Influence of serotonin on anxiety-like behaviour in rat

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Gothenburg 2018

Cover illustration by Robert Pettersson

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ISBN 978-91-7833-143-7 (PRINT)
ISBN 978-91-7833-144-4 (PDF)

Printed in Gothenburg, Sweden 2018
Printed by BrandFactory

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that contributed to this work

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ABSTRACT

It is well-established that serotonin is involved in the regulation of mood and behaviour, partly implied by the therapeutic effect of prolonged treatment with selective serotonin reuptake inhibitors (SSRI) on mood and anxiety disorders. However, the mechanisms behind the paradoxical exacerbation of anxiety described during initial treatment, as well as behind the therapeutic effects of prolonged treatment with SSRIs, remain poorly understood.

In the research described in this thesis, the effects of different manipulations of the serotonin system on anxiety-related behaviour was studied in rat models of contextual conditioned fear.

In Paper I, we investigated whether rats could be classified as more or less 'anxious' based on two behaviours, namely startle and freezing, and to what extent this baseline 'anxiousness' predicted subsequent startle and freezing behaviour in the response to acute administration of an SSRI, escitalopram, with or without further aggravation of fear by so-called contextual conditioned fear conditioning. Startle and freezing correlated and showed good inter-test stability before contextual conditioning and were both enhanced by

escitalopram, where the enhancement of startle was more pronounced in animals with high startle and freezing before contextual conditioning. This resembles the clinical picture, in the sense that anxiety-prone patients can experience worsening of their anxiety symptoms during early medication with SSRIs.

The work of Paper II evaluated whether startle and freezing in the model of Paper I were dependent on serotonin and whether sex was an important factor. Startle and freezing responses were assessed in male and female rats after treatment with a serotonin-depleting agent, para-chlorophenylalanine (PCPA), with or without prior contextual conditioning. The main finding, i.e. that PCPA reduced contextual conditioned freezing solely in male rats, thereby abolishing a sex difference in this parameter, indicates that both sex and serotonin can influence anxiety-like behaviour.

In Paper III, the possible involvement of specific serotonin receptor subtypes for the effect of acute SSRI administration on contextual conditioned freezing was examined. Without exerting any effect on their own, the combination of a 5-HT2A receptor antagonist and escitalopram resulted in a pronounced reduction in freezing behaviour. A remarkable freezing reduction was also observed after administration of any of two agents normally causing a more robust increase in extracellular serotonin than do the SSRIs, i.e. 5-hydroxytryotophan (5-HTP) (a serotonin precursor) or fenfluramine (a serotonin releaser). It is suggested that the effect of 5-HT2A antagonism may be mediated by inhibition of negative feedback leading to higher extracellular serotonin, but it is also possible that it unmasks an anti-freezing effect of escitalopram by blocking freezing-promoting postsynaptic receptors normally activated by the SSRI. The possibility that the anti-freezing effect of escitalopram plus the 5-HT2A antagonist is mediated by postsynaptic 5-HT1A receptors was explored; however, this appeared not to be the case.

The experiments described by Paper IV showed that long-term treatment with escitalopram reduced contextual freezing whereas acute escitalopram administered at a dose causing similar serum levels of the compound did not influence the behaviour. These results mirror the clinical situations and suggest this model as useful for studying mechanisms underlying the effect of short- versus long-term SSRI treatment. It remains unsolved whether the reduction in freezing by long-term SSRI treatment is caused by a negative or positive

influence on extracellular serotonin level; since a powerful reduction of freezing may be obtained both by serotonin-reducing and serotonin-enhancing agents, neither of these options can be ruled out. The finding that the freezing-reduction caused by long-term SSRI could not be reversed by serotonin-enhancing treatment however favours the latter possibility.

In conclusion, the experiments indicate (1) that there are notable similarities between the studied behaviours and human anxiety (2) that intact serotonergic transmission seems important for freezing behaviour and (3) that the 5-HT2A receptor seems to play an important role in the underlying mechanisms.

Keywords: Anxiety, Serotonin, SSRI
ISBN 978-91-7833-143-7 (PRINT)
ISBN 978-91-7833-144-4 (PDF)

SAMMANFATTNING PÅ SVENSKA

Trots att läkemedel som påverkar nivån av signalsubstansen serotonin i hjärnans synapser, till exempel selektiva serotoninåterupptagshämmare (SSRIs), utgör den vanligaste behandlingen mot depression och ångestsjukdomar, har ännu ingen förklaring hittats till hur behandlingen egentligen utövar sin effekt. Kortvarig behandling med dessa läkemedel åstadkommer i sig ingen märkbar antidepressiv eller ångestdämpande effekt, utan kan snarare förvärra viss typ av ångest. Det är först efter fortsatt behandling som de önskade effekterna uppstår och perioden dessförinnan kan vara förenad med mycket lidande. Forskning rörande mekanismerna bakom såväl de ångestförstärkande egenskaperna av tidig behandling som de ångestdämpande egenskaperna av fortsatt behandling med SSRIs är därför angelägen. I detta projekt var syftet att undersöka dessa mekanismer med hjälp av djurförsök i en beteendemodell av ångestsjukdom där rädslobeteende förstärks med hjälp av lätta elektriska fotchocker – så kallad kontextuell konditionering.

I det första delarbetet (Paper I) studerades i vilken grad råttor går att dela upp i mer eller mindre ”ångestbenägna” baserat på två inom forskningen välstuderade rädslobeteenden – startle, det vill säga ryckighet vid oväntat ljud, och freezing, ett slags spänt stillasittande som gnagare kan uppvisa vid hot – samt hur akut SSRI-behandling och kontextuell konditionering påverkar dessa beteenden. Syftet var att undersöka om mänskligt beteende, där ångest kan förvärras vid tidig SSRI-behandling hos vissa individer, går att avspegla i denna modell. Både startle och freezing visade sig vara stabila mellan tester och korrelera med varandra inom tester, vilket kan tyda på att de avspeglar en liknande typ av rädsla hos råttorna. Dessutom syntes en tydlig förstärkning av både startle och freezing efter kontextuell konditionering liksom vid akut SSRI-behandling, vilken var mer kraftfull vad avser startle hos djur som uppvisat mer startle och freezing före behandling. Dessa fynd tyder på att mätning av dessa beteenden utgör en användbar modell för att undersöka mekanismer bakom förvärrad ångest vid tidig SSRI-behandling.

Syftet med det andra delarbetet (Paper II) var att undersöka huruvida startle och freezing uppvisar könsskillnader och i vilken grad de påverkas av avstängd serotoninfrisättning i samma modell som i det första delarbetet. Hannar visade betydligt mer konditionerad freezing, men vid serotoninavstängning minskade

hannarnas freezing till en nivå motsvarande honornas. Dessa fynd är i enlighet med tidigare forskning som visat att serotonin kan upprätthålla vissa könsskillnader i beteende.

Det tredje delarbetet (Paper III) gick ut på att utreda huruvida de serotonerga receptorerna 5-HT1A, 5-HT2A och 5-HT2C kan påverka freezing efter konditionering, samt vilken effekt akut SSRI-behandling har då dessa receptorer blockeras. Medan akut SSRI och blockad av 5-HT2A-receptorn inte utövade någon påtaglig effekt på freezing när de gavs separat åstadkom kombinationen av de båda behandlingarna en kraftig minskning av freezing. Även behandling med andra farmaka som höjer de extracellulära nivåerna av serotonin dämpade freezing. Resultaten indikerar att blockad av 5-HT2A-receptorn möjliggör för SSRI-preparatet att minska freezing antingen genom att förändra balansen mellan freezing-hämmande och freezing-förstärkande receptorer,
eller genom att förstärka effekten av SSRI-medlet på de extracellulära serotoninnivåerna. Resultaten talar för att det kan vara gynnsamt att kombinera SSRI-medel med 5-HT2A-blockerare vid behandling av depression och ångetssjukdom, och att en möjlig förklaring till att långvarig SSRI-behandling dämpar ångest skulle kunna vara att behandlingen leder till nedreglering av 5-HT2A receptorn.

I delarbete fyra (Paper IV) undersöktes effekten av kort- respektive långvarig SSRI-behandling för att belysa om den ångestdämpande effekten av kontinuerlig SSRI-behandling hos människor går att efterlikna hos råttor. I likhet med vad som gäller för ångestsjukdom hos människa dämpades konditionerad feeezing av långvarig men inte kortvarig SSRI-behandling. Som tidigare visats i delarbete II gav även en substans som minskar den serotonerga aktiviteten upphov till minskad freezing, vilket skulle kunna tala för att SSRI utövar sin anti-freezing-effekt genom att nedreglera den serotonerga aktiviteten; tillförsel av en försubstans till serotonin, som ökar extracellulärt serotonin, hos SSRI-behandlade djur kunde dock inte motverka SSRI-behandlingens anti-freezing-effekt. Resultaten talar för att en intakt serotoninsignalering är nödvändig för freezingbeteende, och att denna störs av subkronisk men inte av akut tillförsel av SSRI. Däremot kunde av dessa försök inte klarläggas om SSRI-behandling minskar freezing genom att förstärka eller dämpa den serotonerga aktiviteten, eller genom någon annan mekanism, som t ex att påverka de receptorer som medierar serotoninets effekter.

Sammanfattningsvis tyder våra fynd på att det finns stora likheter mellan det beteende vi studerar och mänsklig ångest, bland annat vad gäller effekt av SSRI-behandling, att ostörd serotoninsignalering är viktig för freezingbeteende, samt att 5-HT2A receptorn verkar spela en viktig roll för dessa mekanismer.

LIST OF PAPERS

I. Pettersson R, Näslund J, Nilsson S, Eriksson E, Hagsäter SM. Acute escitalopram but not contextual conditioning exerts a stronger "anxiogenic" effect in rats with high baseline "anxiety" in the acoustic startle paradigm. Psychopharmacology (Berl), 2015, 232:8.

II. Pettersson R, Hagsäter SM, Eriksson E. Serotonin depletion eliminates sex differences with respect to context-conditioned immobility in rat. Psychopharmacology (Berl), 2016, 233:8.

III. Pettersson R, Hagsäter SM, Näslund J, Holmäng A, Pettersson C, Eriksson E. Antagonism of the 5-HT2A receptor unmasks an anxiolytic effect of acute SSRI treatment in the contextual fear paradigm. *Preliminary manuscript*

IV. Hagsäter SM, Pettersson R, Carlsson B, Karlsson L, Eriksson E. Chronic escitalopram administration reduces contextual conditioned fear. *Preliminary manuscript*

CONTENT

ABBREVIATIONS

ANOVA	Analysis of variance
BNST	Bed nucleus of the stria terminalis
CS	Conditioned stimulus
DMSO	Dimethyl sulfoxide
EPM	Elevated plus maze
GAD	Generalized anxiety disorder
HCl	Hydrochloric acid
5-HT	5-hydroxytryptamine; serotonin
5-HTP	5-hydroxytryptophan
LSD	Lysergic acid diethylamide (hallucinogenic drug) – or – Least significant difference (statistical post-hoc test)
MAO	Monoamine oxidase
MAOi	Monoamine oxidase inhibitor
MDMA	3,4-Methylenedioxymethamphetamine
NaOH	Sodium hydroxide
OCD	Obsessive-compulsive disorder
PCPA	*para*-Chlorophenylalanine
PTSD	Post-traumatic stress disorder
RCF	Relative centrifugal force
SERT	Serotonin transporter
SSRI	Selective serotonin reuptake inhibitor
TCA	Tricyclic antidepressant
TPH	Tryptophan hydroxylase
US	Unconditioned stimulus
VMAT	Vesicle monoamine transporter

1. INTRODUCTION

1.1. Anxiety disorders

As opposed to fear, which is the response to an actual threat or danger, anxiety is characterized by worry, nervousness, or unease about something with an uncertain outcome. Anxiety can be rational but can also, when occurring frequently and beyond control, be a sign of mental illness. A common distinction is between state and trait anxiety, where state anxiety indicates an acute and transient feeling of anxiety and trait anxiety is persistent and part of a person's personality [1].

Anxiety disorders are disorders in which anxiety is the dominating symptom. Together, they comprise the most common class of psychiatric disorders, with a lifetime prevalence of approximately 30%, showing higher prevalence in women than in men [2].

The symptoms additional to anxiety, when the anxiety appears, and the nature of the anxiety, as described in the fifth edition of Diagnostic and Statistical Manual of Mental Disorders (DSM-V), are factors that decide which specific anxiety disorder a patient can be diagnosed with.

- *Panic disorder* is characterized by reoccurring unexpected panic attacks with symptoms such as palpitations, increased heart rate, a feeling of choking, sweating, trembling and shaking. Such a panic attack is often followed by a period during which the person is afraid of getting more panic attacks, which may lead to a changed behaviour where the person avoids social situations.

- *Generalized anxiety disorder (GAD)* is classified by excessive worry about various topics, events and activities, such as health, economy or performance at work. The worry, which typically stretches far back in the person's life, is difficult to control and often accompanied with increased irritability, sleep disturbances, concentration difficulties, fatigue and restlessness.

- *Post-traumatic stress disorder (PTSD)* is caused by having witnessed or experienced a trauma, such as violence, sexual assault or death. A

person with PTSD can re-experience the trauma in intrusive thoughts, nightmares, flashbacks and physical or emotional reactivity after being reminded of the trauma.

- *Phobias* are divided into three subclasses: social phobia, agoraphobia and specific phobias. Social phobia, also referred to as social anxiety disorder, is characterized by fear of underperforming or being humiliated in social situations, for instance when speaking in public or eating in front of others. Agoraphobia is expressed as avoidance of public places and is often the consequence of panic disorder, since the patient does not want to be among other people if experiencing a spontaneous panic attack. Specific phobias are characterized by an irrational fear of specific objects, such as dogs (cynophobia), dental examinations (dentophobia), enclosed spaces (claustrophopia) or water (aquaphobia).

Obsessive-compulsive disorder (OCD) was previously viewed as an anxiety disorder but is, in the recently published DSM-V, classified as a separate disorder. Nonetheless, the condition shares some psychological features, and possibly also some underlying biological mechanisms, with anxiety disorders, and can often be treated with similar therapy.

Acutely, anxiety can often be attenuated with GABA A receptor modulators of benzodiazepine type, which potentiate the action of the inhibitory amino acid γ-aminobutyric acid (GABA) in the brain. However, due to their side effects and addictive properties, they are not preferable as long-term treatment of anxiety disorders [3]. Instead, treatment with certain antidepressants, such as selective serotonin reuptake inhibitors (SSRIs), which unlike benzodiazepines have no clear-cut effect on healthy individuals, no addiction potential and often better efficacy, is regarded a superior treatment strategy [3, 4]. SSRIs inhibit the reuptake of the neurotransmitter serotonin, thereby increasing its extracellular concentration in the brain [5, 6], which points to serotonin being of major importance in the regulation of anxiety.

1.2. Serotonin

1.2.1. History

The history of serotonin research began when Erspamer and Vialli isolated a substance from enterochromaffin cells in the intestines of rabbits and gave it the name enteramine [7]. It was later discovered that a newly found vasoconstricting substance, named serotonin [8], was the same molecule as enteramine and that it was present in the human brain [9] with serotonergic nerve terminals in most parts of the central nervous system [10]. Later, the cell-bodies of the serotonergic neurons could be located to the raphe nuclei of the brain stem [11]. That the substance may have a role in the regulation of mood was first indicated by the finding that the mind-altering substance lysergic acid diethylamide (LSD) [12, 13] had affinity for serotonin receptors [14]. A role of serotonin for the regulation of behaviour was further supported first by the revelation that iproniazid, a former tuberculosis medicine, had antidepressant action and inhibited monoamine oxidase-mediated degradation of serotonin [15, 16], then by the discovery that serotonin reuptake inhibition is one mechanism of action of tricyclic antidepressants [17] and finally by that selective serotonin reuptake inhibitors (SSRIs) also have antidepressant properties [18, 19].

Because of the mechanism of action of serotonin reuptake inhibitors, i.e. increasing the extracellular levels of serotonin by blocking its reuptake, and their therapeutic effect on mood disorders, serotonin was long viewed as a mood-improving substance and mood disorders a result of low serotonin levels. In the light of decades of further research, the mechanisms by which serotonin regulates mood however appear a lot more complex.

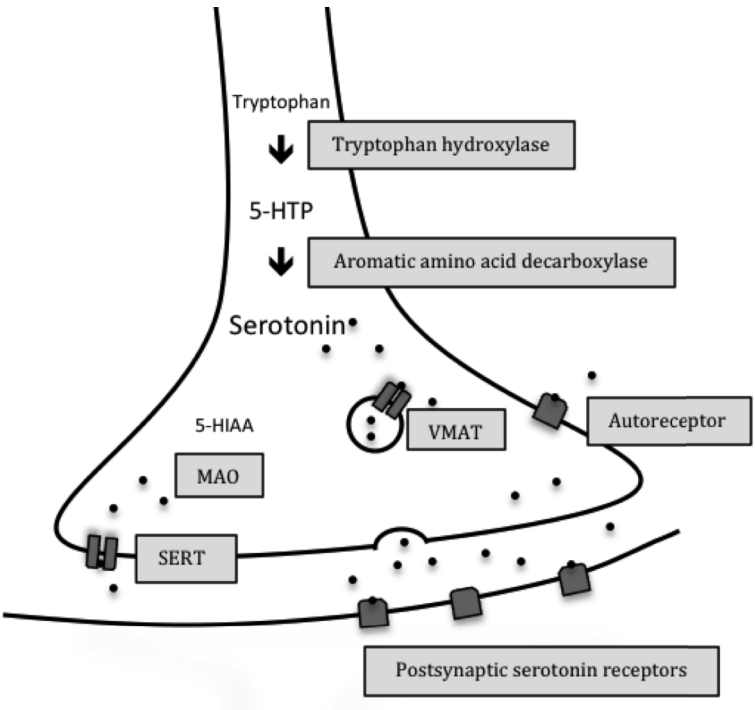
1.2.2. Neuroanatomy

Serotonergic neurons constitute a defined neuronal population with cell bodies located in the raphe nuclei of the brain stem [11]. The projections however reach almost the entire central nervous system including brain areas such as the amygdala, hippocampus, hypothalamus, prefrontal cortex and septum [20]. However, extracellular serotonin levels show variation between brain regions, at baseline as well as after treatment with drugs with an effect on serotonin, indicating diversity in the activity and regulation of serotonergic neurons [6, 21, 22].

1.2.3. Synthesis and metabolism

The synthesis of serotonin occurs in two steps (Figure 1). In the first, which is believed to be the rate-limiting one, the amino acid tryptophan is converted into 5-hydroxytryptophan (5-HTP) by the enzyme tryptophan hydroxylase (TPH). There are two variants of this enzyme, TPH1 and TPH2. TPH2 is only expressed in the brain and is likely the variant with most importance for serotonin synthesis there [23]. In the next step 5-HTP is converted into 5-hydroxytryptamin (5-HT), which is a synonym for serotonin, through the action of the enzyme aromatic amino acid decarboxylase (AADC).

Figure 1. *Image of a serotonergic synapse*



When synthesized in the neuron, serotonin is taken up into vesicles by vesicular monoamine transporter (VMAT). The vesicle containing the transmitter is then stored in the presynapse until activation of the neuron causes release of its content into the synaptic cleft, where serotonin can bind to serotonin receptors on postsynaptic neurons.

There is a continuous active reuptake into neurons by a transport protein called serotonin transporter (5-HTT or SERT). After reuptake, serotonin can be reused or degraded by the enzyme monoamine oxidase (MAO), generally by its isoform MAO-A, and aldehyde dehydrogenase into 5-hydroxyindole acetic acid (5-HIAA). Paradoxically, the other isoform MAO-B, which appears less important for the degradation of serotonin, shows higher presence in serotonergic neurons than MAO-A [24], and no explanation has been found.

All the mechanisms mentioned above can be targeted by pharmacological agents as will be discussed later in this chapter.

1.2.4. Serotonin receptors

Serotonin can mediate its effect through the activation of different serotonin receptors with different distribution in the brain. There are at least 14 subtypes of serotonin receptors, which are grouped into seven subclasses (5-HT1-7). Apart from 5-HT3, which is a ligand-gated ion channel, all subtypes of the serotonin receptor are G-protein-coupled.

All serotonin receptor subtypes can act as postsynaptic receptors on different neurons, but the 5-HT1-family, which exert an inhibitory effect when activated, can also serve as autoreceptors on serotonergic neurons, where they can mediate negative feedback when activated. 5-HT1A receptors can be located in the somatodentritic region, exerting a general inhibitory influence on the firing of the serotonergic neuron, whereas 5-HT1B/D receptors can be found in proximity to serotonergic nerve terminals, mediating a local inhibitory influence on the presynapse with respect to synthesis and release of the transmitter [25].

The density of specific serotonin receptor subtypes varies between brain regions, which may have importance for the regulation of mood and behaviour. For instance, the 5-HT2A receptor has a higher expression in the cortex than elsewhere [26], whereas 5-HT1A is abundant as postsynaptic receptor in the limbic system and cortex [27] and as autoreceptor in the dorsal and medial raphe nuclei [28]. Also, serotonin has different affinity for different serotonin receptor subtypes [29] and the affinity for some of the receptors, such as 5-HT2A, may show variation – referred to as high- versus low-affinity state - which could possibly be of relevance for the function of serotonin under different levels of stress or aversion [30-32].

1.2.5. Manipulations of serotonin activity

1.2.5.1. Enhancement of extracellular serotonin

Inhibition of the SERT-mediated reuptake of serotonin causes an elevation of the neurotransmitter in the synaptic cleft in at least some brain regions [22, 33]. While a number of other drugs, such as tricyclic antidepressants and 3,4-Methyl enedioxymethamphetamine (MDMA), also have SERT-inhibitory properties, it is the sole or dominating mechanism of the SSRIs. A more powerful acute elevation of extracellular serotonin can be achieved by administration of compounds such as fenfluramine, *meta*-Chlorophenylpiperazine (mCPP) and MDMA, that changes the direction of SERT, hence causing a marked release of serotonin [30, 34].

Whereas monoamine oxidase inhibitors (MAOi) can enhance extracellular serotonin by inhibiting its degradation [35], an additional method for enhancement of extracellular serotonin is administration of the serotonin precursor 5-HTP, which unlike serotonin can pass the blood-brain barrier. In the tryptophan hydroxylase-containing neurons of the brain, 5-HTP is converted into serotonin, leading to an increased serotonin release [36, 37]. When used experimentally, 5-HTP is often given in combination with a peripheral amino acid decarboxylase inhibitor, to prevent its conversion before passing the blood-brain barrier.

1.2.5.2. Depletion of serotonin

In order to study the importance of serotonin for a certain mechanism, a useful strategy is to study the mechanism in the absence of the neurotransmitter. There are several interventions by which the level of serotonin can be reduced. Since tryptophan is a precursor to serotonin, one method is to exclude this amino acid from the diet resulting in a moderate reduction in brain serotonin [38, 39]. This method is referred to as tryptophan-free diet or acute tryptophan depletion and can be used in humans [40] as well as in animals [41].

More efficiently, serotonin synthesis can be transiently blocked by inhibition of tryptophan hydroxylase using the compound *para*-chlorophenylalanine (PCPA) [42], resulting in an almost complete depletion of serotonin lasting for several days. This method, which is used in the research described in this thesis, is mostly used in experiments performed on animals due to the risk of side effects.

A third way by which serotonin can be depleted is by using transgenic techniques, for instance knock-out of genes encoding TPH2 or genes essential for the development of serotonergic neurons [43, 44]. This type of depletion is generally irreversible and life-long, which makes it more useful for studying consequences of long-term depletion of serotonin, although depletion can also be induced later in life with so-called knock-down technique [45].

Additional techniques for achieving serotonin depletion include lesions of cell bodies located in the raphe nuclei [46] and infusion of 5,7-DHT, a neurotoxic agent that is believed to predominantly kill serotonergic neurons [47].

1.2.5.3. Receptor antagonists and agonists

A great number of compounds have agonistic or antagonistic properties on different serotonin receptor subtypes. Such compounds, some of which are used clinically, can be used for studying the importance of the receptor with or without other additional interventions, as done in the research described in this thesis.

Serotonin receptor antagonists inhibit postsynaptic receptors, whereas agonists stimulate them. However, activation or blockade of serotonin receptors may also influence the extracellular levels of serotonin. For instance, blockade of the 5-HT1A, 5-HT2A and the 5-HT2C receptors can potentiate the increase in synaptic serotonin that occurs during SSRI administration in certain brain areas of rats, indicating that the receptors, when activated, can exert a negative feedback on serotonergic neurons [28, 48, 49]. 5-HT1A mediates this action directly as an inhibitory autoreceptor on serotonergic neurons and the other receptors via non-serotonergic neurons, by not yet explored mechanisms.

1.3. Serotonin and human anxiety

1.3.1. History of serotonin reuptake inhibition

The probably strongest evidence of serotonin being involved in mood and anxiety disorders is the effect of SSRIs on these conditions. While drugs with inhibition of serotonin reuptake as one of several putative mechanisms of action, so called tricyclic antidepressants, had been used in treatment of depression for

decades, the first drug to selectively act by this mechanism was zimelidine, which was developed as the result of a collaboration between the Swedish Nobel prize winner Arvid Carlsson and the pharmaceutical company Astra Hässle [18]. This drug was however taken off the market shortly after its introduction due to severe side effects – a number of patients had developed a reversible Guillain-Barré-like syndrome. In the meantime another pharmaceutical company, Eli Lily, was developing a drug with a similar mechanism of action under the name of Prozac, with the substance name fluoxetine, which did not display the troublesome side effects [19]. After its launch in 1988, fluoxetine rapidly became one of the most prescribed antidepressants worldwide, partly due to its safety in comparison with previous antidepressants.

It was later discovered that SSRIs also are effective in treatment of anxiety disorders, where the magnitude of the effect depends on for which anxiety disorder they are used [4]; treatment of panic disorders and social phobia generally shows better efficacy than treatment of GAD and PTSD. Also, SSRIs show relatively good efficacy in treatment of OCD.

1.3.2. Synaptic serotonin availability and anxiety

As mentioned, the mechanisms behind the effect of SSRIs in treatment of anxiety disorders and depression are not yet fully understood. Although SSRI administration immediately causes a pronounced elevation of extracellular brain serotonin in at least some brain regions [21, 22], several weeks of treatment are usually required before the onset of the therapeutic effect. Moreover, a common observation is that some symptoms of anxiety can be exacerbated during early treatment of anxiety with SSRIs, sometimes referred to as "jitteriness syndrome" [50, 51]. Interestingly, depressed patients also show increased somatic symptoms of anxiety during the first weeks of SSRI treatment [52], while their symptoms of psychic anxiety, on the other hand, are reduced.

This suggests that short-term elevation of extracellular serotonin under some conditions can enhance some symptoms of anxiety and that other mechanisms activated during long-term serotonin elevation, as achieved by chronic SSRI treatment [5, 53], may counteract this anxiogenic effect, resulting in reduced anxiety. Some theories regarding such mechanisms include down-regulation of the inhibitory 5-HT1A autoreceptors [54], thus increasing the serotonin-releasing capacity of the neurons, or the opposite, reduced serotonin-releasing capacity, for example by down-regulation of tryptophan hydroxylase [55, 56].

In favour of the second possibility, a genetic polymorphism resulting in lower expression of the serotonin transporter, hence possibly higher serotonin level, is associated with anxiety-like personality traits [57, 58]. Also, patients with anxiety disorders may tentatively display higher presynaptic serotonin synthesis in brain areas such as the prefrontal cortex and amygdala and higher brain serotonin turnover, which is all reduced by sustained SSRI treatment [59-62]. Complicating the picture however, a moderate increase in some symptoms of anxiety [40] is also seen after tryptophan-free diet resulting in discretely reduced central serotonin levels [38, 39]. However, when interpreting results from tryptophan depletion studies, one should bear in mind than only a minor part of the tryptophan utilized by the body is used for the formation of serotonin, and that tryptophan is also the precursor of kynurenic acid, with influence on brain neurotransmission [63].

Additional mechanisms that have been proposed for how anxiety may be reduced by long-term serotonin reuptake inhibition include interference with neurotrophins such as BDNF [64] with a possible influence on neuroplasticity, and, which will be discussed further in this thesis, down-regulation of postsynaptic serotonin receptors with anxiety-promoting impact [65-68].

In contrast to mood and anxiety disorders, SSRIs display a fast onset of action in treatment of anger and irritability, e.g. in patients with premenstrual dysphoric disorder, stroke, dementia or brain injury, as well as in the treatment of premature ejaculation [69-71]. Since these are, as judged by animal experiments, likely consequences of an enhanced serotonergic output, there are reasons to believe that SSRIs do elicit a functionally relevant increase in extracellular levels of serotonin already shortly after the onset of treatment, at least in some brain areas.

1.3.3. Serotonin receptor subtypes and anxiety

Most drugs with affinity for serotonergic receptors that are used clinically also bind to a variety of other receptors, making it difficult to determine which of the actions that are central for the response observed. With this in mind, most serotonin receptor subtypes have shown potential importance in mood disorders; the evidence is however particularly strong for two of them: the 5-HT1A and the 5-HT2A receptors [30].

1.3.3.1. The 5-HT1A-receptor

That the 5-HT1A receptor is involved in mood disorders is implied partly by the fact that buspirone, a partial 5-HT1A receptor antagonist, is used with some efficacy in the treatment of GAD [30, 72] and that genetic variation as well as variation in expression level of this receptor may be associated with anxiety, depression and response to antidepressants [73].

Also, down-regulation of 5-HT1A receptors has been indicated following long-term SSRI-treatment in rats [53, 54]. Since the 5-HT1A receptor is present in the brain both as a postsynaptic receptor, e.g. inhibiting the activity of recipient neurons, and as a somatodendritic autoreceptor, inhibiting the activity of serotonergic neurons and thus their serotonin release, a relevant question is which of these receptors that are responsible for the mood-altering effects – many, but not all, researchers propose postsynaptic receptors as the answer [30].

1.3.3.2. The 5-HT2A receptor

Drugs with antagonistic effect on the 5-HT2A receptor as one of several putative mechanisms of action, including ritanserin, mirtazapine and several atypical antipsychotics, have been claimed to exert some efficacy in the treatment of depression, OCD and several anxiety disorders [72, 74-83], especially when given in combination with an SSRI. Also the amount of blocked 5-HT2A receptors in the amygdala during ketanserin treatment is reported to be positively associated with fear recognition in healthy volunteers [84]. Importantly, mirtazapine and ritanserin also display strong affinity for the 5-HT2C receptor. These and other observations suggest that the 5-HT2A receptors, and possibly also the 5-HT2C receptors, may have anxiogenic properties.

Interestingly, a down-regulation of 5-HT2A receptors has been reported following continuous treatment with antidepressants [66, 67, 85], raising the question whether this mechanism is central for their therapeutic effect.

Surprisingly, psychedelic drugs such as LSD and psilocybin, which have strong agonistic effect on 5-HT2A receptors, have, despite their psychotic-like and anxiety-like properties when administered acutely [12, 13, 86-88], shown potential as an antidepressant [30], indicating a dual impact of this receptor on mood.

1.4. Animal models of anxiety

1.4.1. Background

Since mood disorders are diagnosed based on the verbal description of symptoms made by the patient it is impossible to determine to what extent they are experienced by animals. While some symptoms, such as guilt or excessive worry, probably require a rather complex mind, which would exclude a great number of species, the perhaps most fundamental emotions characterizing mood disorders, such as sadness, fear, stress and anger, are visible in the behaviour of some animals and pharmaceuticals are prescribed in veterinary care to inhibit such emotions in pets [89]. Some aspects of behaviour by which an animal expresses emotion are species-specific, whereas other forms of behaviour are shared between numerous species.

Animal behaviour can be used to study possible mechanisms underlying human behaviour in so-called animal models of behaviour, which are classified based on what type of intervention is conducted and which behaviour is studied. For instance, the effect of a pharmacological compound with a known impact on anxiety in humans can be studied in an animal model in which a behaviour with similarities to human anxiety is expressed. The animal model is then referred to as a pharmacological animal model of anxiety. In other types of research the term animal model can also refer to the animal itself, for instance in a disease model where the pathophysiology of a disease is studied.

Animal models of fear, which may be used in research on anxiety and hence sometimes referred to as animal models of anxiety, are often divided into conditioned and unconditioned fear models. In an unconditioned fear model, an innate behaviour is studied with as little interference as possible, whereas in a conditioned model, the behaviour of the animal has been acquired or strengthened.

1.4.2. The quality of an animal model

The quality of an animal model is based on three types of validity [90]. The first type is *face validity*, which means that something similar to the studied phenomenon is also present in humans. Rodents are, partly due to their relative biological resemblance to humans, common research animals. In rodent models

of anxiety, the animals are usually presented to something unpleasant or aversive, which will result in a fear-, avoidance- or defence-related behavioural response that in some regard mirror anxiety in humans (see later chapters).

Animal models often focus on specific aspects of human conditions, especially when modelling psychiatric disorders such as anxiety disorders with a broad spectrum of symptoms. There are distinctions between what type of behaviour or emotions that are expressed in different models. For instance, some argue that an aetiologically based model, in which an unconditioned response (such as avoidance of open areas) is studied, would rather correspond to trait anxiety, whereas a model where a fear response is created, i.e. a conditioned model, would rather suggest state anxiety to be reflected [1, 90].

The second type of validity, and the one most possible to achieve in pharmacological research, is referred to as *predictive validity*, i.e. that a compound with a known effect on anxiety in humans alters the behaviour in the model, while a compound without any effect on anxiety in humans leaves the behaviour unaltered. Thus, a drug that reduces anxiety in humans should also reduce the behavioural response in the model. To this purpose, benzodiazepines, which exert a rapid onset anxiolytic effect in humans, have generally been used to evaluate the predictive validity of animal models of anxiety [1].

The third type, *construct validity*, is the most difficult to achieve in animal models of anxiety. It describes the resemblance concerning the underlying mechanism between the condition in humans and the animal model [90]. Since both the aetiology and the pathophysiology of mood disorders and anxiety disorders are far from understood, the construct validity of animal models of anxiety can only be speculatively discussed. While certain aspects of anxiety disorders, such as fear, can be studied, it would be a great overstatement to claim that the entire pathology of anxiety disorder is explored. Nonetheless, results in a model can be added to previous knowledge and thus contribute to a greater understanding of the disorder.

It is important to underline that the behaviour of rodents differs greatly from that of humans, and that human anxiety and behaviour in an animal model of anxiety are two vastly different things. If a behavioural response to one procedure corresponds to human behaviour, i.e. shows good predictive validity, it does not mean that this could be expected from the response to a different procedure. For example, the fact that benzodiazepines, which suppress anxiety in humans, also suppress a behaviour in an animal model of anxiety, neither

means that this behaviour *is* anxiety, nor that every other compound with anxiety-reducing capacity in humans will inhibit the behaviour in the model in question. Furthermore, results are often difficult to replicate in different species of research animals and even in different strains of the same species.

To summarize, data from animal models of behaviour should be interpreted with great caution, especially when drawing any conclusion about conditions in different species, such as humans. However, these models may nevertheless provide very important information regarding the mechanisms underlying, e.g., psychiatric disorders.

1.4.3. Unconditioned fear

A well-used unconditioned model of anxiety in rodents is the elevated plus maze (EPM), where the animal is placed in the middle of a plus-shaped maze, which is raised above the floor and with two of its arms enclosed by walls and two of them open. Since rodents innately fear open spaces but also tend to explore novel environments, it is thus put in a conflict situation. This model is often favoured because of its face validity and predictive validity; the fear displayed is spontaneous and responds to pharmacological treatment in the same manner as human anxiety. Administration of an anxiety-reducing substance, such as a benzodiazepine, will hence shift the behaviour into a more explorative and less avoidant behaviour, and the animal will hence spend more time on the open arm and less on the enclosed one [91]. Conversely, a stressor or a drug that would increase anxiety will reduce time spent on the open arm in favour of time spent on the enclosed one.

Other examples of frequently used unconditioned fear models are the open field test and the light/dark box.

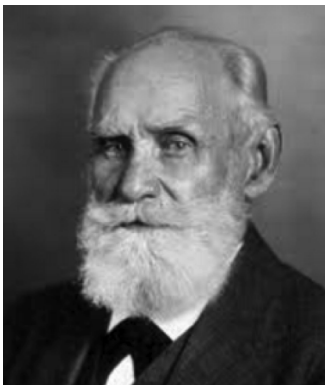
1.4.4. Conditioned fear

1.4.4.1. Types of fear conditioning

So-called classical conditioning was initially developed by the Russian physiologist Ivan Pavlov in the 1920s. It is based on the principle that a neutral stimulus is presented in association with an event that can trigger a biological response [92]. The former is known as conditioned stimulus (CS), while the latter

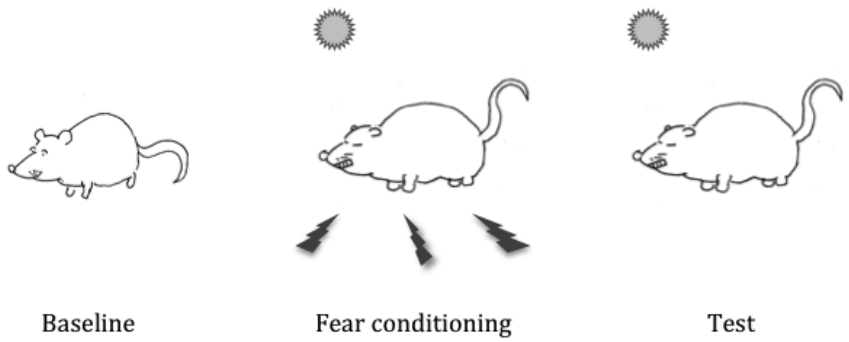
is referred to as an unconditioned stimulus (US). After training, involving repeated simultaneous presentation of the two stimuli, the response initially evoked by the US can be initiated by presentation of the CS without the US. The explanation is that the repeated simultaneous presentation of the two stimuli has resulted in a memory of the response, which can be recalled when only one of the stimuli is present – the response has been conditioned.

Figure 2. *Ivan Pavlov* (public domain)



In fear conditioning (figure 3), a painful or unpleasant event, commonly an electric foot shock, is used as the US. The CS may be a tone or a light, but a specific environment can also be conditioned using a US, a procedure referred to as contextual conditioning [92]. The psychological process that occurs during fear conditioning is referred to as fear acquisition or fear learning. After training, the CS or the context can give rise to fear expression in the form of physiological or behavioural responses. The type of fear response depends on which species is studied. In rodents, freezing and fear-potentiated startle are two fear responses that are often studied following fear conditioning [92, 93].

Figure 3. *Image showing a fear conditioning procedure*



An advantage of conditioned fear is that the underlying neuronal circuitry has been extensively studied [92, 93]. Also, fear conditioning can be performed in humans, which enables direct comparison.

Today, additional models of conditioned fear have been developed, which are not entirely based on classical fear conditioning. These models create a conflict between instinctive behaviour and conditioned fear. Examples of such models are Vogel's conflict test, conditioned place preference and the shock-probe burying test [94-96].

The face validity of conditioning models can be argued to be better for PTSD than for other anxiety disorders, since the fear is acquired rather than spontaneous as in unconditioned models. It is however important not to overstate how much a model reflects the symptoms of a specific anxiety disorder and rather consider the predictive validity concerning the core symptom of anxiety disorders, i.e. anxiety itself. Conditioned fear models can be regarded as having relatively good predictive validity for anxiety-dampening compounds [97, 98].

It is important to consider that animal models of conditioned fear are, at the same time as they are assessments of fear and anxiety, also assessments of memory. For instance, a compound reducing conditioned fear could do so either by specifically inhibiting the fear itself, or by a more general impairment of memory. Thus, a compound that reduces conditioned fear needs to be tried in other models of learning and memory before it can be concluded that the effect is caused by fear- or anxiety-suppressing and not memory-impairing properties of the compound. When analysing a compound such as an SSRI, which has an already well-established effect in humans and has shown no major effect on memory previously [99], the results can perhaps be interpreted a little less cautiously in this regard.

1.4.4.2. Conditioned fear behaviours

Startle

In numerous species a loud sound may trigger a so-called acoustic startle reflex, in which muscles are quickly tensed in response to the sound. This is a reflex that most of us have experienced, for instance in response to a sudden sound effect in an exciting film. The startle reflex can be strengthened through fear conditioning and is then referred to as fear-potentiated startle [93, 97]. The CS used in fear conditioning should not trigger startle on its own but will after fear conditioning strengthen the reflex when presented in conjunction with a loud sound. Similarly, contextual conditioning should result in a potentiation of the

startle reflex to loud sounds when presented in the context. Unlike freezing, the startle reflex in response to loud sounds is normally pronounced also before fear conditioning, enabling baseline assessment, but requiring a more intense conditioning protocol for strengthening of the reflex.

A useful feature of fear-potentiated startle is that it can be studied in humans, commonly as eye-blink reflex, whereby people with anxiety disorder or anxiety-like personality tend to respond stronger than others [100, 101]. This enables direct comparison between results obtained in animal experiments and in humans.

Freezing

Freezing, i.e. complete absence of all body movements apart from breathing motions, is a common response to threat in rodents and can be induced using fear conditioning [92, 102, 103]. During a freezing response the animal typically shows a characteristic posture with an arched back, erected fur, rigged tail and retracted ears. For instance a tone or a light can be used as a CS resulting in freezing behaviour during its presence, and a conditioned context can cause freezing when an animal is placed in it, referred to as contextual freezing or contextual conditioned freezing.

In addition to fear conditioning, the freezing response is expressed following a loud sound, in general immediately after the startle reflex. Typically, rodents that express strong startle response also show more intense freezing between the loud sounds [104], indicating that these behaviours are in some way related.

1.5. Serotonin and experimental anxiety

1.5.1. The effect of pharmacological manipulations of serotonin

1.5.1.1. Enhancement of the serotonergic system

Conditioned fear

In healthy volunteers, acute SSRI administration has been shown to enhance conditioned fear, assessed as fear-potentiated startle, and sustained SSRI

treatment to reduce it [100, 105, 106]. This behaviour has previously shown correlation with trait anxiety [101].

However, the anxiolytic effect of sustained SSRI administration seen in the treatment of patients with anxiety disorder has proven difficult to mirror in animal models of conditioned fear, with only a handful of successful experiments [107-109].

Also acute administration of SSRIs in animal models has rendered varied and inconsistent results; while some experiments have shown increased conditioned fear [109-112], others have shown decreased conditioned fear [113-115], which has also been reported after treatment with 5-HTP [116].

Other models of anxiety

Acute administration of the serotonin-releasing agent fenfluramine has led to both increased [117] and reduced [118, 119] anxiety-like behaviour in rodents. Administration of the serotonin precursor 5-HTP has however shown a more consistent albeit dose-dependent reduction of anxiety-like behaviour in conflict tests [94, 95].

In humans, acute administration both of an SSRI [106] and of fenfluramine [120] has been shown to increase different types of experimental anxiety in some experiments – reduced anxiety in a simulated public speaking test following low-dose fenfluramine administration however being an exception [121].

1.5.1.2. Serotonin depletion

Conditioned fear

The effect of serotonin depletion on conditioned fear has been relatively sparsely studied and the results seem to depend on what method of depletion is used and what type of behaviour is analysed.

In animal models of conditioned fear, the results from transient serotonin depletion are few and inconsistent [119, 122, 123]. In one experiment, fear conditioning was impaired by local infusion of 5,7-DHT into basolateral amygdala [47]. In contrast, lifelong suppression of serotonergic activity by knock-out technique has consistently been shown to increase conditioned fear [43, 124].

A moderate increase in conditioned fear [125] is also seen in humans after tryptophan-free diet, which however induces only a mild reduction in central extracellular serotonin [38, 39] and which may influence also non-serotonergic transmission in brain (see above).

Other models

In other animal models of anxiety, transient PCPA-mediated serotonin depletion has resulted in reduced, increased as well as unchanged anxiety-like behaviour [96, 118, 119, 126]. Also, serotonin depletion mediated by knock-down of TPH2 has resulted in an estrogen-dependent inhibition of anxiety-like behaviour in female rats [45] adding to previous evidence of an interaction between serotonin and sex hormones (see sex differences 2.5.3).

1.5.1.3. Manipulations of serotonin receptors

Conditioned fear

Although several serotonin receptors seem to be involved in conditioned fear behaviour, their exact role is far from understood.

Reduced conditioned fear in healthy participants has been shown following acute administration of ritanserin [127], indicating a role of 5-HT2A or 5-HT2C receptors, or both, in this behaviour.

In animals, blockade of the 5-HT2C receptor has shown anxiety-reducing properties with respect to conditioned fear [110, 112].

While agonists at the 5-HT1A receptor, in line with the therapeutic effect of buspirone on GAD, may reduce conditioned fear [128-130], 5-HT1A antagonists generally leave this behaviour unaltered [123, 130] and may paradoxically inhibit it when co-administered with an SSRI [131-133].

Other models

Knock-out of the 5-HT2A receptor has been shown to reduce anxiety-like behaviour in conflict-based models, such as EPM [134], without affecting conditioned fear. Also, agonists of the 5-HT1A receptor reduces [129] conflict-based anxiety-like behaviour and knock-out of the receptor in genetic mouse

models may increase it [135, 136], adding further support for an anxiolytic impact of the receptor.

In addition, other serotonin receptor subtypes that have been shown to influence anxiety-like behaviour in animal models are the 5-HT1B, 5-HT3, 5-HT6 and the 5-HT7 receptors [130, 137-139]. It is hence possible that serotonin may influence anxiety by its action on multiple receptors, which under some conditions may exert opposite effects.

1.5.1.4. Optogenetic manipulations of the serotonin system

A more novel method to study the importance of serotonin is to selectively and transiently activate or silence serotonergic neurons using optogenetic techniques [112, 140]. In line with the hypothesis that enhanced serotonergic activity exerts an anxiogenic influence, such an intervention has resulted in increased cued and contextual conditioned fear in mice, as well as anxiety-like behaviour of mice and rats in the EPM [112, 140].

1.5.2. The effect of fear and stress on serotonin in animals

Since fear- and stress-related behaviour can be influenced by serotonergic manipulations and anxiety disorders may be associated with altered serotonergic activity, a question that may be asked is if a stressful or fearful situation can affect serotonergic transmission. There are several indications of this being the case. For instance, exposure to a stressful environment, such as a conditioned context [141, 142], or to a stressful stimulus, such as electric foot shocks [143, 144], causes an immediate increase in extracellular serotonin in several brain areas in rats. Interestingly, although long-term SSRI administration causes increased extracellular baseline levels of serotonin [53, 145, 146], it can inhibit foot shock-triggered serotonin release in the prefrontal cortex [144], highlighting the interesting possibility that suppression of stress-evoked peaks in extracellular serotonin could be a mechanism behind the therapeutic effect of SSRIs in the treatment of mood disorders.

1.5.3. Sex differences

There is strong evidence for sex being an important factor behind serotonergic function as well as anxiety. In addition to the fact that anxiety disorders are

more common in women than in men [2, 147] it has been found that serotonergic neurotransmission displays sex difference [148] and is under the influence of sex hormones [45, 147, 149, 150]. Accordingly, behaviours such as sexual activity [151-153] and aggression [151, 154] that are regulated by sex hormones seem to be strongly influenced by serotonin. Moreover, serotonin depletion has been shown to abolish sex differences with respect to anxiety-like EPM behaviour in rats [126]. The mechanisms of this influence of sex or sex hormones on serotonergic activity are however not well understood.

1.5.4. "Anxious" animals

As described, the effects of serotonergic manipulations on experimental anxiety show considerable inconsistency between different experiments. Importantly, the behaviour in anxiety models is very sensitive and for example an altered number or intensity of foot shocks in a fear conditioning paradigm could potentially have great impact on the resulting behaviour. It is not unlikely that the response to a compound such as an SSRI could also be influenced by such alterations, hence contributing to the discrepancy in outcome.

Furthermore, since the aim of using an animal model of anxiety is to mirror anxiety disorders in humans, it is not surprising that the outcome of studies on a group of normal laboratory animals, not taking individual differences between them into consideration, may be poor. In some research, rats displaying a more anxiety-like behaviour have been bred to form a more "anxious" strain [155]. In research presented here (Paper I) an alternative strategy was applied [156, 157], namely to use a normal population of rats but comparing the response between those displaying more and those displaying less anxiety-like behaviour before treatment.

1.5.5. Neuroanatomy of serotonin and anxiety

A number of brain areas have been implicated as important for anxiety. First of all, the amygdala is known to play a central role for fear in general, and conditioned fear in particular [92].

In humans, increased activity in the amygdala appears associated with social anxiety disorder and normalisation of this activity with therapeutic response to sustained SSRI treatment [59], tentatively partly caused by an increased inhibition from the frontal cortex on the amygdala [158, 159]. However, acute administration of

an SSRI may both reduce [160, 161] and enhance [162] amygdala activity during emotional processing in healthy volunteers.

In rats, serotonin receptors of the 5-HT2-type in the basolateral amygdala have been found to be crucial for a conditioned response in a conditioned place preference model [163]. Also, acute SSRI administration can intensify amygdala-dependent conditioned fear by acting on 5-HT2C receptors in the bed nucleus of stria terminalis (BNST) [112, 164, 165]. Furthermore, the activity of these two brain areas is increased after acute SSRI administration also in other animal models of anxiety [166, 167].

As mentioned before, both acute and chronic SSRI treatment increases extracellular serotonin in virtually the entire brain of rats, however with marked regional differences in the concentrations with respect to the magnitude of the effect [6, 21, 22]. Also briefly mentioned previously, microdialysis experiments have shown increased serotonin release in the basolateral amygdala, ventral hippocampus, dorsal periaqueductal grey and prefrontal cortex [141-143] following stressful events, such as foot shocks and exposure to a conditioned context; moreover, chronic SSRI treatment has been found to inhibit such a release in the prefrontal cortex in response to foot shocks [144].

2. AIMS, EXPERIMENTAL OVERVIEW AND RESULTS

2.1. Paper I

One explanation for why data from animal models of anxiety do not always mirror the clinical picture when studying the effect of SSRIs could be that the experiments mostly study a whole group of animals, while the effect of SSRIs are more pronounced in people with anxiety disorders or anxiety-like personality [51]. By assessing their baseline behaviour, animals can be divided into groups with more or less "anxious" individuals – a method which has proven successful in previous experiments [156, 157]. Moreover, assessing behaviour using more than one parameter could provide a better face validity of the model.

The purpose of Paper I was to evaluate (1) whether two fear responses, startle and freezing, can be simultaneously assessed and to what extent they correlate, (2) the inter-test stability of the responses, and (3) whether they can predict the effect of acute SSRI administration and/or contextual fear conditioning in the same setting.

Male Wistar rats first underwent two subsequent tests (test 1 and 2), with two weeks in between, each test consisting of 30 noise bursts after an acclimation period of five minutes. Both startle in response to bursts of white noise and freezing between the noise bursts were measured. The correlation between startle and freezing within each test, as well as the correlation between the tests for each response, were analysed.

On the next day, half of the rats underwent contextual conditioning involving 10 electric foot shocks in the same cage as where test 1 and test 2 had been performed, while the other half was placed in the cage without receiving any foot shocks.

One day later, all rats were subjected to a test session (test 3) identical to test 1 and 2, 60 minutes after one injection with the SSRI escitalopram (10 mg/kg) or saline, and the response to the drug as well as to contextual conditioning was compared between animals with high and animals with low test 1 responsiveness.

Correlation between test 1 and 2 was seen for both startle and freezing. Also, strong correlation between startle and freezing was seen within test 1 and test 2.

Acute escitalopram and contextual conditioning both significantly enhanced startle as well a freezing. Moreover, the animals that had displayed high startle or freezing in test 1 showed a stronger enhancement of startle in test 3 following acute escitalopram administration than animals with low test 1 startle or freezing. No such effect was seen with respect to the freezing parameter.

2.2. Paper II

Since acute elevation of extracellular serotonin mediated by acute SSRI administration resulted in increased startle and freezing in Paper I, it was considered important to study the effect of the opposite, i.e. reduced level of serotonin. Also, previous research has suggested that serotonin can influence anxiety-related behaviour in a sex-dependent manner [126], and it was therefore found relevant to investigate if such a pattern can be found also in this model of anxiety.
The aims of Paper II were hence to analyse whether startle and freezing responses under different fear intensities, i.e. with or without prior contextual conditioning, (1) display sex differences and (2) require normal serotonin availability.

Male and female Wistar rats were first subjected to contextual conditioning as described in Paper I, then treated with either the serotonin-depleting agent PCPA (300 mg/kg) or saline for three consecutive days, whereupon they were tested with simultaneous measurement of startle and freezing as described in Paper I. In a second experiment, a different group of rats underwent the same procedures, but without receiving any foot shocks on the conditioning day.

After contextual conditioning, male rats showed significantly higher freezing than female rats, a sex difference that was completely abolished by PCPA-mediated serotonin depletion, which reduced freezing only in the males. In contrast to unconditioned behaviour (Paper I), startle and freezing showed negative correlation after fear conditioning in males; moreover, males displayed a PCPA-mediated increase in the startle response – probably secondary to the reduction in freezing.

2.3. Paper III

One possible explanation to why acute SSRI administration has given conflicting results in conditioned fear models, sometimes enhancing fear (as was seen in Paper I) and sometimes reducing it, could be that a moderate SSRI-induced increase in extracellular serotonin levels activates both fear-promoting and fear-impairing serotonin receptors, and that small experimental variations can affect the balance between the two types. Two serotonin receptors of the 5-HT2 family – the 5-HT2A and 5-HT2C receptors – as well as the 5-HT1A receptor, have previously been attributed anxiety-related properties; hence we deemed it relevant to study to what extent blocking these receptor subtypes could impact the response to an SSRI.

The first aim was to examine if blockade of the 5-HT2A and 5-HT2C receptors, respectively, might unmask or augment a freezing-reducing effect of an SSRI. The second aim, after this had been shown to be the case with respect to 5-HT2A blockade, was to assess if the anti-freezing effect of the combination of the SSRI and the antagonist could be countered by a 5-HT1A receptor antagonist. And the third aim, for the purpose of comparison, was to assess if a more powerful enhancement of extracellular serotonin than that obtained by an SSRI, caused by the serotonin precursor 5-HTP or the serotonin-releasing agent fenfluramine, influences contextual conditioned freezing and if the possible effects of these agents are modified by simultaneous 5-HT2A antagonism.

Six different experiments were performed using male Sprague-Dawley rats. First, all rats underwent contextual fear conditioning, or a habituation session without foot shocks, in somewhat larger chambers than in Papers I and II, optimal for analysing freezing without concurrent analysis of startle. Seven days after fear conditioning, the animals were subjected to different treatments before freezing was assessed.

In experiment I, II and III the rats were treated with different doses of the 5-HT2A antagonist MDL100907 (0.01, 0.3, 1 and 3 mg/kg) or saline prior to acute escitalopram (1 or 5 mg/kg) or saline administration. In experiment IV, the 5-HT1A antagonist WAY100635 (0.1 or 1 mg/kg) or saline and MDL100907 (1 mg/kg) or saline were administered followed by acute escitalopram (5 mg/kg) or saline administration. In experiment V, the 5-HT2C antagonist SB242084 (0.3 or 1 mg/kg) or saline and then acute escitalopram (5 mg/kg) or saline were administered. In experiment VI, the rats were treated with 5-HTP (100 mg/kg), fenfluramine (5 mg/kg) or saline after the administration of the decarboxylase

inhibitor carbidopa (10 mg/kg, inhibiting peripheral degradation of 5-HTP) or saline and MDL100907 (0.3 mg/kg) or saline.

Whereas, the 5-HT2A antagonist *per se* only reduced freezing modestly and inconsistently, i.e. in some of the experiments only, and escitalopram *per se* had no effect, co-administration of the compounds resulted in a dramatic reduction in freezing. While high doses of escitalopram were required for this effect to be at hand, very low doses of 5-HT2A antagonist were sufficient. The anti-freezing effect of the 5-HT2A antagonist and the SSRI when given in combination could not be blocked with a 5-HT1A antagonist; instead reduced freezing was observed when a low dose of the 5-HT1A antagonist was combined with escitalopram. Of note, higher doses of the 5-HT1A antagonist, when combined with the SSRI with our without 5-HT2A antagonist, induced a sedated-like immobile behaviour not displaying the characteristic features of freezing. The 5-HT2C antagonist had no effect at any dose given, alone or in combination with escitalopram. Fenfluramine and 5-HTP markedly reduced freezing, *per se* as well as in combination with 5-HT2A antagonist; however, 5-HT2A blockade slightly counteracted the freezing-reducing effect of 5-HTP.

2.4. Paper IV

Since acute SSRI administration, in line with clinical observations, had been shown to increase contextual fear (Paper I), a reasonable next step was to investigate if clinical conditions could be resembled also with respect to the impact of long-term treatment, i.e. with reduced contextual fear as the expected outcome. When this had been shown to be the case, and given 1) that we had shown PCPA to display anti-freezing properties (paper II) and 2) that one popular theory states that the beneficial effect of long-term SSRI administration might be due to a dampening of serotonergic activity by down-regulating TPH2 (see above), we also found it relevant to assess if 5-HTP could counter the anti-freezing effect of long-term SSRI and PCPA, respectively.

One purpose of Paper IV hence was to evaluate the possible effect of a three-week treatment with escitalopram on contextual conditioned freezing. Also, the effect of PCPA-mediated serotonin depletion, which had shown a freezing-reducing effect on male rats after contextual conditioning in Paper II, was evaluated in this model to enable comparison with the SSRI treatment. In addition, to further illuminate the underlying mechanisms, the effect of enhancement of extracellular serotonin concentration with 5-HTP was studied

per se and following PCPA or long-term escitalopram treatment.

Male Sprague-Dawley rats were used in five separate experiments, in which the effects of short-term versus long-term treatment with escitalopram, as well as PCPA-mediated serotonin depletion, on contextual conditioned freezing, were analysed using the larger type of chambers used in Paper III. Subchronic treatment of escitalopram was given for a period of three weeks, through food (approximately 24 mg/kg/day) in experiment I and through osmotic minipumps (approximately 10 mg/kg/day) in experiments II and IV. The rats underwent contextual fear conditioning under treatment, one day before freezing testing in experiments I and II or a few days before treatment started, approximately 3.5 weeks before the test, in experiment IV. In experiment III, the rats were given two injections of escitalopram (0.3, 1 or 3 mg/kg) or saline; the first one before the fear conditioning and the second one before the test on the next day. 5-HTP or saline was injected before tests in experiment IV and V. PCPA (300 mg/kg) or saline was given on 3 consecutive days before the fear conditioning and with a forth injection after fear conditioning, i.e. one day before the test, in experiment I. In experiment V, starting 4 days after the fear conditioning, the rats were given PCPA (300 mg/kg) on 3 consecutive days before the test. Serum levels of escitalopram were analysed in experiment II and III.

Whereas subchronic escitalopram treatment reduced freezing (as did PCPA administration) when administered before both conditioning and test, acute escitalopram injections yielding similar serum levels were devoid of effect. Subchronic escitalopram administered before the test also reduced freezing, as did administration of PCPA; 5-HTP influenced none of these two treatment effects.

3. DISCUSSION

3.1. Baseline measurements of startle and freezing (Paper I)

Whereas the observed correlation between startle and freezing has also been described by others [104, 168] the result that each of the parameters correlate between baseline test and a second unconditioned test adds evidence to the notion that the two responses are stable and may reflect a common behavioural phenotype. This, in turn enables characterization of animals as more or less "anxious" based on these responses.

3.2. Prediction of anxiety-like response to acute SSRI (Paper I)

The observation that acute SSRI administration can enhance conditioned fear is supported by some previous reports [110, 112], but deviates from others [113, 115]. The finding that acute SSRI administration enhances startle more in animals with higher baseline startle or freezing suggests that the clinical situation, i.e. that people with symptoms of anxiety before treatment may experience a worsening of the symptoms during early treatment with SSRIs [50, 51], can be mirrored using this paradigm. It may hence be suggested that this model should be beneficial for investigating mechanisms by which SSRIs may provoke anxiety, and also for exploration of the mechanisms underlying an individual susceptibility to such a response. Furthermore, in conjunction with our previous finding that only rats that had spent relatively short time in the open arm of an EPM during a first session responded with reduced open arm-time to acute SSRI-treatment in a second session [157], it suggests enhanced anxiety at baseline predicting aggravation of anxiety upon acute SSRI exposure to be a general phenomenon across anxiety models.

3.3. Sex differences and the influence of serotonin depletion (Paper II)

A reduction in contextual freezing induced by serotonin depletion was displayed exclusively by male rats, thus eliminating the sex difference otherwise seen. This suggests that the fear-enhancing effect exerted by serotonin in the contextual freezing paradigm is sex-dependent, mediating stronger freezing response in males. Interestingly, a similar observation, with a male-specific reduction in anxiety-like behaviour following serotonin depletion, has been made in the EPM paradigm [126]. Our finding further supports that anxiety-like behaviour, like aggression and sexual behaviour, is regulated by sex hormones and that serotonin plays a key role in this regulation [151, 153, 154]. Without further going into detail, it should be considered that the more pronounced anxiety-like behaviour in male rats does not correspond to humans, where anxiety disorders and anxiety-related personality traits are more common in women than in men [2, 169]. The sex difference found in the model could perhaps be explained by that freezing behaviour in response to threat may have had greater importance for males than females during evolution.

3.4. Intensified startle by serotonin depletion in male rats (Paper II)

Contextual fear-potentiated startle assessed in the same test displayed the opposite pattern to the one observed in the freezing parameter, i.e. a PCPA-mediated enhancement solely in male rats. Increased startle in male rats, both conditioned and non-conditioned, during serotonin depletion has been reported previously [122, 170]. Our data, showing reduced freezing as well as a negative correlation between startle and freezing in conditioned males, suggest that the stronger startle response may be a consequence of the reduction in freezing, a phenomenon that has been described also by others [93, 115].

3.5. 5-HT2A antagonism and response to acute SSRI administration (Paper III)

Whereas blockade of the 5-HT2A receptor, a receptor that has previously been associated with fear and anxiety [12, 84], had only a weak effect *per se*, acute SSRI

administration during 5-HT2A blockade resulted in a strong inhibition of freezing, not seen in rats only administrated the SSRI.

Like 5-HT2A, the 5-HT2C receptor has previously been attributed anxiety-enhancing properties, for example in conditioned fear models [110, 112]. However, administration of a 5-HT2C antagonist in this model failed to alter the effect of acute SSRI and had no effect on its own.

These results suggest that medication with SSRIs could have an earlier and perhaps stronger therapeutic effect in anxiety disorder if the 5-HT2A receptors are simultaneously inhibited, and that this may be the explanation to why better efficacy and in some cases faster onset of therapeutic effect have been reported when atypical antipsychotics or mirtazapine have been added to SSRIs in treatment of some anxiety disorders, OCD and depression [74, 78, 81, 82, 171, 172].

There are two different possible explanations to the results obtained. One is that acute SSRI treatment may affect contextual conditioned fear through two pathways; one fear-enhancing pathway, partly mediated by stimulation of the 5-HT2A receptor, and one fear-reducing pathway, and that blocking the 5-HT2A receptor yields enhanced leverage to the latter when the extracellular levels of serotonin are increased due to SERT blockade. It is however, as further discussed below, also possible that 5-HT2A blockade unmasks an impact of SSRIs on freezing by augmenting its effect on the extracellular levels of the transmitter.

Of note, psychedelic drugs such as LSD and psilocybin, which are potent 5-HT2A receptor agonists, have shown promising antidepressant effect [30], despite the anxiogenic effect sometimes produced by acute administration of these substances [12, 13, 86-88]; the impact of the 5-HT2A receptor on mood hence seems dual. One suggestion regarding these apparently conflicting results is that there are two ways by which mood can be improved by pharmacologically manipulating the 5-HT2A receptor, the first being to reduce its anxiety-provoking influence by blocking it, and the second to promote adaptive changes in the response to fear or stress by stimulating it [30]. This theory is however clearly in need of further investigations.

3.6. 5-HT1A antagonism and response to acute SSRI administration (Paper III)

The 5-HT1A receptor has been attributed mood-improving properties [30] and the partial 5-HT1A agonist buspirone displays moderate efficacy in the treatment of GAD [72]. However, blockade of this receptor did not reverse the freezing reduction mediated by the combination of acute SSRI and 5-HT2A antagonist. The freezing-supressing influence of this combination must hence be mediated by one or several other serotonin receptor subtypes.

Furthermore, blockade of the 5-HT1A receptor prior to acute SSRI treatment reduced freezing; at a low dose of the antagonist, the rats displayed increased locomotion, at a higher dose, they displayed a sedated-like state but not freezing; thus, these animals were immobile but without expressing any of the additional characteristics of freezing behaviour (see below).

In any case, 5-HT1A antagonism does not increase contextual freezing during acute SSRI administration in this model, but rather inhibits it, and does not reverse the suppression of freezing generated by 5-HT2A blockade with concomitant acute SSRI administration.

3.7. Acute or short-term SSRI administration: methodological aspects (Paper I, III and IV)

Acute escitalopram administration increased freezing in Paper I, whereas it had no effect in Paper III and IV. Possible explanations to this discrepancy may include that the box was smaller in Paper I, as discussed below, and that freezing was measured in the presence of noise burst. In this vein, we have, in a separate study, shown an SSRI to consistently augment both unconditioned and conditioned freezing in response to noise bursts in rat, using the larger type of box (Hagsäter et al, in press).

An additional difference between the experiments was the dosing; the dose of escitalopram thus was higher in Paper I (10 mg/kg) than in Paper III (1 and 5 mg/kg) and IV (0.3, 1 and 3 mg/kg).

It hence seems as if the anxiety-enhancing features of acute SSRI administration can be observed only under certain experimental conditions, and preferentially

in a subset of anxious animals, which is not surprising since initial SSRI treatment does not worsen all symptoms of anxiety humans, and only in some individuals [52]. Interestingly, while reports on the effect of acute SSRI administration on contextual fear are discrepant [111-113, 115], this treatment does appear to increase auditory fear [110, 112]. It is possible that sound-dependent conditioned fear, corresponds more to the somatic symptoms of anxiety, which are exacerbated by initial SSRI-treatment [52], than conditioned fear without the occurrence of sound, and therefore is more likely to be potentiated by short-term SSRI administration.

3.8. Long-term SSRI administration (Paper IV)

Our finding that long-term escitalopram treatment reduced contextual freezing is in resemblance to clinical conditions, where sustained but not initial SSRI administration can reduce anxiety [52, 173].

Anxiolytic effect of long-term SSRI treatment has previously been difficult to establish in models of anxiety with only a handful of successful reports [107, 109, 174]. The model used here may hence be useful in future research on the mechanisms underlying the anxiety-dampening effects of SSRIs.

3.9. Extracellular serotonin and freezing (Paper I-IV)

The marked reduction in contextual freezing induced by PCPA-mediated serotonin depletion, both with (Paper II) and without (Paper IV) the occurrence of noise bursts, suggests serotonergic transmission to be of major importance for this behaviour. In contrast, enhanced conditioned fear in response to PCPA was reported in a previous experiment performed by Archer et al [119], however with differences with respect to dosage and experimental settings as compared to the present studies. Also, life-long serotonin depletion generated in knock-out mouse models may increase conditioned fear [43, 124]. In other models of anxiety, both anxiogenic [118] and anxiolytic [126, 157] effects of short-term serotonin depletion have been demonstrated. One intriguing possible explanation to these discrepancies could be that serotonin may execute both an inhibitory and an augmenting influence on fear and that its absence hence may allow both an increase and a reduction of fear behaviour to occur, depending on circumstances.

In this view, we observed a reduction of contextual freezing also after a pronounced enhancement of extracellular serotonin induced by the serotonin precursor 5-HTP (Paper III and IV) or a serotonin-releasing agent, fenfluramine (Paper III). An inhibitory impact of 5-HTP on conditioned fear and anxiety-like behaviour in conflict-based models [94, 95, 116, 119] has been reported previously.

Tentatively, serotonin may reduce fear by some receptors, and the activity of these receptors is high when extracellular serotonin is abundant, but absent or low when the serotonin level is normal or only moderately increased (e.g. during acute SSRI treatment), allowing fear-promoting serotonin receptors (e.g. the 5-HT2A receptor) to dominate under such conditions. As said, it is not known which serotonin receptors that mediate the inhibitory serotonergic influence on contextual fear, but the results of paper III suggest that the 5-HT1A receptor is not of importance in this context.

Alternatively, the reason for the anti-freezing influence of drugs causing an excessive, non-physiological increase in synaptic serotonin may be that freezing is dependent on a physiological, stress-induced release that is precisely coordinated in time with other neuronal responses to the stressful stimulus, and that both serotonin depletion and excessive serotonin release disrupt this presumably fine-tuned machinery.

As discussed above, the mechanism underlying the unmasking of an anti-freezing effect of an SSRI by pretreatment with a 5-HT2A antagonist may be explained by a change in leverage for different postsynaptic serotonin receptor populations (possibly in different brain regions): when the anxiogenic influence of the 5-HT2A receptor is out of play, the dominating influence of an SSRI-induced elevation of extracellular serotonin may be enhanced activation of yet unidentified, postsynaptic freezing-reducing receptors.

However, an alternative explanation to the freezing-reducing effect of 5-HT2A antagonism, and one that gains support by the results obtained using fenfluramine and 5-HTP, could be that a negative feedback on serotonergic firing mediated by the 5-HT2A receptor is blocked [175] hence causing a potentiation of the SSRI-induced elevation of extracellular serotonin levels and thus eliciting the freezing-reducing impact of serotonin abundance. Since negative feedback on serotonergic neurons during acute SSRI treatment is also exerted by 5-HT1A and 5-HT2C receptors [28, 49, 175], the relevance of this mechanism is supported by the resulting freezing reduction following a 5-HT1A

antagonist plus acute SSRI, but contradicted by the lack of effect of a 5-HT2C antagonist plus SSRI (Paper III).

Adding to the complexity, in contrast to the freezing-reduction caused by adding a 5-HT2A antagonist to acute SSRI administration, 5-HT2A blockade *counteracted* the freezing reduction generated by 5-HTP administration. 5-HTP has previously been shown to inhibit anxiety-like behaviour in conflict models in a U-shaped fashion, where intermediate doses resulted in a stronger inhibition than higher and lower doses [94, 95], suggesting high but not exaggerated extracellular serotonin levels to supress anxiety. It is possible that the 5-HT2A antagonist blocks negative feedback as described above so that extracellular serotonin, already markedly raised by 5-HTP, reaches a level where freezing is less reduced. It however also cannot be excluded that postsynaptic 5-HT2A receptors may exert a dual influence on anxiety, the pro- versus anti-freezing influence being dependent on extracellular serotonin levels and/or other circumstances. The alleged paradoxical effects of 5-HT2A agonists in depression (see above) may be regarded as support for this possibility.

The fact that both PCPA-mediated serotonin depletion, and increased extracellular level of serotonin inhibit contextual freezing makes it difficult to draw any firm conclusion regarding if the anti-freezing effect of long-term but not short-term SSRI administration revealed in paper IV is best explained as the result of a down-regulation or an up-regulation of serotonergic output. Also, both these descriptions may be false: other possibilities are that the SSRIs modify the balance between different postsynaptic receptors (e.g. by down-regulating the 5-HT2A receptor), or act as serotonin stabilizers, causing a modest increase in baseline extracellular serotonin levels while (in patients) preventing excessive stress-induced release.

There is indeed evidence for a the notion that long-term SSRI treatment down-regulates serotonergic signalling on the presynaptic side; subchronic SSRI treatment is hence reported to down-regulate TPH2 [55, 56], to reduce serotonin synthesis in patients with an anxiety disorder [59] and to reduce serotonin release triggered by aversive stimuli in rats [144].

If down-regulation of TPH2 were a key mechanism, the administration of 5-HTP in our model should however be expected to reverse the freezing reduction induced by long-term SSRI treatment. While we did obtain an indication for such an effect to be at hand when 5-HTP was administered to PCPA-treated animals, no corresponding effect was however observed when 5-HTP was given to SSRI-

treated rats. Whereas this finding argues against the notion that SSRI-induced presynaptic down-regulation is the mechanism of action for the anti-freezing effect, it does not preclude an involvement of a down-regulation of postsynaptic anxiety-promoting receptors. To shed further light on this issue, studies combining behavioural studies with assessment of serotonin release and receptor density obviously are warranted.

Combining methods measuring extracellular serotonin concentration with behavioural parameters as the ones described in this thesis, would be an interesting strategy for analysing influence of serotonin level on contextual freezing.

3.10. Freezing detection: Methodological aspects

In an attempt to attain an objective analysis of freezing [176], automatic scoring was used, taking only complete immobility, but not the additional hallmarks of typical freezing, into account. Additional analyses in our laboratory (not presented here) however indicate that typical freezing behaviour and scored immobility show strong correlation, i.e. otherwise normal animals exhibiting enhanced immobility after exposure to a fear-conditioned context usually also display freezing when immobile. Moreover, in all experiments, it was confirmed by gross observation that rats displaying immobility did show typical freezing behaviour. There were however two situations when this was not the case.

The first exception was seen in PCPA-treated animals in Paper II, that had not been exposed to foot shocks. Thus, the immobility expressed by these animals was lacking the additional characteristics of typical freezing behaviour, and was likely not fear-related. PCPA-treated animals of Paper IV that had not received foot shocks did not display non-freezing immobility, indicating that the behaviour is related to the limited space of the startle box. Therefore, a larger box, which should promote mobility and hence reduce unwanted atypical immobility, was used in Paper III and IV.

A second exception was the immobility of animals treated with a 5-HT1A receptor antagonist in combination with escitalopram or with 5-HT2A antagonist plus escitalopram (Paper III). These animals generally expressed a sedated-like behaviour, with no features of freezing apart from complete immobility.

3.11. Learning and memory versus fear expression (Paper II, III and IV)

Since conditioned fear is a memory-based model, compounds with an effect on memory can influence the expression of conditioned fear. Importantly, serotonin depletion has been associated with impaired working memory in other paradigms [34]. Moreover, 5-HT2A receptor stimulation has been shown to improve, and 5-HT2A receptor inhibition to impair, working memory and associative learning in primates [177-179]. The possibility that these effects have contributed to the results obtained should hence not be overlooked. On the other hand, PCPA has shown fear-reducing properties in other models of anxiety that are not based on learning and memory, such as EPM [126]. Moreover, administration of the 5-HT2A antagonist alone displayed only a moderate impact on contextual freezing; it was only in combination with an SSRI, which itself has no known effect on memory function [99], that freezing was reduced. With respect to the 5-HT1A receptor, this subtype has been shown to impair memory functions [180], so the possibility that an antagonist would inhibit expression of contextual fear by impairing memory hence appears unlikely. Noteworthy, however, is that male rodents show better learning than females [181], which could contribute to why they express more contextual freezing in Paper II.

It should also be noted that the present study addressed the effects of drug exposure either before and during both fear acquisition and expression, or before and during expression only. What possible effect the tested compounds may have when administered during acquisition only was hence not addressed in this thesis.

4. CONCLUDING REMARKS

Although it is well established that serotonin can influence anxiety in humans, it is still not known whether the substance primarily exerts an enhancing or dampening influence. Likewise, it is not established whether the therapeutic effect on anxiety achieved by sustained SSRI treatment is due to intensified or inhibited serotonergic transmission.

An animal model mirroring the effect of SSRIs on humans by exacerbating anxiety during initial treatment in a subset of (anxious) individuals would hence be desirable. The data presented in this thesis (Paper I) suggest simultaneous measurement of startle and freezing in response to loud sounds to be a reliable method for classifying rats as more or less anxious, and indicate that those that are more anxious are also more likely to display an enhancement in fear-potentiated startle when exposed to acute SSRI administration.

Moreover, in experiments focussing on fear-conditioned freezing, we show that long-term unlike acute SSRI administration inhibits freezing, but that such an effect also of acute administration can be unmasked by simultaneous administration of a 5-HT2A antagonist. Complicating the interpretation of these results, both serotonin depletion and excessive serotonergic output also reduced freezing.

Another aspect discussed in this thesis is the relation between serotonin and sex hormones, where a male-specific reduction in contextual freezing was seen following serotonin depletion, thus abolishing a sex difference in this behaviour. Whereas this kind of marked sex difference is probably not at hand in humans, the possibility that the influence of serotonin on mood and behaviour is not identical in the two sexes should not be ignored.

Needless to say, the present results justify a number of follow-up experiments, with the objectives listed below:

- To assess the effects of long-term SSRI treatment in anxious versus non-anxious animals

- To evaluate the effect of long-term and short-term SSRI treatment, as well as 5-HTP and fenfluramine, in female rats

- To utilize the model of Paper I to evaluate which postsynaptic

receptor(s) that mediate the observed anxiety-enhancing effect of acute SSRI treatment

- To examine if there are serotonin-related biochemical differences between 'anxious' and 'non-anxious' rats in the model used in Paper I

- To investigate the effect of antagonists against other receptors than the 5-HT1A on the anxiolytic effect of acute SSRI plus 5-HT2A blockade

- To compare the different treatments in Paper III and IV concerning the effect on extracellular serotonin (using microdialysis)

- To study if the serotonin release associated with conditioned fear can be attenuated by long-term SSRI treatment (using microdialysis)

- To analyse, functionally and by assessment of receptor density, if long-term SSRI treatment down-regulates 5-HT2A receptors in relevant brain areas (as preliminary data suggest that they do)

5. ACKNOWLEDGEMENTS

The **Swedish Science Council**, the **Swedish Brain Foundation**, **Söderberg's Foundation** and **Hållsten's Foundation** are sincerely acknowledged for their financial support.

I would also like to express my deepest appreciation to the people that contributed particularly to the accomplishment of this thesis:

Elias Eriksson, for giving me the opportunity to work in this exciting field of research, for your enviable optimism and cheerfulness at all times, and for challenging my scientific thinking, also when it is less convenient

All **staff** of the **Department of Pharmacology** for memorable times and for making this a working environment to aspire

Melker Hagsäter, for excellent collaboration, for your odd ideas that so often turn out brilliant, and for good advice during my writing process

Daniel Hovey for all helpful suggestions for my thesis, for being a true friend and for ensuring that I get a break for small talk now and then

Daniel Eckernäs, for your chumminess and for social initiatives such as the Game of Thrones evenings

Alexander Lisinski and **Fredrik Hieronymus** for your helpfulness and for making this department more entertaining

Martin Johnsson for your constant good mood and for social initiatives

All other **PhD students**, for fine memories and for maintaining a social and unselfish atmosphere

Jakob Näslund and **Erik Studer,** for introducing me to this area of research, for great collaboration, as well as for good friendship and unforgettable moments, abroad as well as in Sweden

Lina Jonsson, for your friendliness and for sharing your wide knowledge as well as for great memories, such as those from travelling in Japan

Soaleha Shams, for your friendliness, your easy-goingness and for being as generous with your time and help as with your candy

Filip Bergquist as well as my co-supervisor **Hans Nissbrandt**, for sharing your time and enormous knowledge and for introducing me to the world of HPLC

Elisabeth Jerlhag, **Lars Westberg**, **Alana Rejfeldt** and **Thomas Carlsson** for always taking the time, in the middle of more important things, for giving me helpful advice in research

Lars Börjesson for your excellent assistance, your patience, as well as your admirable skills in small-talk

Gunilla Bourghardt, **Ann-Christine Reinhold** and **Inger Oscarsson** for great assistance and for sharing your expertise in laboratory work

Staffan Nilsson, for outstanding collaboration and for explaining statistics better than anyone else I have ever encountered

Björn Carlsson and **Louise Karlsson**, at the Department of Clinical Pharmacology at Linköping University for your collaboration

Axel Holmäng and **Christopher Pettersson**, for your interest in and valuable contribution to this project

All **summer students** over the years, for the assistance and for your interest in our research

My parents, **Fingal** and **Lotta**, for showing interest in my project, for always standing behind me in my life choices and for all help with dogs and children under times of hard work. To see that you enjoyed it made the help even greater

Kicki and **Herbert Strenn** for all the help with the children and for your unbelievable hospitability and kindness during my intense thesis writing in your home

My two dogs, **Ophelia** and **Filifjonka** for always cheering me up and for your demand of walks, giving me a useful break now and then

My best results during this PhD Project – my three wonderful daughters, **Elsa**, **Selma** and **Freya**, for filling my life with joy and always giving me something to look forward to

Nina Strenn, for your magnificence, both as my partner and as the mother of our children, making these years as PhD students into something I could never have dreamt of. Also, your support, help, encouragement and honest remarks truly improved this thesis. I am indefinitely grateful for having you in my life!

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7. APPENDIX: MATERIALS AND METHODS

7.1. Animals

In all experiments described in this thesis, the most well-used rat in animal research, Norwegian brown rat (Rattus Norvegicus), was used as experimental animal. Two different outbred strains were used: Wistar and Sprague-Dawley. Different strains are known to differ in behaviour, which is important to keep in mind. In pilot experiments was found that Wistar rats were favourable for the study of startle, whereas Sprague-Dawley rats were preferable when studying freezing. Therefore, Wistar rats were used in experiments where startle was assessed, while Sprague-Dawley rats were used when freezing was the only behaviour of interest. In Paper II, female and male rats were used, in the remaining Papers only male rats.

The animals were 9-11 weeks at arrival and were housed 3-4 per cage in an animal facility with 12/12 hour light/dark cycle and free access to food and water.

7.2. Contextual fear conditioning (Paper I-IV)

Contextual fear conditioning was used in all experiments presented. For the startle reflex to be strengthened a more robust protocol is required than what is necessary for generating freezing behaviour only. Since startle was studied in Paper I and II more electric foot shocks were given than in Paper III, in which only freezing was studied. Interesting results in the freezing parameter regarding the effect of PCPA in Paper II however prompted us to use a more intense protocol also in Paper IV, despite no startle being studied.

In the contextual conditioning procedure of Papers I and II, the rat was placed in a wire mesh cage attached to a response platform constructed to measure startle amplitude (Startle reflex system®. Med Associates, St Albans, VT, USA). After an acclimation period of five minutes, 10 electric foot shocks with amperage of 0.8 mA, duration of 250 milliseconds and intervals of 90 seconds were delivered through the grid floor of the cage. A protocol of this intensity has been suggested optimal for simultaneous analysis of startle and freezing [168]. In Paper I, the rats had been placed in the chamber twice before the conditioning, without receiving any foot shocks.

Since startle was not measured in experiments of Papers III and IV, the rat was placed in a much larger chamber with a grid floor (Contextual NIR Video Fear Conditioning System for Rat, Med Associates, St Albans, Vermont, USA). After five minutes of acclimation the rat received five (Paper III) or ten (Paper IV) electric foot shocks with amperage of 0.6 mA, duration of 1 second and intervals of 30 seconds.

7.3. Simultaneous startle and freezing analysis (Paper I and II)

Rats were placed in the same wire mesh cage as had been used for contextual conditioning. The startle/freezing test protocol consisted of a five-minute acclimation period followed by a series of noise bursts (95 dB, 20 milliseconds long) with an interval of 30 seconds.

Each startle response of a rat generated a movement of the cage, which was sensed by the response platform, causing an electrical signal to a computer where the amplitude of each response was automatically computed using Startle Reflex Software® (Version 6.00, Med Associates).

The animals were video-recorded during the procedure with monochrome, near infrared video camera and the Video Monitor Software® (Med Associates) and freezing, during the acclimation period and during the period where noise burst were delivered, was measured subsequently by automated scoring of the videos utilizing an in-house composed MATLAB® script, comparing subsequent frames. Freezing was presented as percentage of time spent completely immobile for more than 1 second [182].

7.4. Contextual freezing (Paper III and IV)

The rats were placed in the same box as where they had undergone contextual conditioning. Freezing in this larger conditioning box was measured with the same method as with the smaller box, i.e. by automated scoring of video recordings, the difference being a shorter test period and that no noise bursts were applied.

7.5. Estrous cycle determination (Paper II)

Because the level of female sex hormones are fluctuating during the rat estrous cycle, corresponding to the human menstrual cycle, and these hormones have an impact on rat behaviour, it was important to determine estrous cycle phase in our experiments. This was done by obtaining vaginal smear samples, using distilled (MilliQ) water, from all saline-treated female rats after the startle/freezing test. No samples were obtained from PCPA-treated animals since the drug is known to impair normal estrous cycling. After having air-dried properly, the samples were analysed in light microscope and estrous phase was determined by analysing the morphology of cells and their distribution according to guidelines described by Marcondes and co-workers [183]

7.6. HPLC (Paper II)

For determination of successful serotonin depletion in PCPA-treated animals, analysis of whole brain serotonin was performed using a high-pressure liquid chromatography with electrochemical detection (HPLC-ED), in which substances can be separated based on their chemical and electrochemical properties. Immediately following the startle/freezing tests, randomly selected rats from each treatment group were anesthetized using inhalation of isoflurane and killed by means of decapitation. Brains were then removed and frozen at −80 °C. Later, one hemisphere from each extracted brain was homogenized in a solution and the supernatant was analysed by the HPLC-ED system, where serotonin amount was determined using an ion exchange column, in which the separation of the sample is mediated by van der Waals interactions with charged particles.

7.7. Serum retrieval and serum-escitalopram analysis (Paper IV)

For assessment of serum levels of escitalopram (experiment II and III) animals were anesthetized using inhalation of isoflurane and trunk blood was retrieved immediately following decapitation of the animals. The blood samples were centrifuged (2300 RCF), whereupon serum was removed and frozen (-80 to -20 °C) until analysis. In experiment III, all samples were obtained immediately

after testing, to ensure that serum levels had not changed.

The serum samples were analysed at the Division of Drug Research at the Department of Clinical Pharmacology at Linköping University (Linköping, Sweden) using HPLC with fluorescence detection combined with liquid chromatography-mass spectrometry (LC-MS).

7.8. Drug preparation and administration

The drugs used in these experiments were as follows:

- Escitalopram oxalate (Shodana Labs, Hyderabad, India) used in Paper I, III and IV
- PCPA (4-chloro-DL-phenylalanine-methyl ester hydrochloride, Sigma-Aldrich, St Louise, Missouri) used in Paper II and IV
- MDL100907 (Tocris, Bristol, UK) used in Paper III
- SB242084 (Sigma-Aldrich) used in Paper III.
- WAY100635 (Tocris, Bristol, UK) used in Paper III
- 5-HTP (5-Hydroxy-L-tryptophan, Sigma-Aldrich) used in Paper III and IV
- Carbidopa (S-(-)-Carbidopa, Sigma-Aldrich) used in Paper III
- Fenfluramine (Dexfenfluramine hydrochloride, Tocris, Bristol, UK)

All compounds used for injections were dissolved in 0.9 % sodium chloride, i.e. saline, at a concentration so that the injected volume would be approximately 1 ml. PCPA was injected intraperitoneally (i.p.), the remaining compounds were injected subcutaneously.

MDL100907, carbidopa and 5-HTP were all dissolved while heated and stirred, while adding a few drops 1 M hydrochloric acid (HCl) until dissolved, then a few drops of 0.1 M sodium hydroxide (NaOH) until pH reached 6.4.

SB242084 was dissolved at a concentration of 0.5 mg/ml with 8 % cyclodextrin and 0.48 % citric acid, while heated and stirred, whereupon pH was adjusted to 6.4 using a small volume of 1 M NaOH.

WAY100635 was dissolved at a concentration of 2 mg/ml by the addition of 8 % DMSO during heating and stirring.

For administration of escitalopram through food, rat chow pellets containing the drug, as well as pellets not containing the drug, were prepared by Lantmännen (Kimstad, Sweden). The concentration of drug in the food (0.65 mg/g) was calculated based on desired daily dose (24 mg/kg), estimated amount of food eaten per day and estimated body weight of the rats.

For administration in osmotic minipumps (2ML4, Alzet), 2 ml of escitalopram dissolved in saline, with a concentration (53 mg/ml) based on calculations of desired daily dose (10 mg/kg), estimations of rat body weight (500 g) as well as previous experiments [53], and was injected into each minipump, whereas minipumps of control rats were injected with 2 ml saline. Under anesthesia provided by administration of ketamine and rompun (experiment II) or isoflurane inhalation (experiment IV), the pumps were then implanted subcutaneously in an incision in the neck of the rats, which was then closed by sewing.

7.9. Statistical analysis

All statistical analysis was carried out using IBM® SPSS® Statistics version 20. One-way ANOVA with LSD post-hoc was used for comparisons between groups. For analysis on dual influence, such as the effect of sex versus the effect of PCPA in Paper II, two-way ANOVA was used.

Investigations of correlations were made using Pearson correlation tests.

In Paper I, the delta-values between the second test before treatment and the test after treatment (test 3-test 2) for rats of each treatment group were regressed on values of the first test (test 1) before treatment and the regression slopes of the treatment groups were compared using an interaction term between the value in test 1 and treatment group. In a two-way ANOVA the influence of contextual conditioning and escitalopram treatment on test 3 values was analysed in the whole group, as well as divided into high and low test 1 startle or freezing. To rule out regression towards the mean, i.e. that multiple testing tends to cause a normalization of spread out data, these analyses were adjusted for test 2 value by using it as a covariate.

7.10. Ethics

Since the objectives of this thesis depend on behaviour, the use of live animals for the experiments can unfortunately not be circumvented. However, in accordance with the ”reduce” principle of the ”three R’s” (refine, reduce, replace), the number of rats was limited to what was deemed necessary for the objectives of each experiment. To this purpose, before undertaking larger experiments, pilot experiments, involving a smaller number of animals, had first been performed. The possible knowledge gained and the potential benefit of this knowledge for mankind was always weighed against the discomfort of the animals used and the experiments were planned so that possible harm to the animals was minimized, with the objectives still being attainable.