Targeting the Modulation of Neural Circuitry for the Treatment of Anxiety Disorders

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Abstract—Anxiety disorders are a major public health concern. Here, we examine the familiar area of anxiolysis in the context of a systems-level understanding that will hopefully lead to revealing an underlying pharmacological connectome. The introduction of benzodiazepines nearly half a century ago markedly improved the treatment of anxiety disorders. These agents reduce anxiety rapidly by allosterically enhancing the postsynaptic actions of GABA at inhibitory type A GABA receptors but side effects limit their use in chronic anxiety disorders. The introduction of benzodiazepines nearly half a century ago markedly improved the treatment of anxiety disorders. These agents reduce anxiety rapidly by allosterically enhancing the postsynaptic actions of GABA at inhibitory type A GABA receptors but side effects limit their use in chronic anxiety disorders. Selective serotonin reuptake inhibitors and serotoninnorepinephrine reuptake inhibitors have emerged as an effective first-line alternative treatment of such anxiety disorders. However, many individuals are not responsive and side effects can be limiting. Research into a relatively new class of agents known as neurosteroids has revealed novel modulatory sites and mechanisms of action that are providing insights into the pathophysiology of certain anxiety disorders, potentially bridging the gap between the GABAergic and serotonergic circuits underlying anxiety. However, translating the pharmacological activity of compounds targeted to specific receptor subtypes in rodent models of anxiety to effective therapeutics in human anxiety has not been entirely successful. Since modulating any one of several broad classes of receptor targets can produce anxiolysis, we posit that a systems-level discovery platform combined with an individualized medicine approach based on noninvasive brain imaging would substantially advance the development of more effective therapeutics.
I. Introduction

Anxiety is a recognized symptom of many psychiatric disorders, including generalized anxiety disorder (GAD), social anxiety disorder (SAD), obsessive-compulsive disorder (OCD), and post-traumatic stress disorder (PTSD). Although anxiety is a commonly reported clinical manifestation, it can be severely debilitating if left untreated. According to the Epidemiologic Catchment Area Study, the prevalence of anxiety disorders is about 7% in the adult population of the United States (Regier et al., 1990, 1998). Studies looking at the incidence of anxiety disorders indicate that about 18% of adults in the United States may seek treatment for anxiety in any given year (Kessler et al., 2005b). Women are more likely than men to be diagnosed with an anxiety disorder but how much of this sex difference is due to socioeconomic factors has not been established. That said, the need for a unified theory that will aid the development of targeted anxiolytics could not be more urgent with the emergence of PTSD as a major global public health challenge faced after more than a decade of war.

The importance of an improved approach to treating anxiety is highlighted by the inconsistent results seen with the class of drugs considered to be the contemporary first-line treatment: selective serotonin reuptake inhibitors (SSRIs) and serotonin and norepinephrine reuptake inhibitors (SNRIs). Although these agents have been shown to be beneficial for the treatment of certain anxiety disorders, not all patients achieve an adequate clinical therapeutic response. The existence of “nonresponders” indicates that SSRIs and SNRIs are not the fail-safe solution to treating anxiety clinicians have been looking for. In addition, SSRIs and SNRIs are associated with complications that can limit their use in some patients, including delays in producing the desired clinical reduction in anxiety or even potential worsening of the anxiety (Ravindran and Stein, 2010; Sertraline, 2012; Venlafaxine, 2012; Fluoxetine, 2013), not to mention the risk for the SSRI discontinuation syndrome in noncompliant patient populations. Furthermore, some SSRIs (e.g., citalopram) are not suitable for patients with heart problems (Wang and Pae, 2013).

Another class of drugs, benzodiazepine (BZD) anxiolytics, has played a central role in the pharmacologic management of anxiety disorders for about 50 years. Although not as widely prescribed as in the past, these compounds nevertheless remain an effective alternative to SSRIs. That said, the nonselective BZDs are not devoid of side effects, which can include drowsiness, ataxia, impairment of cognition, as well as the risk of dependence and withdrawal symptoms, including increased anxiety, tremors, and seizures (Noyes et al., 1988; Bennett et al., 1998; Yamawaki, 1999; Allgulander et al., 2003). The identification of subtype-selective GABA receptor–positive allosteric modulators, such as zolpidem, which have proven to be effective in the treatment of patients with insomnia while producing fewer untoward side effects than nonselective BZDs, suggested that similar drugs could be developed for anxiety disorders. Unfortunately, although subunit-prefering compounds are effective for insomnia, they are not yet proven to be any more beneficial for the management of anxiety disorders than the classic BZDs, such as diazepam (Farkas et al., 2013).

Another alternative to enhancing inhibitory neurotransmission mediated by GABA is modulating excitatory glutamatergic neurotransmission mediated by...
N-methyl-D-aspartate (NMDA) receptors. Although NMDA receptors offer a promising therapeutic target for treating anxiety disorders, the efficacy of NMDA receptor modulators developed to date also remains limited (Swanson et al., 2005; Barkus et al., 2010).

Collectively, these findings reveal a clear need for an improved understanding of the neural systems underlying anxiety as well as a more effective approach to developing anxiolytic agents. We believe that three recent developments—the use of noninvasive imaging in human subjects to identify biomarkers implicated in neurologic disorders, and a convergence of pharmacologic effects in human populations of drugs that augment GABAergic tone or serotonergic tone, combined with the development of systems-based approaches to drug discovery—offer a promising path forward. Systems-based drug discovery differs from traditional target-based discovery in that it utilizes an understanding of complex systems to search for potential therapeutic agents, as opposed to identifying agents based solely on interactions with a single target (Desbiens and Farb, 2012).

Research into neuroactive steroids recently revealed an important role for these agents in the pathophysiology of anxiety, both influencing and being influenced by the GABAergic, serotonergic, and other neural systems associated with anxiety and demonstrates both the complexity of the problem and why focusing on a specific target without regard for the entire neural network may actually be counterproductive. This review aims to consider the basic molecular mechanisms by which neurotransmission can be modulated across various neural systems implicated in anxiety disorders, as well as to parse out how best to approach the treatment of specific anxiety disorders based on the neural networks involved in each, with the ultimate goal of developing a systems-based approach to the discovery of novel anxiolytics.

Our understanding of anxiolytics starts with the recognition that at least in some cases, therapeutics appear to effectively interact with specific receptors to modulate apparently simple and conserved neural systems implicated in hypervigilance and fear in both humans and animals. A closer look at the problem of anxiety in humans reveals that complex cognitive processes such as executive function mediated by the frontal lobes also play a role in how subjects respond to environmental stimuli and that a single drug target may not be the most effective approach in the treatment of all types of anxiety. A growing body of research is now also shedding light on the multiple genetic, environmental, and social factors that interact in the development of anxiety disorders (Lépine, 2002; Stein et al., 2002, 2014; Pigott, 2003; Gelernter et al., 2004; Hettema et al., 2005; Porcelli et al., 2012). In addition, it is increasingly evident that a diverse array of neurotransmitters and their respective receptors and transporters, including those for GABA, serotonin (5-HT), and NMDA, play critical roles in the development of anxiety disorders (Swanson et al., 2005; Barkus et al., 2010; Graeff and Zangrossi, 2010). An increased understanding of the neural systems underlying a pathologic response to environmental stimuli that do not induce anxiety in healthy subjects therefore begins with the development of “connectivity maps” that in turn may lead to more individualized approaches to the pharmacological management of various anxiety disorders.

II. Types of Anxiety Disorders

A comprehensive review of anxiety disorders must begin with a definition of the problem. Anxiety per se is generally considered to be synonymous with acute nervousness, fretfulness, and apprehension. Although the words fear, anxiety, and worry can sometimes be used interchangeably, these terms generally represent distinct emotional responses that can be distinguished clinically by intensity and duration. Among healthy individuals, the magnitude of this type of emotional response is proportional to the specificity and intensity of the stimulus. The specificity of the stimulus is determined by its relevance to a particular individual based on his or her current circumstance combined with past life experience with that particular stimulus (i.e., individual sensitivity). For example, exposure to a stimulus that has previously been paired with an adverse life event will produce a different response than will exposure to a novel stimulus. Likewise, fear, fright, flight, and panic are more intense and acute reactions than trepidation, apprehension, and worry and normally occur in close chronological and physical relation to an exposure to the appropriate stimuli. For example, an individual may feel slightly nervous and fretful while waiting to meet a blind date, or may be apprehensive about his or her ability to perform a novel task in public, but fearful of encountering an armed robber while walking down a dark alley alone at night. All of these responses are typically brief and resolve spontaneously with termination of exposure to the respective stimuli.

Fear, an intense fight-or-flight response that occurs in reaction to a real or perceived threat, is a survival response that is in contrast with panic disorder, which is a fight-or-flight response in the absence of any identifiable danger, therefore appearing to be a pathologic condition. Because these symptoms occur spontaneously and without warning, persons with panic disorder will typically avoid situations that their past experience suggests can trigger the onset of an attack. Patients who have difficulty assigning a specific trigger for their attacks may become socially withdrawn.

Worry is synonymous with a more persistent concern or preoccupation with the unknown outcome of future life events. Worrying can be controlled so as to permit normal functioning in social and occupational settings. By contrast, worrying such as that which occurs among patients with GAD is associated with persistent concern...
about routine life experiences, such as job performance or personal relationships, and is severe enough to interfere with the afflicted individual's ability to concentrate and perform activities of daily living.

The clinical anxiety disorders recognized in the fifth edition of the Diagnostic and Statistical Manual of Mental Disorders (DSM-V) include the following: GAD, OCD, panic disorder, acute and chronic PTSD, and the various phobias, including agoraphobia, social phobia, and specific phobia (e.g., fear of flying) (American Psychiatric Association, 2013). Each of these recognized clinical anxiety disorders is unique with respect to the intensity and duration of the anxiety experienced as well as the type of stimuli that can induce it.

A systems-level approach to the development of anxiolytic agents starts with a recognition of the range of anxiety disorders from which patients can suffer, including GAD, SAD, panic disorder, various phobias (e.g., fear of flying), and medication-induced anxiety disorder (DSM-V; American Psychiatric Association, 2013). In addition, disorders that fall under their own category in the DSM-V, such as acute and chronic PTSD, include clinical manifestations, such as hypervigilance, exaggerated startle response, and fear as a component of their diagnostic criteria. Likewise, OCD includes fear of some dreaded event or situation among its diagnostic criteria. Each of these disorders is unique with respect to the intensity and duration of the symptoms experienced by the patient as well as the type of stimuli that can induce these. For example, persons with panic disorder experience recurrent, discrete, and typically brief periods of intense anxiety referred to as “uncued panic attacks” in the absence of any identifiable source of danger. This anxiety is associated with somatic symptoms, such as shortness of breath, chest pain, lightheadedness, and sweating. Persons with panic disorder often have persistent concerns about the causes or consequences of having a panic attack. By contrast, “cued” anxiety attacks and phobias are defined as those in which the sufferer experiences debilitating symptoms in response to a clearly defined object or situation. In the cases of phobias, exposure to the phobic stimulus produces an immediate onset of increased anxiety that is severe enough to interfere with the individual’s ability to function normally in social or occupational settings even when the person recognizes that his or her own anxiety is excessive or unreasonable.

The onset of an anxiety disorder may be insidious or may follow an identifiable life event. For example, acute stress disorder is characterized by symptoms that occur during the first month after exposure to an extremely stressful event that evokes intense fear and/or a sense of helplessness, such as being in an automobile accident or witnessing a murder. Symptoms of acute stress disorder include increased arousal, avoidance of places and events that remind one of the traumatic event, and recurrent intrusive memories or dreams about the event. Although severe enough to interfere with activities of daily living, these symptoms typically resolve spontaneously within about 4 weeks after the event. By contrast, chronic PTSD is characterized by similar symptoms that persist for more than 1 month and, in many cases, can last for years after exposure to a traumatic event. These types of anxiety disorders respond well to treatments that combine virtual reality exposure (VRE) therapy with pharmacologic interventions (Difede et al., 2014).

Unlike incident-evoked anxiety disorders, GAD is characterized by at least 6 months of persistent and excessive apprehension and worry in response to a plethora of different stimuli, including such routine life events as job performance, health of family members, or simply being on time for appointments. The onset of GAD is typically insidious and is often not associated with a readily identifiable precipitating event. Individuals with GAD can be differentiated from those with nonpathological anxiety by two main criteria: 1) they find it difficult to control their worrying, and 2) this perseverative thought process interferes with their ability to function in social and occupational settings.

The clinical manifestations of OCD are unique among the disorders that include fear and anxiety as a clinical manifestation in that the fear is about what will happen if the individual does not perform recurrent thoughts, impulses, or compulsions (e.g., repetitive hand washing) that the individual recognizes to be unreasonable and unrelated to real-life problems but nevertheless finds extremely difficult to stop. These symptoms cause distress, and thus, the affected individual may avoid situations that promote the obsession. For example, a person who obsesses about personal contamination may not leave the house to prevent placing himself in a situation where he might have to shake hands.

III. Brain-Imaging Studies: Assessment of Brain Activation in Patients with Anxiety Disorders

Although a range of clinical diagnostic criteria and assessment tools (e.g., Hamilton Anxiety Rating Scales and the Minnesota Multiphasic Personality Inventory) have historically been used in the differential diagnosis of anxiety disorders, recent research has also shown that various neuroimaging technologies can be employed to identify common and unique brain regions associated with the different disorders. Neuroimaging studies can be used to measure differences in regional cerebral blood flow (rCBF) and for identifying significant changes in binding to specific receptor subtypes and changes in uptake by transporters implicated in these disorders. Although it is premature to say that “silver bullets” targeting specific type A GABA receptor (GABA_A) subtypes or serotonin transporters (SERTs) within particular brain regions are on the horizon, it seems likely that critical advances in brain imaging and
subunit subtype analysis are improving the chances for optimizing therapeutic approaches to patients with anxiety disorders.

A. Functional Magnetic Resonance Imaging as a Method for Biomarker Identification

It is natural to wonder whether serotonergic neurons in the dorsal and median raphe nuclei that project to corticolimbic circuits may be controlled by GABAergic, serotonergic, and neurosteroid modulators. A noninvasive approach for assessing the elements of a potential anxiety connectome is to evaluate the pharmacologic effects of anxiolytics on specific brain regions by determining changes in rCBF associated with anxiety-inducing stimuli—these changes in response to different pharmacologic interventions. Blood oxygen level–dependent (BOLD) functional magnetic resonance imaging (fMRI) can also be used to differentiate between types of anxiety, such as between anxious apprehension (which involves verbal rumination and worry) and anxious arousal (which involves intense fear or panic or both) (Engels et al., 2007).

An fMRI study of healthy subjects with no history of psychiatric illness or treatment revealed that those who also tested positive for a regulatory variant (5-HTTLPR) in the SERT gene (SLC6A4), which was previously associated with reduced transcription of the transporter, had greater amygdaloid reactivity in response to presentation of fearful and angry faces independent of patient sex. These findings suggest that the amygdala can be biased for reactivity based on genetics and that this may represent a susceptibility factor for a pathologic response to stressful life experiences (Hariri et al., 2005). The observation that SSRIs promote downregulation of SERTs (Mirza et al., 2007) also implicates dysregulation of serotoninergic neurotransmission in etiology of certain anxiety disorders. This polymorphism has also been associated with reduced responsiveness to SSRI treatment (Zanardi et al., 2001).

The potential for an anxiolytic effect of 5-HT via attenuation of amygdala activation was shown in a study of healthy subjects treated with the SSRI citalopram for 7 days. Compared with control subjects, the fMRIs of individuals treated with citalopram showed a bilateral reduction in activation of the amygdala, hippocampus, and medial prefrontal cortex in response to presentation of threatening stimuli (Harmer et al., 2006).

Evaluation of fMRI responses to the presentation of pleasant or unpleasant words while monitoring left versus right hemisphere activity indicates that the left side (inferior frontal gyrus) is more active in patients with anxious apprehension, consistent with the fact that this type of anxiety is more verbally mediated. These findings may seem to be discordant with data supporting a left-sided bias in response to pleasant emotion, but positive emotional content preferentially activates different regions of the left hemisphere: the left frontal region (dorsolateral prefrontal cortex) (Engels et al., 2007).

Results from fMRI imaging studies support the clinical impression that GAD patients overreact to both pathology-specific and nonspecific cues and that treatment-induced reduction of anxiety attenuates the response to both types of cues (Hoehn-Saric et al., 2004). In a small cohort of six patients, subjects were initially studied using fMRI while each listened to verbal descriptions of a personal worry or a neutral statement before treatment with citalopram and again after 7 weeks of treatment. Reduced BOLD responses to worry statements were observed in prefrontal regions, the striatum, and insula and paralimbic regions. In addition, contrasts before and after treatment revealed reductions in the differential response that existed between worry and neutral statements. Overall reduction of the BOLD response was most prominent during neutral statements, particularly in the left hemisphere. These findings are consistent with the suggestion that frontolimbic structural connectivity underlies the preservative thoughts as well as the emotional disturbances seen in patients with GAD (Tromp et al., 2012).

An fMRI study of 21 patients (8 males and 13 females) with generalized social phobia, characterized by an exaggerated and pervasive fear and avoidance of scrutiny by others, suggested that activation of the amygdala in response to presentation of angry and fearful faces was attenuated relative to baseline after 12 weeks of treatment with the SSRI sertraline (Phan et al., 2013). All patients received a stable dose (100–150 mg/day) at the time of testing and had been titrated based on their clinical response to treatment. At testing, 14 of 21 patients were deemed to be responsive to sertraline treatment, whereas 7 were considered nonresponders. All treated patients (clinical responders and nonresponders) were included in the study but blood levels of sertraline were not measured. A post hoc analysis looking at differences between responders and nonresponders indicated that the attenuation from pretreatment to post-treatment in amygdala reactivity to fearful faces was significant in responders ($P = 0.01$) but not in nonresponders ($P = 0.22$), as was attenuation in ventromedial prefrontal cortex reactivity to angry faces (responders, $P = 0.02$; nonresponders, $P = 0.29$). Activation of the amygdala in all of the patients before and after treatment was also compared with that of 19 healthy controls (10 males and 9 females). Consistent with reports from other studies, activation of the amygdala in response to fearful faces before treatment was found to be greater in the anxiety patients than in control patients; importantly, this difference was abolished by treatment with sertraline. By contrast, activation of the ventromedial prefrontal cortex in response to angry faces before treatment was found to be significantly lower in anxiety patients than in control patients, and this difference was also abolished by sertraline treatment.
The effects of chronic paroxetine treatment (20 mg/day) versus placebo have also been measured in patients with SAD using fMRI, along with performance of several tasks including a public-speaking task, public exposition of a recorded performance task, and an emotional face-processing task (Giménez et al., 2014). Paroxetine treatment was associated with reduced activation in the insula, thalamus, and anterior cingulate cortex at rest and during performance of the public exposition of a recorded performance task. By contrast, during the emotional face-processing task, paroxetine treatment was associated with an increase in activation of the right amygdala and the insula bilaterally.

Collectively, these findings point to a role for corticolimbic circuits, including the amygdala and medial prefrontal cortex in SAD, and suggest that reductions in the level of activation in these brain regions may prove to be a useful biologic marker for the efficacy of novel anxiolytics.

B. Positron Emission Tomography: Advances and Caveats

rCBF can also be measured using positron emission tomography (PET) scans. Studies of rCBF using PET suggest that paralimbic structures together with the right inferior frontal cortex and subcortical nuclei mediate symptoms across three different anxiety disorders: OCD, simple phobia, and PTSD (Rauch et al., 1997). Oxygen 15–labeled carbon dioxide was used as a tracer to measure rCBF in 23 right-handed adult subjects (8 men and 15 women) meeting criteria for one of these three disorders and relative rCBF measured in the context of subjects' specific symptom provocation paradigms (e.g., exposure of a patient with OCD to a “contaminated” object). Overall, analysis of pooled imaging data revealed activation in the right inferior frontal cortex and right posterior medial orbitofrontal cortex, and bilateral activation in the insula, lentiform nuclei, and brainstem during symptomatic versus control conditions.

The limitations of measuring changes in rCBF as a measure of SSRI efficacy in the treatment of anxiety disorders is hampered by a deficiency of knowledge about the underlying neural circuitry, or functional connectome, that gives rise to feelings of anxiety and pharmacologically induced anxiolysis. For example, when PET techniques were used to assess the effects of SSRI treatment in patients with SAD, the SSRI-treated subjects showed a selective deactivation of the left lateral amygdala not associated with a reduction in anxiety status. By contrast, the left basomedial/basolateral and right ventrolateral regions of the amygdala were deactivated in both SSRI-treated and placebo patients who showed improvement in anxiety symptoms (Faria et al., 2012) (Fig. 1). This latter deactivation was not observed in nonresponders (i.e., subjects who showed no reduction in anxiety scores) irrespective of treatment group, suggesting that this change was mediated by a reduction in anxiety unrelated to SSRI treatment. Thus, SSRI treatment was no more effective than placebo for treating SAD, perhaps due to the difficulty of separating anxiolysis from a placebo effect in conjunction with a small sample size. Unfortunately, the subjects were not tested for the 5-HTTTLPR polymorphism in the 5-HT transporter gene (associated with reduced responsiveness to SSRI treatment; Zanardi et al., 2001), further confounding interpretation of the results.

An advantage of PET versus fMRI as a clinical research tool can be exemplified by a study that observed decreased flumazenil binding in specific brain regions in medication-free persons diagnosed with panic disorder (Malizia et al., 1998). Global reduction in BZD site binding throughout the brain were found in patients with panic disorder (n = 7) compared with controls (n = 8) (Fig. 2A). In addition, several loci thought to be essential in the regulation of anxiety (right orbitofrontal cortex and right insula) were found to have the largest regional decrease in binding. These results are consistent with previous clinical psychopharmacologic evidence of involvement of the GABA<sub>A</sub>R (Hasler et al., 2008) using PET with [<sup>11</sup>C]flumazenil in patients with panic disorder compared with healthy controls, which showed reduced binding potential across regions of the frontal, temporal, and parietal cortices, particularly the dorsal anterolateral prefrontal cortex.

Dynamic three-dimensional PET scans show that the BZD-GABA<sub>A</sub>R may play a role in the pathophysiology of PTSD, a finding that is consistent with previous animal and clinical pharmacological studies (Geuze et al., 2008). In this study, radiolabeled [<sup>11</sup>C]flumazenil was
used as a tracer in nine drug-naïve male veterans with deployment-related PTSD and seven male veterans without PTSD. After a scan of 60 minutes, reduced [11C]flumazenil binding was observed throughout the cortex, hippocampus, and thalamus of the PTSD patients (Fig. 2B). PET techniques have also proven valuable in evaluating variations in the distribution of specific subtypes of the GABAAR within the human brain and the roles of these subunits in mediating different functions. Competition studies in vivo in the rat revealed that [11C]Ro 15-4513 (ethyl-8-azido-5,6-dihydro-5-methyl-6-oxo-4H-imidazo[1,5-a]benzodiazepine-3-carboxylate) uptake was reduced to nonspecific levels only by drugs that have affinity for the $\alpha_5$ subtype, such as flunitrazepam, RY80 (ethyl-8-acetylene-5,6-dihydro-5-methyl-6-oxo-4H-imidazo[1,5-a][1,4]benzodiazepine-3-carboxylate, [ethyl-3H]), Ro 15-4513, and L655,708 [ethyl(13aS)-7-methoxy-9-oxo-11,12,13,13a-tetrahydro-9H-imidazo[1,5-a]pyrrolo[2,1-c][1,4]benzodiazepine-1-carboxylate], but not by the $\alpha_1$-selective agonist zolpidem. [11C]Ro 15-4513 uptake was relatively greater in limbic areas compared with [11C]flumazenil, but lower in the occipital cortex and cerebellum (Lingford-Hughes et al., 2002). The authors concluded that [11C]Ro 15-4513 PET labels the GABA$_A$R containing the $\alpha_5$ subunit in vivo in limbic structures and can be used to further explore the functional role of this subunit in humans. This technique was used to characterize BZD recognition site occupancy in the human brain associated with administration of several $\alpha_2$-selective compounds, such as TPA023B [7-((1,1-dimethylylethyl)-6-(2-ethyl-2H-1,2,4-triazol-3-ylmethoxy)-3-(2-fluorophenyl)-1,2,4-triazolo[4,3-b]pyridazine] and MRK-409 [7-cyclobutyl-6-(2-methyl-2H-1,2,4-triazol-3-ylmethoxy)-3-(2,6-difluorophenyl)-1,2,4-triazolo[4,3-b]pyridazine] (Fig. 3) (Atack et al., 2006b, 2011a,b; Van Laere et al., 2008).

C. Single Photon Emission Computed Tomography

A single photon emission computed tomography (SPECT) study using $^{123}$I-labeled 2b-carbomethoxy-3b-(4-iodophenyl)-N-(3-fluoropropyl)nortropane revealed an increase in dopamine transporter binding in the striatum of SAD subjects treated with escitalopram for 12 weeks (Warwick et al., 2012). These subjects also showed a decrease in anxiety scale scores associated with treatment, but the scores were not correlated with changes in dopamine transporter binding. The scores remained relatively high after therapy, suggesting that treatment may not have been completely successful for all participants. These subjects were also not stratified as responders versus nonresponders, thus limiting the interpretation of the data.

As revealed by SPECT scans using $^{123}$Iiomazenil, patients with panic disorder exhibit decreased benzodiazepine receptor (BZDR) binding in the left hippocampus and precuneus relative to controls (Bremner et al., 2000). Further comparisons of patients with panic disorder who either did or did not have a panic attack at the time of the scan revealed a decrease in BZDR binding in the prefrontal cortex among patients who had a panic attack. These findings suggest that BZDR function in the prefrontal cortex appears to be involved in changes in state-related panic anxiety.

These findings also suggest a role for brain regions implicated in episodic memory function, such as the precuneus. This in turn may be amenable to cognitive behavioral therapy (CBT) and targeting by pharmacologic...
interventions that can enhance extinction of salient memories that trigger panic attacks. The results indicate that the neuronal circuits implicated in panic attacks (i.e., the precuneus) are distinctly different from those implicated in GAD (i.e., more involvement of the frontal lobe).

In another study of depressed patients with or without panic disorder ($N = 18$), radiolabeled iomazenil SPECT scans demonstrated that those patients with panic disorder had a significant decrease ($P < 0.05$) in the regional activity index in the lateral inferior temporal lobes (right and left), medial inferior temporal lobes (left), and inferior frontal lobes (right and left) after 2 hours (Kaschka et al., 1995). The findings may be due to either regional blood flow differences or BZDR effects, but the former hypothesis is confirmed to some extent by similar findings in the scans acquired after 10 minutes. Only hypoactivity in the left lateral temporal region seemed to be independent of the reduced blood flow seen in panic disorders.

A SPECT study in patients with social phobia found that citalopram therapy decreased activity in the left cingulum, the anterior and lateral part of the left temporal cortex, and the anterior, lateral, and posterior part of the left midfrontal cortex (Van der Linden et al., 2000). This broad engagement of cortical areas in anxiety disorders and anxiolysis may be the consequence of a primary abnormality or may be a cause of the disorders.

In some ways, similar to the phenomenon of “kinetic ambiguity” common to the study of enzyme mechanisms and bio-organic chemistry, “systems ambiguity” presents a conundrum for the pharmacologist seeking to translate target-based pharmacology into pharmacotherapeutic effects based on a mechanistic understanding of an anxiolysis connectome.

D. Magnetic Resonance Spectroscopy

Magnetic resonance spectroscopy (MRS) allows for the noninvasive monitoring of levels of GABA as well as other neurochemicals in the human brain (Goddard et al., 2001; Strawn et al., 2013; Zwanzger et al., 2013). MRS has been used to measure GABA levels in patients with PTSD, social anxiety, and panic disorder (Ham et al., 2007; Pollack et al., 2008; Rosso et al., 2014). A study of 14 unmedicated patients with panic disorder and 14 healthy control subjects matched for age and sex revealed a 22% mean reduction of GABA in the occipital lobe of 12 of 14 subjects compared with controls. There were, however, no significant correlations between GABA levels in the occipital lobe and any measures of illness or state anxiety (Goddard et al., 2004). In a study of subjects with PTSD, MRS measurement of GABA levels in the insula revealed that patients had 30% less GABA than healthy controls. This finding could not be accounted for by age or sex. This
difference could also not be accounted for by the difference in the percent gray matter versus white matter proportions within the voxel of interest. In addition there was a significant negative correlation between State-Trait Anxiety Inventory scores and GABA levels (Rosso et al., 2014) (Fig. 4).

MRS can also be used to study acute drug-induced changes in brain glutamate levels. Experimental pharmacologic induction of panic has been used to elucidate the neurobiological correlates of panic attacks. Cholecystokinin-tetrapeptide-4–induced panic attack was studied using MRS and was found to be associated with an increase in glutamate plus glutamine/creatine ratios in the anterior cingulate cortex (Zwanzger et al., 2013). This suggests that a chemically induced (i.e., by cholecystokinin-tetrapeptide-4) panic attack alters the glutamatergic system in the anterior cingulate cortex and may be involved in the pathogenesis of panic attack. Given the activation of precuneus in salient episodic memory–induced panic attack, it is tempting to speculate that a targetable downstream network involving the anterior cingulate cortex susceptible to glutamatergic pharmacotherapy could be an effective approach toward treating panic attack.

By contrast, in patients with PTSD, positive modulators of glutamatergic transmission (e.g., d-cycloserine and acamprosate; see section V.B) have been used in combination with behavioral therapy to extinguish salient memories associated with PTSD.

E. Overview

Taken as a whole, brain-imaging studies implicate the amygdala, prefrontal, insular, limbic, paralimbic, and occipital cortices as well as the basal ganglia more often than other regions in pathologic anxiety. Given the role that the insula plays in integrating sensory, emotional, and cognitive information, it is certainly not surprising to find changes in the level of activation in this brain region. Given that the insula spans from the prefrontal cortex to the posterior parietotemporal lobes and thereby creates extensive connections between the lateral prefrontal cortex, ventromedial prefrontal cortex, orbitofrontal cortex, cingulate, amygdala, bed nucleus of the stria terminalis (BNST), and ventral striatum, it seems reasonable to think of the insula as a node in the anxiety connectome.

These neuroimaging findings also reveal some evidence of lateralization and localization based on how the anxiety-inducing stimulus is presented (e.g., verbally versus visually), suggesting a path forward for differentiating among types of anxiety and tailoring treatment
appropriately based on systems-level analysis of data. For example, a meta-analysis of neuroimaging studies related to emotional processing in SAD, PTSD, and specific phobia suggests that common brain regions (hyperactivation of the amygdala and insula) play a role in these anxiety disorders that can be triggered by specific stimuli. In addition, the results revealed that hypoactivation in the dorsal and rostral anterior cingulate cortices and the ventromedial prefrontal cortex may play a role in the dysregulation of emotions associated with PTSD (Etkin and Wager, 2007).

Human imaging studies in which both PET and fMRI are used to study biomarkers in the same cohort may be the most suitable for identifying relationships between individual variability in receptor binding and brain region activation in response to treatments (although studies employing one technology are also useful). This dual-technology approach was used by Fisher et al. (2006) to study 5-HT$_{1A}$ autoreceptor binding in relation to amygdala activation and by Rhodes et al. (2007) to study the association between 5-HT transporter binding and amygdala activation. Kobiella et al. (2011) took this approach one step further and studied 5-HT transporter polymorphisms in relation to amygdala region activation, further demonstrating the unique power of multimodal neuroimaging studies.

Nonhuman animal imaging using experimental models of anxiety are also helping to parse out inferences concerning the possible differences and similarities underlying various anxiety disorders. One MRS study using a chronic stress–induced depression model revealed a reduction in brain levels of N-acetyl-aspartate, total creatine, and choline-containing compounds in tree shrews exposed to psychosocial stress. In this study, the voxel of interest was the forebrain including parasagittal neocortex, subjacent white matter, and portions of subcortical forebrain structures, including striatum (Czéh et al., 2001). Postmortem analysis showed reduced cell proliferation in the dentate gyrus and a decreased GluR2 expression in the prefrontal cortex. Concomitant administration of a novel compound combining partial dopamine D2 receptor agonism with SSRI activity prevented these changes, suggesting that combining dopamine D2 receptor agonism with SSRI activity may serve as a novel treatment of those anxiety disorders presenting with comorbid depression (Michael-Titus et al., 2008). In another study using fluorodeoxyglucose-PET, three specific characteristics of the anxious phenotype identified in rhesus monkey (hypothalamic-pituitary-adrenal activity, freezing behavior, and expressive vocalizations) were associated with a common elevation of activity in the central nucleus of the amygdala and the anterior hippocampus (Shackman et al., 2013).

However, can these types of imaging data produce useful information for real-life therapy in human patients with a range of anxiety disorders? In patients with SAD, fMRI was used to measure brain activation prior to intervention with CBT and showed that changes in regional activation in response to angry versus neutral faces or emotional versus neutral scenes are an effective predictor of response to CBT (Doehrmann et al., 2013).

IV. Neural Systems and the Neurochemical Basis of Anxiety

In the following sections, we explore the neurochemical components of the brain, placing these findings in the context of a systems-level approach to therapeutics based on the real-time differences in rCBF demonstrated by functional neuroimaging studies and changes in brain levels of GABA and glutamate revealed by MRS.

A. Neural Systems Underlying Anxiety

1. A Connectome Hypothesis. Anxiety is a complex emotional response that involves multiple neurotransmitters acting at various receptors within the limbic system (i.e., hippocampus, parahippocampal gyrus, cingulate gyrus, hypothalamus, and amygdala) and within the paralimbic regions of the cerebral cortex such as the orbital and medial prefrontal cortex (Rauch et al., 1997; Simpson et al., 2000; Pape and Stork, 2003; Phillips et al., 2003; Sah et al., 2003; Yilmazer-Hanke et al., 2003; Zwanzger et al., 2003; Kang-Park et al., 2004; Phan et al., 2004). From a neural systems perspective, fear and anxiety seem to be controlled by two major circuits: those involved in our most primitive innate responses to simple and overt threatening stimuli, and those involved in moderating responses to more complex situations. The prefrontal cortex appears to be the brain region most often implicated in the latter.

The prefrontal cortex in particular is implicated in attenuation of responses to anxiety-inducing stimuli. Lesions to the ventrolateral prefrontal cortex have been associated with increased anxiety in nonhuman primates (Agustin-Pavón et al., 2012). It has been proposed that the amygdala is hyper-responsive to threatening stimuli, whereas the response of the ventromedial prefrontal cortex is a blunted in patients with anxiety disorders, resulting in inadequate regulation of their responses to anxiety-inducing stimuli (Kent and Rauch, 2003; Rauch et al., 2003). This hypothesis is supported in part by functional neuroimaging studies showing that individuals with GAD have an attenuated discriminatory response pattern in the ventromedial prefrontal cortex when presented with threatening versus nonthreatening stimuli (Greenberg et al., 2013).

The amygdala, which receives afferent inputs from the sensory cortex and sends its efferent projections to the hypothalamus, periaqueductal gray matter, locus ceruleus, raphe nuclei, and ventral tegmental area (Sah et al., 2003), appears to be primarily involved in the encoding and consolidation of emotionally charged memories (Isenberg et al., 1999; Roozendaal et al., 2004). Nonhuman animal imaging using experimental models of anxiety are also helping to parse out inferences concerning the possible differences and similarities underlying various anxiety disorders. One MRS study using a chronic stress–induced depression model revealed a reduction in brain levels of N-acetyl-aspartate, total creatine, and choline-containing compounds in tree shrews exposed to psychosocial stress. In this study, the voxel of interest was the forebrain including parasagittal neocortex, subjacent white matter, and portions of subcortical forebrain structures, including striatum (Czéh et al., 2001). Postmortem analysis showed reduced cell proliferation in the dentate gyrus and a decreased GluR2 expression in the prefrontal cortex. Concomitant administration of a novel compound combining partial dopamine D2 receptor agonism with SSRI activity prevented these changes, suggesting that combining dopamine D2 receptor agonism with SSRI activity may serve as a novel treatment of those anxiety disorders presenting with comorbid depression (Michael-Titus et al., 2008). In another study using fluorodeoxyglucose-PET, three specific characteristics of the anxious phenotype identified in rhesus monkey (hypothalamic-pituitary-adrenal activity, freezing behavior, and expressive vocalizations) were associated with a common elevation of activity in the central nucleus of the amygdala and the anterior hippocampus (Shackman et al., 2013).

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The basolateral amygdala (BLA), which is composed of the lateral, basomedial, and basolateral nuclei, plays a central role in this process and sends projections to the medial temporal lobe and nucleus accumbens and septal nuclei via the stria terminalis (Roozendaal et al., 2001; Pelletier and Paré, 2004). Lesions of the BLA or its major efferent pathway, the stria terminalis, interfere with the encoding and consolidation of memories induced by emotional arousal (McGaugh, 2000; Roozendaal et al., 2001). In addition, the effects of glucocorticoids such as RU 28362 (11β,17β-dihydroxy-6,21-dimethyl-17α-pregna-4,6,10-trien-20yn-3-one) on memory consolidation are dependent on an intact BLA nucleus and its efferent fibers that run through the stria terminalis (Roozendaal et al., 2001).

The subunit composition and pharmacological properties of GABAA receptors vary widely between and within brain regions. For example, receptors containing the α2 and α5 subunits, which show pharmacologic profiles similar to the classic BZD II or type 2 receptors, are found in higher concentrations in the amygdala and hippocampus, whereas receptors containing α6 subunits, which are insensitive to BZD anxiolytics, are localized to the cerebellum (Wieland et al., 1992; McKernan and Whiting, 1996; Whiting et al., 1999).

The neuroanatomic distribution of 5-HT receptors is complex, as would be expected based on the pharmacological effects of receptor agonists, antagonists, and SSRIs. The limbic system is critical for the generation of motivational and affective states, with the limbic forebrain containing high levels of 5-HT (an evolutionarily ancient neurotransmitter that has functional ortholog receptors even in the Drosophila fruit fly) (Nichols et al., 2002). The 5-HT fibers that project into the forebrain originate for the most part from the median raphe nucleus and the dorsal raphe nucleus. These projections are heterogeneous in terms of morphology and electrophysiology, representing yet another level of intricacy that could influence treatment outcomes (Hensler, 2006).

The hippocampus is well known to be involved in the formation of new memories. Neurogenesis in the hippocampal dentate gyrus has been implicated in the formation of both spatial memory and anxiety disorders, as well as in the action of SSRIs. The temporal behavior of dentate gyrus granule cells with respect to theta rhythm activity is different between rats with normal and impaired levels of neurogenesis, suggesting that immature neurons could distinctly affect the temporal dynamics of hippocampal encoding (Rangel et al., 2013). Serotonergic antidepressants can reverse the established state of neuronal maturation in adult hippocampal cells, and upregulation of 5-HT1 receptor-mediated signaling may play a role in this process (Kobayashi et al., 2010). The role of dentate granule cell hypoactivity in anxiety and in the mechanism of action of SSRIs was recently suggested based on the observation that challenge-induced hypoactivation of dentate gyrus neurons in mice with high trait anxiety is normalized by fluoxetine treatment (Sah et al., 2012).

Differences in the intrinsic physiology and synaptic inhibition between the mature and immature cell populations could separate the activity of differently aged neurons in a temporal coding regimen, influencing downstream CA3 neuron integration of information conveyed by young and mature granule cells. In line with this hypothesis, fluoxetine treatment induces active somatic membrane properties in a manner reflective of immature granule cells (Kobayashi et al., 2010). These changes do not seem to be explained by an increase in newly minted immature neurons and may be characterized as “dematuration” of mature granule cells. These observations suggest that normalization of hippocampal hypoactivity may serve as a neurobiological marker of anxiolytic effectiveness and successful remission.

2. Optogenetics and Electrophysiology. Optogenetic techniques have been used to further elucidate the role of the amygdala in anxiety. Tye et al. (2011) used viral transfections to study the role of BLA–centrolateral nucleus pathway in the expression of anxiety. Illumination of channel rhodopsins expressed in BLA terminals located at the central nucleus of the amygdala was shown to induce an acute reversible anxiolytic effect. Selective illumination of specific BLA terminals produces a behavioral response opposite that induced by activation of all glutamatergic BLA terminals suggesting that multiple subpopulations or projections of BLA neurons can act in opposition (e.g., direct excitation of centromedial nucleus along with feedforward inhibition of centromedial nucleus) (Fig. 5).

Subsequent studies demonstrated that bilateral optogenetic inhibition of BLA axon terminals in the ventral hippocampus reduces anxiety-related behaviors in mice, suggesting that BLA inputs to the ventral hippocampus may play a role in modulating basal levels of anxiety (Felix-Ortiz et al., 2013). A reduction of hippocampal theta activity has also been implicated in the anxiolysis induced by various classes of anxiolytics, including buspirone (McNaughton et al., 2007; Chee et al., 2014). A reduction of hippocampal theta activity has also been implicated in the anxiolysis induced by various classes of anxiolytics, including buspirone (McNaughton et al., 2007; Chee et al., 2014). McNaughton et al. (2013) proposed that conflict-specific, right frontal activation recorded noninvasively with surface electrodes is a readily obtainable noninvasive human functional homolog of rodent hippocampal-cortical activity that may also be useful as an objective biologic marker of anxiolytic efficacy.

An optogenetics approach has also been used to elucidate the role of the dentate gyrus in the processes of learning and memory, as well as the pathophysiology of anxiety (Kheirbek et al., 2013). Specifically, granule cells in the ventral dentate gyrus suppress innate anxiety but do not affect contextual learning,
whereas those in the dorsal region control the encoding of contextual fear memories but not their retrieval. As noted, findings from this study suggest that disturbances in pattern separation may underlie anxiety disorders in some patients, and therapies targeting the excitability of the ventral dentate gyrus may offer a beneficial approach in this population. These kinds of techniques have also borne fruit in animal models as well, potentially allowing for translation of data from animal models to studies in humans. The connection between hippocampal-mediated learning and anxiety was further elucidated using optogenetic techniques, showing that hippocampal dentate gyrus neurons activated during fear learning in mice can be reactivated later using light stimulation, producing an increased freezing response outside of the original context (Liu et al., 2012).

Optogenetics combined with in vivo electrophysiological recordings may further elucidate activity in the neural networks implicated in anxiety disorders. Optogenetic inhibition of the BNST in mice increased exploration of open spaces in the elevated plus maze test and open field test, whereas stimulation of the BNST with the excitatory channelrhodopsin-2 was associated with increased anxiety in both tests. To elucidate the neural circuitry between the BLA and the anterodorsal BNST, microdrive electrode arrays containing stereotrodes surrounding a fiber-optic were implanted in the anterodorsal BNST of mice expressing channelrhodopsin-2 in the BLA. Excitation of the glutamatergic BLA terminals was associated with increased activity of anterodorsal BNST neurons. Conversely, optically stimulating inputs to the oval nucleus of the BNST induced a net inhibition of neural activity in the BLA, suggesting that the oval nucleus and anterodorsal BNST play antagonistic roles in modulating anxiety (Kim et al., 2013). These findings are consistent with observations that the extended amygdala plays an important role in the neural circuitry implicated in anxiety.

The BNST occupies a central position in the neural circuitry regulating the HPA axis response to stress and thus plays an important role in regulating the neuroendocrine and behavioral responses to stress (Henke, 1984; Dunn, 1987; Fernandes et al., 2002; Walker et al., 2009). The release of neuroactive steroids in this brain region may represent an endogenous homeostatic mechanism for modulating anxiety and restoring normal GABAergic neurotransmission and HPA axis activity after exposure to stress-inducing stimuli (Patchev et al., 1994; Barbaccia et al., 1996; Fernandes et al., 2002). Stressors that activate the BNST also activate serotonergic systems, and changes in 5-HT receptor expression in this region were suggested to play a role in pathologic stress responses to stress (Guo et al., 2009; Hazra et al., 2012). Thus, modulation of the activity of specific 5-HT receptor subtypes in this region may be a novel approach to treating anxiety disorders.

### B. Type A GABA Receptor and Serotonin Receptor Structure/Roles in Anxiety

A disturbance of the nuanced integration of excitatory and inhibitory transmission can occur on a local network level that ultimately affects interconnected domains of larger-order structures linking to the outside world. Unraveling this will undoubtedly involve the action of networks serving as low-pass as well as high-pass filters, all of which are based on the integrated action of multiple transmitters at their respective receptors.
1. GABA. There is general agreement that GABA-mediated inhibitory neurotransmission plays a particularly important role in the modulation of emotional responses to fear-inducing stimuli (Davis and Myers, 2002; Stork et al., 2002, 2003; Walker et al., 2003), and the concept of positive and negative allosteric modulation of fast postsynaptic GABAergic inhibitory neurotransmission by BZDs was central to anxiolytic pharmacology for decades (Choi et al., 1977; Macdonald and Barker, 1978; Chan and Farb, 1985). GABAARs are located primarily in the postsynaptic membrane at synaptic and extrasynaptic sites and mediate the majority of fast postsynaptic inhibitory neurotransmission in the adult central nervous system (CNS) (Rabow et al., 1995; Barnard et al., 1998; Ben-Ari, 2002). The action of GABA on GABAARs can modulate both the frequency and duration of chloride channel openings to maintain the membrane near its resting voltage (Macdonald et al., 1989; Twyman et al., 1989).

The GABAAR is a member of the cys-loop superfamily of receptors, which includes the nicotinic acetylcholine receptors, the glycine receptors, and the 5-HT3 receptors. These receptors all have a characteristic loop formed by a disulfide bond between two cysteine residues (Sieghart and Sperk, 2002). The GABAAR is a pentameric complex, which can be composed from the members of eight recognized families of glycoprotein subunits (α, β, γ, δ, ε, π, θ, and ρ) (Fig. 6). Several of these subunit families are known to include multiple isoforms (e.g., α1–α6 and γ1–γ3), giving rise to at least 19 distinct subunits that, when combined, permit the assembly of a large number of receptor subtypes with variable affinities for GABAAR modulators (Barnard et al., 1998; Smith et al., 2001). Each of these receptor subunits has an extracellular domain, which serves as a ligand-binding site, and four transmembrane domains, which are involved in chloride ion channel structure (Fig. 7).

The majority of GABAARs are composed of two α subunits, two β subunits, and a single γ subunit (Pritchett and Seeburg, 1991; Chang et al., 1996; Graham et al., 1996; Tretter et al., 1997; Wingrove et al., 2002), with the GABA binding site located on the αβ subunit (Casalotti et al., 1986). Residues that contribute to the GABA binding site are F64 on the α subunit and Y157, T160, and Y205 located on the β subunit (Amin and Weiss, 1993; Amin et al., 1997; Baur and Sigel, 2003). Substitution of any of these four amino acids decreases the sensitivity of the GABAAR to activation by GABA and muscimol. However, these same amino acid substitutions do not alter GABAAR activation by pentobarbital, indicating that these three compounds act at distinct binding sites (Amin and Weiss, 1993). A large fraction of GABAARs have a subunit composition of α1β2γ2, and possess many of the pharmacologic properties associated with the classic BZD type 1 receptors.

Compounds that affect GABAAR-mediated responses fall into three categories: agonists, antagonists, and modulators. Agonists, such as muscimol, directly and specifically interact with the GABA recognition site on the GABAAR to induce a response. Competitive antagonists, such as bicuculline, or noncompetitive antagonists, such as picrotoxin, bind to the receptor and block GABA from activating a response via its recognition site. Allosteric modulators bind at modulatory sites and increase, decrease, or null modulate the binding of GABA to the GABAAR, thereby enhancing, inhibiting, or null modulating the ensuing response to GABA (Farb et al., 1984; Chan and Farb, 1985). Chlordiazepoxide and diazepam were the first demonstrated therapeutics to act as positive allosteric modulators at the GABAAR (Braestrup and Squires, 1977; Choi et al., 1977; Möhler and Okada, 1977; Macdonald and Barker, 1978; Gallager and Tallman, 1983; Ferrero et al., 1984). These compounds appear to impart a conformational change in the transmembrane receptor that alters its affinity for GABA and GABA agonists (Czajkowski and Farb, 1986; Lavoie and Twyman, 1996; Boileau and Czajkowski, 1999). Activation of GABAARs with the agonist muscimol also increases the affinity for the receptor of positive allosteric modulators [e.g., diazepam and the triazolopyridine CL 218872 (3-methyl-6-[3-(trifluoromethyl)phenyl]-1,2,4-triazolo[4,3-b]pyridazine)], indicating that this relationship is also reciprocal (Chiu and Rosenberg, 1979; Lippa et al., 1979; Villiger, 1984). Sensitivity to BZDs
appears to be mediated primarily by the $\gamma_2$ and $\alpha_1$, $\alpha_2$, $\alpha_3$, and $\alpha_5$ subunits (Brooks-Kayal and Pritchett, 1993; Boileau and Czajkowski, 1999).

Dissimilar compounds influence GABA$\alpha$Rs differently. For example, although BZDs and barbiturates both act on GABA$\alpha$Rs, electrophysiological studies suggest that the actual mechanism of action is different. These studies indicate that diazepam increases the duration of GABA-induced channel openings or burst by only 9.3%, but increases the number of bursts by 102.5%. In contrast, phenobarbital increases burst duration by 81.9%, but increases the number of bursts by only 6.3% (Twyman et al., 1989). Although the mechanism of receptor modulation has not been fully elucidated, BZDs appear to enhance single-channel burst frequencies by increasing the affinity of GABA for its binding site, whereas barbiturates appear to slow receptor deactivation by stabilizing the interaction between GABA and the GABA$\alpha$R (Mathers, 1985; Twyman et al., 1989; Rabow et al., 1995; Steinbach and Akk, 2001). The binding of BZDs to the allosteric BZD site increases the affinity of the receptor for GABA and enhances GABAergic neurotransmission by increasing the number of chloride channels opened at a given synaptic concentration of GABA, which in turn hyperpolarizes the cell membrane.

The $\alpha$ subunit appears to be intimately related to GABA$\alpha$R sensitivity to the prototypical BZDs and particularly to diazepam, playing an important role in drug binding and concentration response relationships (Pritchett et al., 1989; Pritchett and Seeburg, 1990; Wafford et al., 1993; Smith et al., 2001) via interacting with the $\alpha/\gamma$ interface (Buhr and Sigel, 1997). The $\gamma$ subunit contributes to receptor affinity for specific ligands as well as the single-channel characteristics (Herb et al., 1992; Günther et al., 1995; Benke et al., 1996).

GABA$\alpha$R subtypes that are modulated by BZDs can be divided into those that are diazepam sensitive and those that are not (Lo et al., 1982). BZDRs that recognize the classic 5-phenyl-1,4-BZDs (e.g., diazepam and flunitrazepam) are referred to as “diazepam-sensitive” receptors, whereas those that do not recognize these ligands are referred to as “diazepam-insensitive” receptors (Malminiemi and Korpi, 1989; Hadingham et al., 1996; Knoflach et al., 1996). It was originally thought that there were just two subtypes of BZD-sensitive GABA$\alpha$Rs. The first group, type 1 receptors that are enriched in the cerebellum and globus pallidus, display a higher affinity for the triazolopyridine CL 218872 and the $\beta$-carboline [3H]propyl-$\beta$-carboline-3-carboxylate. By contrast, type II...
receptors, which are found in the superficial layer of the superior colliculus, striatum, and the dentate gyrus of the hippocampus, show low affinity for CL 218872 and [3H]propyl-β-carboline-3-carboxylate (Braestrup and Squires, 1977, 1978; Braestrup and Nielsen, 1981; Young et al., 1981). It has since been shown that there are multiple classes of BZD Rs, reflecting differences in GABA\(_{\text{A}}\)R subunit compositions such as substitution of the \(\gamma_3\) and \(\gamma_2\) subunits.

Overall, it appears that sedation and amnesia associated with classic BZD actions are mediated primarily via \(\alpha_1\) subunit–containing GABA\(_{\text{A}}\)Rs in rodents, whereas reduction of vigilance is mediated primarily via the \(\alpha_2\) and \(\alpha_3\) subunits (Löw et al., 2000; McKerman et al., 2000; Dias et al., 2005), with activation of the \(\gamma\) subunit also playing a role in BZD efficacy (Rudolph et al., 1999). The \(\alpha_2\) subunit is largely expressed in limbic structures implicated in anxiety and fear, including the amygdala and hippocampus. Staining intensities of the \(\alpha_2\) subunit are highest in the accessory olfactory bulb, molecular layer of the dentate, hippocampal area CA3, central and lateral amygdaloid nuclei, septum, striatum, accumbens, and hypothalamus.

The lateral septum has a high concentration of \(\alpha_2\) subunits and has been shown to participate in the regulation various visceral and somatic functions as well as in modulation of anxiety, aggression, and fear responses in rodent models (Pesold and Treit, 1996; Gavioli et al., 1999). Neurons in this region are also not sensitive to zolpidem-induced enhancement of GABAergic inhibition, which has been associated with activation of receptors containing the \(\alpha_1\) subunit (Duncan et al., 1995). Cat odor exposure induces Fos expression in the posterior accessory olfactory bulb, which has also been shown to contain a high concentration of the \(\alpha_2\) subunit (McGregor et al., 2004). Cat odor induction of Fos expression is also seen in the main olfactory bulb and midazolam inhibited Fos expression in this region. Midazolam also inhibited Fos expression in key limbic regions involved in pheromone transduction (medial amygdala and BNST) and defensive behavior (prelimbic cortex, lateral septum, lateral and medial preoptic areas, and dorsal premammillary nucleus).

H101R mice in which the \(\alpha_2\) subunit was rendered insensitive to diazepam by a knock-in point mutation (His101→Arg) show reduced sensitivity to the anxiolytic effects of diazepam. This mutation was not associated with any overt phenotypic changes and the animals expressed all subunits tested (\(\alpha_1\), \(\alpha_2\), \(\alpha_3\), \(\beta_2\), and \(\gamma_2\)) at normal levels and distribution. After administration of diazepam, the \(\alpha_2\) knock-in mice did not show the increase in exploratory behavior (as measured by the amount of time spent and the number of entries into the open arms) on the elevated plus maze that is typically seen in wild-type mice. This difference in behavior could not be attributed to motor impairment because the motor activity in the enclosed arms was similar in \(\alpha_2\) H101R and wild-type mice irrespective of the treatment (Löw et al., 2000).

A subsequent study looked at the behavior of H101R mice in a conditioned emotional response task (Morris et al., 2006). In these experiments, lever pressing for food on a variable interval schedule of reinforcement was suppressed by delivery of a foot shock paired with a conditioned stimulus tone or light. During the conditioned emotional response test session, wild-type and H101R mice were pretreated with diazepam (0, 0.5, 1, and 2 mg/kg) 30 minutes prior to testing. The wild-type mice showed an increase in lever pressing during treatment with diazepam, whereas H101R mice were resistant to the anxiolytic effects of diazepam.

These observations naturally suggest that development of compounds that are selective for those receptor subtypes implicated in anxiety may prove to be extremely fruitful in the management of anxiety disorders. To date, efforts to fine-tune the binding affinity or functional efficacy of compounds that display selectivity or preference for specific receptor subtypes remain promising in rodent experimental animal systems, yet elusive in human clinical trials (Ballenger et al., 1991, 1992; Rudolph et al., 1999; Löw et al., 2000; Sandford et al., 2001; Atack, 2003; Lippa et al., 2005; Rudolph and Möhler, 2006; Atack et al., 2011a,b; Olivier et al., 2013). Additional consideration for targeting therapeutics, which take advantage of the rapid rates of GABA\(_{\text{A}}\)R turnover and the nonlysosomal pathway for degradation (Borden et al., 1984; Borden and Farb, 1988), may also yield greater selectivity and reduced adverse events. Further research directed along a neural-systems level may well resolve the drug discovery puzzle at hand if the receptor subtype and selective modulatory therapeutic can be effectively aligned for the treatment of specific anxiety disorder subtypes.

2. Serotonin. The role of 5-HT in the pathophysiology of anxiety disorders has become increasingly well established, and agents that inhibit the reuptake of this neurotransmitter now represent important components of current approaches to anxiolysis (Graeff and Zangrossi, 2010; Sertraline, 2012; Venlafaxine, 2012; Fluoxetine, 2013). 5-HT is a monoamine whose postsynaptic action is terminated via 5-HT reuptake into the presynaptic nerve terminal via a 5-HT transporter. The efficacy of SSRIs is thus based on augmenting the concentration of 5-HT in the synaptic cleft by inhibiting its clearance (Ressler and Nemeroff, 2000; Nichols and Nichols, 2008).

Effects of 5-HT are mediated by as many as 14 receptor subtypes, including 13 G protein–coupled receptors (GPCRs) and a single ligand-gated ion channel. These receptors are divided into seven distinct classes (5-HT\(_1\) to 5-HT\(_7\)) (Fig. 8); however, postgenomic modifications can produce at least 20 additional GPCRs, leaving more than 30 5-HT receptors that signal through G proteins. In addition, activation may result in variable effects, depending on the brain region in which the receptors...
are expressed and on individual differences in responsiveness mediated by genetic polymorphisms (Raymond et al., 2001; Hoyer et al., 2002; Gray and Roth, 2007).

Although it is tempting to conceptualize the actions of 5-HT at its receptor sites as being either inhibitory or excitatory, the complexity of the signal transduction pathways activated ultimately may confound such an appealing target- and hypothesis-driven approach. That said, 5-HT receptors generally have the following functions (Polter and Li, 2011): 1) 5-HT$_1$ receptors inhibit adenyl cyclase, reduce cyclic AMP, and inhibit protein kinase A (PKA); 5-HT$_1A$ receptors are the prototypical 5-HT$_1$ receptors; 2) 5-HT$_2$ receptors increase inositol trisphosphate, increase intracellular calcium levels, increase diacylglycerol, and activate PKA; 5-HT$_2A$ receptors are the prototypical type 5-HT$_2$ receptors; 3) 5-HT$_3$ receptors are ligand-gated cation channels; 4) 5-HT$_4$ receptors activate adenyl cyclase, increase cyclic AMP, and activate PKA; 5) 5-HT$_5$ receptors inhibit adenyl cyclase, reduce cyclic AMP, and inhibit PKA; and 6) 5-HT$_6$ and 5-HT$_7$ receptors activate adenyl cyclase, increase cyclic AMP, and activate PKA.

The complex distribution and functionality of these receptor subtypes contributes to their unique ability to modulate emotional responses and other bodily functions via the systems into which they feed forward. The 5-HT$_3$ receptor is a ligand-gated ion channel. The remaining subtypes are GPCRs with the latter also being defined as a type A family, rhodopsin-like receptors. Evidence suggests that most, if not all, of the 5-HT GPCRs undergo dimerization, and that dimerization may be necessary for proper functioning of the receptor (Nichols and Nichols, 2008). For example, when inactive 5-HT$_2C$ receptors are coexpressed with a wild-type 5-HT$_2C$ receptor, the inactive receptor is able to inhibit the activity of the wild-type receptor through the formation of a nonfunctional heterodimer, suggesting that dimerization is essential for receptor function (Herrick-Davis et al., 2005). Studies to date suggest that the 5-HT receptors primarily related to anxiety and mood are 5-HT$_1A$, 5-HT$_2A$, and 5-HT$_2C$ (Toth, 2003; Van Oekelen et al., 2003; Mato et al., 2010).

Interestingly, the roles of these receptor subtypes may vary depending on the form of anxiety involved, as outlined in a review by Graeff and Zangrossi (2010). The authors suggest that SSRIs improve symptoms in patients with panic disorder by enhancing the responsiveness of 5-HT$_1A$ and 5-HT$_2A$ receptors in the dorsal periaqueductal gray matter in the midbrain, but improve symptoms in patients with generalized anxiety through the desensitization of 5-HT$_2C$ receptors and, perhaps, through stimulation of 5-HT$_1A$ receptors in the forebrain.

Understanding the effects of 5-HT in anxiety disorders requires attention to variations in transporter reuptake subtypes that may potentially influence the clinical response to SSRIs. Genetic polymorphisms of the 5-HT transporter gene promoter (5-HTTLPR) have been associated with efficacy of antidepressants (Porcelli et al., 2012), as well as with anxiety. For example, a functional polymorphism of the 5'-flanking region of the 5-HT transporter gene (SLC6A4) is associated with differences in anxiety-related personality traits (Heils et al., 1996; Lesch et al., 1996). A 43-bp deletion/insertion in 5-HTTLPR is associated with long “L” (16 repeats) and short “S” (14 repeats) alleles (Heils et al., 1996). There appear to be ethnic differences in the prevalence of these polymorphisms as well, with Caucasians having about 22% S/LG alleles and Asians having about 60% S/LG alleles (Hu et al., 2006). Gene/environment interactions may therefore account for susceptibility to anxiety disorders and for patient response to
pharmacological treatment with SSRIs and SNRIs (Lesch et al., 1996; Nakamura et al., 2000; Hu et al., 2006; Klumpers et al., 2012). The L(A) allele is overtransmitted by 2-fold to patients with OCD (Hu et al., 2006), whereas subjects with at least one short allele exhibit greater fear-potentiated startle than do subjects with the long allele (Klumpers et al., 2012).

Another genetic factor implicated in the clinical response to SSRIs is a polymorphism in the gene that codes for RGS2, a protein involved in neuronal GPCR responses to neurotransmitters including 5-HT. Stein et al. (2014) found that a mutation that reduces expression of RGS2 is associated with a poor clinical response to sertraline treatment.

V. Neuropsychopharmacology of Anxiety Disorders

Different combinations of subunits comprise the GABA<sub>A</sub>, NMDA, and 5-HT receptors, providing tremendous variability in affinity for endogenous and exogenous ligands and thus for the ability of the receptor to modulate neuronal function (Smith et al., 2001; Nichols and Nichols, 2008). Below, we review a range of established and investigational anxiolytic agents, outlining the impact of selective modulation of receptor subtypes where data are available. The pharmacology of drug action is inherently the result of the net outcome of systems-level actions of the neuroactive agent. Therefore, it must be considered that, although subtype-prefering compounds exist, highly selective therapeutics have not yet been developed.

A. Positive Allosteric Modulation of GABAergic Transmission

1. Benzodiazepines and Related Modulators.

Although the actions of BZDs on GABA<sub>A</sub>Rs can reduce anxiety, this therapeutic effect is not without several adverse side effects, including drowsiness and ataxia as well as the risk of dependence and withdrawal reactions (Noyes et al., 1988; Bennett et al., 1998; Yamawaki, 1999; Allgulander et al., 2003). Because of these untoward effects, the use of BZDs for long-term treatment of chronic anxiety disorders such as GAD is undesirable (Allgulander et al., 2003). As indicated previously, side effects and efficacy of anxiolytic effects may be mediated through different α subunits (Löw et al., 2000; McKernan et al., 2000; Dias et al., 2005), providing one impetus for research into the development of more selective compounds.

Partial positive allosteric modulators have a lower intrinsic ability to induce a modulatory effect at given fractional occupancy as compared with full allosteric positive modulators and represent an approach to developing anxiolytics with fewer side effects and toxicity (Atack, 2003). The relationship between fractional receptor occupancy and fractional effect can be assessed by determining the percentage of inhibition of flumazenil binding to GABA<sub>A</sub>Rs and potentiation of GABA-evoked chloride currents. Potentiation of GABA-gated currents by 25% occurs at about 35% receptor occupancy for diazepam and about 45% for triazolam (Facklam et al., 1992b). The benzoquinolizinone Ro 19-8022 ([R]-1-[(10-chloro-4-oxo-3-phenyl-4H-benzo[a]quinolizin-1-yl)carbonyl]-2-pyrrolidine-methanol), a BZDR partial allosteric modulator, produced 25% potentiation when receptor occupancy reached about 95%. However, the imidazobenzodiazepine bretazenil, or Ro 16-6028 ([13aS]-8-bromo-11,12,13a-tetrahydro-9-oxo-9H-imidazo[1,5-a]pyrrolo[2,1-c][1,4]benzodiazepine-1-carboxylic acid 1,1-dimethylethyl ester), which also acts as a partial allosteric positive modulator, did not produce 25% potentiation even at 100% receptor occupancy. These in vitro observations can be related to in vivo responses in animal models, which indicate that bretazenil does not protect against seizures as well as the full allosteric positive modulator diazepam (Brouillet et al., 1991; Haefely et al., 1992). By contrast, most studies in humans and animals indicate that bretazenil can provide anxiolytic effects with less sedation and motor impairments than diazepam (Facklam et al., 1992a; Cole and Rodgers, 1993; Busto et al., 1994). However, there is an increase in symptoms of sedation and deficits in certain measures of attention and executive function (digit symbol) among subjects receiving 0.5 mg bretazenil (van Steveninck et al., 1996).

Although most compounds possessing the β-carboline structure are anxiogenic, the β-carboline abecarnil (isopropyl-6-benzoyloxy-4-methoxymethyl-β-carboline-3-carboxylate) produces anxiolytic effects without substantial sedation (Stephens et al., 1990; Ballenger et al., 1992). Abecarnil exhibits high affinity for the BZD recognition site, although no differences in affinities were observed for recombinant receptors expressed in human embryonic kidney cells containing α<sub>1</sub>, α<sub>2</sub>, or α<sub>3</sub> subunits (Hadingham et al., 1993). Another study shows that abecarnil only partially inhibits diazepam-insensitive binding of [³H]Ro 15-4513 in the cerebellum, suggesting that this site can be differentiated into abecarnil-sensitive and abecarnil-insensitive components (Mehta and Shank, 1995).

Electrophysiological studies indicate that abecarnil potentiates GABA-induced chloride currents less than full allosteric modulators. Interestingly, abecarnil is most effective with the fewest side effects at the lowest dose examined in a double-blind, placebo-controlled clinical trial for treatment of GAD (Ballenger et al., 1991, 1992). However, higher concentrations (>7.5 mg/day) of abecarnil were associated with reports of symptoms and signs of CNS depression including somnolence, difficulty concentrating, and dizziness. These observations suggest that efficacy alone may not account for all of the adverse side effects of BZDs.

The cyclopyrrole pagonclon, another compound believed to act as a partial positive allosteric modulator
of the GABA_A receptor, has also been investigated for its anxiolytic effects. A randomized double-blind study of patients suffering from panic attacks found that this compound provides anxiolytic effects without the typical side effects associated with BZDs (Sandford et al., 2001), but it has not been approved for use in the treatment of anxiety disorders (Bateson, 2003; Atack et al., 2006a; de Wit et al., 2006).

Agents such as imidazenil and SL651498 [6-fluoro-9-methyl-2-phenyl-4-(pyridin-1-yl-carbonyl)-2,9-dihydro-1H-pyrido[3,4-b]indol-1-one], which have a relatively higher affinity for specific GABA_A subunits, such as the α2 subunit, also appear to produce reduced measures of vigilance (a model for anxiety effects in human) with fewer side effects than the classic BZDs (Sieghart, 1994; Griebel et al., 2001; Williams and Akabas, 2001; Collins et al., 2002; Licata et al., 2005). This is consistent with the hypothesis that the α2 subunit-containing receptor subtype, which is largely expressed in the limbic system, may be an important contributor to the subtle positive modulation of GABAergic inhibition necessary to induce a reduction in vigilance in rodents and possibly induce anxiolysis in humans without undesirable side effects (Pritchett et al., 1989; Pritchett and Seeburg, 1990; Löw et al., 2000; Kaufmann et al., 2003; Licata et al., 2005). Yet bridging the gap between convincing results of a therapeutic candidate in animal models for anxiety and its beneficial clinical utility in humans remains an elusive if not daunting objective.

Research into the allosteric modulatory effects of compounds such as zolpidem, which act on the BZD site but do not possess the 7-member ring associated with the structure of the classic BZDs, has yielded compounds with a preference for specific GABA_A subunits (Ghiani et al., 1994; Serra et al., 1994; Drover, 2004). For example, zaleplon, zolpidem, and zopiclone exhibit a modest preference of up to 6-fold for GABA_A receptors containing α1 subunits compared with α2 or α3 subunits using model systems (Drover, 2004), and are effective sedative/hypnotic agents reported to produce less tolerance and dependence than diazepam (Follesa et al., 2002).

Compounds that do not act at GABA_A receptors containing α1 subunits may be useful as nonsedating anxiolytics. Whereas zolpidem and zaleplon appear to lack significant anxiolytic effects and seem to also not have any active metabolites, zopiclone is metabolized in vivo to the active metabolite (S)-desmethylzopiclone, which has been shown to produce anxiolysis with fewer negative effects on locomotor activity than diazepam and alprazolam, suggesting that structurally related compounds may be useful in the treatment of anxiety disorders (Carlson et al., 2001; Fleck 2002).

Ocinaplon (pyrazolo[1,5-a]-pyrimidine) is a novel pyrazolopyrimidine that potentiates GABAergic neurotransmission, increases the response of rats and primates in measures of anxiety (conflict test), and attenuates symptoms among human subjects with GAD as demonstrated by scores on the Hamilton Anxiety Rating Scales (Lippa et al., 2005). Electrophysiological studies indicate that ocinaplon has lower potency and efficacy than diazepam. However, the potentiation of GABA-gated currents in recombinant receptors expressed in Xenopus oocytes observed in this study indicates that ocinaplon has greater efficacy at α1 subunits than at α2 subunits, a finding that on the surface appears to be in conflict with the hypothesis that anxiolysis is mediated by α2 subunit-containing GABA_A receptors.

A compound with comparable binding affinity but different efficacies at the various receptor subtypes can exert its effects predominantly at those receptor subtypes implicated in anxiolysis, rather than having nonspecific effects and/or producing untoward side effects such as sedation (Atack, 2003). Interestingly, although ocinaplon shows selectivity for modulating α1 subunit-containing GABA_A receptors, competitive binding assays using native GABA_A membrane preparations derived from rat cerebellum (wherein α1 subunits are highly expressed) indicate that this compound is also much less potent at inhibiting the binding of flunitrazepam than is diazepam (Lippa et al., 2005). Since the clinical potency of a drug depends on its binding affinity for the target and its efficacy of signal transduction, it is plausible that lower receptor occupancy may contribute to the ability of ocinaplon to produce anxiolysis without inducing sedation.

Alternatively, some positive modulation of GABA_A receptors containing α1 subunits may be necessary to effectively induce anxiolysis in the human nervous system compared with mouse models, wherein even mild dysregulation of higher-order cognitive processes mediated by the frontal lobes has been implicated in controlling threat assessment and anxiety (Cha et al., 2014). Irrespective of which of these hypotheses or others turns out to be the most valid, the development of this promising compound was halted due to reports of elevated liver enzymes in just a few subjects, underscoring the inherent difficulties associated with bringing a novel anxiolytic to market even using a well defined target.

SL651498 is a pyridoindole that preclinical studies suggest acts at the BZD binding site to produce anxiolytic effects qualitatively similar to those of BZDs with reduced ataxia or sedation. This agent is a full positive modulator at recombinant rat GABA_A receptors containing α2 or α3 subunits (approximately 95% enhancement) and a partial positive modulator at receptors containing α1 or α5 subunits (approximately 45% enhancement). Binding-affinity assays indicate that SL651498 does not differentiate between α1 (17 nM), α2 (73 nM), and α3 (83 nM) subtype-containing GABA_A receptors (Griebel et al., 2001), indicating that binding affinity does not contribute to the anxiolytic actions of this drug.

2. Natural Product Modulators. Some natural products may also provide new platforms for discovery. For example,
chrysin, one of the first flavonoids shown to interact with the BZD binding site, initiated the search for natural anxiolytics among traditional medicinal herbs (Medina et al., 1989, 1990, 1997; Wolfman et al., 1994, 1996). A number of other naturally occurring flavonoids and their derivatives, such as K36 (5,7,2’-trihydroxy-6,8-dimethoxyflavone) (Huen et al., 2003), hispidulin (Kavvadias et al., 2004), wogonin (Hui et al., 2000, 2002), and related compounds (Nielsen et al., 1988; Häberlein et al., 1994; Wolfman et al., 1996; Goutman et al., 2003), are allosteric modulators at GABAARs. In a mouse study, wogonin (7.5–30 mg/kg p.o.) was found to produce an anxiolytic response similar to diazepam in the elevated plus maze without accompanying sedation (Hui et al., 2002). K36, a naturally occurring flavonoid, increases entries onto the open arms of the elevated plus maze without changing locomotor activity, sedation, myorelaxation, or motor incoordination in mice (Huen et al., 2003).

3. Increasing GABA Levels. On the basis of all of these results, it would be expected that increasing synaptic concentrations of GABA, either by inhibiting its reuptake or degradation, could be another approach to enhancing GABAergic neurotransmission and reducing anxiety disorders. GABA-transaminase inhibitors, such as vigabatrin (γ-vinyl-GABA) and valproic acid (VPA) (Rando et al., 1981; Gram, 1988), increase synaptic concentrations of GABA by preventing catabolism of GABA (Zwanzger et al., 2001a,b; Nemeroff, 2003). Vigabatrin raises levels of GABA in nerve terminals and inhibits striatal dopamine release (Kushner et al., 1997). However, reports of psychosis and depression associated with vigabatrin suggest that this particular compound may have significant clinical limitations (Levinson and Devinsky, 1999; Ashton and Young, 2003).

In retrospect, elevating GABA in a way that is proportionate to its endogenous levels might be expected to activate the natural distribution of relevant receptor subtypes nonselectively, thus increasing the probability of side effects. A more nuanced, neural systems–level approach might be capable of modulating anxiety in a similar fashion to the effective SSRIs.

B. Modulators of Glutamatergic Transmission

A delicate balance between excitatory and inhibitory neurotransmission is essential to modulation of anxiety and fear responses. Not surprisingly, disturbances of glutamatergic as well as GABAergic neurotransmission have been implicated in anxiety disorders (Jardim et al., 2005).

1. Inhibitors of Glutamatergic Activity. Glutamatergic neurotransmission in the lateral amygdala is essential to the acquisition and extinction of fear-related memories and behavioral responses associated with re-exposure to fear-inducing stimuli (Sajdyk and Shekhar, 1997). When a neutral stimulus is paired with an adverse stimulus such as presentation of a tone paired with delivery of a foot shock, the previously neutral stimulus is soon able to elicit a fear response independently. NMDA receptor antagonists such as d,l-2-amino-5-phosphonopentanoic acid, which prevent induction of long-term potentiation, have also been shown to block the acquisition but not the expression of conditioned fear responses in rodents (Miserendino et al., 1990; Falls et al., 1992). As previously mentioned, the prefrontal cortex appears to be the brain region most often implicated in attenuation of anxiety.

Memantine is another NMDA receptor antagonist that is currently approved for the treatment of Alzheimer’s disease, but there is some evidence that it may also have anxiolytic properties. A study in mice found that high stable doses of memantine (10, 30, and 100 mg/kg per day) produced some improvements in measures of anxiety, including reductions in isolation-induced aggression, escape latency (hidden platform), and wall swimming tendency in the Morris water maze test (Minkevičiene et al., 2008). In addition, several case studies in humans have shown some benefit for memantine in reducing symptoms associated with PTSD, SAD, and GAD (Battista et al., 2007; Schwartz et al., 2012). For example, in a small study of four patients with combat exposure–induced PTSD, treatment with memantine led to consistent improvements in memory as well as depressive and hyperarousalistic symptoms (Battista et al., 2007).

Another study showed that acamprosate, an NMDA and metabotropic glutamate 5 receptor modulator, was effective as a therapeutic for idiopathic and chemical withdrawal–induced anxiety disorders (Kotlinska and Bochenski, 2008; Hertzman et al., 2009; Schwartz et al., 2010; Palucha-Poniewiera and Pile, 2012; Koltunowska et al., 2013). Acamprosate seems to be a safe and effective therapeutic option for patients presenting with comorbid alcoholism. These findings were initially not entirely surprising given that enhancing inhibitory neurotransmission is a well-established method of treating anxiety; thus, it logically followed that if acamprosate attenuated excitatory neurotransmission, it could yield a similar clinical outcome (Berton et al., 1998). However, subsequent research has revealed both enhancement as well as inhibition of network synaptic elements underlying glutamatergic and GABAergic transmission, further emphasizing the need for a larger-scale understanding of drug action in the context of connectivity.

There is also evidence that riluzole, which reduces symptoms of GAD (Pittenger et al., 2008), may exert its effects by inhibiting voltage-gated sodium channels and enhancing astrocytic uptake of extracellular glutamate (Urbani and Belluzzi, 2000; Wang et al., 2004). In an open-label study of GAD patients, 8 of 15 patients treated with riluzole had remission of anxiety over a period of 8 weeks, with a median time to response of 2.5 weeks (Mathew et al., 2005). Thus, therapeutics that inhibit glutamatergic neurotransmission (e.g., riluzole) may prove to be more useful in treating GAD and the hyperarousal seen in PTSD.
2. Potentiators of Glutamatergic Activity. Administration of D-cycloserine, a partial NMDA receptor agonist, has been shown to facilitate extinction of learned fear responses in rodents and of phobias, SAD, and PTSD among patients undergoing behavioral exposure therapy (Walker et al., 2002; Ressler et al., 2004; Woods and Bouton, 2006; Mao et al., 2008; Uppperle et al., 2009; Difede et al., 2014).

A randomized, double-blind, placebo-controlled clinical trial examining the efficacy of a combination of D-cycloserine or placebo and exposure therapy for treatment of SAD revealed that short-term dosing of partial NMDA receptor agonists may also be effective in treating this disorder (Hofmann et al., 2006). This compound seems to be most effective for treating anxiety disorders that respond to “exposure therapy” in which learning not to fear the stimulus facilitates improvement in symptom measures. In a similar study, Difede and colleagues (2014) compared the effectiveness of VRE therapy alone versus the combined treatment with D-cycloserine plus VRE, and found that D-cycloserine augmented the therapeutic response to VRE, suggesting that negative memories may be extinguished using cognitive enhancers. Overall, these observations suggest that compounds that increase glutamatergic neurotransmission are useful in the treatment of certain types of anxiety disorders (e.g., specific phobias) and PTSD wherein re-exposure to a specific salient stimuli can trigger a pathologic response that interferes with activities of daily living (Heresco-Levy et al., 2002; Difede et al., 2014).

3. Neurosteroids as Modulators of Glutamatergic Transmission. The observation that stress-induced anxiety changes glutamatergic neurotransmission (Masneuf et al., 2014) raises the possibility that endogenous mechanisms for modulating the balance of excitatory to inhibitory neurotransmission could underlie the genesis of anxiety. Neurosteroids such as pregnanolone sulfate, which enhances cognition, positively modulates NMDAR function, and stimulates NMDAR trafficking to the cell surface (Wu et al., 1991; Kostakis et al., 2013), may represent one such endogenous mechanism for linking fearful stimuli and the emotional response subject to therapeutic manipulation (Plescia et al., 2013). Neuroactive steroids, such as pregnanolone sulfate and pregnanolone hemisuccinate, which inhibit glutamatergic neurotransmission by negatively modulating NMDAR function (Park-Chung et al., 1994) and may also exhibit mixed function by serving as both a negative modulator of the NMDAR and a prodrug for pregnanolone (a positive modulator of GABAAR) delivery across the blood–brain barrier, may also represent a novel platform for discovery (Weaver et al., 1997, 2000).

C. Dopaminergic Transmission

In a recent study by Zweifel and colleagues (2011), cue conditioning was found to be significantly impaired in knockout mice lacking functional NMDA receptors on dopaminergic neurons of the ventral tegmental area. This impairment was associated with development of a GAD-like phenotype, suggesting that dopaminergic neurotransmission plays a role in this anxiety disorder. Interestingly, increased anxiety is among the symptoms associated with dopamine agonist withdrawal in humans (Nirenberg, 2013). The interplay between the glutamatergic and dopaminergic systems is well studied in drug addiction, wherein motivation for drug seeking is mediated, in part, by this relationship. Nucleus accumbens and prefrontal cortex dopaminergic and glutamatergic systems have been implicated in anxiety disorders and addiction (Ahmadi et al., 2013) and it has been suggested that the D2/D3 receptor agonist quinpirole may induce compulsive checking behavior in rodents by enhancing dopaminergic activity (Alkhhatib et al., 2013). The role of motivation in OCD is well established and plays a key role in the ability of an individual to override his or her pathologic responses to anxiety-inducing stimuli; it is not uncommon for patients with OCD and phobias to temporarily overcome their fears to avoid life-threatening events.

In contrast with the D2/D3 receptor agonist quinpirole, the anxiolytic buspirone has been shown to block D3 receptors in primates (Kim et al., 2014). However, the role that this mechanism plays in attenuating anxiety is not entirely clear since the compound also acts at serotonin and adrenergic receptors (Skolnick et al., 1984).

D. Epigenetics of Anxiolysis: Histone Deacetylase Inhibitors

Combining histone deacetylase inhibitors with CBT can also facilitate extinction of learned fear responses in humans and animals (Heinrichs et al., 2013; Kuriyama et al., 2013). Administration of VPA at a dose of 400 mg/kg to human subjects was shown to augment acquisition and delayed offline consolidation of extinction training (Kuriyama et al., 2013). The unexpected effect of VPA on acquisition could be due to in part to an anxiolytic effect associated with enhanced GABAergic neurotransmission mediated by inhibition of GABA transaminase by VPA at this dose.

VPA seems to be rather nonspecific in terms of mechanisms of action, at least at present. For example, in addition to inhibition of histone deacetylases, VPA also increases levels of brain-derived neurotrophic factor and cAMP response element-binding protein in the hippocampus and amygdala (Jeon et al., 2006; Casu et al., 2007; Jornada et al., 2010). A case of familial bipolar disorder with comorbid anxiety has been studied as part of the National Institute of Mental Health Human Genetics Initiative and was found to be associated with the genes that code for the extracellular signal-regulated kinase/mitogen-activated protein kinase and cAMP response element-binding protein–regulated intracellular signaling pathways (Kerner
et al., 2013). Although further research in this area is needed, these findings suggest that behavioral modification therapy could be combined with drugs that modulate gene expression to facilitate extinction of the fear-inducing stimuli associated with specific phobias and PTSD.

E. Modulators of Serotonergic and Noradrenergic Transmission

Several studies reveal the potential benefits of SSRIs over BZD pharmacotherapy of certain types of anxiety. For example, it has been shown that patients who present with comorbid symptoms of depression and anxiety may respond better to SSRIs, whereas those who suffer from anxiety alone may respond better to BZDs. In addition, patients suffering from GAD respond well to BZDs, whereas those with PTSD and OCD have a relatively better clinical response to SSRIs (Rickels and Rynn, 2002; Varia and Rauscher, 2002; Blanco et al., 2003; Davidson, 2003).

Paroxetine was the first SSRI to receive approval from the US Food and Drug Administration for the treatment of anxiety disorders and was superior to placebo in short-term clinical trials among patients with GAD (Allgulander et al., 2003). The SSRI fluoxetine is widely used for the treatment of patients who are comorbid for depression and anxiety (Cassano et al., 2004). It seems unclear whether the anxiolytic effects of fluoxetine may paradoxically be due entirely to enhancement with GAD (Allgulander et al., 2003). The SSRI fluoxetine is widely used for the treatment of patients who are comorbid for depression and anxiety (Cassano et al., 2004). It seems unclear whether the anxiolytic effects of fluoxetine may paradoxically be due entirely to enhancement with GAD (Allgulander et al., 2003).

F. Neuroactive Steroids as Neuromodulators of Transmission

Neuroactive steroid modulation of GABAergic neurotransmission in the central amygdala has been implicated in anxiety (Wang et al., 2007). There is some evidence that fluoxetine increases brain levels of the neurosteroid allopregnanolone, a positive allosteric modulator of GABA$_\text{A}$Rs (Matsumoto et al., 1999), and it has been suggested that the anxiolytic effects of fluoxetine and possibly other SSRIs (e.g., paroxetine) are related to this effect on allopregnanolone (Uzunova et al., 1998; Griffin and Mellon, 1999; Khisti and Chopde, 2000). This hypothesis is supported by a recent study in a mouse model of social isolation, which found that an SSRI-induced increase in allopregnanolone significantly contributes to the anxiolytic effects of SSRI treatment (Pinna, 2010). It is therefore of interest to take a closer look at the mechanisms of action and modulatory sites of neuroactive steroids, such as allopregnanolone.

The search for an endogenous system of new targets for modulation has been intensive, and neuroactive steroid-based options offer some promise. Selye (1970) first demonstrated that endogenous steroids and their metabolites can produce rapid effects on CNS activity, including modulation of GABAergic neurotransmission associated with anxiety (Lambert et al., 2001, 2003; Yang et al., 2002).

The potential effects of neuroactive steroids on the development of anxiety disorders can be observed during puberty, a developmental stage associated with increased levels of reproductive hormones and onset of many psychiatric disorders, including GAD, social anxiety, and panic attacks (Hayward and Sanborn, 2002; Kessler et al., 2005a; Merikangas et al., 2009, 2010; Reardon et al., 2009; Van Oort et al., 2009). Anxiety symptoms have been found to increase from middle to late adolescence (Van Oort et al., 2009), with a particularly high prevalence of all anxiety disorders reported among adolescent girls (Merikangas et al., 2009; Leikanger et al., 2012; Legerstee et al., 2013).

Of particular interest in this setting are the neuroactive steroids allopregnanolone (in humans and rats) and pregnanolone (in humans only), metabolites of the reproductive hormone progesterone that are also produced in the brain in response to stress (Purdy et al., 1991; Girdler et al., 2001). Allopregnanolone decreases in the cerebral spinal fluid of women with PTSD (Rasmusson et al., 2006), but increases in depressed patients receiving SSRIs (Uzunova et al., 1998). Moreover,
administration of sodium lactate and cholecystokinin-tetrapeptide to persons with panic disorder—induced attacks decreases plasma concentrations of both pregnanolone and allopregnanolone, and increases the concentration of the functional antagonist isomer 3β,5α-tetrahydroprogesterone (Ströhle et al., 2003). Together these findings suggest that changes in endogenous brain levels of neuroactive steroids associated with age, sex, stress, and administration of SSRIs may play a role in the onset and clinical response to treatment in certain anxiety disorders.

Supporting this hypothesis are findings related to the effects of allopregnanolone on gonadotrophin hormone-releasing hormone, the primary chemical messenger implicated in the onset of puberty and sexual maturation (Moguilevsky and Wuttke, 2001). Allopregnanolone has been found to suppress the release of hypothalamic gonadotrophin hormone-releasing hormone via allosteric modulation of GABAARs (Calogero et al., 1998; Sim et al., 2001). Although this inhibition is increased by allopregnanolone administration before puberty and in adulthood, it is paradoxically reduced during puberty, leading to increased excitability of pyramidal cells in hippocampal region CA1. This effect appears to be due in part to inhibition of α4-containing GABAARs, which are expressed at higher levels than normal in the CA1 region of the hippocampus during puberty.

It has also been shown that GABAARs of the α4β2δ subtype, which have a δ subunit instead of a γ subunit, play a role in tonic inhibition in areas such as the dentate gyrus and cortex (Wisden et al., 1992). GABAAR-mediated conductance is normally inhibitory; however, the reversal potential of GABAAR-mediated postsynaptic current in dentate gyrus granule cells is “positive” to the resting membrane potential, making membrane hyperpolarization of GABAARs unlikely. Inhibition of shunting appears to play a role in overcoming this process, such that nonhyperpolarizing inhibitory conductance reduces the depolarizing effect of postsynaptic potentials by decreasing proximal membrane resistance (Staley and Mody, 1992).

In the dentate gyrus and cortex, the GABAergic current is inward (i.e., chloride flux is outward) (Staley and Mody, 1992; Gullidge and Stuart, 2003); thus, inhibition in these areas is enhanced by allopregnanolone. However, in the CA1 region, the current is normally outward (Alger and Nicoll, 1982); thus, increased expression of α4β2δ GABA receptors paradoxically results in allopregnanolone attenuating rather than enhancing inhibition. The reduction in current generated by allopregnanolone at α4β2δ GABAAR is dependent upon the presence of arginine 353 in the intracellular loop of α4, where it may serve as a chloride modulatory site (Shen et al., 2007). This polarity-dependent decrease in inhibition mediated by allopregnanolone may have important implications for how we approach the treatment of anxiety disorders in the future.

The modulation of GABAergic neurotransmission by neuroactive steroids is mediated by interactions with allosteric sites on GABAARs (Lambert et al., 2001, 2003; Yang et al., 2002; Hosie et al., 2006). The interaction of certain neuroactive steroids with GABAAR is stereoselective, suggesting that the binding sites for these compounds are of a specific dimension and shape (Harrison and Simmonds, 1984; Park-Chung et al., 1994; Covey et al., 2000). There is also evidence that the transmembrane domains play a role in neuroactive steroid–mediated modulation of GABAARs (Hosie et al., 2006) (Fig. 9), and that the effects of neuroactive steroids on GABAARs may be associated with its actions at δ subunit–containing receptors (Mihalek et al., 1999, 2001; Spigelman et al., 2003; Steill et al., 2003). Although changes in these receptors have not been associated with alterations in behaviors considered indicative of vigilance or possibly

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**Fig. 9.** Neurosteroid activity at the GABAAR is determined by α-subunit M1 domain residues. (A) The M1 through the end of M2 murine α1 and β2 subunits were replaced with the corresponding sequence from the resistance to dieldrin (RDL) subunit, forming the chimeras αR and βR, respectively, which were previously shown to have very low sensitivity to potentiation and to lack direct activation by micromolar concentrations of neurosteroids. Polar residues present only in α1 (blue) are in bold, with Thr236 and Gln241 (red) highlighted. The transmembrane domains are boxed. The pharmacology of receptors containing the modified α subunit was then compared with that of wild-type receptors using whole-cell recordings from human embryonic kidney cells. (B) THDOC concentration–response curves for direct activation (red) and for potentiation (blue) of EC10 (concentration causing 10% of maximal response) GABA currents expressed as a percentage of the maximum GABA response, at wild-type α1β2γ2 (squares) and chimeric αRβ2γ2 (circles) receptors showing that potentiation and direct activation by THDOC and allopregnanolone is abolished on receptors incorporating αR. (C) Structures of ALLOP and THDOC. ALLOP, allopregnanolone; THDOC, tetrahydrodeoxy corticosterone. Reprinted with permission from Hosie et al. (2006).
anxiety in animals (Mihalek et al., 1999), evidence strongly suggests that a change in subunit subtype from \( \alpha_1\beta_2\gamma_2 \) to \( \alpha_4\beta_2\gamma_2 \) increases receptor desensitization and decreases GABAergic inhibition, a process that may play a role in epilepsy (Roberts et al., 2005, 2006; Lund et al., 2008; Grabenstatter et al., 2012). Could such a change in subunit composition also underlie increased anxiety and serve as a biomarker to identify patients resistant to existing anxiolytic therapeutics? Could switching of receptor subunit subtypes—perhaps involving the \( \alpha_4 \) subunit, which is insensitive to BZD positive modulators—be a control point in anxiolysis as in epilepsy?

Both allopregnanolone and pregnenolone have been implicated in the prevalence of anxiety disorders in men and women (Le Mellédo and Baker, 2004), and a range of preclinical studies suggest potential mechanistic rationales for the anxiolytic effects of these neuroactive steroids. It has been shown that allopregnanolone exhibits anticonflict effects pharmacologically at 8 mg/kg in rats (Brot et al., 1997), and that it attenuates certain effects of caffeine in rats indicative of vigilance or anxiety (Jain et al., 2005). Pregnanolone potentiates electrophysiological responses to and binding of muscimol to GABA\( _A \)Rs (Majewska et al., 1988; Wu et al., 1993; Park-Chung et al., 1994), and exhibits sedative hypnotic properties via its likely interaction with GABA\( _A \)Rs (Wang et al., 2001). Overall, these observations suggest that allopregnanolone and pregnenolone act as endogenous modulators of GABAergic neurotransmission in vivo (Lambert et al., 2001, 2003; Yang et al., 2002; Ströhle et al., 2003). In support of the hypothesis that allopregnanolone and pregnenolone have anxiolytic properties, two small studies suggest that the plasma concentrations of pregnenolone sulfate, a precursor of these steroids, is lower in males with generalized social phobia or GAD (Semeniuk et al., 2001; Heydari and Le Mellédo, 2002). Administration of pregnenolone is associated with an increase in allopregnanolone and reduced activity in the amygdala and insula, as well as a reduction in self-reported anxiety (Sripada et al., 2013) (Fig. 10).

Because they have poor bioavailability and can potentially be metabolized to hormonally active steroids, endogenously occurring neuroactive steroids have limited therapeutic use in patients. Synthetic analogs of neuroactive steroids, which are more resistant to metabolism and better able to cross the blood–brain barrier, are now being investigated for potential use as anxiolytics, anesthetics, and anticonvulsants (Weaver et al., 1997; Gasior et al., 1999). One synthetic neurosteroid, pregnanolone hemisuccinate, produces sedation in mice and rats (Weaver et al., 1997), raising the possibility that it acts either by inhibiting the NMDA receptor or by crossing the blood–brain barrier and undergoing metabolism to pregnanolone. Synthetic neurosteroids bearing a hemisuccinate group are more resistant to hydrolysis than the corresponding sulfate esters and are partly unionized at physiologic pH, allowing ready passage across the blood-brain barrier.

Ganaxolone (3-hydroxy-3-methyl-5-pregnane-20-one), an orally active synthetic analog of allopregnanolone, is a positive allosteric modulator of GABA\( _A \)R that has shown promising basic and early stage clinical outcomes and is currently under investigation as a novel treatment of epilepsy and PTSD (Carter et al., 1997; Gasior et al., 1999; Reddy and Rogawski, 2012). Another

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**Fig. 10.** Functional MRI studies showing that administration of PREG (400 mg) is associated with a reduction in activity in regions associated with generation of negative emotion. (A) Compared with placebo, pregnenolone administration decreased activation in right amygdala and right insula. (B) Compared with placebo, pregnenolone administration increased dorsal medial prefrontal cortex activation during appraisal. The percent signal change is displayed next to each figure. PBO, placebo; PREG, pregnenolone administration group. Reprinted with permission from Sripada et al. (2013).
synthetic analog of allopregnanolone, Co 3-0593 (3ß-ethenyl-3a-hydroxy-5a-pregn-20-one), was found to have anxiolytic effects comparable to BZDs after both subcutaneous and oral administration in rodents. An absence of tolerance to this drug is suggested by the fact that its effects were maintained even after chronic administration (Wieland et al., 1997).

Although these data suggest that anxiety disorders may be amenable to treatment with synthetic neurosteroids or through modulation of the synthesis of endogenous neuroactive steroids, the more compelling value of these agents to date has been the insights they have provided into the pathophysiology of anxiety, both influencing and being influenced by the GABAergic, serotonergic, and other neural systems in a range of anxiety disorders.

VI. Conclusions

“Personalized medicine” is now a familiar concept, not only in the scientific literature but also in the popular media. The concept is so familiar, in fact, that some are now questioning whether the term has outlived its usefulness, implying both too much and too little: too much in the sense that unique treatments are not being developed for every individual patient, and too little in the sense that personalized medicine is what clinicians already aspire to every time they treat a patient (Katsnelson, 2013). Regardless of the term we use to describe this aspiration, it is certainly true that the more we are able to tailor treatments to a patient’s individual profile, the greater the chance of clinical success. In the cases of mental disorders such as anxiety or depression, the idea of individualized approaches to therapy take on even greater urgency, given the uniquely personal and complex mental processes that underlie the disorders.

We believe that an adequate response to these challenges will require a systems-based approach to research. Although traditional target-based discovery has shown success in identifying new drugs against previously validated targets, the reductionist nature of this approach often leads to late-stage failure when the drug is assessed in increasingly complex systems. By contrast, a systems-based approach may speed the drug discovery process by focusing less on drug interactions with single targets and more on systems-level assays representative of the disease (Desbiens and Farb, 2012).

A “pharmacologic connectome” is emerging based upon which disorder-specific novel therapeutic agents for anxiety can be developed (Fig. 11). Functional neuroimaging studies are revealing the brain regions and nuclei implicated in specific anxiety disorders and are providing important clues about which pharmacological interventions are the most appropriate for which disorders. The use of drugs that enhance extinction is gaining acceptance for the treatment of specific phobias and the intrusive memories associated with PTSD (Rothbaum et al., 2014). Compounds that inhibit excitatory neurotransmission or enhance inhibitory neurotransmission are proving to be more effective for treating GAD and the hypervigilance associated with PTSD (Mathew et al., 2005). At the same time, we are beginning to understand that because of genetic polymorphisms, some patients may not respond to SSRIs and SNRIs (Zanardi et al., 2001). The opportunity to apply this growing body of knowledge to the development of novel therapeutics for anxiety disorders has not been greater.

Neuroactive steroids, which modulate various neurotransmitter pathways and are a locus for the neural crosstalk involved in anxiety disorders, may prove to be an important tool for advancing a systems-based approach to drug development. The unique place of neuroactive steroids in this setting is given further support by a growing body of evidence showing that these agents are important for both improving the survival of neurons and increasing the number of newly formed neurons (Charalampopoulos et al., 2008).
The results reviewed in this article suggest that current research into brain activation and therapeutic targeting of GABA, 5-HT, and NMDA receptor subunits has not produced a silver bullet for treating anxiety that optimizes efficacy and reduces risk of adverse events. Moreover, true personalized medicine in patients suffering from various anxiety disorders would require a comprehensive and multifaceted approach beyond analysis of neurologic activation. This could include assessment of patient demographics, clinical presentation, family history, recent genetic polymorphisms, and biomarkers, as well as treatment options encompassing not only pharmacotherapy but also psychotherapeutic approaches such as CBT and behavioral activation therapy. Nevertheless, research into the brain regions and variations in receptor subunits engaged in anxiety provides reason for optimism that the pathophysiology underlying this complex mosaic of perceptions will lead to treatments better tailored to the individual needs of patients suffering from a range of anxiety disorders based on results from noninvasive brain imaging and improved therapeutics.

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