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Anxiety and Depression: Mouse Genetics and Pharmacological Approaches to the Role of GABA_A Receptor Subtypes

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Abstract

GABA_A receptors mediate fast synaptic inhibitory neurotransmission throughout the central nervous system. Recent work indicates a role for GABA_A receptors in physiologically modulating anxiety and depression levels. In this review, we summarize research that led to the identification of the essential role of GABA_A receptors in counteracting trait anxiety and depression-related behaviors, and research aimed at identifying individual GABA_A receptor subtypes involved in physiological and pharmacological modulation of emotions.

Keywords

GABA_A receptor; anxiety; depression; benzodiazepines; development

1. Introduction

GABA_A receptors are ionotropic receptors mediating fast inhibitory neurotransmission in the CNS. GABA_A receptors are heteropentamers made up from a subunit repertoire of 19 different subunits (α 1-6, β 1-3, γ 1-3, δ , ϵ , theta, π , ρ 1-3) (Olsen and Sieghart, 2009). Most GABA_A receptors are pentamers composed of α , β , and γ subunits, typically containing two α subunits, two β subunits, and one γ subunit. Over 90% of all GABA_A receptors include the γ 2 subunit, making it the most abundant GABA_A receptor subunit in the CNS. The activity of these ionotropic receptors is modulated by a variety of clinically-used drugs, including benzodiazepines, barbiturates and general anesthetics (Rudolph and Mohler, 2004), as well as endogenously-produced neurosteroids (Hosie et al., 2007).

Benzodiazepines (e.g. chlordiazepoxide and diazepam) were introduced into clinical practice more than 50 years ago and display anxiolytic, sedative-hypnotic, anticonvulsant and muscle relaxant properties, with major drawbacks being the development of tolerance and physical dependence. These drugs bind to and modulate the activity of GABA_A receptors that contain the α subunits α 1, α 2, α 3, or α 5, a β subunit and a γ subunit, typically γ 2. While barbiturates and general anesthetics also modulate GABA_A receptors consisting of only α and β subunits, a γ subunit is required for modulation by benzodiazepines (Pritchett et al., 1989). Neurosteroids are natural allosteric modulators of GABA_A receptors, with α and β

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subunits contributing to the neurosteroid binding site. They modulate both γ - and δ -containing GABA_A receptors (Hosie et al., 2007).

Benzodiazepines have replaced barbiturates in the treatment of anxiety disorders and insomnia largely because they are safer in overdose. This effect may be related to the fact that the action of benzodiazepines is self-limiting, i.e. the maximum effect that can be achieved with benzodiazepines is not higher than the effect that could be achieved with a high concentration of GABA alone. In contrast, the action of barbiturates is not self-limiting in that the drug effects surpass the level that can be achieved by GABA. However, while the introduction of benzodiazepines provided a series of useful pharmacologic actions for more effective treatments of specific disorders, these drugs also have effects which – depending on the indication -- are generally viewed as being clinically disadvantageous.

For example, while the anxiolytic action of diazepam (Valium[®]) is observed at lower doses than the sedative-hypnotic action of this same drug, sedation can be a problem even at low doses when benzodiazepines are used as daytime anxiolytics. One obvious question that arises is whether the different actions of benzodiazepines, in particular anxiolysis and sedation, are pharmacologically separable. Since benzodiazepines are positive allosteric modulators of GABA_A receptors, examination of their pharmacological effects can therefore teach us what functional changes are induced when these receptors are modulated. It is reasonable to assume that such experiments can also provide information about the physiological functions of GABA_A receptors.

However, the similarity of the different GABA_A receptor subtypes, as defined by their α subunits, has made it difficult to synthesize subtype-specific compounds, and even now, no absolute subtype-specific compounds are available. Consequently, identification of the pharmacological function of individual subtypes was largely achieved by introducing point mutations into GABA_A receptor subunits which rendered them insensitive to either benzodiazepines (Crestani et al., 2002; Low et al., 2000; McKernan et al., 2000; Rudolph et al., 1999) or general anesthetics like etomidate (Jurd et al., 2003; Reynolds et al., 2003) and isoflurane (Borghese et al., 2006; Sonner et al., 2007; Werner et al., 2011). The physiological functions of GABA_A receptor subtypes have also been studied in loss-of-function experiments in receptor subunit knockout mice (Rudolph and Mohler, 2004). In general, one can assume that if the positive modulation of a receptor subtype yields a certain behavioral effect, e.g. benzodiazepine-induced anxiolysis acting via $\alpha 2$ -containing GABA_A receptors in wild type mice, that negative modulation, or a knockout of this subtype, might lead to opposite behavioral effects, e.g. increased anxiety. In this sense, studies of the effects of positive modulation of GABA_A receptor subtypes also provide clues about the physiological function of the individual receptor subtypes. Here, we provide an overview about studies in which genetically modified mice have been used to elucidate the potential roles of GABA_A receptors in general, and of specific GABA_A receptor subtypes in anxiety and depression.

2. GABA_A receptors and anxiety

2.1. The $\gamma 2$ heterozygous knockout mouse: a preclinical model of trait anxiety

The first global GABA_A receptor knockout mouse that was generated was the $\gamma 2$ subunit knockout which, as expected, led to a 94% reduction in benzodiazepine binding sites (Gunther et al., 1995). Although the homozygous $\gamma 2$ knockout ($\gamma 2^{-/-}$) is lethal in the perinatal period, behavioral experiments with rare surviving animals demonstrated that diazepam was behaviorally inactive (Gunther et al., 1995). Additionally, the single channel main conductance level was reduced in these animals, a result that is consistent with *in vitro*

observations in recombinant receptors indicating that the $\gamma 2$ subunit is essential for proper channel conductance and the presence of benzodiazepine sites (Gunther et al., 1995).

Therefore, to characterize the physiological function of this receptor, heterozygous $\gamma 2$ knockout ($\gamma 2^{+/-}$) mice were studied. These mice exhibit, on average, a 20% reduction of benzodiazepine binding sites (Crestani et al., 1999), an effect that was most pronounced in the cortex (e.g., frontal cortex; -23%), the hippocampus (e.g., CA1 -35%; CA3, -28%) and in the lateral septum (-30%). Regions with a below-average decrease included the dentate gyrus (-15%), striatum (-6%), globus pallidus (-13%) and most of the amygdala. Synaptic clusters in CA1 and dentate gyrus were also decreased in these mice. It is noteworthy that the reduction of benzodiazepine binding sites in the hippocampus is much greater than that observed in the amygdala, potentially supporting the theory put forward by McNaughton and Gray that changes in septo-hippocampal function can underlie both normal and pathological changes in anxiety (McNaughton and Gray, 2000).

Behaviorally, $\gamma 2^{+/-}$ mice showed increased reactivity to ethologically-based anxiogenic stimuli in that they explored the novel compartments of the free-choice exploration (FCE) test less and explored the open arms of the elevated plus maze (EPM) and the lit portion of the light/dark box (LDB) less than wild types (Crestani et al., 1999). From these observations, it was determined that $\gamma 2^{+/-}$ mice have an anxiogenic-like phenotype. Additional studies with these genetically modified animals revealed that the phenotypic changes observed in the above-mentioned paradigms could be reversed following administration of low doses of diazepam that would ordinarily have no effect in wild type animals in these assays (Crestani et al., 1999). When considered in the context of clinical data, this reversal corresponds to the observation that humans with high-anxiety scores are more sensitive to the anxiolytic action of benzodiazepines (Glue et al., 1995; O'Boyte et al., 1986). It is interesting to note though that while rare surviving $\gamma 2^{-/-}$ knockout mice are behaviorally insensitive to diazepam (Gunther et al., 1995), $\gamma 2^{+/-}$ mice are actually more sensitive to diazepam than wild type mice. Thus, there is no functional behavioral continuum when comparing $\gamma 2^{-/-}$, $\gamma 2^{+/-}$, and wild type mice.

The $\gamma 2^{+/-}$ mice were also studied in a series of fear conditioning experiments to examine nondeclarative and declarative forms of memory. First, there was no genotypic difference between $\gamma 2^{+/-}$ and wild type mice in either delay or contextual fear conditioning paradigms. These nondeclarative memory tasks are at least in part amygdala-dependent and the lack of a phenotypic difference between wild types and mutants was likely due to the fact that GABA_A receptors in the amygdala were only minimally reduced in the $\gamma 2^{+/-}$ mice (Crestani et al., 1999).

However, when the $\gamma 2^{+/-}$ mice were examined in a trace fear conditioning paradigm, a hippocampal-dependent declarative memory task, they exhibited increased levels of freezing during test compared to wild types (Crestani et al., 1999). This behavioral effect observed in the $\gamma 2^{+/-}$ mice may have resulted from the reduction of GABA_A receptors in the hippocampus generated by the mutation. Similarly, when tested in an ambiguous cue discrimination task, where a tone served as a conditioned cue and a light served as a partial (ambiguous) cue, wild type mice froze significantly more to the conditioned cue than to the partial cue (an expected result), but the $\gamma 2^{+/-}$ mice exhibited equal amounts of freezing in the presence of both cues (Crestani et al., 1999); this response pattern remained consistent when the tone and light were counterbalanced. The lack of cue discrimination and heightened responsiveness to an ambiguous cue observed in the $\gamma 2^{+/-}$ mice is consistent with the clinical observation that humans with increased levels of anxiety may be more likely to perceive ambiguous situations as threatening.

In the step-through passive avoidance test, $\gamma 2+/-$ mice took significantly longer to re-enter the compartment in which a footshock had been administered 24 hours before compared to wild types, a result which is also interpreted as a heightened response to a fearful or threatening situation (Crestani et al., 1999). Lastly, $\gamma 2+/-$ mice were behaviorally indistinguishable from wild types in the Morris water maze and the Y maze spontaneous alternation tests and electrophysiological studies showed that LTP was also unchanged in the mutants demonstrating that spatial learning and memory were not altered in these mice (Crestani et al., 1999).

Collectively, these results demonstrate that $\gamma 2+/-$ mice more actively avoid harmful situations and that their explicit memory bias for threat-associated cues results in heightened sensitivity to negative associations. Although environmental factors and inherent differences in parental behaviors as a function of murine strain can impact the behavior of offspring (Caldji et al., 2004), the $\gamma 2+/-$ and wild type mice in the behavioral studies cited in this review both arise from wild type x heterozygous breeding pairs and therefore not only have the same parents, but are reared under the same environmental conditions. The phenotypic differences described are unlikely to be attributed to environmental and/or strain nurturing differences. In summary, $\gamma 2+/-$ mice can be considered as a preclinical model of anxiety (e.g. generalized anxiety disorder) with good face validity, as well as the potential for good construct and predictive validities, suggesting that a dysfunction in the GABA_A receptor system may be an important or even causal event in the development of anxiety disorders in humans.

However, as previously mentioned, the majority of GABA_A receptors (approximately 94% of all benzodiazepine-sensitive GABA_A receptors) contain the $\gamma 2$ subunit (Gunther et al., 1995). As such, a heterozygous knockout of the $\gamma 2$ subunit is predicted to affect the subunit composition and properties of many different GABA_A receptor subtypes. While the *in vivo* work described above clearly shows that a generalized GABA_A receptor deficit is causally linked to an anxiety-like phenotype in mice, it does not provide information about the reduction of which specific GABA_A receptor subtype, as defined by the α subunit, is underlying the anxiety-related phenotype exhibited by the $\gamma 2+/-$ mice.

Similarly, while the above average downregulation of hippocampal GABA_A receptors might indicate that this brain region plays a role in emotional responding, the mutation in the $\gamma 2+/-$ mice is global and the work does not provide any formal proof for the role of hippocampal GABA_A receptors, or for GABA_A receptors in other brain regions, in anxiety regulation. Lastly, since the $\gamma 2+/-$ mutation is present throughout embryonic development, it is not clear whether the observed phenotype is due to a reduction of GABA_A receptors in adult animals or whether GABAergic dysfunction during development is sufficient to cause this phenotype.

2.2. GABAergic deficits in development contribute to anxiety-related behaviors in adulthood

The GABA_A receptor deficit generated in the global $\gamma 2+/-$ knockout mice is present throughout embryonic and postnatal development but it remained unclear after the initial phenotypic characterization as to whether the developmental regulation of the knockout was occurring during the pre- or postnatal periods, or both. Previous work in other animal models, the 5-HT_{1A} receptor knockout and conditional rescue mice, revealed that the postnatal deletion of this receptor in the first two weeks of life is crucial for the development of an anxiety-like phenotype in adult animals; this phenotype cannot be reversed when the 5-HT_{1A} receptor is then selectively rescued in adulthood (Gross et al., 2002). Conversely, if the receptor is present during the first two postnatal weeks, and subsequently silenced during adulthood, mice do not develop an anxiety-like phenotype demonstrating that the neural

circuitry that subserves anxiety-like responses in adulthood is formed within a critical postnatal period (Gross et al., 2002). Since the behavioral rescue of the global 5-HT_{1A} receptor knockout mice was achieved with a conditional transgene that was expressed primarily in cortex and hippocampus, but not in the midbrain raphe nuclei (the CNS source of serotonergic neurons), expression of 5-HT_{1A} receptors in these forebrain regions was likely sufficient for the rescue (Gross et al., 2002).

Along similar lines, Luscher and collaborators performed experiments designed to answer two questions relevant to the involvement of GABA_A receptors in the development of anxiety-like behaviors: a) whether a forebrain-restricted heterozygous deletion of $\gamma 2$ starting after the first two postnatal weeks would be sufficient to induce an anxiety-like phenotype, and b) whether a forebrain-restricted heterozygous deletion of $\gamma 2$ beginning during embryonic development would have different behavioral outcomes (Earnheart et al., 2007). To this end, they crossed mice carrying a $\gamma 2$ floxed allele with two different cre lines: EMX1-cre mice, which express the cre transgene in glutamatergic forebrain neurons from embryonic day 10 onward (Iwasato et al., 2000; Iwasato et al., 2004), and with CamKII α -cre mice (line 2834) that express cre in forebrain glutamatergic neurons beginning around postnatal day 17 (Schweizer et al., 2003).

Using a series of ethologically-based tasks to assess novelty detection and anxiety-like behavior (i.e., FCE, EPM, LDB) the authors observed that mice with a heterozygous $\gamma 2$ deficit present throughout pre- and postnatal development (EMX1-cre / $\gamma 2^{\text{floxed/wt}}$) exhibited an anxiogenic-like phenotype (Earnheart et al., 2007) similar to that observed in the global constitutive $\gamma 2$ knockout mice (Crestani et al., 1999). In contrast, when the $\gamma 2$ deficit was induced more than two weeks after birth (CamKII α -cre / $\gamma 2^{\text{floxed/wt}}$), an anxiogenic-like phenotype did not emerge in these behavioral paradigms and these mice were behaviorally indistinguishable from wild types (Earnheart et al., 2007). The distribution of the expression of the two cre transgenes is not absolutely identical, e.g. EMX1-cre is expressed in neurons and glia, while CamKII α -cre is expressed only in neurons. However, the $\gamma 2$ subunit of the GABA_A receptor was undetectable in astrocytes (Earnheart et al., 2007). These results provide strong evidence that a forebrain-specific neuronal $\gamma 2$ deficit generated during embryonic development contributes to the development of an anxiogenic-like phenotype; these observations are similar to the behavioral observations reported for the 5-HT_{1A} knockout mouse (Gross et al., 2002). Together, the findings from work on GABA_A and 5-HT_{1A} receptors indicate that the first two weeks of life are crucial for the development of normal anxiety-related behavior.

2.3. GABA_A receptor subtype(s) modulate anxiety-related behaviors

The findings described in the previous section highlighted the importance of the GABA_A receptor system in anxiety-related behaviors. In this section, we will review studies using either genetic and/or pharmacological approaches to identify the functions of GABA_A receptor subtypes as defined by their α subunits in the modulation of anxiety-related behaviors. In order to identify the GABA_A receptor subtype that mediates the anxiolytic-like effect of diazepam (and thus of benzodiazepines in general), two complementary approaches have been used: a pharmacological approach based on the use of subtype-specific compounds and a genetic approach based on the generation of mice harboring genetic mutations that rendered individual subtypes insensitive to the benzodiazepine diazepam.

The pharmacological approach is dependent on the availability of compounds that have sufficient selectivity for one or more GABA_A receptor subtypes. The effects of such compounds thus provide information on the function of the GABA_A receptor subtype that is targeted by the drug. Currently available subtype-selective compounds include the $\alpha 1$ -selective agonist zolpidem (Pritchett and Seeburg, 1990), the $\alpha 1$ -selective antagonist β -CCT

(Huang et al., 1999), the $\alpha 2$ -, $\alpha 3$ -, and $\alpha 5$ -partial agonist L-838,417 (McKernan et al., 2000), and the $\alpha 2$ -, $\alpha 3$ -partial agonist TPA023 (also known as MK0777) (Atack et al., 2006; Lewis et al., 2008).

However, the main challenge to this approach is that so far no absolute subtype-specific compounds are available. As such, results with compounds that only have a relative selectivity for individual subtypes must be interpreted with caution since a biological effect might be mediated by a GABA_A receptor subtype that is different from the subtype that the compound is selective for. For example, the $\alpha 1$ -selective antagonist β -CCT blocks the anxiolytic-like actions of diazepam in the LDB (Griebel et al., 1999) and punished responding (Shannon et al., 1984), and chlordiazepoxide in the EPM (Belzung et al., 2000). Since this drug blocked the anxiolytic-like action of the two different benzodiazepines used in the above-mentioned behavioral paradigms, it was concluded that the anxiolytic-like actions of diazepam and chlordiazepoxide were mediated by $\alpha 1$ -containing GABA_A receptors (which were also referred to as “BZ1” at that time). Since then, more data has emerged demonstrating that this receptor subtype does not play a role in benzodiazepine-induced anxiolysis (Low et al., 2000; McKernan et al., 2000). Nevertheless, a plethora of useful information has evolved from the study of subtype-selective compounds.

Thus, mouse genetic strategies were devised to identify the functions of individual GABA_A receptor subtypes. A successful approach was to render individual GABA_A receptor subtypes insensitive to diazepam by introducing histidine to arginine point mutations at a conserved position in the relevant α subunits [i.e., $\alpha 1$ (H101R), $\alpha 2$ (H101R), $\alpha 3$ (H126R), and $\alpha 5$ (H105R)] in mice. In such knock-in mice, diazepam had an anxiolytic-like effect in the EPM and LDB tests in $\alpha 1$ (H101R) (Rudolph et al., 1999), $\alpha 3$ (H126R) (Low et al., 2000), and $\alpha 5$ (H105R) (Crestani et al., 2002) mice, but not in $\alpha 2$ (H101R) mice (Low et al., 2000) indicating that $\alpha 2$ -containing GABA_A receptors modulate anxiety levels and are necessary for the anxiolytic-like action of diazepam. Additional support for this also came from the behavioral examination of $\alpha 1$ and $\alpha 3$ global knockout mice: both knockouts exhibit a profile of anxiety-like behavior in the EPM that is indistinguishable from wild types with diazepam eliciting an anxiolytic-like response in both mutants (Kralic et al., 2002) (Yee et al., 2005). These data are consistent with role of $\alpha 2$ -containing GABA_A receptors in anxiety responses.

The conclusion that $\alpha 2$ -containing GABA_A receptors modulate anxiety levels is also supported by behavioral experiments using partially subtype-selective compounds. In particular, L-838,417, a partial agonist at $\alpha 2$ -, $\alpha 3$ -, and $\alpha 5$ -containing GABA_A receptors, and an antagonist at $\alpha 1$ -containing GABA_A receptors (McKernan et al., 2000), is anxiolytic in the EPM but has no sedative effect (McKernan et al., 2000). Moreover, TPA023 (also known as MK0777) which is a partial agonist at $\alpha 2$ - and $\alpha 3$ -containing GABA_A receptors, is anxiolytic in the EPM, fear-potentiated startle, and conditioned suppression of drinking tests in rats. This compound also reduces conditioned emotional responding in squirrel monkeys but is not sedative in either of these species (Atack et al., 2006). While the Phase II studies that examined the efficacy of this drug in treating generalized anxiety disorder were terminated prematurely due to preclinical toxicity (development of cataracts) (Mohler, 2011), a combined analysis of these studies revealed that the compound is anxiolytic (i.e. a significantly greater reduction of the Hamilton Anxiety Rating Scale (HAM-A) scores relative to baseline) but not sedative in humans (Atack, 2010) providing proof-of-principle that anxiolysis without sedation can be achieved by targeting selected GABA_A receptor subtypes in humans.

TPA023 has also been shown to reverse ketamine-induced working memory deficits in rhesus monkeys (Castner et al., 2010). In one small study with 15 schizophrenic patients,

TPA023 improved some cognitive functions, and EEG recordings revealed increased frontal γ band power (Lewis et al., 2008). However, no beneficial effect on cognition was observed in another larger study using the same compound (Buchanan et al., 2011). Potentially, an $\alpha 2/\alpha 3$ -selective compound with higher efficacy than TPA023 (11% at $\alpha 2$, 21% at $\alpha 3$, compared to chlordiazepoxide (Atack et al., 2006) would be useful for this indication.

A potential caveat for developing non-sedating $\alpha 2/\alpha 3$ -selective agents is that humans apparently have a higher propensity to develop sedation than experimental animals. For example, MRK-409 (also known as MK-0343) was anxiolytic and non-sedating in rodents and primates, but sedative in humans at <10% receptor occupancy at $\alpha 1$ -containing GABA_A receptors (Atack, 2010); it appears that even minimal activity of a compound at $\alpha 1$ -containing GABA_A receptors can result in sedation in humans.

Another subtype-selective compound is TP003, which has been reported to be highly selective for $\alpha 3$ -containing GABA_A receptors *in vitro* (Dias et al., 2005). This compound has an anxiolytic-like action in rats tested in the EPM and in squirrel monkeys when conditioned emotional responding was evaluated (Dias et al., 2005). However, a potential caveat is that there is no independent confirmation of the high $\alpha 3$ -selectivity of this compound; this is notable since the rat EPM results appear to be at odds with the observation in $\alpha 2$ (H101R) point mutant mice that diazepam also acts at $\alpha 3$ -containing GABA_A receptors but does not elicit an anxiolytic-like response at the doses tested (Low et al., 2000). Furthermore, it is also important to note that a 75% receptor occupancy is needed for the anxiolytic-like action of TP003 whereas a 25% receptor occupancy is sufficient for the anxiolytic-like action of chlordiazepoxide (Dias et al., 2005). This suggests that the anxiolytic-like action of chlordiazepoxide is not (or at least not primarily) mediated by $\alpha 3$ -containing GABA_A receptors.

The claim that $\alpha 3$ -containing GABA_A receptors play a role in anxiety has also been made in a publication studying the anxiogenic-like properties of inverse agonists at the benzodiazepine site. It has been reported that the “inverse agonist selective for $\alpha 3$ subunit-containing GABA_A receptors”, $\alpha 3$ IA, has anxiogenic properties in the EPM (Atack et al., 2005). These authors interpreted the inverse agonistic action of the compound as being mediated by $\alpha 3$ -containing GABA_A receptors. However, in the same publication, the efficacy of $\alpha 3$ IA (measured as percent maximal modulation) in recombinant $\alpha \beta 3 \gamma 2$ GABA_A receptors has been reported as $-43 \pm 2\%$ for $\alpha 1$, $-34 \pm 5\%$ for $\alpha 2$, $-45 \pm 5\%$ for $\alpha 3$, and $-4 \pm 4\%$ for $\alpha 5$ (Atack et al., 2005). Given the substantial inverse agonistic modulation of $\alpha 2$ -containing GABA_A receptors, one cannot exclude the possibility that the anxiogenic-like properties of $\alpha 3$ IA are largely, or entirely, mediated by $\alpha 2$ -containing GABA_A receptors.

Further support for a role of $\alpha 2$ -containing GABA_A receptors in the modulation of anxiety comes from experiments with $\alpha 2$ global knockout mice. In a conditioned emotional response paradigm, the benzodiazepine diazepam and the barbiturate pentobarbital have an anxiolytic-like action in wild type mice but not in $\alpha 2$ global knockout mice (Dixon et al., 2008). Additionally, L-838,417 has an anxiolytic-like action in $\alpha 2$ (H101R) point mutant mice in the same paradigm, which was interpreted as showing that $\alpha 3$ -containing GABA_A receptors mediate anxiolytic-like actions (Morris et al., 2006). However, it has neither been shown *in vitro* nor *in vivo* that L-838,417 has no action, or is an antagonist, at the benzodiazepine site of $\alpha 2$ (H101R)-containing GABA_A receptors. For example, bretazenil, a partial BZ site agonist, still has agonistic actions at $\alpha 2$ (H101R)-containing GABA_A receptors *in vitro* (Benson et al., 1998), a result demonstrating that not all agonists at the BZ site are inactive at the mutated receptors.

Results from mice examined in a stress-induced hyperthermia (SIH) paradigm, a test of autonomic hyperactivity, showed that chlordiazepoxide blocked core body temperature increases (i.e. SIH) in wild type and $\alpha 2$ (H101R) mice when subjected to cage changing stress (Dias et al., 2005). This suggests that this effect is independent of $\alpha 2$ -containing GABA_A receptors (Dias et al., 2005). While administration of TP003 subsequently reduced SIH in both wild type and $\alpha 2$ (H101R) mice, it should be kept in mind that the effect of this drug on $\alpha 2$ (H101R)-containing GABA_A receptors has not been examined in recombinant receptors. Given that this paradigm assesses autonomic hyperactivity, and since $\alpha 3$ is the only α subunit expressed in neurons involved in autonomic regulation (e.g. noradrenergic neurons of the brain stem), it is conceivable that the SIH-blocking effect of benzodiazepine-site ligands (which may be independent of the effect of benzodiazepines on affective appraisal) is mediated by $\alpha 3$ -containing GABA_A receptors.

Lastly, an independent study in rats showed that the $\alpha 1$ -selective (but not $\alpha 1$ -specific) antagonist β -CCT did not reverse the SIH-blocking effects of diazepam and zolpidem (an $\alpha 1$ -selective agonist) while it antagonized the hypothermic effect of these compounds (Vinkers et al., 2009). This finding is consistent with the interpretation that the SIH-blocking effects of diazepam and zolpidem are not mediated by $\alpha 1$ -containing GABA_A receptors while hypothermia is mediated by these receptors (Vinkers et al., 2009). Moreover, TP003 dose-dependently attenuated SIH (in contrast to the study by Dias et al. with mice (Dias et al., 2005)) and locomotor responses in rats, providing additional evidence for a role of $\alpha 2$ - or $\alpha 3$ -containing GABA_A receptors in attenuating SIH (Vinkers et al., 2009).

As mentioned previously in the Introduction of this review, tolerance and physical dependence are drawbacks of benzodiazepines. A study with point-mutated knock-in mice showed that concomitant activation of $\alpha 1$ - and $\alpha 5$ -containing GABA_A receptors is required for the development of tolerance to the sedative effect of diazepam (van Rijnsoever et al., 2004). Tolerance also develops to the anti-hyperalgesic action of diazepam but not to the antihyperalgesic action of L-838,417, an $\alpha 2/\alpha 3/\alpha 5$ partial agonist and $\alpha 1$ antagonist (Knabl et al., 2008), which might point to a role for $\alpha 1$ in tolerance development. To our knowledge, the contribution of individual GABA_A receptor subtypes to the development of tolerance to other pharmacological actions of diazepam such as anxiety and to withdrawal symptoms have not been studied in mutant mice.

In summary, the results from studies using the complementary genetic and pharmacologic methodological approaches have helped to shed light on the different functions subserved by individual GABA_A receptor subtypes. The overall findings from these studies strongly suggest a role for $\alpha 2$ -containing GABA_A receptors in the anxiolytic-like action of diazepam, while the potential role of $\alpha 3$ -containing GABA_A receptors underlying anxiety-like functions is on weaker footing experimentally.

3. GABA_A receptors and depression

Anxiety and depression are frequently comorbid (Kessler et al., 2005; Regier et al., 1998). Illness severity is often greater in patients with comorbid depression and anxiety and treatment responses are generally slower, or even diminished, than for those patients suffering from either disorder alone (Fava et al., 2008; Gorman, 1996). The question thus arises whether the pathogenesis of these two disorders are related. One neurotransmitter system that may be involved is the GABAergic inhibitory system, particularly since growing evidence is showing that GABAergic deficits are associated with major depression (Luscher et al., 2011), and that some select benzodiazepines (alprazolam and adinazolam) have been shown to have anti-depressant effects in humans (Amsterdam et al., 1986; Petty et al., 1995). Proton magnetic resonance spectroscopy has revealed that depressed subjects have

significantly lower GABA concentrations in the occipital cortex compared to healthy controls, a decrease that was particularly striking in subjects with melancholic depression (Sanacora et al., 2004); treatment with either fluoxetine or citalopram increased occipital GABA concentrations (Sanacora et al., 2002). Additionally, preclinical data discussed in a recent comprehensive review (Luscher et al., 2011) suggest that norepinephrine and serotonin reuptake inhibitors potentiate GABAergic neurotransmission which may be due in part to an increased synthesis of neurosteroids (see also 3.3.).

3.1. $\gamma 2$ heterozygous knockout: a mouse model for anxious depression

Luscher and colleagues examined whether heterozygous $\gamma 2$ knockout mice ($\gamma 2^{+/-}$) might be a preclinical model for anxious depression. When challenged in the forced swim test (FST), a rodent model of behavioral despair operationally defined by a subject's immobility following inability to escape, $\gamma 2^{+/-}$ mice became immobile faster and spent significantly more time immobile compared to wild types. In the novelty-suppressed feeding test (NSFT), a conflict-based test sensitive to antidepressant treatment, the latency to begin eating in a novel environment following food deprivation was substantially increased compared to wild type mice. Together, these results show that a deficit in $\gamma 2$ -containing GABA_A receptors can give rise to depression-related phenotypes (Earnheart et al., 2007).

Similar results were also observed in these behavioral tests when the $\gamma 2$ deletion was generated during prenatal development and restricted to the forebrain (EMX1-cre/ $\gamma 2^{\text{floxed}}$ /wt). In contrast, there were no behavioral differences between wild types and mice in which the $\gamma 2$ deficit was induced after postnatal day 17 (CamKII α -cre/ $\gamma 2^{\text{floxed}}$ /wt). These data demonstrate that a depression-like phenotype can be developmentally induced and regionally restricted similar to the anxiogenic-like phenotype observed in these same mice (Earnheart et al., 2007).

As a follow up to these findings, Luscher and collaborators then asked whether the behavioral deficits in their $\gamma 2$ mutant mice could be reversed by the tricyclic antidepressant desipramine and the selective serotonin reuptake inhibitor fluoxetine, two drugs used to treat depression in humans. Even though neither of these drugs directly targets the GABAergic system (i.e. acting via GABA_A or GABA_B receptors), strikingly, chronic treatment with each of these antidepressants resulted in normalizing the behaviors of the $\gamma 2^{+/-}$ mice to wild type levels in a variety of depression-related tasks. For example, administration of both desipramine and fluoxetine restored the latency to begin eating in the NSFT to wild type levels (Shen et al., 2010). Chronic desipramine treatment led to a normalization of the duration of immobile behavior in the FST and tail suspension test (TST) whereas chronic fluoxetine did not have such an effect (Shen et al., 2010). The $\gamma 2^{+/-}$ mouse model indicates that a GABAergic deficit is sufficient to elicit phenotypic changes reminiscent of anxiety and depression in mice. It is currently unknown whether a $\gamma 2$ -deficit in humans is sufficient to cause anxiety and depression. If this can be proven, $\gamma 2^{+/-}$ mice might represent an "etiological" model of anxiety and depression. Currently, these mice can be considered a clear "pathophysiological" model of anxiety and depression.

3.2. Developmentally-regulated GABAergic deficits contribute to depression-related behaviors in adulthood

Using their conditional $\gamma 2$ knockout lines described above, Luscher and collaborators then investigated whether the depression-related behaviors in $\gamma 2^{+/-}$ mice are related to the reduction of GABA_A receptors in forebrain neurons during development. Strikingly, the embryonic reduction of $\gamma 2$ in EMX1-cre/ $\gamma 2^{\text{floxed}}$ /wt also generates a depressive-like phenotype in these animals (i.e., FST and NSFT), while reduction of $\gamma 2$ after the second postnatal week in the CamKII α -cre/ $\gamma 2^{\text{floxed}}$ /wt mice did not have an effect (Earnheart et

al., 2007). These results suggest that increased expression of behavioral despair and increased sensitivity to novelty in a conflict-based task in adulthood has developmental origins. Moreover, reduced adult hippocampal neurogenesis, specifically impaired differentiation, maturation, or survival of adult-born hippocampal neurons, was observed in both $\gamma 2^{+/-}$ and EMX1-cre/ $\gamma 2^{\text{floxed/wt}}$ mice but not in CamKII α -cre/ $\gamma 2^{\text{floxed/wt}}$ mice (Earnheart et al., 2007). Thus, it is possible that these differences in hippocampal neurogenesis might be an essential factor underlying the different behavioral phenotypes of these individual global and conditional $\gamma 2$ knockout mice.

In addition to a variety of behavioral features, depression in humans is also associated with hyperactivity of the hypothalamic-pituitary-adrenal (HPA) axis as reflected by increased serum glucocorticoid levels. Corticosterone levels in all three $\gamma 2$ mouse lines (i.e. global $\gamma 2^{+/-}$, EMX1-cre/ $\gamma 2^{\text{floxed/wt}}$, CamKII α -cre/ $\gamma 2^{\text{floxed/wt}}$) were measured before and after a stressor to determine whether a similar endocrine pattern could be observed as a preclinical, physiological hallmark (Shen et al., 2010). Baseline corticosterone levels in adults from all three lines were significantly elevated compared to wild types indicating that HPA axis hyperactivity was independent of whether the $\gamma 2$ deficit was developmentally regulated. However, the developmental timing of HPA hyperactivity correlates with the presence of an anxious- and depressive-like phenotype (Shen et al., 2010).

Chronic desipramine, but not fluoxetine, treatment normalized both the elevated corticosterone levels and the behavioral phenotype in $\gamma 2^{+/-}$ mice. In humans, melancholic depression is characterized by HPA axis hyperactivity and poor responsiveness to fluoxetine (Petty et al., 1995). Based on their preclinical studies, Luscher and collaborators conclude that developmental deficits in GABAergic inhibition in the forebrain cause behavioral changes that reflect depressive symptomology and have suggested that the $\gamma 2^{+/-}$ mice may serve also as a preclinical model of melancholic depression (Shen et al., 2010).

3.3. δ -containing GABA_A receptors and postpartum depression

GABA_A receptors are allosterically modulated by endogenously produced neurosteroids such as 3 α ,5 α -tetrahydroprogesterone (3 α ,5 α -THP) and 3 β ,5 α -tetrahydroprogesterone (3 β ,5 α -THP); these are high-affinity modulators of extrasynaptic $\alpha 4\beta \delta$ -containing GABA_A receptors (as well as others) and are rapidly induced by stress. In depressed patients, 3 α ,5 α -THP plasma concentrations were decreased while 3 β , 5 α -THP plasma concentrations were increased, a disequilibrium that was normalized by fluoxetine (Romeo et al., 1998) suggesting that neurosteroid synthesis may play a potential role in the pathophysiology of depressive disorders.

During pregnancy, there is a large increase of progesterone-derived neurosteroids leading to downregulation of δ and $\gamma 2$ subunits and to a significant decrease in tonic and phasic inhibition; this decrease rebounds immediately postpartum. In the FST, postpartum δ subunit knockout mice ($\delta^{-/-}$) more rapidly became immobile and remained immobile longer compared to virgin $\delta^{-/-}$ mice and both virgin and postpartum wild types. Likewise, in a sucrose preference test, postpartum $\delta^{-/-}$ mice had a lower preference for sucrose than the other three groups. Attack latencies in a resident-intruder assay did not differ between groups but digging, burrowing, and circling, which may reflect anxiety-like behaviors, were increased in the postpartum δ knockouts (Maguire and Mody, 2008). These data, together with the fact that the δ subunit is strongly expressed in a variety of brain regions (e.g. dentate gyrus) (Fritschy and Mohler, 1995; Pirker et al., 2000), indicate that δ -containing GABA_A receptors may play a role in postpartum depression and anxiety.

3.4. Use of genetic approaches to identify specific GABA_A receptor α subunits involved in modulating depression-related behaviors

Luscher and colleagues have provided key preclinical evidence demonstrating that developmentally- and spatially-regulated GABAergic deficits are sufficient to cause a depressive-like phenotype in adult mice. However, given that the $\gamma 2$ subunit is expressed in the majority of GABA_A receptors, it remained unknown as to which GABA_A receptor, as defined by its α subunit, was responsible for these phenotypic changes; this situation is reminiscent of that seen in the identification of subunits underlying anxiety-like responses.

Due to the naturally-occurring regional expression of different GABA_A receptor subtypes (Fritschy and Mohler, 1995; Pirker et al., 2000), it was hypothesized that $\alpha 3$ -containing GABA_A receptors could be involved in depression-related behaviors given their location in brainstem monoaminergic neurons. Specifically, these receptors could mediate GABAergic inhibition of the dopaminergic (Yee et al., 2005), serotonergic, and noradrenergic systems. To test the involvement of $\alpha 3$ -containing GABA_A receptors in behavioral despair, $\alpha 3$ global knockout mice ($\alpha 3^{-/-}$) were generated (Yee et al., 2005) and tested in the FST (Fiorelli et al., 2008). These mice maintained active swimming behavior much longer and were significantly less immobile than controls throughout the test thereby exhibiting an antidepressant-like phenotype (Fiorelli et al., 2008). Given that the behavioral profile of the $\alpha 3^{-/-}$ mice is exactly the opposite of that observed in the $\gamma 2^{+/-}$ mice when challenged in the FST, it appears that $\alpha 3$ -containing GABA_A receptors are not responsible for the depressive-like phenotype of the $\gamma 2^{+/-}$ mice and that these same receptors may actually play a pro-depressant-like role *in vivo*. It is also noteworthy that the $\alpha 3^{-/-}$ mice also do not exhibit heightened anxiety-like behavior (Yee et al., 2005) indicating that the lack of $\alpha 3$ -containing GABA_A receptors is also not responsible for the anxiogenic-like phenotype of the $\gamma 2^{+/-}$ mice.

Recently, we have examined whether $\alpha 2$ -containing GABA_A receptors are key players subserving depressive-like behaviors (Vollenweider et al., 2011). $\alpha 2$ -containing GABA_A receptors are highly expressed in distinct brain areas including the amygdala, hippocampus, and nucleus accumbens (Fritschy and Mohler, 1995; Kaufmann et al., 2003; Pirker et al., 2000), anatomical structures which are thought to play a role in depression. Additionally, given its role in anxiety-related behaviors this specific GABA_A receptor subtype may serve as a link between comorbid depression and anxiety. In order to evaluate whether $\alpha 2$ -containing GABA_A receptors are involved in modulating depressive-like behavior, we generated heterozygous ($\alpha 2^{+/-}$) and homozygous ($\alpha 2^{-/-}$) $\alpha 2$ global knockout mice and examined them in the NSFT, FST and TST in comparison to wild type littermates (Vollenweider et al., 2011).

In the NSFT, $\alpha 2^{+/-}$ but not $\alpha 2^{-/-}$ mice showed an increased latency to bite, and an increased latency to sit and eat a food pellet indicating a profile of increased sensitivity to novelty (Vollenweider et al., 2011). This observation is reminiscent of the lack of a functional continuum between $\gamma 2^{-/-}$, $\gamma 2^{+/-}$ and wild type mice (see above, Section 2.1.). Although the reasons for this remain unknown, it is possible that there could be compensatory changes in the $\alpha 2^{-/-}$ mice that are not present in $\alpha 2^{+/-}$ mice that account for the phenotypic differences in this test. In the FST and TST, $\alpha 2^{-/-}$ mice were considerably more immobile than wild types, a result that was not due to baseline differences in locomotor activity (Vollenweider et al., 2011). Thus, these findings suggest a profile of increased behavioral despair in the $\alpha 2^{-/-}$ mice. Given that these mice were generated by heterozygous breedings, it is unlikely that the phenotypic differences between $\alpha 2^{-/-}$, $\alpha 2^{+/-}$ and wild type mice ($\alpha 2^{+/+}$) mice are due to environmental or parental behavior differences.

Collectively, the findings on $\alpha 2$ global knockout mice identify $\alpha 2$ -containing GABA_A receptors as a key subtype mediating antidepressant-like actions *in vivo*, but the role of $\alpha 2$ -containing GABA_A receptors in depression-like behavior should also be studied in other behavioral paradigms with applications to the human condition, such as the chronic social defeat test (Avgustinovich et al., 2005; Berton et al., 2006), before drawing further conclusions. However, the identification of a specific GABA_A receptor subtype in depressive-like behavior suggests that it may be a target for therapeutic intervention.

4. Outlook

Clinically-used benzodiazepines are non-subunit-selective allosteric modulators of GABA_A receptors and are frequently prescribed as anxiolytics but they are generally not considered to have useful antidepressant actions. Interestingly though, selected benzodiazepines, specifically alprazolam and adinazolam have been shown to have antidepressant effects in humans similar to widely prescribed antidepressants in major depressive disorder (Amsterdam et al., 1986; Petty et al., 1995). It is tempting to speculate that these benzodiazepines exert this effect at least in part via $\alpha 2$ -containing GABA_A receptors. We posit that $\alpha 2$ -selective agonists would not only serve as non-sedating anxiolytics (see above), but also potentially as fast-acting antidepressants. Additionally, $\alpha 2$ -containing GABA_A receptors in the spinal cord have also been shown to mediate antihyperalgesic actions (Knabl et al., 2008; Knabl et al., 2009); an $\alpha 2$ -selective agonist could also be suitable for the treatment of chronic pain which is frequently associated with anxiety and depressive symptoms (Dersh et al., 2002).

While classical monoamine-based antidepressants take weeks or months to exert their therapeutic effects, an $\alpha 2$ -selective agonist is predicted to have a rapid onset of antidepressant action. A fast-acting antidepressant is a much-needed treatment option that can serve to more immediately relieve severe depression, particularly during the initial phase of treatment, until monoamine-based antidepressants develop their clinical effects. An $\alpha 2$ -selective agonist could potentially be used either in combination with currently available antidepressants or as a stand-alone therapy. If further experiments confirm that $\alpha 3$ -containing GABA_A receptors have a pro-depressant-like action and $\alpha 2$ -containing GABA_A receptors have an antidepressant-like action, this would have consequences for drug design, in that an $\alpha 2$ -selective agonist would be expected to have stronger antidepressant-like actions than a combined $\alpha 2/\alpha 3$ -selective agonist. To our knowledge, it is unknown whether any of the currently existing $\alpha 2/\alpha 3$ -selective agonists have antidepressant-like actions in animals.

In summary, recent use of genetic, behavioral and pharmacological techniques has helped to clarify the general role of the GABA_A receptor system and the roles of individual GABA_A receptor subtypes in the regulation of emotions. Together, these findings provide useful directions for the development of novel therapeutic approaches for the treatment of anxiety and depression.

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Highlights of the review

- GABA_A receptors prevent anxiety- and depression-like behavior.
- Mouse genetic evidence reveals a role of the $\alpha 2$ subunit in anxiety and depression
- The experimental for an involvement of $\alpha 2$ and $\alpha 3$ subunits is discussed.

Figure 1. Developmental role of $GABA_A$ receptors in anxiety and depression

Schematic depicting the adult phenotype of $\gamma 2^{+/-}$ mice when $GABA_A$ receptors ($GABA_A$ Rs) are downregulated in the forebrain at different time points during development. (A) Downregulation beginning on embryonic day 10 caused an anxiogenic-like and pro-depressant-like phenotype along with HPA axis hyperactivity in adult animals (Earnheart et al., 2007). (B) Postnatal downregulation beginning on postnatal day 17 also caused HPA axis hyperactivity but did not impact anxiety- and depressive-like behaviors in adults (Shen et al., 2010).

Figure 2. Behavioral profiles of adult $\alpha 2$ knockout mice and their wild type littermates in a conflict-based task and tests of behavioral despair

(A) $\alpha 2^{+/-}$ mice took significantly longer to begin eating a food pellet in the Novelty Suppressed Feeding Test (NSFT) than $\alpha 2^{+/+}$ littermates. (B) $\alpha 2^{-/-}$ mice spent more time immobile in the Forced Swim Test (FST) and (C) Tail Suspension Test (TST) than $\alpha 2^{+/-}$ or $\alpha 2^{+/+}$. **p < 0.01; $\alpha 2^{+/-}$ = homozygous $\alpha 2$ knockout mice; $\alpha 2^{+/-}$ = heterozygous knockout mice; $\alpha 2^{+/+}$ = wild type mice. Figure modified from (Vollenweider et al., 2011) with permission.

Figure 3. Distribution and functions of the GABA_A receptor $\alpha 2$ subunit

Left panel: immunohistochemical distribution of the $\alpha 2$ subunit in a parasagittal brain section. Reproduced from (Mohler et al., 2002) with permission. $\alpha 2$ is expressed e.g. in hippocampus, dorsal and ventral striatum, cerebral cortex, and olfactory bulb. Right panel: $\alpha 2$ -containing GABA_A receptors are proposed to reduce anxiety and depression levels. Furthermore, spinal $\alpha 2$ -containing GABA_A receptors are proposed to reduce chronic inflammatory or neuropathic pain (Knabl et al., 2008). Thus, potentially positive allosteric modulation of $\alpha 2$ -containing GABA_A receptors could be useful for treating chronic pain comorbid with anxiety and depression.