

Serotonin in Fear and Anxiety

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ABSTRACT

That the neurotransmitter serotonin (5-HT) has a central role in fear and anxiety is supported by numerous experimental and clinical studies. Arguably the most illustrative example is the effect of serotonergic-acting drugs, and in particular the selective serotonin reuptake inhibitors (SSRIs), in the treatment of anxiety disorders. Interestingly, long-term administration is required to induce a dampening effect on anxiety, while on the contrary acute administration can aggravate the symptoms in susceptible individuals.

Freezing behaviour is a well-established measure of fear and anxiety, foremost assessed in studies performed on rodents. The bulk of the experiments presented in this thesis investigate the effect of pharmacological manipulations of the serotonin system on conditioned and unconditioned freezing behaviour.

In paper I, the importance of an intact serotonergic neurotransmission in fear conditioning was explored. The serotonin system was compromised by administration of the serotonin-depleting agent *para*-chlorophenylalanine (PCPA). PCPA impaired both acquisition and expression of conditioned fear without imposing an effect on memory consolidation, supporting the notion that fear-induced release of serotonin primarily promotes rather than dampens fear conditioning.

In paper II, the effects of 5-HT_{2A} receptor agonism and antagonism alone and in combination with administration of an SSRI were investigated. The first main finding was that while administration of neither the 5-HT_{2A} receptor antagonist MDL 100907 nor the 5-HT_{2A} receptor inverse agonist pimavanserin consistently reduced expression of conditioned freezing, a marked reduction of fear was observed after administration of either drug combined with an SSRI. The second main finding was that administration of

both a selective agonist for the 5-HT_{2A} receptor and the psychedelic drug psilocybin reduced expression of conditioned freezing, an effect that was totally abolished by co-administration with MDL 100907. The experiments demonstrated that the 5-HT_{2A} receptors have the ability to modulate fear expression in both directions, putatively involving 5-HT_{2A} receptor populations in different areas of the brain.

In paper III, the effects of chronic and acute administration of an SSRI were compared. Chronic administration but not acute administration induced a dampening effect on anxiety. Since these findings mirror the clinical situation, context-conditioned freezing could supposedly be applied in animal studies on the mechanism of action for the sluggish effects of SSRIs in anxiety disorders.

In paper IV, the effect of acute administration of an SSRI was evaluated in a model of unconditioned fear. Acoustic noise bursts constituted the unconditioned stimulus. The SSRI increased the expression of unconditioned fear. Unconditioned models are tentatively related to panic disorder, the condition in which aggravation of anxiety after acute administration of an SSRI is most pronounced, suggesting that noise burst induced freezing presumably could be a useful tool in preclinical studies on the anxiety-provoking effects of SSRIs.

In paper V, the effect of 5-HT₆ receptor manipulations on gut motility was explored. It was found that 5-HT₆ receptor antagonists reduced defecation in both stressed (fear conditioned) and non-stressed animals while an agonist for the same receptor was void of effect. This mechanism could putatively be utilized in the treatment of irritable bowel syndrome with diarrhea (IBS-D).

In summary, the studies on rats presented in this thesis suggest that i) intact serotonergic transmission is required for both acquisition and expression of conditioned fear, ii) that the 5-HT_{2A} receptor has an important role in modulating conditioned fear, iii) that chronic administration of an SSRI, in contrast to acute administration, reduces conditioned fear, iv) that acute administration of an SSRI increases unconditioned fear and v) that 5-HT₆ receptor antagonism impairs gut motility.

Keywords: serotonin, fear, anxiety, SSRI, IBS

SAMMANFATTNING PÅ SVENSKA

Signalsubstansen serotonin (5-HT) har en central roll vid rädsla- och ångesttillstånd vilket stöds av ett stort antal experimentella och kliniska studier. Det kanske mest slående exemplet är den effekt serotoninmodulerande substanser, och i synnerhet de selektiva serotoninåterupptagshämmarna (SSRIs), har vid behandling av ångestsjukdom. För att erhålla en gynnsam effekt krävs att dessa preparat tas under en längre tid medan ångestsymptomen hos vissa individer tvärtom (övergående) kan förvärras vid behandlingsstart.

Frysning beteende (*freezing behaviour*) är ett mått på rädsla och ångest som i synnerhet är välstuderat i gnagare. Merparten av experimenten som presenteras i denna avhandling undersöker effekten av farmakologiska manipulationer av serotonin systemet på konditionerat och icke-konditionerat frysning beteende.

I delarbete ett (paper I) studerades betydelsen av intakt serotonin funktion för kontextuell konditionering. Hjärnans serotonin nivåer reducerades med hjälp av substansen *para*-chlorophenylalanine (PCPA). PCPA hämmade både konditioneringen och uttrycket av konditionerad rädsla men uppvisade inga minnesstörande effekter, vilket stödjer uppfattningen att det serotonin som frisätts vid negativ stimulering förstärker snarare än försvagar konditioneringseffekten.

I delarbete två (paper II) undersöktes effekten av 5-HT_{2A}-receptoragonism- och antagonism enskilt eller i kombination med escitalopram (ett SSRI-preparat). Det första huvudfyndet var att varken behandling med 5-HT_{2A}-receptorantagonisten MDL 100907 eller med den inversa 5-HT_{2A}-receptoragonisten pimavanserin dämpade konditionerad rädsla, vilket däremot kombinationen av endera drog och escitalopram gjorde. Det andra huvudfyndet var att en selektiv agonist för 5-HT_{2A}-receptorn, samt den psykedeliska substansen psilocybin, påtagligt minskade uttrycket av konditionerad rädsla, en effekt som totalt upphävdes vid samtidigt administrering av MDL 100907. Försöken visar att 5-HT_{2A}-receptorer kan

modulera uttrycket av konditionerad rädsla, i båda riktningarna, en effekt som kan antas involvera receptorpopulationer i flera olika delar av hjärnan.

I det tredje delarbetet (paper III) jämfördes effekten av akut och kronisk behandling med escitalopram på konditionerad rädsla. Kronisk behandling, till skillnad från akut behandling, uppvisade en ångestdämpande effekt. Att dessa fynd motsvarar det man ser vid klinisk behandling talar för att konditionerad rädsla kan användas som en experimentell modell för att studera den fördröjda ångestdämpande effekten hos SSRI-preparat.

I delarbete fyra (paper IV) studerades effekten av akut administrering av escitalopram på okonditionerad rädsla i form av korta ljudstötter med hög amplitud. De råttor som hade behandlats med SSRI-preparatet uppvisade mer frysningsbeteende jämfört med kontrollerna. Modeller som studerar okonditionerad rädsla anses kunna representera paniksyndrom, vilket är den ångestsjukdom där man tydligast sett en förvärring av ångestsymptom vid behandlingsstart med SSRI. Således skulle den studerade modellen potentiellt kunna användas för prekliniska studier av den akuta ångestinducerande effekten hos SSRI-preparat.

I delarbete fem (paper V) undersöktes effekten av 5-HT₆-receptor-manipulationer på tarmmotilitet. Olika 5-HT₆-receptorantagonister hämmade tarmmotilitet hos både stressade (konditionerad rädsla) och ostressade djur medan en 5-HT₆-receptoragonist inte uppvisade någon effekt. Denna mekanism skulle potentiellt kunna utnyttjas vid behandling av colon irritabile.

Sammanfattningsvis indikerar fynden på råttor i dessa delarbeten i) att intakt serotoninfunktion behövs för både konditionering och uttryck av konditionerad rädsla, ii) att 5-HT_{2A}-receptorn har en viktig roll i moduleringen av konditionerad rädsla, iii) att kronisk behandling med SSRI, till skillnad från akut behandling, minskar konditionerad rädsla, iv) att akut behandling med SSRI ökar okonditionerad rädsla och v) att 5-HT₆-receptorantagonism reducerar tarmmotilitet.

LIST OF PAPERS

- I. Hagsäter SM, Pettersson R, Holmång A and Eriksson E, "Serotonin depletion reduces both acquisition and expression of context-conditioned fear", *Submitted*
- II. Hagsäter SM, Pettersson R, Pettersson C, Atanasovski D, Näslund J and Eriksson E, "A complex impact of the 5-HT_{2A} receptor on conditioned fear revealed by systemic administration of different 5-HT_{2A} ligands including pimavanserin and psilocybin", *Submitted*
- III. Pettersson R, Hagsäter SM, Carlsson B, Karlsson L and Eriksson E, "Chronic but not acute administration of escitalopram reduces context-conditioned fear", *Submitted*
- IV. Hagsäter SM, Thorén J, Pettersson R and Eriksson E, "Selective serotonin reuptake inhibition increases noise burst-induced unconditioned and context-conditioned freezing", *Acta Neuropsychiatrica*, 2019, **31**, 46-51
- V. Hagsäter SM, Lisinski A and Eriksson E, "5-HT₆ receptor antagonism reduces defecation in rat: A potential treatment strategy for irritable bowel syndrome with diarrhea", *European Journal of Pharmacology*, 2019, **864**, 172718

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ABBREVIATIONS

5-7-DHT	5,7-dihydroxytryptamine
5-HT	5-hydroxytryptamine; serotonin
5-HTP	5-hydroxytryptophan
5-HTT	Sodium-dependent serotonin transporter (same as SERT)
BAn	Basal amygdalar nucleus
BBB	Blood brain barrier
BLA	Basolateral complex (of the amygdala)
BNST	Bed nucleus of the stria terminalis
BZD	Benzodiazepine
CEl	Central lateral division (of the amygdala)
CEm	Central medial division (of the amygdala)
CEn	Central nucleus (of the amygdala)
CFS	Conditioned fear stress
CNS	Central nervous system
CS	Conditioned stimulus
dPAG	Dorsal periaqueductal gray
DRN	Dorsal raphe nucleus
ECs	enterochromaffin cells
GAD	Generalized anxiety disorder
GI	Gastrointestinal
IBS	Irritable bowel syndrome
IBS-D	Irritable bowel syndrome with diarrhea
LAn	Lateral amygdalar nucleus
LSD	Lysergic acid diethylamide
MAOA	Monoamine oxidase A
MAOB	Monoamine oxidase B
MAOI	Monoamine oxidase inhibitor
MRN	Medullary raphe nuclei
NA	Noradrenaline; norepinephrine

NAc	Nucleus accumbens
OCD	Obsessive compulsive disorder
PAG	Periaqueductal gray
PCPA	<i>para</i> -chlorophenylalanine
PD	Panic disorder
PMAT	Plasma membrane monoamine transporter
PTSD	Post-traumatic stress disorder
RCF	Relative centrifugal force
SERT	Sodium-dependent serotonin transporter (same as 5-HTT)
SNRI	Serotonin–norepinephrine reuptake inhibitor
SSRI	Selective serotonin reuptake inhibitor
TCA	Tricyclic antidepressant
TPH	Tryptophan hydroxylase
vlPAG	Ventrolateral periaqueductal gray
vPAG	Ventral periaqueductal gray
US	Unconditioned stimulus

FEAR AND ANXIETY

Fear and anxiety in humans

Fear and anxiety are unpleasant emotions that, evolutionary, have saved the subject from dangerous situations. There are differences between fear and anxiety with respect to triggers, duration and circuitry (Davis et al. 2010). Fear is induced by an immediate or imminent threat, whereas anxiety is induced by a threat that is uncertain or is distal in space or time (Davis et al. 2010, LeDoux and Pine 2016). Fear triggers active defence responses while anxiety induce arousal and vigilance and while fear is diminished once the threat is gone, anxiety can last for longer durations. However, the emotions are related in that they share certain common symptoms. Hence, it can be difficult to tell whether a subject is experiencing fear or anxiety in a certain situation and the words are often used interchangeably (LeDoux 2017).

Symptoms of anxiety are commonly separated into psychological (cognitive) anxiety and somatic anxiety (Hamilton 1960). In the traditional “fear center” model, the feelings of fear are thought to originate from the same subcortical areas that control the behavioural and physiological responses to fear. This view has been challenged, and a “two-system” framework has been proposed in which the fear circuitry is divided into one cortical *cognitive circuit* and one subcortical *defensive survival circuit* (LeDoux and Pine 2016). This division stresses that the words fear and anxiety should be used exclusively for their respective mental states (LeDoux 2017).

Pathological fear and anxiety in humans

Dysregulated fear responses can manifest themselves as pathology in humans, i.e. anxiety disorders (Parsons and Ressler 2013). In adults, according to DSM-5, these

comprise specific (simple) phobia, agoraphobia, panic disorder (PD), generalized anxiety disorder (GAD), substance-induced anxiety disorder and anxiety disorder due to another medical condition (American Psychiatric Association 2013). Obsessive-compulsive disorder (OCD) is no longer classified as anxiety disorder, but is relevant to mention as it responds to the same kind of pharmacological treatment.

Studies on comorbidities among different mental disorders have suggested that fear and anxiety cluster into two different clinical entities: *fear* (social phobia, simple phobia, agoraphobia and PD) and *anxious-misery* (major depressive episode, dysthymia and GAD), respectively (Krueger 1999, Vollebergh et al. 2001). In human experimental anxiety (Grillon et al. 2019), the general perception is that unconditioned models, such as simulated public speaking, are related to social phobia and PD (Graeff et al. 2003, Siepmann and Joraschky 2007, Zuardi et al. 2013). Similarly, conditioning models, such as aversive conditioning to tones and fear potentiated startle, are supposedly related to GAD (Graeff et al. 2003, Siepmann and Joraschky 2007).

There are three main classes of drugs that are used to treat anxiety disorders: 5-HT_{1A} receptor agonists, benzodiazepines (BZDs) and selective serotonin reuptake inhibitors (SSRIs) (Millan 2003). Only BZDs have an immediate effect and 5-HT_{1A} receptor agonists are only effective in treating GAD and only moderately so (Fulton and Brogden 1997, Howland 2015, Sheehan et al. 1990).

Fear and anxiety in animal research

Throughout this thesis, when not stated otherwise, statements on fear and anxiety in animals are referring to the defensive survival circuit (LeDoux and Pine 2016), that is, a non-emotional state of consciousness.

Similar to humans, if an animal can clearly identify a threat the reaction can be regarded as fear, whereas if it cannot, it can be regarded as anxiety (Walker et al.

2003). While fear is a direct response to a specific threat, anxiety describes a vaguer experience.

Fear and anxiety are difficult to separate in animals. In an experimental situation, it can be hard to determine whether an animal has identified the threat (indicating fear) or if it is reacting to the experimental situation (indicating anxiety). Animals cannot be queried whether they are experiencing fear or anxiety and neither is it possible to tell how their emotions qualitatively resemble human feelings (LeDoux and Pine 2016).

Researchers have divided fear into different categories, depending on the situation or context in which fear is studied, where some subdivisions have been denoted anxiety.

One division is based on that different types of threats activate different circuits in the brain. Some have suggested the amygdala to control fear (short term, *phasic fear*) and the bed nucleus of the stria terminalis (BNST) to control responses closer resembling to anxiety (long term, *sustained fear*) (Davis et al. 2010, LeDoux and Pine 2016, Miles et al. 2011, Walker et al. 2003, Walker and Davis 2008).

Fear can also be divided into *innate fear* and *learned fear*. Innate fear include fear of predators, aggressive conspecifics, pain or species specific threats in the environment (Blanchard and Blanchard 1989). An innate fear-inducing episode will also be remembered in order to improve performance in similar situations in the future, i.e. the context or other neutral cues will be associated with the unpleasant event (Silva et al. 2016). This is referred to as learned (or conditioned) fear (LeDoux 2003).

Yet another separation is based on which behavioural responses that are evoked, where active avoidance (i.e. attempting to leave a potentially dangerous situation) corresponds to fear and approach (e.g. behavioural inhibition or risk assessment) corresponds to anxiety (McNaughton and Corr 2004).

Finally, fear in animals (and in humans) can be divided into *trait anxiety* (*sustained fear*, i.e. anxious personality) and *state anxiety* (*transient fear*, essentially analogous to fear) (Millan 2003). To explore trait anxiety, specific strains, genetic models and inter-individual differences within one strain can be utilized.

To conclude, there is no uniform definition of what is fear and what is anxiety in animals and what the terms are thought to represent is dictated by the circumstances.

Indicators of fear and anxiety

Humans and animals

Feelings of fear and anxiety are typically adjoined by behavioural responses and autonomic adaptations (Gross and Canteras 2012, LeDoux and Pine 2016). These behavioural and physiological signs are assessed as indicators of fear and anxiety. Several behaviours are shared between humans and rodents, such as increased vigilance, startle reflex and freezing (Grillon et al. 1993, Hagenaaers et al. 2014). Many autonomic changes are displayed in common, for instance increased heart rate, decreased salivation, augmented urination and increased defecation (Davis 1992). Since freezing behaviour and increased defecation are the two main indicators of fear that are exploited in this thesis, they are further described below.

Freezing behaviour

Freezing behaviour, a defensive reaction elicited by fear, is one of the most studied fear associated responses (for a review see Hagenaaers et al. 2014). It is readily displayed by small mammals such as rodents, but also by humans, which has gained more attention in recent years (Hagenaaers et al. 2012, Hermans et al. 2013). Freezing is thought to have several evolutionary advantages, such as better avoiding predators (since predators are more likely to attack moving prey), increasing attention and improving preparation for fight-or-flight response.

Freezing was first described as a type of immobility (Griffith 1920, Riess 1945) and the definition that is widely used today defines the behaviour as crouching in a tense body posture (Blanchard and Blanchard 1969), with complete absence of movements except for respiration (Fanselow 1980, Fanselow 1984). Freezing is mostly, but not necessarily, expressed in a sitting posture (Bolles and Collier 1976). It can be evoked by a large number of fearful stimuli, both innate and learned (Blanchard and Blanchard 1969, Blanchard and Blanchard 1972, Bolles and Collier 1976, Conti et al. 1990, Fanselow 1980, Miyata et al. 2007, Phillips and LeDoux 1992, Rosen et al. 2008, Wallace and Rosen 2000). The neural circuitry of freezing is discussed in a later paragraph.

Increased defecation

Changes in bowel habits are one type of physiological adaptation that is induced by fear. Rodents react with increased defecation and humans with diarrhea (Davis 1992). The suggested evolutionary gain is that reduced body weight facilitates escape from a predator. Increased defecation has been observed in response to both innate and learned fear (Antoniadis and McDonald 2000, Blanchard and Blanchard 1969, Ennaceur et al. 2006, Ferre et al. 1995, Funatsu et al. 2007, Sutherland and McDonald 1990). The neural circuitry of defecation is discussed in a later paragraph.

Animal models of fear and anxiety

Introduction

Animals are used as proxies for humans in situations where research cannot be conducted on humans for practical or ethical reasons. The nature of this research can be either basic or aiming towards clinical applications in humans. In animal models of fear and anxiety the animals are typically subjected to a fearful situation, where the fear-inducing method and the studied parameter(s) vary between models.

Below follows a description of the validity of animal models followed by a brief presentation of the animal models used in this thesis.

Validity

The word validity is utilized to denote the transferability of a model (Bourin 2015, Lezak et al. 2017). Validity can be divided into several types, where three variants are commonly discussed in relation to animal models.

In research aiming to shed indirect light on human conditions, *predictive validity* is unquestionably the most important. If drug A (but not drug B) induces effect C in humans, drug A (but not drug B) should induce effect D in the animal model. In principal a model can have good predictive validity independent on the similarities between C and D. However, in the case of the animal models of fear and anxiety, if a drug reduces anxiety in humans it typically induces an anxiolytic-like response in the animals. Drugs that are effective in dampening fear and anxiety in humans, i.e. 5-HT_{1A} receptor agonists, BZDs and SSRIs, are commonly used to assess the predictive validity of animal models of fear (Millan 2003). However, there is no single model of fear and anxiety that displays full predictive validity, which has advocated the use of multiple tests in the screening of psychoactive compounds (Olivier et al. 1994).

For *face validity*, the same stimulus should produce the same behavioural and autonomic responses in the animal model as in humans.

Construct validity is fulfilled when the neuropharmacological and neuroanatomical basis of fear are similar in the animal model and in humans.

Noise burst-induced unconditioned freezing

One fearful stimulus that can evoke freezing behaviour is acoustic noise (Kiernan and Cranney 1992, Plappert et al. 1993) of the same type that is utilized to elicit a startle reflex (Brown et al. 1951, Davis et al. 1993, Kurtz and Siegel 1966). The

noise is not delivered constantly, but in the form of short bursts, separated by a fixed or random time interval (in paper IV a fixed 30 s interval between bursts was used). Freezing, during presentations of noise bursts, and startle reflex are known to correlate (Borszcz et al. 1989, Leaton and Borszcz 1985, Pettersson et al. 2015).

Conditioned freezing

Pavlovian conditioning is the process in which animals learn to associate different stimuli (Pavlov 1927). Pavlovian fear conditioning, a type of learned fear, is the process in which a neutral stimulus (i.e. conditioned stimulus, CS) is associated with an innate threat (i.e. unconditioned stimulus, US) (LeDoux 2000, Rescorla 1988). By presenting the CS and the US together, the biological responses evoked by the US will eventually be evoked by the CS presented alone. The US is often electric foot shocks (Blanchard and Blanchard 1969, Davis and Astrachan 1978, Fanselow 1980), while a few reports utilize acoustic noise bursts as the US (Cranney 1987, Kiernan and Cranney 1992, Young et al. 1991). The CS can be either a discrete cue (such as a light or a tone) (Bolles and Collier 1976, Hunt et al. 1994) or contextual (i.e. conditioning to the experimental conditions and the testing apparatus) (Fanselow 1980, Fanselow and Kim 1994).

Conditioned freezing has been considered to display predictive validity, based on that most studies on 5-HT_{1A} receptor agonists, BZDs and SSRIs have been demonstrated to either block or reduce the freezing response (Conti et al. 1990, Fanselow and Helmstetter 1988, Li et al. 2001). Both animals and humans display freezing (Hagenaars et al. 2014), involving the same brain systems (Fendt and Fanselow 1999), which advocates that both face validity and construct validity are present.

Conditioned fear stress-induced defecation

Rats subjected to conditioned stress display increased colonic motility (Goldstein et al. 1996, Gue et al. 1991, Stam et al. 1996) and psychological stress is an important factor in the pathophysiology of irritable bowel syndrome (IBS) (Stasi

et al. 2012). Based on these observations, conditioned fear stress (CFS)-induced defecation was proposed as a model for IBS with diarrhea (IBS-D) (Funatsu et al. 2007, Hirata et al. 2008). The CFS protocol is a context conditioning protocol where the quantified parameter is colonic transit time or number of stools. In addition, freezing can readily be assessed in the model, which makes it feasible to determine if an observed effect on defecation is secondary to reduced anxiety or not.

CFS-induced defecation displays predictive validity, demonstrated by that both 5-HT₃ receptor antagonists and loperamide reduce CFS-induced defecation in rats (Funatsu et al. 2007, Hirata et al. 2008), as well as symptoms of IBS-D in humans (American College of Gastroenterology Task Force on Irritable Bowel Syndrome 2009). Regarding construct validity, much of the circuitries are similar in rodents and humans (Browning and Travagli 2014), but there are some differences. For example, humans retain faeces and stop activity to defecate, whereas rats do not (Callaghan et al. 2018). Stress initiates similar propulsive effects in both humans and rodents indicating face validity (Davis 1992).

Neuroanatomy of fear and anxiety

Introduction

At first it was believed that all types of threatening situations triggered all types of fear responses (Bolles 1970, Fanselow 1994). More recently fear responses have been separated into (at least) three different circuits: *fear of pain*, *fear of predators* and *fear of aggressive members of the same species* (Gross and Canteras 2012). The pathway denoted fear of pain includes both conditioned and unconditioned fear (displayed as freezing behaviour) (Herry and Johansen 2014, Johansen et al. 2011, LeDoux 2000, Maren 2001) and will be discussed in more detail below. Fear of predators and fear of aggressive members of the same species are not within the scope of the manuscripts included in this thesis and those circuits will not be discussed further.

The brain processes fear similar to a computer; it takes in data, processes them and puts out a result. The input comes from the sensory system and the output is transmitted to the brain stem that initiate the behavioural responses and the autonomic adjustments previously discussed (LeDoux 2000). The amygdala, located near the temporal pole, has a central role in the brain's processing of fear (Davis 1992), illustrated by the blunting of emotional reactivity observed in the Klüver-Bucy syndrome (i.e. impairment of the amygdala) (Aggleton 1993, Lilly et al. 1983). The amygdala was first described as a nucleus in the early 19th century (Burdach 1819), and during 100 years of sequential subdivisions it detailed out into approximately 12 different regions (Johnston 1923) that are largely relevant today (Swanson and Petrovich 1998).

Circuitry of fear conditioning and freezing behaviour

The central components of the circuitry that controls conditioned and unconditioned fear expressed as freezing behaviour are the amygdala, the BNST and the periaqueductal gray (PAG) (Fendt and Fanselow 1999, LeDoux 2000, Walker and Davis 2002). Several other areas of importance are interconnected with these, for example the medial prefrontal cortex, the hippocampus and the hypothalamus (Herry and Johansen 2014, Tovote et al. 2015). Freezing in response to direct threats is primarily relayed in the amygdala, whereas freezing to uncertain threats (indicating anxiety) involves both the amygdala and the BNST (LeDoux and Pine 2016).

Sensory input is processed in the (cortical) basolateral complex of the amygdala (BLA), which contains two nuclei: the lateral amygdalar nucleus (LAn) and the basal amygdalar nucleus (BAn) (Pitkanen et al. 1997, Swanson and Petrovich 1998, Swanson 2000). Animals with BLA impairment are not capable of fear conditioning (Amorapanth et al. 2000, Maren 1999, Oliveira et al. 2004). The LAn is the input station where the CS and US signals converge (Gross and Canteras 2012, Herry and Johansen 2014, Johansen et al. 2011, LeDoux and Pine 2016, Parsons and Ressler 2013, Rogan et al. 1997, Tovote et al. 2015).

The (striatal) central nucleus of the amygdala (CEn) is comprised of several nuclei, of which two prominent subdivisions are the central medial division (CEm) and the central lateral division (CEl) (Swanson and Petrovich 1998). The CEm is under tonic inhibitory control by the CEI (Ciocchi et al. 2010). The BLA transmits excitatory signals to the CEn. Most of these projections are directly from the LAN to the CEI, while some signals are first relayed in the BAN (Swanson and Petrovich 1998).

The expression of several defensive behaviours, including freezing, are initiated by activation of the PAG of the midbrain. In conditioned fear, GABAergic (inhibitory) output from the CEm projects to GABAergic interneurons in the ventrolateral periaqueductal gray (vlPAG) (Tovote et al. 2016). In unconditioned fear, output from the amygdala projects to the dorsal periaqueductal gray (dPAG) (Brandao et al. 2008, Isosaka et al. 2015, Oliveira et al. 2007), in some cases via the hypothalamus (Gross and Canteras 2012). Freezing induced by electric stimulation of the ventral periaqueductal gray (vPAG) decreases when the stimulus is interrupted, while freezing induced by stimulation of dPAG persists for a long time after cessation of the stimulus (Oliveira et al. 2004, Vianna et al. 2001a). Inactivation of the PAG (Johansen et al. 2010), and the vPAG specifically (De Oca et al. 1998, Vianna et al. 2001b), reduces expression of both conditioned and unconditioned fear (e.g. freezing behaviour). The relationship between behaviour elicited by the dPAG and the vlPAG (i.e. innate and learned fear) appears to be hierarchical, where learned fear is suggested to predominate (Magierek et al. 2003). However, certain cell populations in the CEn appears to be able to modulate this relation (Isosaka et al. 2015). Finally, the PAG relays information back to the amygdala, demonstrated by that inactivation of the PAG attenuates US-evoked responses in LAN neurons, putatively resulting in impaired acquisition of conditioned fear (Johansen et al. 2010, Kim et al. 2013).

Among several functions the BNST regulates light enhanced startle (Walker and Davis 1997), freezing induced by predator odour (Fendt et al. 2003) and Pavlovian fear conditioning (Avery et al. 2016, Goode and Maren 2017). The BNST is located in the ventral forebrain and has extensive and far-reaching connections, including

both inhibitory and excitatory output signals (Jennings et al. 2013). Accordingly, depending on placement, electric stimulation of the BNST can both increase and decrease contextual fear responses (Goode and Maren 2017). The BNST receives excitatory input from the BAn, and lesions of the BLA tend to reduce or eliminate both phasic and long-lasting fear responses, even with the BNST intact. Notably, these circuits pass through CEn (Davis and Whalen 2001). The BNST has strong reciprocal connections with the CEn (Nagy and Pare 2008) and innervates both the vPAG (Gray and Magnuson 1992) and the dPAG (Nagy and Pare 2008).

The BNST is involved in conditioning to a context but not to a discrete CS, implying that the BNST regulates long-term fear responses whereas the CEn mediates short-term fear responses (Davis et al. 2010, LeDoux and Pine 2016, Walker et al. 2003, Walker and Davis 2008). Recently this view has been revised, suggesting that the BNST rather is modulating reactions to conditioned fear that lacks temporal predictability (Goode et al. 2019, Goode et al. 2020, Hammack et al. 2015, LeDoux and Pine 2016).

Neuroanatomy of defecation

The enteric nervous system is responsible for regulating and controlling the gastrointestinal (GI) tract (Browning and Travagli 2014). The intestines have a high level of autonomy, but also receive extrinsic control from the central nervous system (CNS) through the parasympathetic and the sympathetic nervous system.

The parasympathetic nervous system exerts both inhibitory and excitatory influence on the GI tract. The sections from the oesophagus to the proximal colon are innervated by the vagus nerve. The distal colon and the rectum are innervated by preganglionic neurons in the dorsal intermediolateral column of the lumbosacral spinal cord (Browning and Travagli 2014). The supraspinal regulation of colonic motility and defecation, through the same preganglionic neurons, is executed by the Barrington's nucleus located in the pons (Pavcovich et al. 1998, Rouzade-Dominguez et al. 2003). Colonic motility is increased when this

nucleus is stimulated. The Barrington's nucleus is interconnected with neurons at all supraspinal levels, where the densest projections come from the PAG, the lateral hypothalamus and the medial preoptic nucleus (Callaghan et al. 2018, Browning and Travagli 2014, Valentino et al. 1994).

The main influence of the sympathetic nervous system is inhibitory. The sympathetic preganglionic neurons that innervate the colon are primarily located on the spinal L2-L5 levels (Browning and Travagli 2014).

Damage to the amygdala and the hippocampus impairs fear-induced defecation, although it is not fully disclosed whether these areas pose a direct control on defecation or if the observed effects are secondary to reduction of fear (Antoniadis and McDonald 2000, Sutherland and McDonald 1990).

SEROTONIN

Discovery

5-hydroxytryptamine (5-HT, or serotonin) was first discovered in rabbit enterochromaffin cells (ECs) (Erspamer 1935, Erspamer and Vialli 1937), at that time denoted enteramine. A decade later, a novel vasoconstrictor substance was extracted from beef serum and named serotonin (a “serum” factor that affected blood vessel “tonus”) (Rapport et al. 1948). Enteramine and serotonin were found to be the same substance (Erspamer and Asero 1952), followed by the discovery of serotonin in the brain (Twarog and Page 1953), leading to the proposal that serotonin was a neurotransmitter. Serotonin is found in most unicellular organisms and is highly expressed in plants (Azmitia 2007).

Neuroanatomy

The human brain contains more serotonergic neurons compared to other species, but they only constitute a small part (1/1000000) of the neurons of the brain. However, the relative number of axon terminals that are serotonergic is much higher (Audet et al. 1989). The cell bodies of the serotonergic neurons are largely found in the brainstem reticular formation within the boundaries of the raphe nuclei, whereas their projections cover virtually the entire CNS (Azmitia 1986, Charnay and Leger 2010, Tork 1990).

The serotonergic projections in humans and primates are restricted and myelinated, whereas they in rodents are predominantly diffuse and unmyelinated (Azmitia and Gannon 1983). Hence, in the rat brain the serotonergic neurons have been suggested to function largely through volume transmission, whereas more discrete targets are established in the human brain.

The serotonin cell bodies were first divided into 9 clusters (Dahlstrom and Fuxe 1964), and later assembled into different nuclei (Jacobs and Azmitia 1992, Tork 1990). There are two main groups of serotonergic nuclei which are separated anatomically and morphologically (Tork 1990). The rostral group, that comprises the caudal linear nucleus, the dorsal raphe nucleus (DRN), the median raphe nucleus and the supraleminiscal serotonergic cell group, projects primarily rostrally to the forebrain, the thalamus and the hypothalamus. The caudal group, that comprises the nucleus raphe obscurus, the nucleus raphe pallidus and the nucleus raphe magnus, also denoted the medullary raphe nuclei (MRN), projects caudally (to the spinal cord) and ventrally (to the cerebellum).

Function in animals

Brain serotonin is involved in many behavioural activities such as eating, sleeping, locomotion, learning and memory, aggression and sexual activity (Azmitia 2007). It also has a role in the regulation of temperature, respiration and hormone secretion. Several of these functions act in concert.

ECs in the intestine produce the major fraction of serotonin in the body. In addition to functions in the intestine, such as regulating gut motility, serotonin produced by these cells operates extrinsically as a hormone, for example supporting bone formation (Gershon 2013).

The platelets, in which serotonin stimulates aggregation and vasoconstriction, cannot produce their own 5-HT but collect 5-HT produced by the ECs from the blood (Adnot et al. 2013). In fact, most of the body's serotonin is circulating in platelets (Berger et al. 2009). Other peripheral effects of serotonin released from platelets and mast cells, most notably the effects of 5-HT in activating immune responses, are outside of the scope of this thesis.

Synthesis and metabolism

Serotonin is produced in low quantities in animals, and in higher animals the production is restricted to a few cells (i.e. ECs, mast cells and neurons).

In the first step of the synthesis, the essential amino acid tryptophan is hydroxylated into 5-hydroxytryptophan (5-HTP) by tryptophan hydroxylase (TPH1 and TPH2). The conversion of tryptophan into 5-HTP is the rate-limiting step (Fitzpatrick 1999), whereas the amount of available tryptophan will determine the serotonin level. 5-HTP and tryptophan can pass through the blood brain barrier (BBB), whereas serotonin cannot. Additionally, only TPH2 is expressed in the brain (Walther et al. 2003). In the second step, 5-HTP is decarboxylated to 5-HT by aromatic amino acid decarboxylase (AADC).

The vesicle monoamine transporter (VMAT2) transports 5-HT into vesicles. In response to action potentials, the vesicles release 5-HT into the synaptic cleft. The major uptake of 5-HT to the presynaptic neuron is through the sodium-dependent serotonin transporter (SERT) (Aggarwal and Mortensen 2017). Low-affinity monoamine transporters, i.e., the plasma membrane monoamine transporter (PMAT) and the organic cation transporter (OCT3), also transport 5-HT into neurons (Courousse and Gautron 2015, Lloyd et al. 2019). The PMAT is located on both post and presynaptic neurons, whereas its presence on glial cells is disputed (Wang 2016). Supposedly the PMAT is important in clearing the synapse of released neurotransmitters when their primary route of uptake is compromised (e.g. by SSRIs) (Zhou et al. 2007).

5-HT is oxidised into 5-hydroxyindole acetaldehyde by monoamine oxidase (MAOA and MAOB). MAOA is more selective for 5-HT, but serotonergic neurons and astrocytes contain predominantly MAOB (Westlund et al. 1985, Youdim et al. 2006). Aldehyde dehydrogenase (ALDH2) oxidises 5-hydroxyindole acetaldehyde into 5-hydroxyindole acetic acid (5-HIAA), which is the main metabolite of 5-HT.

Serotonin receptors

There are 14 known types of serotonin receptors (Hannon and Hoyer 2008, Nichols and Nichols 2008). These are divided into 3 protein-coupled receptor families, the $G_{i/o}$ -protein-coupled receptors (5-HT_{1A}, 1B, 1D, 1E, 1F, 5A, 5B), the $G_{q/11}$ -protein-coupled receptors (5-HT_{2A}, 2B, 2C), the G_s -protein-coupled receptors (5-HT₄, 6, 7) and one ligand-gated ion channel (the 5-HT₃ receptor). The $G_{i/o}$ -protein-coupled receptors are inhibitory, while the rest are excitatory.

Most serotonin receptors are expressed postsynaptically (Hoyer et al. 2002, Nichols and Nichols 2008), while the 5-HT_{1B} and the 5-HT_{1D} receptors are also located presynaptically in the axon terminals functioning as autoreceptors. The 5-HT_{1A} receptors are both postsynaptically situated on non-serotonergic neurons and expressed on the soma and the dendrites of serotonergic neurons where they also serve as autoreceptors.

Pharmacological manipulations

There are several drugs by which the serotonergic system can be manipulated pharmacologically. Some of these are used in the clinic and some only experimentally.

Increase of extracellular serotonin

Extracellular serotonin can be increased in a number of ways. Firstly, by decreasing the degradation of serotonin using first generation antidepressants such as the irreversible monoamine oxidase inhibitors (MAOIs), that inhibit both MAOA and MAOB, or the selective reversible inhibitors of MAOA (Shulman et al. 2013). Secondly, by blocking the SERT (Artigas 2013), a mechanism exploited by the SSRIs, the tricyclic antidepressants (TCAs), the serotonin-norepinephrine reuptake inhibitors (SNRIs) and to some degree by several other drugs. Of interest to mention are for instance the antidepressant vortioxetine (Sanchez et al. 2015)

and the opioid tramadol (Ogawa et al. 2014). Thirdly, by supplementation with exogenous 5-HTP (Turner et al. 2006). Fourthly, by various serotonin releasing agents, such as 3,4-methylenedioxymethamphetamine (MDMA), *para*-chloroamphetamine (PCA), *meta*-chlorophenylpiperazine (mCPP) and fenfluramine, that increase extracellular serotonin by various mechanisms, e.g. by acting as substrates (competitive uptake) or reversing the function of the SERT (Fuller et al. 1988, Miller 2011).

Reduction of extracellular serotonin

Extracellular serotonin can be reduced efficiently by using *para*-chlorophenylalanine (PCPA), which selectively and irreversibly inhibits tryptophan hydroxylase, hence impairing the synthesis of serotonin (Koe and Weissman 1966). Another method to reduce extracellular serotonin, which strictly is not a pharmacological manipulation, is through dietary restriction of tryptophan, the precursor of serotonin (Biggio et al. 1974, Gessa et al. 1974, Lieben et al. 2004). However, tryptophan depletion is far from depleting, but rather decreases the amount of available serotonin. Moreover, tryptophan is not restricted to being a precursor of serotonin and non-serotonergic effects of tryptophan depletion should be considered. A final method is to use neurotoxins, such as 5,7-DHT, by which serotonergic neurons can be selectively killed (Baumgarten and Bjorklund 1976, Baumgarten and Lachenmayer 2004).

Manipulation of serotonin receptors

Numerous drugs manipulate the serotonergic receptors, where only a subsection with relevance to this thesis will be mentioned. Below some serotonin receptor ligands that are or have been used clinically or as recreational drugs will be presented.

There are drugs that function primarily through (partial) agonism at the 5-HT_{1A} receptor (e.g. the anti-anxiety drug buspirone) (Blier and Ward 2003, Sanchez et

al. 2015) and those that display partial 5-HT_{1A} receptor agonism as one of many possible mechanisms of action (e.g. the antidepressant vortioxetine).

Antipsychotics exert their main effect by blocking the dopamine receptor D₂, but also have affinity for serotonin receptors, where antagonism of the 5-HT_{2A} receptor is believed to augment their therapeutic effects (Meltzer and Massey 2011). This is especially true for the second generation antipsychotics (SGAs) (e.g. clozapine and olanzapine) and particularly so for the atypical antipsychotic drug lurasidone, which has 60-fold higher affinity for the 5-HT_{2A} receptor compared to the D₂ receptor (Corponi et al. 2019, Leucht et al. 2013, Moller 2005). Pimavanserin, which may reduce psychosis in patients with Parkinson's disease, is an inverse agonist at the 5-HT_{2A} receptor that has no significant binding to dopamine receptors (Howland 2016).

Conversely, psychedelic drugs such as lysergic acid diethylamide (LSD), psilocybin and mescaline are agonists at the 5-HT_{2A} receptor (Glennon et al. 1984, Halberstadt 2015, Lopez-Gimenez and Gonzalez-Maeso 2018), and their psychedelic effects can be blocked by co-administration of a 5-HT_{2A} receptor antagonist (Schmid et al. 2015, Vollenweider et al. 1998).

While the SSRIs are highly selective for the SERT, some of them (e.g. fluoxetine) display some antagonistic action, primarily to the G_{q/11}-protein-coupled receptors (and foremost the 5-HT_{2C} receptor), while notable affinity to these receptors (the 5-HT_{2A} and the 5-HT_{2C} receptors) is displayed by the TCAs (Palvimaki et al. 1996). The serotonin receptor antagonist ritanserin and the so-called noradrenergic and specific serotonergic antidepressant mirtazapine have affinity (as antagonists) for the 5-HT_{2A} and the 5-HT_{2C} receptors, while the antihypertensive agent ketanserin functions as a nonselective 5-HT_{2A} receptor antagonist (Anttila and Leinonen 2001).

With special regards to the clinical practice of diseases of the GI-system, 5-HT₃ receptor antagonists (e.g. ondansetron) are used to decrease gut motility, while 5-HT₄ receptor agonists (e.g. prucalopride) are used to increase gut motility (Beattie

and Smith 2008, Costedio et al. 2007, Garsed et al. 2014, Gore et al. 1990, Sajid et al. 2015, Sanger 2008, Stasi et al. 2014, Zheng et al. 2017).

Apart from the serotonin receptor ligands mentioned above, there are many, more or less selective, agonists and antagonist for the different serotonin receptor subtypes at the disposal of preclinical researchers that, however, have yet not been clinically introduced.

All drugs that are used in the manuscripts presented in this thesis are listed in the materials and methods section.

SEROTONIN IN FEAR AND ANXIETY

Serotonin and anxiety in humans

Background

The observation that LSD antagonizes constrictive serotonin-induced action of smooth-muscles in the periphery made Gaddum suggest a role of serotonin in the human brain of relevance for psychosis (Gaddum 1953). In the same decade, it was proposed that mental illnesses were caused by an imbalance of serotonin in the brain (Woolley and Shaw 1954), including suggestions of opposing roles of serotonin and norepinephrine (NA) (Brodie and Shore 1957). The MAOI iproniazid, developed for treatment of tuberculosis, was shown effective in treating depression (Loomer et al. 1957, Smith 1953), followed by the same observation for the TCA imipramine (Azima and Vispo 1958, Kuhn 1957). However, the antidepressant effect of these compounds was primarily thought to be mediated through an influence on NA and it took some years before the importance of the impact of these compounds on serotonin was suggested (Carlsson et al. 1968) and finally disclosed by the report of an effect of members of the SSRIs class on depression and anxiety (Carlsson 1981, Evans et al. 1986).

Treatment of anxiety with serotonergic drugs

The ability to treat panic attacks with imipramine was demonstrated in the early 60's (Klein and Fink 1962). The TCAs and the MAOIs immediately gained popularity in the treatment of depression but it took nearly two decades after an effect on anxiety was first demonstrated before these compounds gained interest in the clinic (Modigh 1987).

Today, the SSRIs are the first line of treatment for anxiety disorders (i.e. GAD, social phobia and PD) and OCD (Eriksson and Humble 1990, Zohar and

Westenberg 2000). The MAOIs are generally effective as well. The TCA with the strongest serotonergic profile, clomipramine, is more effective than less serotonergic TCAs in PD and OCD (Modigh 1987). The MAOIs and the TCAs are rarely preferred over the SSRIs in treatment of anxiety due to more side effects and higher toxicity (Barbey and Roose 1998). Other serotonergic-acting drugs that can be used in treatment of anxiety disorders are the SNRIs and the 5-HT_{1A} receptor agonists. The SNRIs have been demonstrated effective in GAD (Katzman 2009), and venlafaxine, an SNRI, which is comparable to the SSRIs in terms of SERT occupancy (Owens et al. 2008), is assumingly effective in PD, social phobia and OCD. The 5-HT_{1A} receptor agonists are moderately effective in GAD but not in PD (Fulton and Brogden 1997, Howland 2015, Sheehan et al. 1990). Although not used in the clinic, long-term administration of 5-HTP has also been reported to alleviate anxiety (Kahn and Westenberg 1985, Kahn et al. 1987).

Acute increase of extracellular serotonin

None of the drugs of the above-mentioned classes are immediately effective in treatment of anxiety and the full clinical effect takes several weeks to obtain. Conversely, the SSRIs and the TCAs can paradoxically increase anxiety in susceptible subjects upon initialization of treatment (Coplan et al. 1992, Pohl et al. 1988, Rammsayer and Netter 1990, Sinclair et al. 2009). This aggravation is worst in the first few days and typically disappears within two weeks (Modigh 1987). Since many SSRIs are highly selective to the SERT (Hyttel 1994, Lundberg et al. 2012), and blockade of the SERT increases extracellular serotonin in several brain areas (discussed in a later paragraph), it has been suggested that the observed initial aggravation of anxiety may be caused by increased extracellular serotonin.

The serotonin-releasing agent fenfluramine acutely increases anxiety in both patients suffering from PD (Targum and Marshall 1989) and, to a much lesser extent, in healthy volunteers (Brauer et al. 1996), while others report decreased unconditioned fear, assessed in a simulated public speaking test (Hetem et al. 1993). Oppositely, chronic administration of fenfluramine has been suggested to reduce the number of panic attacks in PD patients (Solyom 1994). Acute

administration of mCPP induces panic attacks in PD patients but not in GAD patients or in healthy controls (Van Veen et al. 2007).

Acute administration of 5-HTP has been reported to be void of effect or to alleviate anxiety in healthy volunteers and patients suffering from PD (den Boer and Westenberg 1990, Maron et al. 2004, Schruers et al. 2002, van Vliet et al. 1996).

Reduction of serotonin

Although the effect of PCPA administration on anxiety *per se* has never been explored in humans, PCPA has been tested on healthy volunteers (Cremata and Koe 1966), been used for symptomatic treatment of carcinoid syndrome (Engelman et al. 1967) and evaluated in schizophrenic patients (Casacchia et al. 1975, DeLisi et al. 1982) and patients suffering from Parkinson's disease (Van Woert et al. 1972). Moreover, PCPA administration has been found to reverse the antidepressant effect of serotonergic drugs (Shopsin et al. 1976). Treatment with PCPA comes with marked side effects, including mental disturbances, personality changes, uneasiness, fatigue, dizziness and allergic eosinophilia, leading to cessation of therapy in nearly 50% of tested patients (Sjoerdsma 1970). To what extent PCPA may enhance or dampen anxiety in humans, however, remains unclear.

A less potent way to reduce serotonin is through dietary restriction of tryptophan (Biggio et al. 1974, Young 2013). Moderate effects of tryptophan depletion on anxiety have been reported in a few studies, including increased generalized anxiety (Klaassen et al. 1998), increased anxiety but not fear (Robinson et al. 2012) and attenuated responses to a cue indicating an upcoming aversive event (Hindi Attar et al. 2012).

Manipulation of serotonin receptors

Buspironone has displayed anxiolytic-like properties in conditioned, but not in unconditioned models, in accordance with that the drug is effective in GAD and

not in PD (Guimaraes et al. 1989, Hellewell et al. 1999). Agonists for the 5-HT_{2A} receptor, including psychedelic drugs such as LSD, mescaline and psilocybin, induce mild subjective anxiety in some subjects and can trigger panic attacks (Barrett et al. 2016, Carbonaro et al. 2016, Griffiths et al. 2006, Hoffman 1980). After the acute effects have subsided, on the other hand, they have potential therapeutic benefits, which have gained accumulating interest in recent years (Carhart-Harris et al. 2016, Carhart-Harris and Goodwin 2017, Griffiths et al. 2008, Mithoefer et al. 2016). The 5-HT_{2A} receptor antagonist ritanserin has demonstrated an effect on GAD in some but not all studies (Bressa et al. 1987, Westenberg and den Boer 1989), and has never been approved for this or any other indication. Ritanserin is reported to increase unconditioned and decrease conditioned fear (Guimaraes et al. 1997, Hensman et al. 1991), and ketanserin reduces sensitivity to exposure to fearful facial expressions (Hornboll et al. 2013).

Genetic variations in humans

In humans, the promotor region of the 5-HTT (i.e. the SERT) gene comprises a polymorphism commonly denoted 5-HTTLPR (i.e. 5-HTT gene-linked polymorphic region) that exists in a long (L) and a short (S) allele variant (Lesch et al. 1996). The efficacy of the transcription is impaired for the S allele, tentatively leading to lower expression of the transporter and consequently to reduced re-uptake of 5-HT (Greenberg et al. 1999). The S allele is associated with increased amygdala responses to aversive stimuli (Lesch et al. 1996) and enhanced fear conditioning (Brocke et al. 2006, Garpenstrand et al. 2001, Lonsdorf et al. 2009). It has also been noted that the S allele carriers display greater reactivity in response to fearful faces (Hariri et al. 2002, Hariri et al. 2005). However, as the number of studies has accumulated, the strength and the importance of the association between the 5-HTTLPR and anxiety has been challenged, where an estimation made a few years ago approximated the polymorphism to account for around 1% of the variance in amygdala activation (Murphy et al. 2013).

Serotonin and anxiety in rodents

Microdialysis studies

There is a large body of evidence that fear and anxiety enhance extracellular serotonin. Expression of conditioned fear leads to increased serotonin release in the amygdala (Yokoyama et al. 2005) including the BLA (Zanoveli et al. 2009), the nucleus accumbens (NAc) (Fulford and Marsden 2007), the dPAG (Zanoveli et al. 2009) and the prefrontal cortex (Hashimoto et al. 1999, Yoshioka et al. 1995). In the same vein, exposure to various other stressors has been shown to enhance release of serotonin in several brain areas (Kawahara et al. 1993, Rueter and Jacobs 1996, Tanaka et al. 1983), and specifically in the hippocampus (Amat et al. 1998b, Hajos-Korcsok et al. 2003), the NAc (Fulford and Marsden 2007), the CEn (Hassell et al. 2019, Li et al. 2014, Mo et al. 2008), the BLA (Mitsushima et al. 2006), the dPAG (Amat et al. 1998b) and the prefrontal cortex (Dazzi et al. 2005, Miyata et al. 2007).

Acute administration of SSRIs increases extracellular 5-HT concentrations in both the raphe nuclei (Malagie et al. 1995) and several brain regions containing serotonin nerve terminals, including the CEn, the hippocampus, the hypothalamus, the NAc, the striatum and the frontal cortex (for reviews see Fritze et al. 2017, Fuller 1994). Acute administration of fenfluramine increases the extracellular concentration of 5-HT in the hypothalamus, the NAc and the prefrontal cortex (Arrant et al. 2013, Baumann et al. 2014, Laferrere and Wurtman 1989) and acute administration of 5-HTP increases the extracellular concentration of 5-HT in the NAc and the hypothalamus (Baumann et al. 2011, Gartside et al. 1992).

Chronic administration of SSRIs non-uniformly increases extracellular serotonin in various brain areas (Wegener et al., 2003). This includes the frontal cortex, where extracellular serotonin, inversely, is reduced during the first few days of long-term treatment (Fritze et al., 2017).

Finally, investigating the effect of SSRI administration on the response to aversive stimuli, it was found that chronic administration, but not acute administration, impairs serotonin release in the prefrontal cortex triggered by foot-shock stress (Dazzi et al. 2005), hence, potentially exerting a dampening effect on anxiety.

Acute increase of extracellular serotonin

The reported effects of acute administration of SSRIs prior to acquisition and expression of context-conditioned fear have been variable, a drug-induced reduction in freezing observed in some studies but not consistently (Gravius et al. 2006, Hashimoto et al. 2009, Inoue et al. 1996, Li et al. 2001, Montezinho et al. 2010, Pettersson et al. 2015, Santos et al. 2006). On the contrary, in fear conditioning to a cue (a tone) acute SSRI administration is reported to increase both acquisition and expression of fear (Burghardt and Bauer 2013).

There are reports on acute administration of fenfluramine to decrease acquisition (Archer et al. 1982) and 5-HTP to decrease expression (Inoue et al. 1996) of context-conditioned freezing. Likewise, in our laboratory, acute administration of either fenfluramine or 5-HTP was found to impair both acquisition and expression of context-conditioned freezing (to be published).

In several other models of anxiety, such as conflict tests, SSRIs enhance anxiety (Borsini et al. 2002). There are reports on fenfluramine both increasing (File and Guardiola-Lemaitre 1988) and decreasing (Miyata et al. 2007) expression of anxiety.

Reduction of serotonin

The most studied mode of serotonin depletion used in animal experiments is systemic administration of the serotonin synthesis inhibitor PCPA. PCPA does not affect learning or long-term memory, but impairs short-term memory, reduces reactivity to emotional stimuli and increases reactivity to aversive stimuli, pain and cutaneous stimulation (Dringenberg et al. 1995, Hritcu et al. 2007, Jensen et

al. 2014, Koe and Weissman 1966, Korzeniewska-Rybicka and Plaznik 2001, Ogren and Berge 1984, Tenen 1967).

PCPA induced serotonin depletion reduces expression of conditioned fear to both context (Pettersson et al. 2016) and to auditory cues (Johnson et al. 2015, Nikaido et al. 2016), although some studies have failed to unfold an effect (Inoue et al. 1996, Masuda et al. 2013).

The net effect of 5-7-DHT injection into the amygdala is enhanced expression of unconditioned and reduced expression of context-conditioned fear (Izumi et al. 2012). Unconditioned fear is also enhanced when 5-7-DHT is injected into the BLA specifically, but reduced when injected into the CEn (Macedo et al. 2002). Fear acquisition to a tone is impaired when 5-7-DHT is injected into the BLA (Johnson et al. 2015).

In other models of anxiety the results of serotonin depletion are mixed (Angrini et al. 1998, Kubala et al. 2008, Miyata et al. 2007, Naslund et al. 2013, Soderpalm and Engel 1990, Treit et al. 1993), where in some cases a confounding dampening effect on locomotion can be suspected (Dringenberg et al. 1995, Tenen 1967).

Manipulation of serotonin receptors

The serotonin receptors most studied with respect to the regulation of anxiety in both humans and animals are the 5-HT_{1A}, the 5-HT_{2A}, the 5-HT_{2C} and the 5-HT₃ receptors (Bauer 2015, Borsini et al. 2002, Griebel 1995). By studies applying local infusions of receptor ligands, it has been revealed that the same receptor can have opposite effects on anxiety in different brain areas (Hammack et al. 2009), and by optogenetic experiments that the effects can differ even between different cell groups within the same area (Isosaka et al. 2015).

Systemic administration of 5-HT_{1A} receptor agonists reduces acquisition and expression of context-conditioned fear (Inoue et al. 2011). Antagonists for the 5-HT_{1A} and the 5-HT_{2C} receptors have shown no effect on either acquisition or

expression while antagonists for the 5-HT₃ receptor reduce expression of context-conditioned fear in some studies (Inoue et al. 2011, Masuda et al. 2013). Antagonists for the 5-HT_{2A} receptor have been void of effect in some studies (Inoue et al. 2011, Masuda et al. 2013), while one study reported reduced conditioned freezing in rats bred to display high freezing (Carioca High) and increased freezing in rats bred to display low freezing (Carioca Low) (Leon et al. 2017). Additionally, genetically modified mice with disruption of 5-HT_{2A} receptor signalling, do not differ from controls with regards to expression of context-conditioned fear (Weisstaub et al. 2006).

Turning to other models of anxiety, selective stimulation of the presynaptic 5-HT_{1A} receptors tends to induce anxiolytic-like effects, while manipulations of the postsynaptic 5-HT_{1A} receptors have shown disparate results (Homberg 2012, Stiedl et al. 2000, Stiedl et al. 2015, Zmudzka et al. 2018). Drugs that stimulate the 5-HT_{2A} and the 5-HT_{2C} receptors can induce anxiogenic effects, but sometimes impair anxiety, tentatively explained by different sites of activation (Bauer 2015, Hammack et al. 2009, Homberg 2012, Zmudzka et al. 2018). The majority of studies on antagonist for the 5-HT_{2A}, the 5-HT_{2C}, and the 5-HT₃ receptors report either no effect or anxiolytic-like effects (Bauer 2015, Homberg 2012, Zmudzka et al. 2018).

Some additional examples are discussed in the paragraph on the role of serotonin in the neuroanatomy of fear and anxiety.

Genetic manipulations

Mice with increased extracellular serotonin by knockout of SERT display higher levels of anxiety-like behaviour compared to normal mice (Jansen et al. 2010, Wellman et al. 2007). Accordingly, mice with overexpression of SERT exhibit impaired fear learning (Barkus et al. 2014, Bocchio et al. 2015).

In contrast to what has been reported on PCPA and 5,7-DHT in rat, mice that are deprived of serotonergic transmission by knockout of either TPH2 (Gutknecht et

al. 2015) or transcription factor required for early development of serotonergic neurons (Dai et al. 2008, Kiyasova et al. 2011) are reported to display increased conditioned fear.

There are a great number of studies that investigate the effect of knockout of specific serotonin receptor types, of which only a subsection will be mentioned here (for a review see Zmudzka et al. 2018). The 5-HT_{1A} receptor knockout mice have been reported to exhibit increased anxiety-like behaviour (Olivier et al. 2001, Ramboz et al. 1998). Genetically modified mice with disruption of 5-HT_{2A} receptor signalling do not differ from controls with regards to conditioned fear, but they have displayed reduced anxiety in other behavioural models of anxiety (Weisstaub et al. 2006). Similarly, reduced anxiety-like behaviour has been observed for 5-HT_{2C} receptor knockout mice (Heisler et al. 2007).

Serotonin in the neuroanatomy of fear and anxiety

The main serotonergic input to the amygdala, the dPAG and the BNST is from the DRN (Vertes 1991).

Both CS and US elicit serotonin release in the BLA (Bauer 2015, Bocchio et al. 2016). In rats, the majority of the serotonergic projections to the amygdala are to the BLA (primarily the BAn) but a smaller portion targets the CEI (Asan et al. 2013, Bocchio et al. 2016). The latter projection is denser in non-human primates (Bauman and Amaral 2005). In BAn the effect of 5-HT is primarily inhibitory, acting on 5-HT_{2A} receptors situated on GABAergic interneurons (McDonald and Mascagni 2007).

Increased serotonin in the forebrain, i.e. the amygdala and the medial prefrontal cortex, stimulates defensive responses to uncertain threats, related to anxiety (Graeff and Zangrossi 2010). Interestingly, inhibition of 5-HT_{2A} receptors in the BLA appears to stimulate innate/unconditioned fear and dampen

learned/conditioned fear (Macedo et al. 2007), while the opposite has been reported for 5-HT_{2A} receptors in the CEn (Isosaka et al. 2015).

Increased serotonin in the dPAG inhibits responses to direct threats, related to fear (Graeff and Zangrossi 2010). This influence has been attributed to 5-HT_{1A} receptors on excitatory interneurons and 5-HT_{2A} receptors on inhibitory interneurons, where the net effect in both cases (since the 5-HT_{1A} receptor is inhibitory and the 5-HT_{2A} receptor is excitatory) is dampened defensive responses (Brandao et al. 2008, Oliveira et al. 2007).

The primary target for serotonergic input to the BNST is the anterolateral cell group (Commons et al. 2003, Guo et al. 2009). The proposed net effect of local 5-HT release in the anterolateral BNST is reduced anxiety (Hammack et al. 2009), and similarly reduced anxiety has been reported for optogenetic activation of 5-HT terminals in the lateral BNST in mice (Garcia-Garcia et al. 2018). The serotonin receptors that are most densely expressed in this area are the 5-HT_{1A}, the 5-HT_{2A} and the 5-HT₇ receptors. Stimulation of 5-HT_{1A} receptors in the anterolateral BNST decreases anxiety whereas stimulation of 5-HT_{2A} (and 5-HT₇) receptors increases anxiety (Hammack et al. 2009).

Serotonin and defecation

Serotonin, SERT and several types of 5-HT receptors are abundant in the GI tract and are important in the regulation of the gut's function (Beattie and Smith 2008, Costedio et al. 2007, Stasi et al. 2014). Signals from the enteric nervous system (ENS) and the parasympathetic nervous system (either directly or via the ENS) induce serotonin release from the ECs (Stasi et al. 2014). Stimulation of 5-HT₃ and 5-HT₄ receptors in the intestine increases motility, and diarrhea is a common side effect of SSRI treatment (Spigset 1999).

As previously discussed, parasympathetic neurons in the dorsal intermediolateral column of the lumbosacral spinal cord regulate colonic and rectal motility

(Browning and Travagli 2014). In addition to the central control exerted by the Barrington's nucleus, the function of these neurons is modulated by direct serotonergic projections from the raphe nucleus (Hentall et al. 2006, Jones and Light 1992, Marlier et al. 1991). Increased colorectal motility is seen after both electric stimulation of the MRN (Nakamori et al. 2018a) and intrathecal administration of serotonin into the lumbosacral spinal cord (Nakamori et al. 2018b). The latter effect is putatively mediated by stimulation of 5-HT₂ and 5-HT₃ receptors.

The Barrington's nucleus is connected with numerous areas that are under serotonergic control, at all hierarchical levels of the brain (Browning and Travagli 2014, Callaghan et al. 2018, Valentino et al. 1994). Gut motility is increased in response to fear, and since serotonin influences fear and anxiety, defecation is indirectly affected by serotonergic activity in this way.

To conclude, serotonin influence defecation both directly in the intestine, directly through the parasympathetic nervous system (through serotonin nerve terminals in both the brain and in the spinal cord) and indirectly through modulation of fear.

AIMS, EXPERIMENTAL OVERVIEW AND RESULTS

Paper I

The influence of a transmitter on a specific behaviour can be explored in a number of ways. A reasonable approach is to study the effect of removal of the transmitter on the behaviour in question. This can be achieved with several methods, e.g. by blocking the synthesis, using neurotoxins, genetic or optogenetic manipulations. While some methods are capable of examining the function of specific brain areas or cell populations, a straightforward way to assess the overall influence of a transmitter on fear conditioning is through depletion.

Although serotonin depletion has been applied in several behavioural paradigms, there are few studies on the effect of serotonin depletion on context-conditioned fear. The purpose of this study was to evaluate the effect of serotonin depletion in several stages of the fear conditioning process, i.e., fear acquisition, memory consolidation and fear expression.

Rats were depleted of serotonin using PCPA (300 mg/kg) administered on either 4 (experiment I) or 3 (experiments II-V) consecutive days. The effect of serotonin depletion on I) both acquisition and expression, II) acquisition only, III) memory consolidation, IV) expression only and V) unconditioned animals was evaluated.

Serotonin depletion before acquisition and/or expression impaired context-conditioned fear. No effects were seen on memory consolidation or in unconditioned animals.

Paper II

The purpose of this paper was to explore the possible impact of systemically administered ligands exerting agonism, antagonism or inverse agonism on the 5-HT_{2A} receptor with respect to conditioned fear. The experiments were partly prompted by apparently contradictory suggestions from clinical reports that both 5-HT_{2A} antagonists (when combined with an SSRI) and 5-HT_{2A} agonists (such as psilocybin) may be beneficial for the treatment of anxiety disorders.

Whereas the 5-HT_{2A} receptor has predominantly been attributed anxiogenic-like properties in studies targeting specific areas of the brain (Hammack et al. 2009, Izumi et al. 2012, Marcinkiewicz et al. 2016), the effects of systemic selective inhibition and stimulation of the 5-HT_{2A} receptor with regards to context-conditioned fear have been poorly explored. Since serotonin depletion (paper I) impaired context-conditioned fear, and since the 5-HT_{2A} receptor appears mainly anxiogenic, we speculated that systemic 5-HT_{2A} receptor blockade may mimic the effect of PCPA with respect to fear conditioning. Given the possibility that SSRIs at acute administration may cause activation of both anxiogenic and anxiolytic-like receptor subtypes, we also wanted to test if blocking the putatively anxiogenic 5-HT_{2A} receptor might unmask an anti-freezing effect of the reuptake inhibitor not seen when the 5-HT_{2A} receptor is not antagonized. And, finally, we wanted to assess the effect of psilocybin and other 5-HT_{2A} receptor agonists in this paradigm.

Experiment I tested the effect of the 5-HT_{2A} receptor antagonist MDL 100907 administered at different doses (0.003, 0.03, 0.1, 0.3 and 3 mg/kg). Experiment II evaluated the effect of MDL 100907 (0.01 and 0.3 mg/kg) administered alone and in combination with escitalopram (5 mg/kg). Experiment III investigated the effect of the 5-HT_{2A} receptor inverse agonist pimavanserin (0.3 mg/kg) alone and in combination with escitalopram (5 mg/kg). Experiment IV examined the effect of the 5-HT_{2C} receptor antagonist SB 242084 (0.3 and 1 mg/kg) alone and in combination with escitalopram (5 mg/kg). Experiment V evaluated the effect of MDL 100907 (3 mg/kg) alone and in combination with escitalopram (5 mg/kg) in the elevated plus maze. Experiment VI evaluated the effect of administration of

the 5-HT_{2A} receptor agonists 25CN-NBOH (3 mg/kg) and TCB-2 (1 mg/kg) alone and in combination with MDL 100907 (1 mg/kg). Experiment VII tested the effect of the psychedelic drug psilocybin (2 mg/kg) alone and in combination with MDL 100907 (1 mg/kg).

With the exception of one low dose of MDL 100907 (0.03 mg/kg), neither MDL 100907 nor pimavanserin significantly affected freezing in any of the experiments when administered alone. On the other hand, when co-administered with escitalopram, both MDL 100907 and pimavanserin markedly reduced freezing. In contrast, adding a 5-HT_{2C} receptor antagonist to the SSRI did not reveal any freezing-reducing effect. Administration of 25CN-NBOH, TCB-2 and psilocybin all induced a powerful reduction of freezing, an effect that could be reversed by co-administration with MDL 100907 in the case of 25CN-NBOH and psilocybin, but not in the case of TCB-2.

Paper III

In humans, chronic, but not acute administration of SSRIs is effective in treatment of anxiety disorders (Nutt et al. 1999). An animal behavioural model for studies on the effects of SSRIs would preferably mirror this situation.

The primary purpose of this study was to evaluate the effect of chronic administration of an SSRI on context-conditioned fear and, secondly, to compare that effect to the acute effect of the same drug.

In experiment I, rats were treated with escitalopram through osmotic minipumps (approximately 10 mg/kg/day). Twenty days after the initiation of treatment rats were fear-conditioned and tested on two consecutive days. In experiment II, rats received acute administration of escitalopram (0.3, 1 and 3 mg/kg) prior to fear conditioning and test on two consecutive days. Serum levels of escitalopram were analyzed in both experiments.

As in humans, chronic administration of escitalopram displayed an anxiety-reducing effect. On the other hand, acute administration, at doses yielding similar serum concentrations, did not affect the expression of context-conditioned fear.

Paper IV

Freezing behaviour can be evoked by both fear conditioning (learned fear) and by direct presentation to an US (innate fear). These fear-inducing pathways are thought to be relevant for the clinical manifestations of GAD and PD, respectively. Escitalopram increases freezing in rats subjected to acoustic noise bursts (Pettersson et al. 2015), putatively mirroring the anxiogenic effects of acute administration of SSRIs seen in susceptible humans (Sinclair et al. 2009). Co-administration of an SSRI and a 5-HT_{2C} receptor antagonist has been reported to block anxiogenic effects of acute SSRI administration (Burghardt et al. 2007).

The aim of this study was to assess the effects of acute administration of an SSRI in a model utilizing acoustic noise bursts as US and to test if co-administration with a 5-HT_{2C} receptor antagonist would disrupt this effect.

Experiment I assessed the effect of escitalopram (10 mg/kg) on unconditioned freezing and acquisition of context-conditioned fear. Experiment II assessed the effect of escitalopram (10 mg/kg) and the 5-HT_{2C} receptor antagonist SB 242084 (0.5 mg/kg), alone and in combination, on unconditioned freezing and (mild) conditioned fear.

Noise burst presentation induced freezing behaviour in saline-treated controls but did not induce a conditioned response. Escitalopram increased freezing during US presentations and also unmasked an anxiogenic effect on conditioned fear. SB 242084, alone or in combination with escitalopram, did not affect freezing behaviour.

In addition, it was noted that the amount of freezing displayed by rats at subsequent testing was highly correlated, i.e. it was the same animals that displayed high or low freezing at multiple tests, indicating a potential use of noise burst-induced freezing as a model to study trait anxiety.

Paper V

When evaluating the influence of different 5-HT receptor antagonists on conditioned freezing, it was noted that administration of a 5-HT₆ receptor antagonist reduced the number of faecal boli produced during the test. This behaviour, i.e. stress-induced defecation, has been proposed as a model for IBS-D (Funatsu et al. 2007, Hirata et al. 2008). An influence of 5-HT₆ receptor antagonism on defecation is mentioned in one previous report (Finn et al. 2007), but has otherwise been overlooked.

The purpose of this study was to explore the potential effect of 5-HT₆ receptor manipulations on defecation in (1) non-stressed rats and (2) rats subjected to conditioned fear stress.

The first experiment evaluated the effect of the 5-HT₆ receptor antagonist SB 399885 (5 mg/kg) and the 5-HT₆ receptor agonist WAY 208466 (10 mg/kg) on defecation in non-stressed animals. Faecal boli were collected during 7 h. The second, third and fourth experiment assessed the effects of the 5-HT₆ receptor antagonists SB 399885 (1, 3, 10 mg/kg), SB 271046 (5 mg/kg) and SB 258585 (20 mg/kg) and the 5-HT₆ receptor agonist WAY 208466 (10 mg/kg) on stressed-induced defecation.

While all tested 5-HT₆ receptor antagonists reduced the faecal output in both non-conditioned and conditioned rats, administration of the 5-HT₆ receptor agonist had no effect. Neither drug displayed an impact on context-conditioned fear.

DISCUSSION

Serotonin depletion and conditioned fear (paper I)

Serotonin is released in several brain areas in response to exposure to both US and CS. PCPA-induced serotonin depletion reduced both acquisition and expression of context-conditioned fear. Hence, the net effect of this release, in both stages of the fear conditioning process, appears to promote rather than dampen fear conditioning. This assumption is in agreement with that administration of the neurotoxin 5,7-DHT into the amygdala (Izumi et al. 2012, Johnson et al. 2015) and destruction of the median raphe nucleus (Borelli et al. 2005, Melik et al. 2000) impair both acquisition and expression of conditioned fear. On the other hand, studies on transgenic mice, i.e. that are deprived of serotonergic transmission, have oppositely described both increased acquisition (Dai et al. 2008) and increased expression (Gutknecht et al. 2015, Kiyasova et al. 2011) of conditioned fear.

In previous studies on expression of context-conditioned fear, serotonin depletion has been reported to reduce (Izumi et al. 2012, Pettersson et al. 2016) or not affect (Inoue et al. 1996, Masuda et al. 2013) freezing. However, direct comparisons even between these studies are compromised by differences in the behavioural protocols and serotonin depletion procedures, and more observations are warranted.

While prior reports on the effect of PCPA on acquisition of context-conditioned fear, to the best of our knowledge are lacking, local depletion of serotonin in the BLA impairs conditioning to a tone (Johnson et al. 2015) and intact BLA function is required for fear acquisition (Amorapanth et al. 2000, Maren 1999, Oliveira et al. 2004), suggesting that the observed effect of PCPA could be mediated in the amygdala.

The impaired fear acquisition of PCPA-treated rats is not likely due to decreased sensitivity to cutaneous stimulation (Dringenberg et al. 1995, Koe and Weissman 1966) or pain (Bodnar et al. 1981, Korzeniewska-Rybicka and Plaznik 2001, Ogren and Berge 1984), and neither due to an effect on learning and long-term memory (Buhot et al. 2000, Hritcu et al. 2007, Santucci et al. 1996, Tenen 1967).

One possible explanation to our results may be that the amount of freezing displayed during acquisition will impact the amount of freezing displayed during expression of conditioned fear, involving a feedback mechanism from the PAG to the LAn (Johansen et al. 2010, Kim et al. 2013). PCPA administration increases the active avoidance response to aversive stimuli, such as jump reactivity and startle response (Pettersson et al. 2016, Tenen 1967). This is in accordance with that active avoidance is facilitated by a circuit from BAn to NAc, on which decreased serotonin levels in the BAn would have an augmentative influence (Ramirez et al. 2015). Additionally, the drug reduces the emotional reactivity (Tenen 1967), which makes the animals less likely to exhibit responses (e.g. freezing) that compete with the active response.

To conclude, the results of the experiments presented in this manuscript are in agreement with the view that the net influence of the increased serotonin in different brain regions observed during both acquisition and expression of conditioned fear enhances rather than dampens fear conditioning (Bauer 2015, Borsini et al. 2002, Sinclair et al. 2009).

Effects of escitalopram on fear (papers II-IV)

Acute administration

Escitalopram increased unconditioned noise burst-induced freezing. An anxiety-exacerbating effect of acute SSRI administration has been observed in susceptible humans (Rammsayer and Netter 1990, Sinclair et al. 2009) and in several animal models of anxiety (Bauer 2015, Borsini et al. 2002) including conditioning to a cue (Burghardt et al. 2004, Burghardt et al. 2007). However, co-administration with a

5-HT_{2C} receptor antagonist did not, as has been previously demonstrated in cued conditioning (Burghardt et al., 2007), inhibit the anxiogenic effect of the SSRI.

Most humans do not react to acute SSRI administration with increased anxiety. Since the serotonergic system is under tonic inhibition in its ground state, it is reasonable to suggest that the SSRIs have little effect on fear in a neutral situation. In an aversive situation, inducing enhanced serotonin release (Amat et al. 1998a, Zanoveli et al. 2009), SSRIs will however augment the increase in extracellular serotonin in activated brain areas, which might lead to enhanced anxiety in humans and animals (Bauer 2015, Borsini et al. 2002, Sinclair et al. 2009).

The CEn and the BNST supposedly mediate fear responses to direct and temporal uncertain threats, respectively (Davis et al. 2010, Goode et al. 2019, Goode et al. 2020, Hammack et al. 2015, LeDoux and Pine 2016, Miles et al. 2011, Walker et al. 2003, Walker and Davis 2008). The exacerbated anxiety after acute SSRI administration in the noise burst-induced freezing experiments is consistent with the role of the CEn pathway.

Acute SSRI administration has been proposed to have an anxiety-reducing influence on context-conditioned fear (Burghardt and Bauer 2013, Inoue et al. 2011). However, the reports are far from unanimous, and there are inconsistencies between reports regarding the effective dose (Hashimoto et al. 1996, Inoue et al. 1996, Montezinho et al. 2010, Muraki et al. 1999) and the importance of timing between fear conditioning and test (Hashimoto et al. 1996, Muraki et al. 1999). The outcome may also be dependent on the intensity of the US (Santos et al. 2006) and even if animals are of the same strain, origin is presumably a factor. Furthermore, it has been demonstrated that differences in the protocols can result in that different fear circuitries are activated (Goode et al. 2020). Escitalopram did neither increase nor decrease context-conditioned freezing in any of the experiments presented in this thesis (papers II and III). In total, taking unpublished data into account, escitalopram *per se* has at most a weak and inconsistent effect on freezing in this paradigm when using the current protocol.

The context-conditioning experiments are putatively involving the pathway engaging the BNST. Increased 5-HT in the BLA would tentatively reduce signals to the BNST (thus dampen anxiety). Stimulation of the BNST can both increase and dampen context-conditioned fear (Goode and Maren 2017), while local injections of 5-HT in the anterolateral BNST reduce anxiety (Hammack et al. 2009). Hence, the effect on anxiety induced by increased extracellular 5-HT in the BNST may be highly dependent on the situation at hand, which might explain different outcomes with different experimental settings.

Although an intuitive relation is not a prerequisite for predictive validity, we endorse the idea that acute administration of an SSRI to a rat in a conditioning paradigm would resemble acute administration in humans. Supporting this view, acute SSRI administration increased freezing in the unconditioned paradigm (the CEn pathway), but not in the conditioned paradigm (involving the BNST), which is in agreement with that aggravation of anxiety after acute administration of an SSRI in humans is foremost expressed in PD patients (Sinclair et al. 2009). Since, the behavioural paradigms mirror the clinical findings they could putatively be used in preclinical studies on acute effects of SSRI administration.

Chronic administration

There are relatively few studies addressing the effect of (sub)chronic SSRI administration (Li et al. 2001, Spennato et al. 2008, Zhang et al. 2000), and to the best of our knowledge there is no previous report in which repeated but not single SSRI administration reduces context-conditioned fear, i.e. mirrors the effect of SSRI on anxiety in humans (Nutt et al. 1999).

Several mechanisms have been proposed on how SSRIs dampen anxiety. Chronic administration of SSRIs increases the basal level of extracellular serotonin (Fritze et al. 2017), while at the same time the serotonin release to an acute stressor is impaired (Dazzi et al. 2005). Similar observations have been made with respect to fenfluramine (Zolkowska et al. 2008), tentatively attributing to the drug's alleged beneficial effects in the treatment of PD (Solyom 1994).

One potential contributing mechanism of action is through down regulation of the gene encoding TPH2, putatively normalizing pathological overexpression of the gene (Abumaria et al. 2007, Dygalo et al. 2006). In the same line, prolonged SERT inhibition may lead to desensitization of anxiety-inducing postsynaptic receptors (Attar-Levy et al. 1999, Licht et al. 2009, Quested et al. 1997, Yatham et al. 1999).

To conclude, the anxiety-reducing effect of chronic SSRI administration on context-conditioned fear mirrors the clinical situation, suggesting that the paradigm could be utilized in preclinical studies on the mechanism of action of repeated administration of SSRIs in anxiety disorders.

5-HT_{2A} receptor antagonism as add-on to an SSRIs

The observation that neither the 5-HT_{2A} receptor antagonist MDL 100907 nor the 5-HT_{2A} receptor inverse agonist pimavanserin, systemically administered, consistently reduced context-conditioned fear when administered alone indicates that 5-HT_{2A} receptor activation is not a prerequisite for normal fear conditioning to take place.

This notwithstanding, co-administration of either drug with escitalopram, however, markedly impaired freezing. This is well in line with clinical studies on depression and OCD indicating that while compounds that selectively (Fava et al. 2019, Papakostas et al. 2020) or non-selectively (Blier et al. 2010, Carey et al. 2012, Carpenter et al. 2002, Corya et al. 2006, Maes et al. 1999, Marek et al. 2003, Rapaport et al. 2006) block the 5-HT_{2A} receptor lack a therapeutic effect of their own, they may augment the therapeutic effect of an SSRI.

5-HT_{2A} receptors in several areas of the brain have been attributed a role in fear. For example, blockade of the 5-HT_{2A} receptors in the BLA has been suggested to decrease learned/conditioned fear (Macedo et al. 2007), and since the context-conditioning paradigm used in this study is assumed to induce anxiety with temporal uncertainty (i.e. regulated by the BNST) (Goode et al. 2019, Goode et al. 2020, Hammack et al. 2015, LeDoux and Pine 2016), it is plausible that the BNST

is the site where the anxiety reducing combination of an SSRI (stimulating anxiety dampening 5-HT_{1A} receptors) and a 5-HT_{2A} receptor antagonist (preventing the SSRI to simultaneously stimulate anxiety amplifying 5-HT_{2A} receptors) is exerting its effect (Hammack et al. 2009).

To summarize, although the 5-HT_{2A} receptor antagonists do not impact freezing *per se*, systemic inhibition of the 5-HT_{2A} receptors can unmask a freezing reducing effect of an SSRI, suggesting that the 5-HT_{2A} receptors become activated when exposed to the elevated extracellular serotonin levels following the SSRI, and that they, in this situation, may exert an anxiogenic influence. Moreover, the results lend some support for the theory mentioned in the previous section that 5-HT_{2A} receptor down-regulation may be a mechanism of action for long-term SSRI administration. The synergism observed could putatively be utilized in the treatment of anxiety disorders.

5-HT_{2A} receptor agonism (paper II)

Further support for the notion that the 5-HT_{2A} receptors, though not an indispensable prerequisite for normal conditioned freezing to occur, may profoundly impact freezing when activated, was obtained from the studies addressing the effects of 5-HT_{2A} receptor agonists. However, in this situation, enhanced activation led to a reduction in freezing, i.e. an impact opposite to what seemed to be the case when an SSRI was combined with a 5-HT_{2A} receptor antagonist or inverse agonist.

The experiment with 25CN-NBOH thus demonstrated that systemic stimulation of the 5-HT_{2A} receptor induced an anxiety-reducing effect in the context-conditioned fear paradigm that was blocked by MDL 100907. Likewise, it was demonstrated that the impaired freezing observed after administration of psilocybin was blocked by the same antagonist, which is in agreement with previous studies suggesting this receptor to be involved in the mechanism of action of both psychedelics in general (Glennon et al. 1984) and psilocybin in particular (Vollenweider et al. 1998).

Regarding a potential site of action, the anxiety dampening effect is presumably mediated by 5-HT_{2A} receptors situated in the dPAG (Brandao et al. 2008, Oliveira et al. 2007).

MDL 100907, however, did not reverse the freezing effect observed after TCB-2 administration. Although TCB-2 displays high affinity, and has been advocated to be selective, for the 5-HT_{2A} receptor (Fox et al. 2010, McLean et al. 2006), to this date no radioligand binding assays have been performed on this compound, and the selectivity of the drug has been questioned (Di Giovanni and De Deurwaerdere 2018).

To conclude, stimulation of the 5-HT_{2A} receptor reduced context-conditioned fear, supporting the view that this mechanism could be clinically relevant in the treatment of psychiatric disorders (Carhart-Harris and Goodwin 2017, Griffiths et al. 2008, Mithoefer et al. 2016) and that context-conditioned fear could be a useful model for preclinical investigations on such drugs. Additionally, it was demonstrated that TCB-2 impaired freezing through a separate mechanism, which requires further studies to be disclosed.

State and trait anxiety (paper IV)

The response to acoustic noise bursts reflects state anxiety, i.e. a reaction to a direct threat. Rats subjected to bursts display another interesting feature, namely large inter-individual differences that are stable over several tests. It has been suggested that more receptive animals display an inherent quality of sensitivity, that can be utilized to investigate trait anxiety (Leon et al. 2017, Millan 2003, Naslund et al. 2015, Pettersson et al. 2015).

Hence, depending on how the experiments are designed, and how the data is analyzed, noise burst-induced freezing can be used to investigate both state and trait anxiety.

Manual vs automated scoring of freezing (papers I-V)

Manual and automated scoring of freezing are highly correlated under normal conditions (Luyten et al. 2014). Our experience is that in more complicated situations (e.g. when an animal displays signs of sedation or is turned away from the camera), both methods are impaired. The advantage with automatic scoring is that it is user independent. Differences in scoring between different observers can be circumvented through various procedures, but a more serious problem is the difficulty in scoring blindly in situations where the animals might display behavioural signs that hint which treatment they have received. The advantage with manual scoring, if done correctly, is that it can discriminate between freezing and other types of immobility. There are semi-automated approaches (Amorim et al. 2019, Meuth et al. 2013), which however share the benefits and the drawbacks of both methods.

All experiments presented in this thesis utilize automated scoring supplemented with gross observations to exclude deviant animals, displaying behaviours such as resting and signs of sickness.

5-HT₆ receptors and defecation (paper V)

Administration of 5-HT₆ receptor antagonists reduced both CFS-induced and unconditioned defecation, while stimulation of the same receptor had no effect in either model. Stress-induced defecation is sometimes reported as a (secondary) measure of anxiety. In this study, none of the tested 5-HT₆ receptor antagonists affected freezing, indicating that the observed effect on defecation was not secondary to a reduction in fear.

The prerequisites support that the observed effect is centrally mediated, since there are no reports on a functional role of the 5-HT₆ receptors in the intestine (Hirst et al. 2003). However, the present study does not reveal the site of action.

CONCLUDING REMARKS

The role of serotonin in the regulation of anxiety in animals and humans is undisputed, although it is still largely unknown how this influence is exerted.

The results of the experiments with PCPA (paper I) imply that the net influence of serotonin on conditioned fear would be anxiogenic, since both acquisition and expression of fear were impaired rather than augmented by serotonin depletion. At the same time, the freezing-reducing effects of 5-HT_{2A} receptor agonism (paper II), as well as by fenfluramine and 5-HTP (to be published) demonstrate that pharmacological stimulation of the serotonergic system may also reduce the expression of conditioned freezing. Tentatively these two apparent opposite influences are exerted in different brain regions; although not explained by the experiments presented in this thesis, there is thus a body of evidence supporting that increased serotonin in the (basolateral) amygdala augment anxiety, while increased serotonin in the BNST, and foremost the PAG, dampens anxiety.

This thesis also demonstrates that administration of an SSRI in the studied models of unconditioned and conditioned fear mirrors clinical situations. In the conditioning model (paper III), which is thought to resemble anxiety in GAD, chronic but not acute administration of an SSRI reduced anxiety, in line with what is seen in humans. The increased anxiety observed after acute SSRI administration in the unconditioned model (paper IV) may, on the other hand, reflect the exacerbation of anxiety upon initial treatment observed in patients with PD. Thus, both models may be valid paradigms for preclinical studies aiming to reflect human conditions.

Finally, the effect of the 5-HT₆ receptor in stress-induced defecation (paper V), which is used as a model for IBS-D, was evaluated. It was demonstrated that antagonism of this receptor markedly reduced defecation in rats. To test the usefulness of this treatment in a clinical study seems warranted.

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APPENDIX: MATERIALS AND METHODS

Animals

All rats used in this thesis were of the Sprague Dawley strain, 9-10 weeks at arrival, obtained from Taconic (Borup, Denmark) or Janvier (Le Genest-Saint-Isle, France). The animals were kept in an animal facility under controlled conditions: temperature 21-23°C, humidity 55-65%, 12h light-dark cycle and with food and water supplied ad libitum. Rats were kept in groups of 3-4 except during experimental procedures.

Behavioural experiments

Fear conditioning apparatus

The same fear conditioning system (MD-VFC-NIR-R, Med Associates, St Albans, VT, USA) was utilized in papers I-V. It consisted of 3 identical sound-attenuating cubicles, each containing a fear conditioning chamber measuring 305 x 241 x 210 mm. A white background noise of 60 dB was supplied during all experimental sessions.

Context-conditioned fear with foot shocks as US (papers I-III and V)

Context-conditioned fear was induced by electric foot shocks through the grid floor of the fear conditioning chambers. After a habituation period of 5 min rats received either 5 (papers I, II and V) or 10 (paper III) shocks (1 s duration, 0.6 mA, 30 s inter-shock interval). There was no significant difference in the conditioned response between the two protocols (pilot data, unpublished). Freezing was assessed during the first 5 min after replacement in the conditioning chamber, except for 10 min in paper V in order to obtain a larger sample of faecal boli.

Noise burst-induced freezing (paper IV)

After a habituation period of 5 min rats were subjected to 20 presentations of white noise bursts (0.2 s duration, 95 dB, 30 s inter-stimuli interval), and the same protocol was used for repeated testing. The direct response to the unconditioned stimuli was evaluated, as well as the (weak) conditioned response.

Automatic assessment of freezing

Freezing behaviour was assessed through an in-house developed procedure. The fear conditioning system stored videos recorded in parallel in merged Windows Media Video (WMV) files. The files were split using WMV Stream Editor version 1.0 (Open Source Software) and subsequently the resolution of the videos was converted from 320 x 240 px to 80 x 64 px using HandBrake version 0.9.5 and 1.2.0 (Open Source Software). The resolution was reduced to boost computational speed and it did not impair the assessment of freezing. Immobility was quantified using Matlab version 7.11 and 9.5 (Mathworks, Natick, MA, USA). The total absolute difference in pixel intensity (for each px) between two frames (3 fps) was calculated. Only pixel pairs displaying a difference above a certain level were accepted to cancel out noise and minor movements, such as respiration. If the total absolute difference of all pixel pairs was less than a fixed cut-off value for at least 3 consecutive frames (i.e. 1 s), that period was counted as freezing (Anagnostaras et al. 2010, Maren 1998). The automatically assessed freezing was validated by manual gross observations.

Stress-induced defecation (paper V)

Stress-induced defecation was assessed using the context-conditioned fear protocol with foot shocks as US. Seven days after fear conditioning, the rats were reintroduced to the conditioning chamber and the number of faecal boli produced during 10 min was calculated.

Defecation in non-stressed rats (paper V)

Following drug administration, in order to discriminate the number of faecal boli produced by each rat, they were placed individually in single housing cages. After the experiment the animals were returned to their home cages.

Drugs

The following drugs were used in the manuscripts presented in this thesis:

- 25CN-NBOH (paper II) – 5-HT_{2A} receptor agonist
- Escitalopram oxalate (papers II-IV) – SSRI
- MDL 100907 (paper II) – 5-HT_{2A} receptor antagonist
- PCPA (paper I) – tryptophan hydroxylase (TPH) inhibitor
- Pimavanserin (paper II) – 5-HT_{2A} receptor inverse agonist
- Psilocybin (paper II) – 5-HT_{2A} receptor agonist (unselective)
- SB 242084 (papers II and IV) – 5-HT_{2C} receptor antagonist
- SB 258585 (paper V) – 5-HT₆ receptor antagonist
- SB 271046 (paper V) – 5-HT₆ receptor antagonist
- SB 399885 (paper V) – 5-HT₆ receptor antagonist
- TCB-2 (paper II) – 5-HT_{2A} receptor agonist (unselective)
- WAY 208466 (paper V) – 5-HT₆ receptor agonist

All drugs were purchased from Tocris Bioscience (Bristol, UK) except for escitalopram that was purchased from Shodhana Labs (Hyderabad, India), PCPA and SB 242084 that were purchased from Sigma-Aldrich (St Louis, MO, USA), pimavanserin that was purchased from MedChem Express (Monmouth Junction, NJ, USA) and psilocybin that was purchased from Chiron Corporation (Emeryville, CA, USA).

25CN-NBOH, escitalopram, PCPA, SB 399885 and WAY 208466 were dissolved in 0.9% saline solution. MDL 100907, pimavanserin, psilocybin and TCB-2 were dissolved in 0.9% saline solution applying a few drops of 1M hydrochloric acid (HCl). SB 242084 was dissolved in 0.9% saline solution with 8% cyclodextrin and

0.48% citric acid. SB 258585 and SB 271046 were dissolved in 0.9% saline solution with 1% or 20% DMSO, respectively. If required, the solutions were pH adjusted (>6) using sodium hydroxide (NaOH) prior to administration.

Drugs were administered intraperitoneally (papers I and V), subcutaneously (papers II-IV) or through osmotic minipumps (Alzet, 2ML4, Cupertino, CA, USA) (paper III). Injections were given at the following concentrations: 25CN-NBOH (3 mg/kg), escitalopram (paper II: 5 mg/kg; paper III: 0.3, 0.5 and 1 mg/kg; paper IV: 10 mg/kg), MDL 100907 (0.003, 0.03, 0.01, 0.1, 0.3, 1 and 3 mg/kg), PCPA (300 mg/kg on 3-4 consecutive days), pimavanserin (0.3 mg/kg), SB 242084 (paper II: 0.3 and 1 mg/kg; paper IV: 0.5 mg/kg), psilocybin (2 mg/kg), SB 258585 (20 mg/kg), SB 271046 (5 mg/kg), SB 399885 (1, 3, 5 and 10 mg/kg), TCB-2 (1 mg/kg) and WAY 208466 (10 mg/kg). The minipumps were filled with 2 ml of either escitalopram (53 mg/ml) or 0.9% saline solution and implanted subcutaneously under anaesthesia with ketamine and xylazine. The escitalopram concentration was set to deliver 10 mg/kg/day to a rat of 500 g (Ceglia et al. 2004).

Serum-escitalopram analysis (paper III)

Rats were anesthetized with isoflurane and decapitated so that trunk blood could be collected. Samples were kept cold (<2 h) until centrifugation (2300 RCF). Serum was extracted and frozen (-80°C to -20°C) until analysis.

Assessment of serum content of escitalopram (with LC-MS/MS) was performed at the Division of Drug Research at the Department of Medical and Health Sciences at Linköping University (see paper III for in-depth description).

Statistical analysis

Analyses were performed using SPSS Statistics version 19.0 (IBM Corp, Armonk, NY, USA). For normal distributed data comparisons between groups were made using Student's t-tests (2 groups) or one-way analyses of variance (ANOVA)

followed by Fisher's Least Significant Difference post hoc tests (>2 groups). For non-normal distributed data comparisons between groups were performed using Mann-Whitney U tests, preceded by Kruskal-Wallis tests (>2 groups). Correlations in paper IV were assessed using Spearman's tests. In experiment V, a follow up analysis using a linear model with drug dose included as a covariate was performed.

Ethics

All procedures were approved by the Animal Ethics Committee in Gothenburg and carried out in accordance with institutional guidelines.

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