals were first subdivided as in the biochemical experiment described above, and then fed either control chow or chow containing the SSRI escitalopram for five weeks, whereupon they received an injection of another SSRI, paroxetine, 1 h before being again tested in the EPM.

We also wanted to investigate if serotonin serves to uphold inter-individual differences in anxiety-like behaviour, i.e. if these are abolished by serotonin depletion; hence we explored to what extent the well-established anxiolytic effect of p-CPA in the EPM is dependent on baseline temperament[81,181]. To this end, animals were subdivided as in the experiments described above and, three weeks later, administered an irreversible inhibitor of TPH2, *p*-CPA, for three days before being subjected to a new EPM session.

In the first experiment, we observed the anxiogenic-like effect of acute paroxetine to be restricted to LO animals, hence accentuating the difference between LO and HO rats. This effect was counteracted by pre-treatment with escitalopram, which by itself also reduced the tendency for HO animals to spend time on the open arms, again attenuating group differences. In the second experiment, serotonin depletion by way of *p*-CPA abolished differences between HO and LO animals by exerting an anxiolytic-like effect in the latter group only.

3. Receptors mediating the acute anxiogenic effect of SSRIs (paper II)

The receptor subtypes mediating an acute exacerbation of anxiety by SSRIs are largely unknown, although there exists some evidence for a role of 5-HT2C receptors in this context[182,183]. We wanted to investigate the possible involvement of two less investigated serotonin receptor subtypes, 5-HT6 and 5-HT7, as well as of the 5-HT2C receptor, in relation to an acute SSRI-induced increase in EPM-assessed anxiety.

Two experiments were conducted. Animals were subdivided on the basis of their EPM performance in the same manner as in papers I and III and again tested in the EPM after three weeks. In experiment I the possible ability of the 5-HT6 antagonist SB399885 to block the anxiogenic effect of paroxetine was tested, while in experiment II the possible effects of the 5-HT2C antagonist RS102221 and the 5-HT7 antagonist SB269970 were explored.

In both experiments we could replicate our earlier finding that the acute anxiogenic effect of an SSRI is confined to LO animals. Whereas this effect could be blocked by prior administration of a 5-HT6 antagonist, 5-HT2C or 5-HT7 antagonists were ineffective.

4. Serotonin and sex differences in anxiety-like behaviour (paper III)

In humans, there exist considerable sex differences with respect to the trait neuroticism[149] and also with respect to the prevalence of mood and anxiety disorders[4]. There are likewise considerable differences between the sexes with respect to performance in animal models of anxiety (though, as discussed above, partly opposite to those seen in humans)[160,184].

Many lines of evidence point to an important role for serotonin in mediating or modulating behavioural effects of sex steroids and to uphold sex differences in behaviour. We thus wanted to investigate the role of serotonin in maintaining sex differences in EPM-assessed anxiety-like behaviour. Male and female Wistar rats were first subjected to two sessions in the EPM, two weeks apart, in order to study if the sexes habituated differently to the maze. After another four weeks, animals were

administered the serotonin-depleting agent *p*-CPA for three days and again tested in the EPM.

We could confirm earlier observations that females are significantly less prone to display high anxiety-like behaviour and that this difference held regardless of to what extent the animals had prior experience to the maze. We could also demonstrate sex differences in anxiety-like behaviour to be serotonin dependent.

Thus, while depleting brain serotonin to the same degree in both sexes, *p*-CPA did not affect indices of anxiety-like behaviour in the EPM in females while exerting a strong such effect in males. *p*-CPA was however not devoid of behavioural effect in female rats; a dimorphism in general locomotor behaviour (that had emerged after habituation) insofar that females exhibited higher overall locomotion was hence abolished by serotonin depletion as a result of *p*-CPA reducing locomotion in females but not in males.

5. Androgens, serotonin and temperament (paper IV)

Human[185] as well as animal studies[82,186] point to an important role of androgens in traits related to fear and neuroticism. We were therefore interested in investigating whether the differences observed in paper I between LO and HO rats could be partly mediated by circulating androgens, and to what extent an earlier observed anxiolytic-like influence of androgens is dependent on intact serotonergic neurotransmission.

Male Wistar rats were pre-tested with respect to EPM behaviour as in paper I and then castrated. After four weeks, the animals were again tested in the EPM, followed two weeks later by serotonin depletion by way of *p*-CPA and a third EPM session after which the untreated animals were sacrificed and the brains taken out for assessment of TPH2 mRNA levels.

We observed an anxiogenic-like effect of castration that was selective to the HO group and hence effectively abolished group differences. Subsequent *p*-CPA treatment counteracted this effect, indicating that an effect of circulating androgens on anxiety-like behaviour may be partly mediated through the serotonin system. We could also replicate our earlier finding that animals exhibiting higher levels of anxiety-like behaviour express higher levels of raphe TPH2. Furthermore, we observed a significant interaction between baseline temperament and gonadectomy insofar that a gonadectomy-induced decrease of TPH2 in LO animals, but an increase in HO

animals, led to an abolishment of temperament differences regarding TPH2 levels in the gonadectomized group.

6. Gene expression changes induced by acute serotonin elevation and depletion (paper V)

Despite the fact that numerous studies exist where changes in gene expression after manipulation of the serotonergic system have been studied, it remains largely unknown to what extent short-term changes in extracellular levels of serotonin affect the transcription of various genes of importance for serotonergic function.

To shed further light on this, we subjected male Wistar rats to either short-term treatment with an SSRI, paroxetine, or serotonin depletion induced by *p*-CPA, and assessed the effects of these treatments on the expression of a large number of serotonin-related genes in the raphe region, hypothalamus, amygdala, striatum, hippocampus and frontal cortex. After correction for multiple analyses we found surprisingly few significant effects; paroxetine however induced a robust down-regulation of TPH2 in the raphe region while *p*-CPA up-regulated BDNF in several regions. Whereas some receptor genes were up-regulated by serotonin depletion, *p*-CPA induced a marked reduction in the expression of the 5-HT6 receptor in the frontal cortex. With respect to brain regions, subacute administration of an SSRI appeared to exert greater influence in the hypothalamus, amygdala and striatum than in the frontal cortex.

7. Acute anxiogenic effects of SSRIs in depression trials (paper VI)

Though efficacious in treating anxiety disorders upon prolonged administered, the SSRIs may sometimes exacerbate anxiety during the first days of treatment, especially in patients with panic disorder[103]. This phenomenon, that has been variously termed "activation" or "jitteriness syndrome", has however been recognised to occur also in patients with depression[187,188]. Estimates of prevalence vary widely and no real consensus exists as to what symptoms should be included. A connection through akathisia with increased suicidality has been proposed[113,115].

Using a database comprising patient-level data from >8000 adults with major depressive disorder who had participated in company-sponsored placebo-controlled

trials, we performed analyses to test for the likelihood of an SSRI-induced worsening on six HAMD-17 items included in the "jitteriness syndrome" according to a review by Sinclair and co-workers[106]. We also tested for changes in mean scores on these items. Analyses were limited to the two first weeks of treatment.

Of the six items investigated, patients receiving SSRIs were significantly more likely to report worsening on two items at week 1; "late insomnia" and "somatic anxiety", and higher mean rating for one of these, i.e. “somatic anxiety”. As increased GI symptoms could tentatively partly explain an SSRI-induced increase in "somatic anxiety", such complaints included also in the definition of the “somatic anxiety” item, we re-ran the analysis with patients reporting GI worsening excluded; it however remained more common for patients given an SSRI than for those given placebo to report enhanced “somatic anxiety”. At week two, the effect on "somatic anxiety" had vanished, hence supporting this reaction to be transient in nature. Of note is that the item “psychic anxiety” was rated as less severe in the SSRI-treated group already after one week of treatment.

8. SSRI effects on suicidal behaviour in depression trials (paper VI)

There exist a controversy as to whether SSRIs can induce or exacerbate suicidal ideation in some patients during the first few weeks of treatment[113,115,189]. Using the same dataset as described above, we wanted to investigate if we could find support for such a notion.

To this end, we performed analyses to test for differences in the likelihood of an SSRI-induced worsening on item 3, suicidality, on the HAMD-17 scale, and also analyzed mean scores for this item, with focus on the two first weeks of treatment. We however chose to continue the analysis to week 6 in order to preclude the presence of any long-latency suicidogenic effect of the active treatment.

We did not find any support for an increase in the likelihood of worsening with respect to suicidal ideation in SSRI-treated patients, and also no increase in the mean scores for this item in the SSRI-treated group. On the contrary, we observed a significant reduction in suicidal ideation in SSRI-treated patients, that was present already at week 1, and which persisted and grew in strength throughout the weeks examined.

DISCUSSION

Serotonin and temperament differences

There is no consensus as to whether serotonin should be regarded as mainly facilitatory or dampening on anxiety. In paper I in this thesis we find inter-individual variation in anxiety-like behaviour in male Wistar rats to be accentuated by increased serotonin release and abolished by serotonin depletion. Furthermore, we find rats selected for high anxiety-like behaviour to be characterised by a greater potential for serotonin synthesis and/or release, as indicated by biochemical findings. Possibly, this increase in serotonin activity in "anxious" animals could be of a compensatory nature, serving to dampen anxiety generated by non-serotonergic neurons, but one would then expect serotonin depletion to *enhance*, not abolish, the behavioural differences. In short, we therefore conclude that our data support the view that serotonin acts mainly to promote anxiety.

This is in line with results indicating heightened TPH2 levels in animals in which anxiety-like behaviour has been induced by non-pharmacological methods[176,190-192] as well as with results from various genetic models[51,193]. The notion that LO rats are characterized by a stronger serotonergic innervation also gains indirect support from a report[194] showing rats bred for high anxiety to display enhanced SERT binding in hippocampus and enhanced serotonin release in the same brain region when exposed to a stressor (in the form of an EPM session) in conjunction with serotonin reuptake inhibition.

The fact that the anxiogenic-like effect of SSRIs also in rats would seem to be limited to animals displaying high levels of "anxiety" is well in line with findings both in clinical populations[104,179] and in healthy volunteers[107,180]. The effects of long-term SSRI treatment seem to be of a stabilising nature; while exerting an anxiogenic-like effect in HO rats it prevented an anxiogenic-like effect of acute SSRI administration in LO animals.

Receptors mediating an anxiogenic-like influence of serotonin

An acute anxiogenic effect of acute serotonin release by SSRIs and other agents has been known to occur in some patients receiving these drugs. We wanted to investigate the serotonin receptors mediating a putative animal model of this response.

We replicated our finding that only "anxious" animals are susceptible to an anxiogenic effect of acute SSRIs and also demonstrated that this effect can be blocked by 5-HT6 but not by 5-HT2C or 5-HT7 antagonists. We hence conclude that the 5-HT6 receptor subtype is involved in the acute anxiogenic effect of SSRIs in the EPM paradigm.

Our suggestion that acute effects of SSRIs may partly by mediated by 5-HT6 receptors are in line with a report by Svenningsson and co-workers[195] reporting a 5-HT6 receptor antagonist to block the effects of fluoxetine in the tail suspension test, and also with respect to fos expression, while exerting no effect on these parameters *per se*.

Our observations are however to some extent in contradiction to earlier results where 5-HT2C antagonists have been shown to be able to block an anxiogenic influence of acute SSRI administration[182]. Possible explanations for this discrepancy may include various methodological aspects such as differences between the tests used to assess anxiety; our use of animals selected as anxious, the influence of animals having habituated to the maze[196] etc.

Serotonin and sex differences in anxiety-like behaviour

There exist sex differences in regards to anxiety as well as to serotonergic transmission. We can in paper III demonstrate differences in anxiety-like behaviour in rats to disappear after serotonin depletion; we thus find sex differences in EPM-assessed anxiety-like behaviour in rat to be serotonin-dependent.

This is unlikely to be due to *p*-CPA being more efficacious in depleting brain serotonin in males, as no sex difference existed in regards to degree of depletion. Furthermore, depletion was not devoid of behavioural effects in females; a robust reduction of locomotion as reflected by entries into the closed arms were hence observed.

Androgens, serotonin and temperament differences

Both androgens and serotonin have been demonstrated to be important for variation in anxiety-related traits in humans as well as for anxiety-like behaviour in rodents. How they interact is however unknown. We here demonstrate that variation in anxiety-like behaviour in male rats is dependent on circulating male androgens, with "brave" animals becoming less so after castration. This effect is however abolished by serotonin depletion, indicating that an anxiolytic effect of circulating androgens is mediated through an influence on serotonergic neurotransmission, most likely a reduction in serotonergic output.

This would indicate that serotonin and androgens interact in a more direct manner with respect to the regulation of EPM performance than in the case of their regulation of sexual and aggressive behaviour. With respect to the latter aspects of behaviour, they hence appear to influence the behaviour in question in a parallel manner, *p*-CPA not being able to abolish the behavioural effects of castration[86].

Gene expression changes induced by short-term manipulation of serotonin levels

Interpretation of gene expression findings in relation to the serotonergic system would be facilitated by a greater understanding of the putative responsiveness of gene expression to manipulation. We here find that short-term depletion or elevation of synaptic serotonin have relatively minor effects on the expression of a number of serotonergic genes in six brain areas. Regarding short-term SSRI treatment we only detected an effect on raphe TPH2 expression, likely a compensatory down-regulation. The most striking effects induced by *p*-CPA were an up-regulation of the BDNF transcript levels in several areas and a down-regulation of the 5-HT6 receptor in frontal cortex. We conclude that, for most of the genes investigated, any adaptive mechanisms present at the early stages of changes in extracellular levels of serotonin would likely not involve an altered regulation of transcription.

Increased anxiety and suicidal behaviour when initiating SSRI treatment

Though effective in treating anxiety disorders, SSRIs can sometimes induce increased anxiety early in treatment, but there is uncertainty regarding the nature as well as the prevalence of this phenomenon. There is also a long-standing controversy about a

possible increase in suicidal behaviour upon the initiation of treatment with these drugs. In paper VI we find a modest increase in the SSRI group of somatic but not psychic anxiety that was limited to the first week. We also find no support for an increased suicidogenic influence of SSRI but instead observe lower scores of suicidal ideation in SSRI-treated patients when compared to the placebo group already after one week of treatment. It is however important to note that suicidal ideation at baseline often is an exclusion criterion when conducting placebo-controlled depression trials and the studies investigated thus generally only included patients with low baseline suicidality; hence we cannot make any statements as to possible SSRI effects in patients with marked suicidal behaviour already at the start of treatment. Needless to say, the observation that SSRI-treated subjects, regarded as a group, displayed lower mean suicidal ideation already after one week of treatment also does not exclude the possibility that an anxiety-provoking effect may occur in some patients, but is masked by the opposite effect occurring in others. Our results however refute the claim than an SSRI-induced increase in suicidal ideation should be a common phenomenon.

Worth pointing out is that we observed a significant *lowering* of not only suicidality but also psychic anxiety after one week of treatment. Though beyond the scope of this paper, this observation has bearing on the subject of the time to therapeutic effect of these drugs. The early positive effect on these items thus indicates that the alleged delay with respect to onset of action for the clinical effect antidepressants may to some extent be an artefact based on the use of HAMD-17 sum score to measure treatment effect, early side-effects captured by this instrument tentatively masking an early improvement. Though weak in comparison to the effect at week 6, the fact that such a symptom-reducing effect exists already after one week of treatment is of considerable interest from a pre-clinical perspective since it refutes the view that the mechanism of action of SSRIs must be one that requires weeks of treatment (such as the formation of novel neurons).

SAMMANFATTNING PÅ SVENSKA

Avhandlingens huvudtema är förhållandet mellan hjärnans serotonerga neurotransmission och ångest. Den kan kort beskrivas som ett försök att besvara frågan om serotonin framförallt skall ses som en ångestskapande eller -dämpande faktor, med ett särskilt fokus på serotoninets roll för att upprätthålla skillnader i ångest mellan såväl som inom könen samt svara på frågan varför en del men inte alla patienter rapporterar ökad ångest i initialskedet av behandling med selektiva serotoninåterupptagshämmare (SSRI:s).

Avhandlingen består av sex artiklar där de fyra första rör djurstudier som behandlar serotoninets roll i att modulera skillnader såväl mellan som inom könen vad gäller ångestliknande beteende hos råtta. Den femte artikeln rör effekter på uttryck av ett antal för serotoninsystemet viktiga gener av akut höjning respektive sänkning av hjärnans serotoninnivåer. Den sjätte artikeln är en studie av registerdata från ett stort antal läkemedelsprövningar av SSRI-preparat för behandling av depression. Här undersöker vi frekvens och egenskaper hos en ibland, framförallt hos patienter med ångestsjukdom, sedd paradoxal ångesthöjande effekt som kan ses tidigt i SSRI-behandling. Dessutom undersöker vi i vilken mån stöd finns för farhågor om en ökning av självmordstankar och -beteende i samband med inledning av SSRI-behandling.

I artikel I undersöker vi effekter av akut serotoninhöjning och -sänkning på ångestliknande beteende hos råttor. Vi finner här att ju mer rädda djur varit i ett tidigare test, desto mer svarar de med ökat ångestliknande beteende när de får en dos av ett SSRI. Det här är ett fynd med klara paralleller i den akuta ångesthöjande effekt som ibland kan ses i människor med ångestdiagnoser. Vi kan också demonstrera att skillnaderna mellan djur som varit rädda och djur som varit djärva i ett tidigare test helt slås ut om serotoninsystemet aktiveras på farmakologisk väg med serotoninsynteshämmaren *para*-klorofenylalanin (*p*-CPA). Vi kan slutligen demonstrera att hög grad av ångestliknande beteende hos råttor associerar med neurokemiska fynd som indikerar en högre serotonerg aktivitet, inte minst har mer rädda djur högre nivåer av enzymet tryptofanhydroxylas 2 (TPH2), vilket bestämmer syntestakten för serotonin.

I artikel II undersöker vi vilka serotoninreceptorer som kan ligga bakom denna akuta höjning av ångestliknande beteende och finner stöd för att det är en receptor vid man 5-HT6. Vi kan även bekräfta våra fynd i artikel 1 vad gäller att känslighet för SSRI

enbart gäller mer rädda djur.

I artikel III undersöker vi serotoninets roll i att upprätta könsskillnader i ängslighetsliknande beteende. Vi kan replikera andras fynd att råtthonor generellt är mer djärva än råtthanar samt demonstrera att den här skillnaden helt försvinner efter serotoninuttömning med *p*-CPA. I artikel IV studerar vi hur manliga könshormoner påverkar variation i temperament och finner att skillnader mellan djärva och rädda hanråttor försvinner vid kastration såtillvida att djärvare råttor då uppvisar högre ångestliknande beteende. Vi visar sedan att denna effekt slås ut om djuren också behandlas med serotoninsynteshämmaren *p*-CPA; detta tolkar vi som att manliga könshormoner bidrar till skillnader i temperament genom att vara en hämmande faktor för serotoninets ångestsfrämjande effekt.

I artikel V finner vi att akut serotoninhöjning via tre dagars SSRI-behandling samt serotoninuttömning med *p*-CPA har små effekter på mRNA-nivåer hos ett antal viktiga serotonerga gener. Detta tolkar vi som att gener i serotoninsystemet är relativt okänsligt även för stora förändringar i serotoninnivåer och att eventuella kompensatoriska mekanismer troligen framförallt inte finns på genuttrycksnivå.

I artikel VI finner vi att SSRI ökar kroppsliga ångestsymptom under den första behandlingsveckan men att dessa försvinner till vecka två. Vi finner också att vad gäller psykiska ångestymptom så minskar de hos SSRI-behandlade redan vid vecka ett. Vi finner inget stöd för en ökning av självmordstankar eller -beteende i vårt material; tvärt om finner vi här, liksom för psykisk ångest, en skyddande effekt som finns redan efter en veckas behandling. Viktigt att påpeka är att man inte brukar inkludera patienter som uppvisar suicidalitet i studier där de kan riskera att få placebo; vi kan således inte uttala oss om situationen hos patienter med hög suicidalitet redan vid behandlingsstart.

ACKNOWLEDGEMENTS

Financial support for my thesis work was obtained from the by the **Swedish Science Council**, the **Brain Foundation** and **Söderberg's Foundation**. In particular we would like to thank professor **Bertil Hållsten** for encouraging interest and generous support.

Further, I wish to offer my heartfelt thanks to all the following:

My supervisor **Elias** for good cheer and kindness, generosity with your time as well as with your considerable knowledge, confidence in me, impressive patience with my 'research ADHD' and for introducing me to the wonderful world of the monoamines. You are an inspiration and I am sure that I will not fully appreciate all the effort you have put into me until I get PhD students that don't do as they are told of my own.

My co-supervisor **Lars** for kindness and sagacious advice (especially about strategies for handling main supervisors).

Erik, for being a great friend, co-worker, room-mate and sparring partner in political discussions during these years.

Nina and **Sara**, for humouring me when I drop by to talk way too long on the way to the coffee machine and not making faces when I whish you a 'good morning' around noon or so.

Daniel, for endless patience in matters relating to staplers. (And for all your other excellent qualities as well.)

Fredrik and **Daniel** for having just as bad humour as I do, as well as for having great taste in computer and board games and beer, among other things.

Martin for many great conversations and for your considerable proficiency in organising parties.

Johan Fredrik for entertaining stories about hunting, going on tour with choirs and the caprices of the Landsting.

Robert and **Nina** for, among other things, bringing the Dachshund, being excellent travelling companions and organising great parties.

Gunilla, **Ann-Christin** and **Inger** for kindness and for all your help in the lab and at EBM.

Melker, **Alana**, **Patrik**, **Mike**, **Britt-Marie**, **Agneta**, **Hans**, **Jonas**, **Elisabet**, **Petra**, **Richard**, **Dinko**, **Carl**, **Therese**, **Sofia**, **Jaroslav**, **Elin**, **Johanna**, **Anna**, **Jesper**, **Camilla**, **Ghazaleh**, **Carl-Henrik**, **Anna-Lena**, **Daniel**, **Christer** and all the rest, past and present, at the department of Pharmacology. One couldn't possibly hope for better co-workers!

Staffan, for all your help in matters of statistical arcana.

Petronella, **Caroline**, **Karolina** and **Anna** for appreciating the darker subcultures and for combining this with good cheer, beer and grilled cheese.

Elin and **Catrin** for being such great friends and for making med school much more enjoyable. I miss you!

Arvid and **Fredrik**, for so many laughs and happy memories.

Jacob, **Alexandra**, **Agnes**, **Lisa**, **Karin**, **Frida**, **Elin**, **Maria**, **Martina**, **Gabriella**, **Malin**, **Lovisa**, **Sassa**, **Giovanni**, **Anders**, **Daniel**, **Björn** and **Linda** for being such wonderful friends.

My cousin **Albert** for introducing me to research back in 2000, as well as for getting me to switch to Apple.

Jessica, for the illustrations and for turning what otherwise would have been a very stressful and tedious spring into something else entirely.

My parents, **Alice** and **Mikael** and my little sister **Karin** for your endless love and support.

APPENDIX: MATERIALS AND METHODS

ANIMAL STUDIES (PAPERS I-V)

Ethics

All procedures were carried out with approval of the local ethics committee and in accordance with institutional guidelines.

Animals

Wistar rats were used, aged 10-11 weeks at arrival. Animals were housed under controlled conditions: temperature 21-22°C, humidity 55-65% and a 12-h light/dark cycle (lights on at 6 AM). Food (standard chow) and water were available ad libitum except during experiments.

Drugs

p-CPA (Sigma-Aldrich, St Louis, MO, USA) was dissolved in 0.9% saline and administered i.p. as one injection of 300 mg/kg per day for three days with the last injection being given 24 h before animals were tested in the EPM (papers I, III-V) or euthanasied (paper V). Escitalopram oxalate (Shodana Labs, Hyderabad, India) was admixed into food pellets (Lantmännen, Kimstad, Sweden) at a concentration (0.65 g/kg) aimed at providing a daily dose of 25-30 mg/kg. Paroxetine hydrochloride (Jai Radhe Chemicals, Ahmedabad, India) was dissolved in 0.9% saline and administered s.c. at a dose of 10 mg/kg 1 h (40 min in paper II) before an EPM session (papers I-III & V) or x2/day for three days, the last injection being given on the morning of euthanasia (paper IV). SB-399885, SB-269970 and RS-102221 Tocris, Bristol, UK) was dissolved in 0.9% saline and administered at doses of 1, 1 and 2 mg/kg respectively, 45 min before animals were tested in the EPM (paper V).

Behavioural tests (papers I-IV)

The EPM apparatus (Med Associates, St. Albans, VT, USA) was made of black acrylic plastic with closed arms measuring 10x50x40 cm and open arms measuring 10x50x1

cm. Tests were carried out between 09.00 and 16.00 and at a light level of 40 lux provided by a single incandescent matte light bulb positioned 0.8 m above the centre.

Temperament groups (papers I, II, IV) were created in the following manner: the 1/3 most prone to spend time on the open arm was taken to constitute one group presumably characterised by low “anxiety” (high open arm, HO) and the 1/3 most prone to avoid it constituting another, tentatively more "anxious", group (low open arm, LO). The middle group was always excluded from all further analyses in order to avoid misclassification of animals belonging to this group but being close to one of the extreme groups.

Biochemistry

Dissection (papers I, III, IV and V)

Animals were sedated with isoflurane and then decapitated by way of guillotine. Brains were extracted immediately after decapitation and tissue samples were immediately frozen on dry ice and stored at -80°C.

Gene expression analyses (papers I, IV and V)

Individual samples of brain tissue were homogenized in Qiazol (Qiagen, Hilden, Germany) using a TissueLyzer (Qiagen). Total RNA was extracted with an RNeasy Lipid Tissue Mini Kit (Qiagen) using a QiaCube (Qiagen). RNA quality and quantity were assessed by spectrophotometric measurements (Nanodrop 1000, NanoDrop Technologies, USA). For cDNA synthesis, 4000 ng of total RNA was reversely transcribed using random hexamers (Applied Biosystems, Sundbyberg, Sweden) and Superscript III reverse transcriptase (Invitrogen Life Technologies, Paisley, UK) according to the manufacturer's description. Recombinant RNaseout® Ribonuclease Inhibitor (Invitrogen) was added to prevent RNase-mediated degradation. All cDNA reactions were run in duplicate and the products pooled for further analysis.

Real-time qPCR was performed by means of TaqMan® Custom Arrays using TaqMan probe and primer sets for target genes and reference genes chosen from an on-line catalogue (Applied Biosystems). The sets were factory-loaded into the 384 wells of TaqMan® Array (papers I and IV), each port being loaded with cDNA corresponding to 500 ng total RNA combined with nuclease free water and 50 µl TaqMan® Gene Expression Master Mix (Applied Biosystems) to a final volume of 100 µl. The TaqMan Arrays were analyzed using the 7900HT system with a TaqMan Array Upgrade (Applied Biosystems). Thermal cycling conditions were 50°C for 2 min and 94.5°C for 10 min, followed by 40 cycles of 97°C for 30 s and of 59.7°C for 1 min. All reactions

were run in duplicate and reactions were excluded if the CT values of the duplicates differed by more than 5%.

In paper IV a slightly different approach was used. TaqMan probe and primer sets for TPH2 and two control genes were ordered from the on-line catalogue. The sets were loaded onto 384 well plates by a pipetting robot with each well being loaded with cDNA corresponding to 60 ng of RNA, 4.5 µl nuclease free water, 0.5 µl TaqMan® assay and 5 µl TaqMan® Gene Expression Master Mix (Applied Biosystems) to a final volume of 10 µl. The plates were analysed using the QuantStudio 12 k Flex (Life Technologies, Carlsbad, CA, USA). Thermal cycling conditions were 50°C for 2 min and 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and of 60°C for 1 min. All reactions were run in triplicate and in the presence of several non-template controls.

The most stable pair out of four (in experiment IV, two) reference genes was calculated using the NormFinder algorithm (http://moma.dk/normfinder-software) and used to normalize expression levels in areas examined. Gene expression values were calculated based on the $\Delta\Delta C_t$ method.

Western Blot analysis of TPH2 levels (paper I)

A user-developed protocol available at the Qiagen homepage (http://www.qiagen.com/products/rnastabilizationpurification/rneasysystem/rneasylipidtissuemini.aspx#Tabs=t2) describing the isolation of proteins from fatty tissue samples treated with QIAzol Lysis Reagent was used. Briefly the protein pellet was solubilized in buffer (500 µL) containing 8 M urea and 50mM dithiothreitol. Samples were tip sonicated on ice for four times with four seconds bursts followed by four seconds rest. Samples were however not heated to 95 °C for 3 minutes, as suggested in the protocol, in order to avoid carbamylation of proteins by the urea buffer.

After centrifugation for 15 min at 10000×g, the protein containing supernatants were transferred to new tubes and total protein concentration was quantified using the Pierce 660 nm protein assay kit (Thermo Fisher Scientific, Rockford, Illinois). All samples were analyzed in duplicate by fluorescent Western blotting. Protein samples containing 30 µg total protein in 1×NuPAGE LDS sample buffer (Invitrogen) was applied to SDS-polyacrylamide gel electrophoresis (PAGE) on 4-12% NuPAGE Bis-Tris gels (Invitrogen) with 1× MES buffer (Invitrogen) for 40 min at 200V. Proteins were electrophoretically transferred to low fluorescent PVDF membranes (Millipore, Billerica, MA) in the XCell II Blot module (Invitrogen) using 1×NuPAGE transfer

buffer (Invitrogen) containing 20% methanol at 35V for 1h. Membranes were washed with TBS-Tween (0.01%) and blocked with 1×Roti-Block (Carl Roth, Karlsruhe, Germany) for 15 min followed by 2 % BSA (Sigma-Aldrich, St Louis, MO, USA) in TBS-Tween for 15 min. The blocked membranes were transferred to the SNAP i.d. protein detection system (Millipore) and blocked with 2% BSA. Membranes were immunoprobed with a polyclonal rabbit anti-TPH2 antibody (TPH2, NB-100-74555, Novus Biologicals, Cambridge, UK) (1:300) in 0.5×Roti-Block in TBS-Tween and incubated at room temperature for 30 min.

A negative membrane with identical samples loaded was incubated without TPH2 antibody in 0.5× Roti-Block in TBS-Tween for 30 min with the purpose of subtracting any background created by the secondary antibody. Membranes were extensively washed with TBS-Tween and incubated with a fluorescent Cy3 conjugated goat anti-rabbit IgG secondary antibody (1:500, Millipore) in 1% BSA in TBS-Tween for 15 min at room temperature. They were then thoroughly washed in TBS-Tween followed by three washes in TBS. The immunoreaction was visualized using a VersaDoc MP 4000 (BioRad, Hercules, CA, USA) CCD camera equipped with a Cy3 excitation filter. The intensity of total protein extracts were analyzed on Coomassie stained gels to confirm equal staining intensity of the protein extracts to assure equal loading of the gels. Semi-quantitative analysis of protein expression was performed by densitometry using the ImageLab v.3.0 software. Each sample was run in duplicate the mean intensity of which was used. All samples were normalised versus a control sample present on each membrane.

Statistical analyses

T-tests or ANOVA with LSD post-hoc test were generally used to test for differences between treatment and/or temperament groups. In the case of non-normal distributions data were first log-transformed or non-parametric tests were used (paper II). ANCOVA was used to test for interaction effects (paper I and IV). Analyses were performed using SPSS for Mac, version 21 (Chicago, IL, USA)

HUMAN STUDIES (PAPER VI)

Ethics

The Regional Ethical Review Board of Gothenburg, Sweden, has reviewed the study protocol and issued an advisory opinion stating no objection to the conduct of this study.

Statistical analyses

We employed SAS for Windows, version 9.4 (SAS Institute, Cary, NC, USA) for the PROC GLIMMIX and PROC MIXED programs and SPSS for Mac, version 21 (Chicago, IL, USA) for all other statistical procedures.

For the comparison of the two treatment groups with respect to the likelihood of symptom aggravation from baseline to visit 1 and visit 2, respectively, a generalized linear mixed model using a binary response and logit link with study, treatment, time (week) and the time-by-treatment interaction included as categorical predictors, and item score at baseline included as a covariate, was used. The correlated nature of the weekly estimates within patients was modelled as a repeated effect using an unstructured covariance matrix; if the model did not converge using the unstructured covariance matrix we instead specified an autoregressive heterogeneous covariance matrix.

The parameter under study being the proportion of patients who experienced a worsening of the specific symptom as compared to their baseline rating, as such we excluded cases already displaying a maximum score on the item in question at baseline since any further decline could not possibly be detected by the rating instrument. Since the definition of the item somatic anxiety in HDRS includes gastrointestinal complaints, and since it is well established that SSRIs may induce gastrointestinal side effects that are not associated with heightened anxiety, we repeated the analyses regarding this particular item after omitting all patients showing a worsening of the HDRS item specifically addressing gastrointestinal complaints (item 12).

For the comparison of mean differences at visits 1 and 2 we used a linear mixed model. The model included the same categorical predictors and covariates as the binary response model above, and again the correlated observations was modelled as a

repeated effect using an unstructured covariance matrix when possible and an autoregressive heterogeneous one when not. As in the binary response model, the analysis regarding somatic anxiety was repeated after excluding all subjects displaying a simultaneous worsening of gastrointestinal symptoms.

To assess change throughout the entire treatment period for the two items displaying higher mean ratings in SSRI-treated patients than in those given placebo at visit 1, i.e. somatic anxiety and gastrointestinal complaints, the same linear mixed model as described above but extended to six weeks duration was employed. Notwithstanding the fact that there were no indices of an SSRI-induced aggravation at visit 1, the same technique was used also to assess changes in suicidal ideation throughout 6 weeks of treatment.

The chi square test of independence was used to explore possible differences in early drop-out rates between SSRI- and placebo-treated subjects. As many studies did not contain information on time of drop-out we chose to use absence of a visit during the first and second week post-randomization as a proxy measure for early drop-out.

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