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Olivier, Jocelien D A; Olivier, Berend

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# Translational Studies in the Complex Role of Neurotransmitter Systems in Anxiety and Anxiety Disorders

Jocelien D. A. Olivier and Berend Olivier

## Introduction

Anxiety disorders are among the most prevalent psychiatric disorders. In DSM5, anxiety disorders include separation anxiety disorder, selective mutism, specific phobia, social anxiety disorder, panic disorder, agoraphobia, and generalized anxiety disorder. The impact of anxiety disorders is high; studies in Europe [1] or the USA [2] estimate anxiety disorders at approximately 14% and 22%, respectively, of the population. Moreover, anxiety disorders are often comorbid with other psychiatric disorders, including mood and substance abuse disorders. Anxiety disorders are quite heterogeneous, and the individual disorders (e.g., panic disorder versus phobia or generalized anxiety disorder) are quite distant from each other. No laboratory blood test or brain scan or any other biomarker is yet available to distinguish between either disorder. The complex classification of the various anxiety disorders complicates or even hinders the finding and development of new psychoactive drugs, notably because one-to-one translation to preclinical animal models of such anxiety disorders is extremely difficult to realize, if not impossible. Although a classification in intermediate phenotypes [3] that are based on neurobiological mechanisms probably underlying pathological anxiety processes has been suggested, this has not led to novel, innovative treatments for anxiety yet. Another hypothesis is

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J. D. A. Olivier

Department of Neurobiology, Groningen Institute for Evolutionary Life Sciences (GELIFES), University of Groningen, Groningen, The Netherlands

B. Olivier (✉)

Department of Neurobiology, Groningen Institute for Evolutionary Life Sciences (GELIFES), University of Groningen, Groningen, The Netherlands

Department of Psychopharmacology, Utrecht Institute for Pharmaceutical Sciences, Utrecht University, Utrecht, The Netherlands

Department of Psychiatry, Yale University School of Medicine, New Haven, CT, USA  
e-mail: [B.Olivier@uu.nl](mailto:B.Olivier@uu.nl)

based on the assumption that dysfunctional neurotransmitter systems underlie disturbances in the regulation of anxiety processes in the CNS. However, dysfunctional neurotransmitter systems are difficult to prove, and a direct relationship between certain neurotransmitter systems (e.g., the serotonergic or GABA-ergic system) is difficult to establish. Actually, drugs influencing these systems and exerting anxiolytic properties were found serendipitously (benzodiazepines; BDZ) or developed initially for other purposes (selective serotonin reuptake inhibitors; SSRIs) many decades ago. Since 2007, no new anxiolytic agents have been approved by the US Food and Drug Administration [4], and SSRIs (and to a lesser extent serotonin-norepinephrine reuptake inhibitors; SNRIs) remain the mainstay for the treatment of anxiety disorders (often augmented or preceded by certain BDZs for a limited time period). Not all patients respond well to serotonergic therapy, either due to bothersome side effects or treatment resistance.

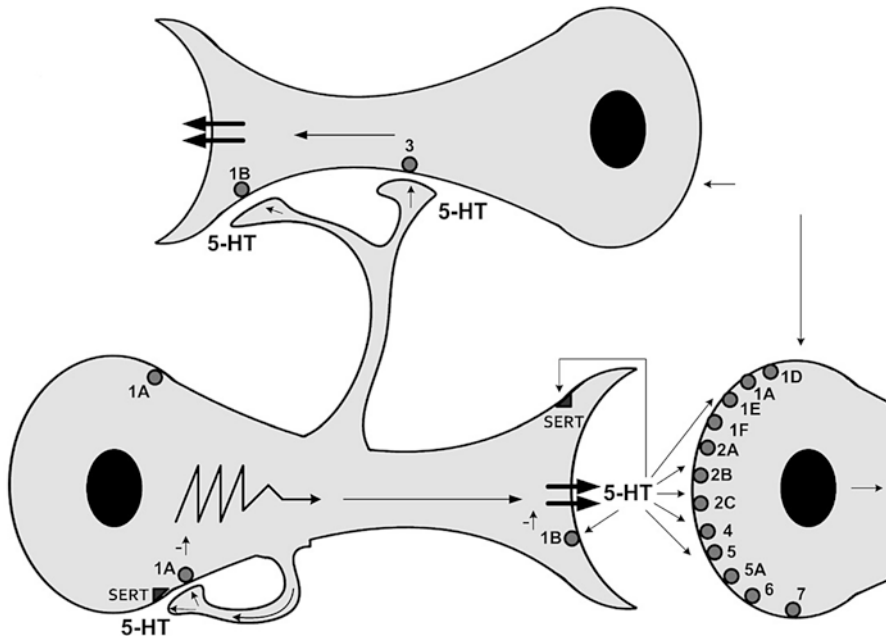
The last decades' intense efforts have been made both in preclinical and clinical studies, to find and test other mechanisms targeting anxiety-involved mechanisms in the CNS in order to detect new and/or improved treatments for the various anxiety disorders.

In the following parts, the existing anxiolytics and putative new promising anxiety targets will be described. First, using a translational approach, serotonergic targets, in particular the serotonergic transporter and 5-HT<sub>1A</sub>- and 5-HT<sub>2</sub> receptors, will be outlined, followed by the involvement of the GABA<sub>A</sub> system in anxiety and anxiety disorders. Some new potential targets will be shortly introduced (CRF<sub>1</sub> receptor, neurokinin<sub>1</sub> (NK<sub>1</sub>) receptors, glucocorticoid receptors, and glutamatergic receptors [5, 6].

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## The Serotonin Transporter (5-HTT)

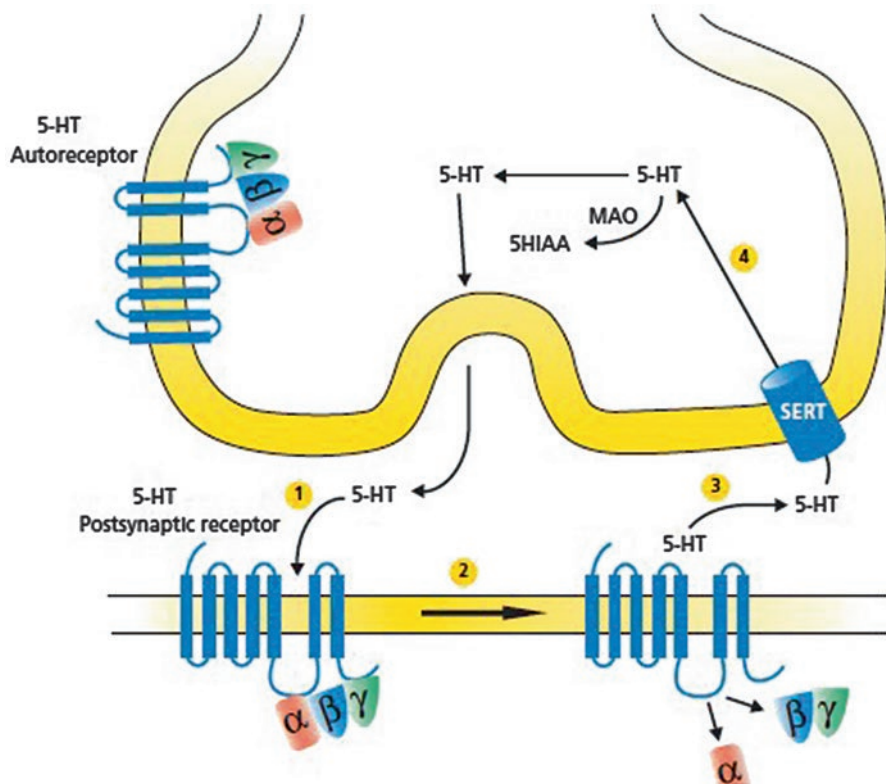
There are at least 14 different serotonin receptor types (5-HT<sub>1</sub> (A,B,D,E,F), 5-HT<sub>2</sub> (A,B,C), 5-HT<sub>3</sub>, 5-HT<sub>4</sub>, 5-HT<sub>5</sub> (A,B), 5-HT<sub>6</sub>, and 5-HT<sub>7</sub>). Except for the 5-HT<sub>3</sub> receptor, all 5-HT receptors are G-protein coupled. Signaling via these G-protein-coupled serotonin receptors is extremely diverse, and we are still largely in the dark how these various 5-HT receptors operate in and contribute to the extremely complex interaction in the various ongoing functions in the CNS. This diversity in combination with a complex distribution of the various receptors in the brain seems to bring the serotonergic system in an important position to modulate various functions in the brain. Disruption of various aspects of serotonergic neurotransmission contributes to changes in vulnerability or even psychopathology, including depression, anxiety disorders, schizophrenia, and others [7]. All 5-HT receptors are postsynaptically located on non-serotonergic neurons (heteroreceptors), but 5-HT<sub>1A</sub> and 5-HT<sub>1B/1D</sub> receptors are also present as autoreceptors on the soma (5-HT<sub>1A</sub>) or synaptic area (5-HT<sub>1B/1D</sub>) of serotonergic cells (Fig. 8.1). The serotonin transporter (5-HTT) is localized both in the synaptic area and on the cell bodies of 5-HT neurons (Fig. 8.2). If serotonergic neurons fire, 5-HT is released into the synaptic cleft where it exerts its action on nearby pre- and postsynaptic serotonergic receptors. The 5-HTT is a



**Fig. 8.1** Cartoon showing a serotonergic neuron and two postsynaptic non-serotonergic neuron (top and right). The various 5-HT receptors and the 5-HTT are schematically localized on these three neurons. 5-HT<sub>1A</sub> receptors are present as somatodendritic autoreceptors and postsynaptic heteroreceptors. 5-HT<sub>1B/1D</sub> receptors are present as presynaptic receptors in the synaptic cleft and as postsynaptic heteroreceptors. The 5-HTT is present in the presynaptic part and at the soma of the serotonergic neuron. All other receptor(type)s are postsynaptically located

key regulator of 5-HT signaling. The reuptake of serotonin by serotonergic neurons via the 5-HTT is the main mechanism for clearing extracellular 5-HT after its release; this leads to the termination of 5-HT signaling and recycles 5-HT in the neuron for subsequent release via uptake by the vesicular monoamine transporter (VMAT2). Another major route to end serotonergic activity is via uptake of synaptic 5-HT by surrounding glial cells where it is degraded to its main metabolite 5-hydroxyindole acetic acid (5-HIAA) by monoamine oxidase-A (MAO-A).

SSRIs are presently considered as the first-line pharmaceutical treatment for anxiety disorders, whereas SNRIs are considered second- or third-line treatments [8]. In a meta-analysis the differential efficacy of SSRIs and SNRIs and placebo in the treatment of anxiety disorders (together with depression, obsessive-compulsive disorder (OCD) and post-traumatic stress disorder) were estimated in children and adolescents [8]. SSRIs and SNRIs were about equally effective in treating anxiety disorders and more beneficial than placebo. Well-known, but still remarkable, is the placebo effect. This is a general observation in psychiatric treatments, both in pediatric and adult applications [9]. Notwithstanding this influential factor, it is generally accepted that psychotropic drugs that inhibit the 5-HTT in the CNS, exert anxiolytic activity in various patient groups with diverse anxiety and fear disturbances.



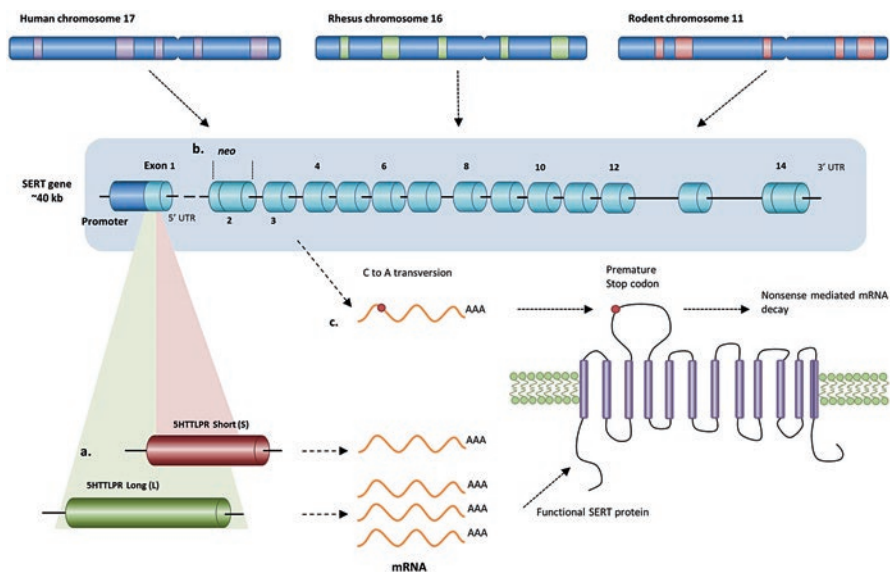
**Fig. 8.2** Schematic representation of serotonin (5-HT) in the terminal and synapse. G-protein-coupled receptors are located presynaptically (5-HT autoreceptors 5-HT<sub>1A/1B</sub>) or postsynaptically (5-HT<sub>1/2/3/4/5/6/7</sub> receptors). **1:** 5-HT is released from the presynaptic neuron and binds to a heterotrimeric G-protein postsynaptic receptor. Heterotrimeric G-protein complexes contain an  $\alpha$ ,  $\beta$ , and  $\gamma$  subunit, which in an inactive state are bound to GDP. **2:** 5-HT acts on postsynaptic receptors and induces a change in the conformation of the postsynaptic receptor. GDP is phosphorylated to GTP and binds to the  $\alpha$  subunit, which subsequently becomes active. The  $\beta$  and  $\gamma$  subunits are freed. **3:** Extracellular 5-HT is taken up by the 5-HTT into the presynaptic neuron. **4:** Back in the presynaptic neuron 5-HT is broken down by MAO to 5-HIAA (occurs also extracellularly) or is being stored in vesicles for future release. MAO, monoamine oxidase; 5-HIAA, 5-hydroxyindole acetic acid; SERT, serotonin transporter. Figure adapted from Vinkers et al. [113]

For an extensive time, the 5-HT transporter has been implicated in processes involved in anxiety mechanisms. Blocking the 5-HTT leads to an acute rise in the synaptic concentration of serotonin. However, anxiolytic efficacy (similarly to depression) only emerges after longer treatment (weeks to months), indicating that long-term plasticity changes in presumed anxiety mechanisms have to take place. It is still largely unknown what those mechanisms are and how they come about. But the pathogenesis of anxiety disorders (and particularly GAD) is clearly influenced by many factors; moreover, many complex interactions between biological factors, experimental influences, and psychological mechanisms are involved [10]. In

linkage studies and non-hypothesis-driven genome-wide association studies (GWAS) in large populations with various anxiety disorder problems, an association with the 5-HTT was never found [10], although some evidence emerged that a highly interconnected molecular network around GAD is part of the anxiety spectrum of disorders [11]. Candidate gene studies on putative individual nodes of this putative anxiety network have generated considerable evidence in favor of the serotonergic system and its impact on anxiety-related endophenotypes [10, 12].

Polymorphisms in the 5-HTT-gene and its associated transcriptional control area influence the activity of the serotonergic system [13]. Although focusing on anxiety, the 5-HTT gene and its variations (SLC6A4), producing a 5-HTT protein, are associated with several human behavioral and neurological traits but also with various medical disorders (e.g., myocardial infarction, pulmonary hypertension, irritable bowel syndrome, and sudden infant death syndrome) [14]. This indicates the important role of serotonergic neurotransmission in various aspects of our healthy and diseased biology. The emerging developmental role of serotonin [15] in psychiatric disorders, including anxiety-related ones, suggests that developmental disturbances in changing serotonin levels during critical periods in development (windows) may have long-lasting effects on brain function, particularly on later anxiety-related behaviors in adulthood [16, 17].

Most research involving the role of the 5-HTTs in psychiatry including anxiety disorders has been performed in adult populations. Lower 5-HTT expression and function in genotypes with the S-allele of the 5-HTTLPR (5-HTT length polymorphism) are associated with anxiety and depression [18, 19]. Subsequently, the 5-HTTPRL and associated variations in coding and noncoding regions of the 5-HTT-gene have been associated with many aspects of psychiatric disorders [14, 20]. The human 5-HTT is located at chromosome 17 (17q11.2) and consists of 14 exons spanning around 40kB; the emerging 5-HTT protein of 630 amino acids comprises 12 membrane domains (see Fig. 8.3). The expression of the human 5-HTT gene is modulated by a length variation (L and S variants) in the 5-HTTLPR and two SNPs in the same region (rs25531 and rs25332), all located upstream of the start of transcription. The single-nucleotide polymorphism rs25531 subdivides the L-allele into L<sub>A</sub> and L<sub>G</sub> alleles; the L<sub>A</sub> allele is associated with increased expression of activity compared to L<sub>G</sub> or S-alleles. A variable number of tandem repeat (VNTR) polymorphisms, called STin2, are present in intron 2, together with various SNPs influencing the activity of the 5-HTT protein. This polymorphism is tri-allelic, with 9-, 10-, or 12-repeat alleles. The latter allele is associated with enhanced 5-HTT expression [21]. It seems possible that individuals with low 5-HTT expressing genotypes, when exposed to SSRIs, have higher 5-HTT occupancy and saturation, theoretically associated with increasing central and peripheral serotonin availability. This might manifest itself as more side effects [21]. The 5-HTT is an extremely important modulator of the serotonergic neuronal system; duration and magnitude of serotonergic neurotransmission is largely steered by 5-HTT activity, thereby fine-tuning 5-HT signaling. Dysfunctions in these signaling pathways have been related to several psychiatric disorders and traits, including anxiety. Although initially, the S- and L-variants of the 5-HTTPRL were associated with differential vulnerability to neuropsychiatric disorders (or as a



**Fig. 8.3** Different alterations in the human, rhesus, and rodent 5-HTT gene resulting in changed transcription levels of 5-HTT. (a) Humans and rhesus macaques have short or long alleles for the 5-HTTLPR resulting in either lower or higher transcription levels, respectively. Since rodents do not carry an orthologue of this polymorphism, knockout of the 5-HTT can be achieved by (b) replacing exon 2 with a neo cassette (mice) or by (c) inducing a premature stop codon in exon 3 (rats) resulting in the absence of functional 5-HTT protein. (Figure adapted from Houwing et al. [56])

predictor of response to SSRIs), recent findings show a far more complicated pattern, where various polymorphisms inside the promoter region influence the activity of the 5-HT reuptake properties of the 5-HTT [22].

The story of modulation of serotonergic transmission via 5-HT mechanisms reveals the complexity of factors involved in the genetic-function coupling of any psychiatric disorder. The identification of specific gene variants and their involvement in anxiety is quite troublesome. It is increasingly clear that these variations may influence “intermediate biological phenotypes” in concert with other (background) genes, epigenetic factors, and environmental and developmental factors. Such complex interactions likely seem to contribute to the risk or resilience to develop certain psychiatric conditions. Using intermediate phenotypes may enable the search for associations between specific candidate genes (e.g., for 5-HTT) that mediate between a modulating allele and a complex disease phenotype [23].

Various studies on the 5-HTTLPR-promoter area are testing gene X environment and gene X gene interactions [24–27]. In general, S-alleles of 5-HTTLPR are associated with enhanced risk for a variety of psychiatric disorders, including anxiety. Caspi et al. [28] hypothesized the S-allele as a “risk” or “vulnerability” allele; the role of the L-allele is less clear although it has been suggested as risk factor for development of psychopathological traits [29]. Because L- and S-alleles are

common to each human, and psychopathology does not happen in every individual, it is assumed that our genome includes “protective” alleles (e.g., CRF<sub>1</sub>-receptor variants) rendering individuals resilient to pathology [30–32].

Genes certainly contribute to the risk to develop anxiety disorders and underlie anxious traits. No candidate genes have emerged playing a primary role in the expression for a vulnerability to anxiety or anxiety disorders [33, 34]. Meta-analysis of genotypes implicated in mental disorders with varying levels of heritability has indicated that for anxiety disorders, only three genes have been identified that contribute to anxious phenotypes (in contrast to, e.g., 50 genes for schizophrenia [33]). The former study did not implicate the 5-HTT and associated promoter area (5-HTTPRL) as a candidate gene involved in anxiety disorders. These complex findings indicate the difficulties in discovering new targets for anxiolytic drugs. This reflects that such complex neurobehavioral traits or disorders like the anxiety disorders are not caused by single genes, but probably by disturbances in extensive networks comprising of numerous parts and pathways that are created by many biological mechanisms.

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## Preclinical Evidence for the 5-HTT in Anxiety

Human linkage and association studies have not identified genes that contribute influentially in the modulation of anxiety or anxiety disorders; therefore, animal pathology and models might be of considerable importance in studying the involvement of genes in anxiety disorders as well. Human anxiety pathology is also present to a certain degree in animal pathology [35], and studies in animal anxiety models have considerably enhanced our knowledge of the neurobiological mechanisms involved in anxiety. Animal models can be very helpful in delineating putative genes involved in either anxiety or anxiety-associated traits [12, 36]. In order to progress in our search for fundamental mechanisms involved in anxiety processes and those in anxiety disorders, we need a translational approach; data from anxiety research, including genetic and environmental factors, should lead to fundamental research in animal models but also the reverse. The availability of cell-specific and inducible knockout and knock-ins, optogenetic technology [37], and, more recently, the Crispr/Cas 9 technology [38] enables selective operations in cellular mechanisms and circuit functions that are coupled to the gene’s function [37]. These techniques are now starting to be applied in animal models of fear and anxiety.

Numerous preclinical anxiety and fear tests have been developed and used over time [39–41]. Animal tests and models of anxiety are based on face validity (is it measuring something analogous to human anxiety symptoms?), predictive validity (is it sensitive to clinically efficacious anxiolytics?), and construct validity (are the same pathophysiological mechanisms involved as in human anxiety disorders?). Till up now, none of the currently available anxiety tests or models does meet all of these criteria unequivocally. Griebel and Holmes [6] give an extensive review on preclinical models and tests used in anxiety and fear research. Many animal tests and models of anxiety are based on natural behavior patterns in rodents (often rats and mice



[42]). Such, often ethologically derived models include “approach-avoidance” tasks [40]. Animals are typically exposed to aversive situations like an open field, elevated plus maze or light/dark box. The level of anxiety is measured by the amount of avoidance of the aversive environment (open area, light). Besides the “unconditioned” procedures, several “conditioned” paradigms are also used to model anxiety disorders, including conflict procedures like the Vogel-lick test, defensive burying, four-plate test, and fear-potentiated startle. Various other paradigms are used to measure anxiety levels including physiological parameters [43], social interaction, predator stress, and stress-induced vocalizations [44]. Over the last decades, several models were introduced to investigate the role of the 5-HTT and adaptive 5-HT signaling in the *in vivo* action of SSRIs. In a transgenic knock-in mouse model, a single amino acid substitution (M172) in one of the 14-membrane domains led to diminished activity of SSRIs in emotional behavior, although the recognition of 5-HT and its clearance were not affected [45]. 5-HTT-overexpressing mice showed reduced anxiety levels [46, 47] and enhanced 5-HT<sub>2A/2C</sub> receptor functioning [48]. This 5-HTT-overexpressing model in combination with the effects associated with 5-HTT knockout (5-HTT<sup>-/-</sup>) mice might shed light on the compensatory effects observed on the life-span but also on those after chronic SSRI administration. 5-HTT<sup>-/-</sup> mice [49] and rats [50] have been created, and in both genotypes, extracellular 5-HT in the brain is enhanced [14, 51]. These enhanced extracellular 5-HT levels are associated with neurodevelopmental changes in 5-HT synthesis and metabolism [14]. The 5-HTT<sup>-/-</sup> rodent model has been regarded as a rather extreme example of the human 5-HTTLPR polymorphism, and as such, animals have difficulties to cope with stress and display anxiogenic behavior [52–55]. Heterozygous 5-HTT knockout rodents (5-HTT<sup>+/-</sup>) might be seen as a better model for the human 5-HTTLPR model, because the reduced 5-HTT expression is more or less comparable to the expression in human S-allele carriers. 5-HTT<sup>+/-</sup> animals have reduced 5-HTT expression and function, with 40–50% less 5-HTT protein levels [49, 51]. However, few neurochemical changes are present in 5-HTT<sup>+/-</sup> rodents compared to wild types (5-HTT<sup>+/+</sup>) [56]. Several serotonergic parameters, like basal extracellular levels and intracellular 5-HT levels, are not affected [51, 57], although often when challenged, 5-HTT<sup>+/-</sup> animals differ from 5-HTT<sup>+/+</sup> animals [56]. 5-HTT<sup>+/-</sup> mice did not differ in anxiety levels from 5-HTT<sup>+/+</sup> mice in an open field or in novelty-suppressed feeding [58]. There is evidence that the 5-HTT genotype may determine the stress response to the environment, particularly during early life stress (ELS) [56]. It has as yet to be established what mechanisms possibly underlie the 5-HTT gene variation X ELS interaction and specifically how that may lead to enhanced anxiety. Whether the 5-HTT<sup>+/-</sup> rodent is a good animal model to study the effects of anxiolytic drugs in situations where the animal is challenged toward showing anxiogenic behavior has to be awaited.

In wildtype mice and rats, often measured in different strains, SSRIs display anxiolytic activity [6]. In humans, SSRIs are also the first-line pharmaceutical agents to treat generalized anxiety disorder (GAD), post-traumatic stress disorder (PTSD), panic disorder (PD), and social anxiety disorder (SAD). Although none of the presently available animal anxiety tests and models specifically models for the

specific human anxiety disorders, many tests/models have properties that predict for specific aspects present in the various anxiety disorders. Although in the early years (1960s till 1990s) of pharmacological treatment of anxiety disorders, BDZs were often first-line treatment, SSRIs have taken this position later on, mainly due to several safety issues (dependence liability, memory disturbances, tolerance, sedation). In an extensive summary of all published results in animal experiments on various pharmacological targets [6], SSRIs exerted in approx. 40% anxiolytic activity, were ineffective in 40% of the studies, and had 20% anxiogenic activity (in over 400 publications). Similar to human anxiety disorder treatment, the effects in animal tests and models are inconsistent. However, most, if not all, animal experimentation is performed using acute studies, whereas anxiolytic activity of SSRIs in humans occurs after at least 4–6 weeks of chronic treatment. Many patients do not respond to SSRIs, and disturbing side effects such as sexual dysfunction, sometimes associated with an initial transient period of enhanced anxiety, have contributed to a reduced acceptability of SSRIs in daily clinical practice.

In our own research, we mainly concurred with this preclinical anxiolytic profile of SSRIs. In a behavioral paradigm for screening putative anti-panic drugs, SSRIs like fluvoxamine and clomipramine (a TCA with predominant 5-HTT blockade) inhibited conditioned ultrasonic distress vocalizations (USV) in adult male rats [59]. SSRIs were also anxiolytic in guinea pig isolation calls [60], whereas in an approach-avoidance ambivalent situation in rats, SSRIs were inactive [61]. In a mouse model of anticipatory anxiety, stress-induced hyperthermia, antidepressants including SSRIs are inactive [62]. SSRIs are also inactive in the fear-potentiated startle [63]. In an ultrasonic vocalization model in isolated rat pups, SSRIs appeared anxiolytic [64].

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## The 5-HT<sub>1A</sub> Receptor and Anxiety

The anxiolytic activity of SSRIs that in some way is caused by the enhanced 5-HT release due to blockade of the 5-HTT must be effectuated by the effects of the released 5-HT on one or more 5-HT receptors; an important candidate is the 5-HT<sub>1A</sub> receptor (5-HT<sub>1A</sub>R). Already in 1979 it was found that buspirone, a partial 5-HT<sub>1A</sub>R agonist (and dopamine D<sub>2</sub>R antagonist) exerts mild anxiolysis in human anxiety patients, notably in GAD [65]. This could be confirmed in rodent models of anxiety [7, 66]. Clinically, development of new 5-HT<sub>1A</sub>R agonists for anxiety disorders (e.g., ipsapirone, gepirone, tandospirone, flesinoxan) failed. However, 5-HT<sub>1A</sub> receptors remain a clinically attractive target implied in anxiety and depression [7, 57, 66–69]. 5-HT<sub>1A</sub>Rs are inhibitory G-protein-coupled receptors and present as autoreceptors on serotonergic neurons and as heteroreceptors (Fig. 8.1) on non-serotonergic neurons (e.g., GABA-ergic). The somatodendritic 5-HT<sub>1A</sub> autoreceptors regulate the serotonergic tone via feedback inhibition in concert with the 5-HTT [7]. Postsynaptic 5-HT<sub>1A</sub> heteroreceptors are abundantly distributed and expressed in the brain although restricted to specific and in high density in limbic areas like hippocampus and various parts of the frontal cortex. Autoreceptors are present in

the dorsal and median raphe nuclei. There is evidence from genetic and imaging studies in humans that 5-HT<sub>1A</sub>R density or regulation is associated with anxiety [70]. A C(-1019)G polymorphism (rs6295) in the promoter region of the 5-HT<sub>1A</sub>R gene (*Htr1a*) was associated with mood-related variables like amygdala reactivity [71]. Increased 5-HT<sub>1A</sub> autoreceptor expression was associated with enrichment of the G-allele; postsynaptically the C-allele was enhanced [72]. In panic disorder with agoraphobia patients [73], variations in the rs6295 polymorphism had functional consequences in defensive behavior, amygdala activity, and neural plasticity, suggesting that differentiation in 5-HT<sub>1A</sub>R activities plays a role in anxiety and fear.

Griebel and Holmes [6] summarized the effects of 5-HT<sub>1A</sub>R agonists in animal models of anxiety and fear. Of the 855 studies reported, approx. 65% of 5-HT<sub>1A</sub>R agonists exerted anxiolytic activity, 30% was inactive and 5% anxiogenic. Moreover, knocking-out (KO) the 5-HT<sub>1A</sub>R in three mouse strains [74–76] led to enhanced anxiety in most standard anxiety tests. This unanimous finding in different labs that inactivation of all 5-HT<sub>1A</sub>R genes led to more anxious mice is rather striking. Later research found, however, that the anxious phenotype was dependent on the paradigm applied [77, 78] and one strain (but not the other two) of 5-HT<sub>1A</sub>R KO mice had reduced sensitivity to anxiolytic effects of diazepam, associated with changes in particular  $\alpha$ -subunits of the GABA<sub>A</sub>- benzodiazepines (BDZ) R complex [79]. Apparently, such a dysfunction of the GABA<sub>A</sub>-BDZ complex is not a prerequisite for the “anxiogenic” phenotype of 5-HT<sub>1A</sub>R KO mice. The enhanced anxiety in the 5-HT<sub>1A</sub>R KO mouse could not be antagonized by SSRIs [80]. Moreover, overexpression of 5-HT<sub>1A</sub>Rs reduced anxiety [81]. These data strongly support a role for 5-HT<sub>1A</sub>Rs in anxiety and fear processes. Further research on differential roles of pre- and postsynaptic 5-HT<sub>1A</sub>Rs in anxiety, using rescue experiments on forebrain (postsynaptic) 5-HT<sub>1A</sub>Rs, indicated a critical role for the latter in development of the anxiogenic phenotype [82]. Pharmacological blockade of 5-HT<sub>1A</sub>Rs during early development, but not in adulthood, enhanced anxiety in normal mice [83, 84]. The complex regulation of anxiety processes during development and adulthood again indicates the complexity of the neural substrates, including genetic regulation, environmental influences, and its interactions. Simple relationships between a certain receptor (in this case 5-HT<sub>1A</sub>R) and the complexity involved in the regulation of anxiety and fear but also its pathology are highly unlikely. However, rodent models (genetic and behavioral) are extremely useful in trying to untangle the different underlying parts and functions.

In our own (preclinical) research, both partial and full 5-HT<sub>1A</sub>R agonists displayed anxiolytic activity. The azapirones (buspirone, ipsapirone, tandospirone, and zalospirone) are partial 5-HT<sub>1A</sub>R agonists. Flesinoxan is a potent and selective full 5-HT<sub>1A</sub>R agonist [66]. 5-HT<sub>1A</sub>R agonists have limited anxiolytic activity in animal anxiety models based on release of suppressed behavior (both conditioned and unconditioned procedures like Geller-Seifter conflict, Vogel-lick test, four-plate, open field, elevated plus maze, light-dark box and social interaction test). In general 5-HT<sub>1A</sub>R agonists are very active in animal models of stress-evoked behaviors both in conditioned and unconditioned situations like fear-potentiated startle, shock-induced USV, conditioned USV, defensive burying, stress-induced hyperthermia,

rat pup, and guinea pig pup isolation calls [66]. Biased 5-HT<sub>1A</sub>R agonists [85, 86] display either selectivity for 5-HT<sub>1A</sub> autoreceptors (F13714) or 5-HT<sub>1A</sub> heteroreceptors (F15599). However, both compounds had anxiolytic activity in several anxiety tests [87], not solving a potential role distribution between pre- and postsynaptic 5-HT<sub>1A</sub>Rs in anxiety, although evidence is gathering that postsynaptic 5-HT<sub>1A</sub>Rs, e.g., in the prefrontal cortex [88], may play a conclusive role.

Buspirone is worldwide marketed as anxiolytic, whereas tandospirone is only available in Japan and China. Flesinoxan was tested in phase III clinical trials as a potential anxiolytic (and antidepressant), but did not show superior activity compared to placebo and was subsequently abandoned. Although buspirone (and to a lesser extent tandospirone) are clinically effective anxiolytics, 5-HT<sub>1A</sub>R agonists have not emerged as a breakthrough and successor of BDZ in the treatment of anxiety disorders.

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## Other 5-HT Receptors and Anxiety

Agonists and antagonists for practically all 5-HT receptors have been synthesized over the last 30 years and subsequently also tested in (mostly simple) anxiety tests. Griebel and Holmes [6] summarize results on frequently tested compounds, including 5-HT<sub>2</sub>R antagonists, 5-HT<sub>3</sub>R antagonists, and others. In 314 experiments 5-HT<sub>2</sub>R antagonists were around 43% anxiolytic and 5-HT<sub>3</sub>R antagonists (232 experiments) in 65%. Żmudzka et al. [87] essentially confirmed these data on 5-HT receptors and anxiety. Although in the 1990s the 5-HT<sub>3</sub>R antagonists were considered the anxiolytics of the future based on animal results, they did not confirm their anxiolytic profile in human anxiety disorders [89]. Thus far, no promising new serotonin receptor ligands have been introduced to the market as new anxiolytics.

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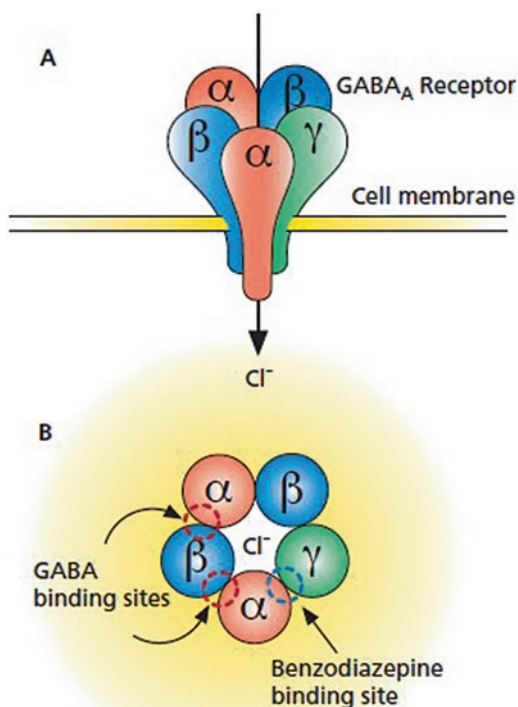
## GABA<sub>A</sub> Receptors (GABA<sub>A</sub>R) and Anxiety

GABA<sub>A</sub> ( $\gamma$ -aminobutyric acid, type A) receptors are the molecular targets of BDZ, chemical structures that were serendipitously found in the 1950s and were developed as the main anxiolytics of the ensuing decades. Besides anxiolysis, the classical BDZs also exerted sedation, epileptic seizure suppression, muscle relaxation, and hypnotic effects [90]. It took over 20 years before the molecular target of the BDZ, the GABA<sub>A</sub> receptor (GABA<sub>A</sub>R) was discovered [91]. The GABA<sub>A</sub>R is the target of clinically important anxiolytics, including BDZ, and is an ionotropic inhibitory receptor [90]. Although BDZs are excellent anxiolytics both in man and animals and are very safe in overdosing, it was realized that long-term use carries risks of tolerance, physical and psychological dependence, and withdrawal signs after discontinuation. Moreover, adverse side effects of sedation and cognitive slowing were found [92]. As a consequence, the SSRIs (and to a lesser extent the SNRIs) are now the first-line pharmacological treatment option for many anxiety disorders, whereas the BDZs are prescribed as second- or third-line treatment if

antidepressants are ineffective and cognitive behavioral therapy has failed [4]. Notwithstanding the reluctance to prescribe BDZs, its use is still high in many countries; more than 5% of adults in the USA are prescribed BDZ each year [93].

Ionotropic GABA<sub>A</sub>Rs are ubiquitous in the brain and are crucial in the temporal precise activity of neuronal circuitry and synchronized oscillatory activity of neuronal populations [94]. The diversity of this inhibitory neurotransmission regulating these networks is on one side regulated by the large variety in interneurons but also by the extremely diverse group of GABA receptors on the receiving side. GABA<sub>A</sub>R consist of five subunits that surround a central pore in a pentameric composition (Fig. 8.4). When the endogenous ligand (GABA) binds to the GABA<sub>A</sub>R, chloride ions flow into the neuron, leading to hyperpolarization of the cell membrane and inhibition of cell firing. GABA<sub>A</sub>R are synaptically and extrasynaptically localized and molecularly very heterogeneous via variable subunit composition (Fig. 8.4). This heterogeneity determines the physiological/pharmacological properties [90, 95]. The various subunits ( $\alpha_{1-6}$ ;  $\beta_{1-3}$ ;  $\gamma_{1-3}$ ;  $\delta$ ,  $\epsilon$ ,  $\theta$ , and  $\rho_{1-3}$ ) make up, although not randomly, the GABA<sub>A</sub>R. Most GABA<sub>A</sub>Rs are composed of two  $\alpha$ , two  $\beta$ , and one  $\gamma$  subunit. GABA binds to the GABA site, which is formed by  $\alpha$  and  $\beta$  subunits, and the BDZ binding site by one of the  $\alpha$  subunits ( $\alpha_{1,2,3, \text{ or } 5}$ ) and a  $\gamma_2$  subunit. Circa 60%

**Fig. 8.4** Schematic picture of the GABA<sub>A</sub> receptor. A shows a global picture of the 2  $\alpha$ , the 2  $\beta$ , and the  $\gamma$  subunits and the direction of the chloride influx. Fig. 1B shows a top view with the subunits arranged around a central pore, the chloride channel. The GABA and BDZ binding sites are indicated. (Figure adapted from Vinkers and Olivier [104])



of GABA<sub>A</sub>Rs are of the  $\alpha_1\beta_2\gamma_2$  subtype, 15–20% of  $\alpha_2\beta_3\gamma_2$ , 10–15% of  $\alpha_3\beta_n\gamma_2$ ; the rest has variable subunit composition [96].

GABA<sub>A</sub>R subtype selective compounds and mouse models with subunit point mutations have generated promising insight in the contribution of the different subunits in the different clinical effects of BDZ [90]. BDZ do not open the chloride channel in absence of the endogenous ligand GABA. Different pharmacological agents act at different GABA<sub>A</sub>R sites, including GABA itself and various GABA<sub>A</sub>R agonists (e.g., muscimol) or antagonists (e.g., bicuculline). Classical BDZ (e.g., chlordiazepoxide and diazepam) binds to the GABA<sub>A</sub>-BDZ modulatory site. Other drug classes, including alcohol, barbiturates, and neurosteroids, also bind to the GABA<sub>A</sub>R. The allosteric binding site for BDZ is always formed by two  $\alpha$ -subunits ( $\alpha_{1, 2, 3, \text{ or } 5}$ ), two  $\beta$ -subunits, and the  $\gamma_2$  subunit. Only if the GABA receptor is activated, stimulation of the BDZ site modulates the channel opening (frequency and/or time). Ligands at the BDZ binding site are allosteric modulators and modify the efficacy and/or the affinity of GABA in a positive way (positive allosteric modification-PAM), negative way (NAM), or not (neutral), by stabilizing different three-dimensional conformations of the GABA<sub>A</sub>R complex. The potency of a certain ligand is determined by its specificity for a certain receptor subtype and affinity/efficacy modulation [97]. BDZ have anxiolytic, sedative, hypnotic, muscle-relaxant, and anticonvulsant properties. If anxiolysis is the primary indication, sedative-hypnotic properties are unwanted. Classic BDZ are nonselectively activating  $\alpha_{1,2,3, \text{ and } 5}$  subunits and bring automatically these unwanted effects. Extensive research, mainly in genetically engineered mice where certain GABA<sub>A</sub>R $\alpha$  subunits (GABRA) were made insensitive to diazepam binding, leaving the GABA site intact, illustrated that different subunits represent different functions. The broad therapeutic (and side) effects of BDZ are due to activation of  $\alpha$ -subunits [90, 98]. Extensive preclinical work implicated  $\alpha_2$  and  $\alpha_3$  subunits in the modulation of anxiety, but intensive efforts (partial agonists, inverse agonists) have not yielded new and highly specific anxiolytics. Moreover, the main negative effects of all GABA<sub>A</sub>R agonistic ligands (PAMs), i.e., tolerance and addiction, are still present in these new ligands [99], and none of these drugs has advanced to the market. A big outstanding question is whether activation of all  $\alpha$ -subunits leads to tolerance/addiction or activation of only one specific subunit [100]. Also the once promising development of the so-called Z-drugs (zolpidem, zaleplon, and zopiclone) did not yield anxiolytics without tolerance/dependence problems. Interestingly, pregabalin, a structural analogue of GABA, is anxiolytic, although via a non-BDZ-like mechanism. Pregabalin is approved in 2007 for anxiety disorders in the EU, but not in the USA [101]. There is some evidence that activation of  $\alpha_1$ -subunits is essential in the addictive potential of BDZ ligands [102, 103], but processes underlying tolerance development are complex and endpoint-dependent [104]. All these failures have probably been a major reason why pharmaceutical companies have left the development of innovative drugs for anxiety disorders that target the GABA<sub>A</sub>R-BDZ complex.

## Other Mechanisms and Anxiety

Although only preclinically investigated, many pharmacological mechanisms seem to be involved in the modulation of anxiety and fear, and intense efforts have tried to find new mechanisms outside the GABA<sub>A</sub>R-BDZ complex and the serotonergic system; no new clinically effective drugs have emerged thus far. The situation since 2012 (described by Griebel and Holmes [6]) has not changed. Still most preclinical drug discovery is focused on neuropeptides and glutamate targets. Preclinically, CRF<sub>1</sub>R antagonists, CCK<sub>2</sub>R antagonists, and neurokinin<sub>1,2</sub>R antagonists have anxiolytic-like activity, but no clinically successful drugs have emerged [6]. Several findings strongly implicated the glutamatergic system in anxiety and anxiety disorders [105]. Glutamate neurotransmission is complex due to the large number of signaling receptors and the wide and diverse distribution in the CNS. Metabotropic glutamate receptors (mGluRs) play a role in the modulation of anxiety in preclinical studies. Various ligands for mGluR subtype receptors have been tested clinically but failed to reach the market. Ketamine was recently found to have anxiolytic activity in social anxiety disorder [106] and refractory anxiety disorder patients [107] but also in animal anxiety models [108]. Ketamine, an NMDA (N-methyl-D-aspartate) R antagonist, exerts a rapid onset of antidepressant action in depressed patients and is particularly effective in anxious depression [109]. Most clinical studies utilize intravenous ketamine administration, which is a severe limitation for therapeutic use. Moreover, ketamine has serious side effects and abuse potential, which limits its widespread use as rapid onset therapeutic [110]. Further research into the efficacy of one of the enantiomers of ketamine (R,S) or one of its metabolites (e.g., 2R,6R)-hydroxynorketamine [111, 112]) may lead to a new, fast-acting anxiolytic (and antidepressant) treatment.

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## Conclusions

Discovery and development of innovative anxiolytics is severely hampering. Existing anxiolytics are developed decades ago and are still the therapeutics of choice. Moreover, the lack of new effective drug targets is not only disappointing but does indicate how the future treatment of the enormous population of CNS-disorder patients is in jeopardy. The reasons for this lack of progress are manifold and are, besides, e.g., regulatory, financial, and marketing reasons, also due to our limited knowledge of the mechanisms and pathophysiology of the various anxiety disorders. We simply lack the knowledge on what is wrong in the brains of anxious people (normal and disordered). It is also not clear yet whether DSM5-based anxiety disorder classification is justifying the underlying disturbed processes in the brain. Whether the new classification of psychiatric diseases, the Research Domain Criteria (RDoC) project, as part of the 2008 NIMH strategic plan's call for new ways of classifying mental illnesses—based on dimensions of observable behavior and neurobiological measures—will be helpful in the research process has to be awaited. Translational research, with exchanging theories and data coming from

clinical and preclinical research, is extremely urgent, because intrusive investigations of the human brain are impossible. In this endeavor, genetic and genomic approaches are part of the spectrum of contributing factors. The initial idea of identifying individual genes causing the disease very soon appeared an illusion. It becomes increasingly clear that anxiety disorders, probably similar to the fundamental mechanisms involved in anxiety and fear processes in the brain, are caused by the (inter)actions of hundreds of genes including environmental factors (epigenesis) and gene X gene interactions. In the present contribution, main focus has been on three druggable targets, 5-HTT, 5-HT<sub>1A</sub> receptor, and GABA<sub>A</sub> receptor, whereas other approaches have been summarized. Although the therapeutics, acting via these targets were found before the mechanisms involved were unraveled, it is still uncertain whether or how these targets are involved in normal and diseased anxiety processes. In case of the serotonergic anxiolytics, the slow onset of action points to indirect effects leading to plasticity changes in certain systems in the brain that finally lead to reduced anxiety. In case of the GABA<sub>A</sub> receptors, a direct anxiolytic effect is found which indicates primary mechanisms directly influencing anxiety processes. Close translational collaboration between fundamental academic and discovery research might lead to badly needed breakthroughs in the development of drugs, not only for anxiety disorders but also other brain diseases.

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