

Basic Pharmacologic Mechanisms Involved in Benzodiazepine Tolerance and Withdrawal

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Abstract: Benzodiazepines are widely prescribed for the treatment of anxiety and sleep disorders. Although safe, tolerance develops rapidly to their sedative activity and more slowly to their anticonvulsant activity. In animals anxiolytic tolerance has also been measured. Abrupt cessation of benzodiazepine treatment leads to symptoms of withdrawal. The mechanisms responsible for these phenomena are not known. Benzodiazepines act via GABA_A receptors, but do not appear to produce tolerance and dependence by simple downregulation of receptor number. GABA_A receptors are hetero-oligomers comprised of multiple subunits encoded by a multigene family. The molecular effects of long-term benzodiazepine exposure are reviewed and a model is presented that draws on results from a number of research groups working in this area.

INTRODUCTION

The benzodiazepines are widely prescribed for the treatment of anxiety and sleep disorders. These agents are extremely safe, but tolerance develops rapidly to their sedative activity, while their potent anticonvulsant effects are compromised within one to two months such that they are not used for the prophylactic treatment of epilepsy. In animal models tolerance to their anxiolytic activity can also be measured. On abrupt withdrawal from benzodiazepine exposure, patients can experience a number of symptoms indicative of a dependent state and physical aspects of the withdrawal phenomena can be reproduced in animals. The benzodiazepines produce their therapeutic effects by interaction with GABA_A receptors, but it appears that tolerance and dependence are not due to a simple downregulation of these receptors.

GABA_A receptors are heteromeric proteins comprised of related subunits encoded by a large gene family. The pharmacological characteristics of particular GABA_A receptor subtypes are dependent on their subunit composition. Long term exposure to benzodiazepines causes differential changes in the expression of GABA_A receptor genes. Our working hypothesis is that tolerance to the effects of chronic treatment with benzodiazepines is associated with the expression of aberrant GABA_A receptors in the mammalian brain. Likewise, withdrawal phenomena manifest when inappropriate GABA_A receptors have to function in the absence of drug following abrupt cessation of treatment. Evidence indicates that the regulation of GABA_A receptor expression is complex and thus seems likely to be governed by multiple mechanisms. In this article I will present our current model and briefly review the relevant literature describing work both from our laboratory and that of others.

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THE BENZODIAZEPINES

Benzodiazepines are the therapeutic agents most often used in the treatment of generalized anxiety disorder and insomnia, and they also find utility in the treatment of panic disorder [1-3]. These conditions have a significant lifetime prevalence in all developed countries [4,5]. The economic burden to society of anxiety disorders is significant: in the USA it has been estimated that anxiety costs society over \$40 billion/year [6,7] which is more than any other mental disorder including schizophrenia [7]. Given the chronic nature of many of the conditions for which benzodiazepines are prescribed, coupled with the findings that tolerance [8-10] and physical dependence [5,9,11] develop upon long-term exposure, there has been much debate, in both the scientific literature and the lay media, as to whether their long-term use should be severely limited [2,3,10,12-21].

Discovery and Introduction into the Clinic

In the mid 1950's Leo Sternbach and his colleagues at Roche embarked upon a chemical synthesis and pharmacological screening program to identify novel tranquilizers [22,23]. Intending to explore the benzheptoxdiazine class of molecules and various derivatives, Sternbach's group serendipitously synthesised a 1,4-benzodiazepine [22-24]. The first of these compounds, chlordiazepoxide, proved to be pharmacologically interesting. In preliminary screening it showed anticonvulsant, muscle relaxant and "taming" effects in mice and monkeys, combined with low toxicity. Two and a half years later, in 1960, chlordiazepoxide (Librium) was introduced into clinical use heralding a new age in the pharmacotherapy of anxiety disorders [22,23]. After another three years, a more potent benzodiazepine, diazepam (Valium), was introduced [23] and the benzodiazepines were found to be effective and safe, thereby replacing the barbiturates which had previously been widely used as the first line treatment for anxiety disorders [8].

Following on from the development of Librium and Valium a number of other 1,4-benzodiazepines and their derivatives were synthesised and pharmacologically evaluated, some of which found their way into the pharmaceutical armamentarium while others found utility as research tools. Most of these compounds were agonists, but some, most notably flumazenil (Ro 15-1788), antagonised the actions of the classical benzodiazepines [25]. Other compounds were also identified that, while not being 1,4-benzodiazepines or derivatives thereof, clearly interacted with the benzodiazepine site. Some of these were also agonists, such as zopiclone [26] and zolpidem [27], and have been introduced into clinical use.

Clinical and Behavioural Properties of the Benzodiazepines

The classical benzodiazepines display a wide variety of behavioural effects in experimental animals and in man. They can act as anticonvulsants, sedatives/hypnotics, anxiolytics and muscle relaxants, they produce anterograde amnesia and are used as anaesthesia adjuncts or pre-anaesthetics [28]. Differences in their pharmacokinetic profiles have largely determined whether a particular benzodiazepine is prescribed for the treatment of sleep or anxiety disorders.

In addition to distinct pharmacokinetic properties, it has become apparent that different benzodiazepine-site ligands display differing efficacies. The first benzodiazepines on the market were essentially full agonists which displayed the complete spectrum of pharmacological properties in a dose dependent manner. Higher doses were required for the sedative, hypnotic and myorelaxant properties whereas lower doses only produced anticonvulsion and anxiolysis [8]. The discovery of a variety of benzodiazepine-site ligands that differ chemically from the benzodiazepines and which display a range of efficacies allowed correlations to be made between *in vivo* receptor binding (a measure of receptor occupancy) and behavioural properties. For example, Haefely and colleagues analysed a number of benzodiazepine-site ligands to determine the relationship between fractional receptor occupancy with their ability to potentiate GABA-stimulated chloride flux [29] in order to determine the influence of the former property on their effects in various animal behavioural tests [30]. They found that low doses of the full agonist diazepam achieved low receptor occupancy [29] and anxiolysis [30]; high doses gave rise to full receptor occupancy [29] and sedation [30]. In contrast, partial agonists, such as the bretazenil [30], were anxiolytic but did not produce sedative effects even at full receptor occupancy [30].

The benzodiazepines produce a number of side-effects. Interestingly, the side-effect profile of the acute effects of benzodiazepines varies depending upon the specific therapeutic goal [17]. Thus, acute sedative effects are beneficial when treating sleep disorders but are viewed as a side effect to be avoided when benzodiazepines are prescribed as anxiolytics. It is therefore sensible to consider the acute side-effect profile in terms of the clinical goals. Many of the problems of long-term benzodiazepine treatment are common

to a number of therapeutic applications and will therefore be considered separately.

Pharmacotherapy of sleep disorders normally requires the shortening of sleep latency without sedative effects the following day, which would have a deleterious affect on the normal day-to-day functioning of the patient [31]. Thus, a full agonist benzodiazepine-site ligand with a rapid onset of action, to induce sleep quickly, and a short half-life, to limit effects the next day, are the preferred therapeutic. Consideration of the pharmacodynamic and pharmacokinetic properties of metabolites is also necessary, however, as some metabolites have long half-lives and are active, sharing pharmacodynamic properties with their parent compounds [32]. Respiratory depression can be a problem, particularly in patients with chronic obstructive airways disease [28,33].

Anxiety disorders are often treated with benzodiazepines [1,34] which provide rapid relief from symptoms and are well tolerated compared to many other anxiolytic agents, thereby obviating problems with compliance [17]. Treatment of generalised anxiety disorder is normally achieved with benzodiazepine ligands that have long half-lives in order to maintain fairly constant receptor occupancy throughout the day. Sedation is not normally a desired outcome and therefore transient high levels of drug are avoided. Panic disorder can also be treated with benzodiazepines but with higher doses than those used for generalised anxiety disorder [35,36]. Alprazolam is often used to treat panic disorder and has been shown to have a faster therapeutic onset than non-benzodiazepine therapeutics such as mono-amine oxidase inhibitors and selective serotonin reuptake inhibitors [37-39]. Although benzodiazepines have given some ground in recent years to the serotonin selective reuptake inhibitors as the first line choice of medication for treatment of panic disorder, they still form the preferred treatment for generalised anxiety disorder and simple phobia [3].

Benzodiazepines are powerful anticonvulsants, but prophylactic treatment of epileptic disorders is generally contraindicated because of tolerance development to their anticonvulsant effects [40]. Recent studies suggest, however, that intermittent use for the control of breakthrough seizures does not result in tolerance development [41]. Benzodiazepines are used extensively in the acute control of status epilepticus [40,42,43]. They also find utility as fall-back medication, in particular the 1,5-benzodiazepine clobazam, for patients that appear refractory to other treatment regimes [41,42,44-46]. Acute febrile convulsions are effectively terminated by benzodiazepines such as diazepam [47] but it is not clear whether repeated treatment diminishes the recurrence of seizures [47-49]. Lorazepam has also been found to be useful for the treatment of alcohol abuse induced seizures [50,51].

While benzodiazepines by themselves do not induce anaesthesia they are often used as adjuncts via premedication [52-55]. The spectrum of pharmacological properties they exhibit are well suited for this purpose in that muscle relaxation, sedation and the reduction of acute anxiety all assist in preparing the patient for surgical procedures: in addition appropriate formulations for intravenous delivery are available [54]. The anterograde amnesic properties of the

benzodiazepines are also desirable in this context, decreasing the likelihood of patients remembering events associated with surgery thereby reducing anticipatory anxiety should further surgical procedures be required. Midazolam, with its short duration of action, tends to be the preferred benzodiazepine [53]. Benzodiazepines are also widely used to induce conscious sedation (often in combination with an opioid analgesic) for procedures that do not require full anesthesia such as endoscopy [56] or oral surgery [57-59]. While benzodiazepines with short half-lives tend to be preferred for use as adjuncts, in order to minimise post-operative effects such as sedation, the introduction of the benzodiazepine antagonist flumazenil into the clinic [25,60] gave the anaesthetist the ability to accurately and rapidly control the arousal time either intra- or post-operatively [61,62] and to rapidly terminate paradoxical reactions of benzodiazepine agonists [63]. Flumazenil has a relatively short half-life, however, and therefore recurrent sedation may occur if it is used to speed recovery from the action of the longer half-life benzodiazepines such as diazepam.

The myorelaxant properties of the benzodiazepines have been known for a long time although the mechanism is not absolutely clear. Alterations in motoneuron presynaptic inhibition in the spinal cord [64,65] have long been thought to be behind their relaxant properties but other mechanisms have been suggested [66]. Diazepam is used to treat spasticity [64,67,68] and stiff-man syndrome [69,70].

The abuse liability of benzodiazepines is well recognised, from the date-rape drug Rohypnol (flunitrazepam), also known as roofies [71], to the use of benzodiazepines among opioid abusers [72-74], although the degree of the abuse problem is still a matter of some controversy [14-16,20,21]. Overdose with benzodiazepines is also a significant problem, and not just in the western hemisphere [75,76]. Flumazenil has a special role not only as a tool for arousal control during anaesthesia but also in the emergency room to negate the pharmacological effects in benzodiazepine agonist poisoning [77,78]. The problem, in this context, of the short half-life of flumazenil has led some emergency room and intensive care unit physicians to use continuous infusion of flumazenil for treatment of benzodiazepine overdose [79-81].

Tolerance and Dependence

The long-term use of benzodiazepines is a widely accepted fact [5,16,20] but whether this is appropriate is disputed [14-16,20,21]. Patients do not appear to escalate their doses [20,21], but long term users of benzodiazepines can suffer from a number of problems including cognitive deficits evinced by poor performance in motor coordination and memory problems, even sometime after the drug has been discontinued [52,82]. By far the most widely reported problems of long-term benzodiazepine usage, however, lie in the development of tolerance and dependence [8,10,83]. Tolerance is defined as a decrease over time in the ability of the drug to produce the same degree of pharmacological effect. Dependence, sometimes referred to as physical dependence, is the state produced by continued drug exposure such that removal of the drug causes a discontinuation syndrome which can be reversed if the drug

is readministered. It has been noted that a small proportion of patients may be at greater risk than the general population for developing dependence [10,84-87].

Tolerance to the sedative effects of classical benzodiazepines has been widely reported in both animals [9,88,89] and man [90,91] with those of shorter duration of action being regarded as more likely to produce this effect [90,91]. In addition to the potential for dependence [92,93], patients treated for insomnia with the benzodiazepines sometimes experience rebound insomnia, on abrupt discontinuation of the medication, to a degree that is worse than the initial symptoms [90].

Tolerance to the anxiolytic effects has been shown in various animal studies [94,95], although not by all authors [9,96], and when detected it appears to occur at a slower rate than that of sedative tolerance [88]. Further, it has been difficult to demonstrate tolerance to the anxiolytic [8] or anti-panic effects of benzodiazepines [97,98] in man. In fact, when treated for generalised anxiety disorder patients develop tolerance to the sedative effects without there being a reported decrease in anxiolytic efficacy [8]. This may not be the case, however, for high potency benzodiazepines such as alprazolam [99,100]. The development of dependence is a serious concern amongst clinicians treating patients for anxiety disorders, particularly as long-term benzodiazepine consumption (months to years) in these individuals is not uncommon [5,8,20,28]. Dependence manifests upon benzodiazepine discontinuation and symptoms can include rebound anxiety. The treatment of benzodiazepine dependence has been attempted by psychological and pharmacological strategies [10,86,101].

One of the most important facets of tolerance development is the differential timescale of its development. Tolerance to the sedative effects of benzodiazepines occurs more rapidly than to the anticonvulsant effects, with the anxiolytic effects in animals at least, occurring after an even longer time period [8,9,88]. These observations suggest that different benzodiazepine targets or different signalling processes may be involved in the development of tolerance to each of these behavioural parameters. Further, some groups have managed to develop treatment regimes in experimental animals in which anticonvulsant tolerance develops in the absence of any withdrawal phenomena on cessation of treatment [102,103]. The separation of these phenomena suggest that they may also manifest via different pathways.

Site and Mechanism of Action

Although the benzodiazepines were introduced into the clinic in 1960, it was not readily apparent how or where they acted. Largely ignored at the time, it was reported in 1967 that diazepam potentiated presynaptic inhibition in spinal cord as measured by an increase in the dorsal root potential [65] where GABA was later shown to be the active neurotransmitter [104]. Haefely and colleagues used electrophysiological means to demonstrate that benzodiazepines facilitate GABA-mediated transmission in the CNS, although at this time it was not clear whether they

acted via a pre- or post-synaptic mechanism [105-107]. Using cultured neurons it was demonstrated that the benzodiazepines potentiated the actions of GABA, shifting the GABA-dose response curve to the left without altering the maximum response obtainable [108,109]. This allosteric interaction between the GABA and benzodiazepine binding sites forms the basis of the so-called GABA-shift, in which *in vitro* binding of radiolabelled benzodiazepine-site ligands is potentiated by GABA or muscimol [110-112].

Specific high affinity binding sites for diazepam were demonstrated in mammalian brain and the affinity of a series of benzodiazepines for these sites was found to correlate with their therapeutic efficacy. This led to the conclusion that these binding sites were the pharmacological target through which the drugs produced their effects [113,114]. GABA or GABA-site agonists were found to increase benzodiazepine binding in a bicuculline sensitive manner, suggesting that the effects were mediated via the GABA_A receptor [110-112]. Likewise, GABA binding was increased in the presence of benzodiazepines [115] and benzodiazepine binding sites showed a pattern of distribution in the CNS that most closely matched those of the GABA_A receptor as determined with radiolabelled muscimol, a GABA_A receptor-specific ligand [116,117]. Subsequent studies found that the benzodiazepine receptor co-purified with the GABA_A receptor and that the benzodiazepine binding site is a modulatory site on the GABA_A receptor allosterically coupled to the GABA-agonist site [118,119].

Most significantly, occupation of the benzodiazepine site in the absence of GABA does not cause activation of the receptor. Benzodiazepines were found to act by primarily increasing the frequency of the GABA-gated chloride channel opening in the presence of GABA [120] in contrast to the barbiturates which, at low concentrations enhance GABA action by increasing the open channel lifetime [120]. At high concentrations barbiturates directly gate the channel in the absence of GABA [121]. This is the main reason why the benzodiazepines have superseded the barbiturates as anxiolytics of choice as the latter can directly activate the GABA_A receptor at high doses rendering them potentially dangerous.

THE GABA_A RECEPTOR

-Aminobutyric acid (GABA) is the major inhibitory neurotransmitter in the central nervous system. It localises to 30% of synapses [122] and largely mediates its effects via the GABA_A receptor [123]. GABA_A receptors are fast-acting neurotransmitter receptors with an integral Cl⁻ channel and a number of allosteric binding sites [124,125]. Clinically, the most important of these is the benzodiazepine site [8,126], as compounds that act there are widely used in the treatment of a variety of neurological and psychiatric disorders [127]. Other allosteric modulatory sites on the receptor include those for barbiturates, steroids and channel blockers, such as picrotoxin [124,125,128]. Ethanol has also been shown to modulate GABA_A receptor function [129-133]. The rich pharmacology of the GABA_A receptor, coupled with it being a target for therapeutic agents, has made it the subject of intense research activity.

Multiplicity of Subunits

A large family of GABA_A receptor genes has been revealed by classical recombinant DNA cloning studies and more recently additional subunits have been identified as a spin-off from the project to sequence the human genome. Subunits are grouped by amino-acid sequence identity into several classes, α , β , γ , δ , ϵ , ζ , and θ , with up to six isoforms in a class [124,134-138]. Many of these subunits are present as orthologues in a variety of vertebrate species [139,140] and, with the nicotinic acetylcholine, glycine and 5HT₃ receptor subunits, they form a ligand-gated ion channel superfamily [141-143]. Further, alternate splice variants of some subunits exist [139,144-148]. Interestingly, GABA_A receptor subunit genes appear to be localised to a small number of discrete gene clusters in the mammalian genome [136,149] and some authors have suggested that a degree of coordinate gene regulation may exist within individual clusters [136,150-152].

Heterogeneity of Benzodiazepine Sites

Initial studies suggested that benzodiazepine binding sites are homogeneous throughout the mammalian CNS [113,114] but the triazolopyridazine CL218872 was found to exhibit a higher affinity for the benzodiazepine recognition sites in the cerebellum than those in the hippocampus [153]. This led to the classification of benzodiazepine sites as either BZI and BZII, both of which display the same affinity for the classical benzodiazepines (such as diazepam) but with CL218872 exhibiting a higher affinity for BZI over the BZII sites.

Another group of ligands, the α -carboline-3-carboxylic acid esters, that interact with the benzodiazepine site but are of a different structural class, were subsequently shown to also be able to distinguish between BZI and BZII receptors [154]. Interestingly, however, the ethyl ester (α -CCE) was found to possess convulsant and anxiogenic properties, characteristics that are opposite to the behavioural effects of the classical benzodiazepines which are anticonvulsant and anxiolytic [155-157]. Around the same time a selective benzodiazepine antagonist was discovered. Ro 15-1788 (flumazenil) was found to inhibit specific high-affinity [³H]-diazepam binding in rat brain membrane preparations, as well as various behavioural and electrophysiological effects of classical benzodiazepines [25]. It is now apparent that there are essentially three classes of ligand for the benzodiazepine receptor: positive modulators (agonists), which are anticonvulsant and anxiolytic; negative modulators (inverse agonists), which are convulsant and anxiogenic, and neutral modulators (antagonists), which produce no effects alone but are able to block the effects of the positive and negative modulators [25,158-160]. The older terminology that utilises the terms agonists and antagonists to describe the actions of the benzodiazepines is falling into disfavour because the benzodiazepines do not possess any intrinsic efficacy in the absence of GABA and should therefore be solely regarded as modulators. Compounds are now available which span the complete efficacy spectrum from full positive to full negative modulators. The situation, however, has become even more complex with the discovery that GABA_A receptors comprise a receptor family and that the

pharmacology of a particular benzodiazepine-site ligand, i.e. the efficacy of a benzodiazepine modulator is exquisitely dependent upon the receptor's subunit composition.

Subunit Determinance of Pharmacology

The identification of a large gene family encoding GABA_A receptor subunits has revealed a molecular substrate for benzodiazepine binding site heterogeneity. Further, it is now well established that the pentameric receptor can be formed by the combination of multiple subunits into a large variety of hetero-oligomers. *In vitro* expression studies, primarily using *Xenopus* oocytes or HEK293 cell expression systems, have revealed that the pharmacological properties, including those of the benzodiazepine site, are exquisitely dependent upon the subunit composition of a given receptor [124,125,128,135-137].

A molecular correspondence has been demonstrated with the early pharmacological classification of BZI and BZII receptors. In the presence of a α_1 and a β_2 subunit, $\alpha_1\beta_2$ subunits generate a BZI phenotype while the α_2 , α_3 or α_5 subunits produce that of BZII [161-163]. In contrast, the α_4 and α_6 subunits combine with a β_1 and a β_2 subunit to produce receptors that are insensitive to classical benzodiazepines such as diazepam but still recognise Ro15-4513 [164,165]. The three β -subunit isoforms confer differing functional properties [135,137]. Thus, benzodiazepine ligands with high affinity for β_2 -subunit-containing receptors often have an increased efficacy for potentiation of GABA-gated currents [166-169]. Receptors containing the β_1 subunit are not recognised by the neutral modulator flumazenil (Ro15-1788) or Ro15-4513 but those in which β_2 is replaced by β_3 have a reduced affinity for classical benzodiazepine positive modulators, though little change is seen for the neutral modulators [166,170-174]. Further, methyl α -carboline-3-carboxylate is a negative modulator on $\alpha_2\beta_1\beta_2$, $\alpha_3\beta_1\beta_2$ and $\alpha_2\beta_1\beta_1$ combinations, but a positive modulator on an $\alpha_3\beta_1\beta_1$ combination, indicating differential cooperative interactions between α and β subunits. Thus, the particular α - and β -subunit isoforms present largely determine the binding and functional properties associated with the benzodiazepine site. Finally, the α_6 subunit combines with β_1 and β_2 subunits *in vivo* to form functional GABA receptors that do not recognise benzodiazepines [175].

Receptor Subtypes

Structural studies on members of this ligand-gated ion channels superfamily, including the GABA_A receptors, indicate that they exist as pentamers of hetero-oligomers arranged in a pseudosymmetrical array surrounding an integral ion channel pore. Given this pentameric structure [170,176-178], the number of possible subunit combinations that could be formed *in vivo* is over 500,000 [135]. Evidence exists, however, for only a small fraction of this number [135-137,179-181]. It is clear that most GABA_A receptors, in particular those with associated benzodiazepine sites, comprise α_1 , α_2 and β_1 subunits [136,137,179-181].

In vitro expression of recombinant receptors suggests that this plethora of receptor subtypes is not redundant. Subunit

composition influences both the affinity and the efficacy of agents that act on the GABA_A receptor [124,125,135,137]. Different GABA_A receptor subunit combinations expressed in heterologous cells display distinct GABA EC₅₀ values (e.g. [182,183]). Functionally distinct native receptor subtypes have now been identified in the hippocampus [182,184-186], dorsal root ganglia [187] and cerebellum [186,188]. Thus, the functional differences seen *in vitro* likely reflect *in vivo* differences. Interestingly, it has been proposed recently that some GABA_A receptors are extrasynaptic and are believed to be responsible for tonic inhibition resulting from GABA that leaks out from the synapse where phasic inhibition predominates [189-196]. In granule cells of the cerebellum it appears that α_6 and β_2 subunits may play major roles in this tonic inhibition [194].

Most recently the *in vivo* pharmacological role of some of the β subunits has been elucidated in more detail. Möhler and colleagues [197] introduced a single point mutation into the native murine β_1 -subunit gene, rendering that subunit insensitive to diazepam but preserving its responses to GABA. These mice demonstrated altered behavioral responses to diazepam indicating that the β_1 subunit is essential for the benzodiazepine-induced sedation, amnesia and, to a lesser extent, seizure protection. Further studies by the same group utilising the same approach indicate that the β_2 subunit plays a major role in mediating the anxiolytic effects of benzodiazepines [198].

Consequences of Heterogeneity on Benzodiazepine Classification

It has already discussed above how the terminology of benzodiazepine-site ligands has moved away from one based on the terms of agonist and antagonist. The traditional classification of benzodiazepines has further been compromised by the discovery that different benzodiazepine-site ligands display differing efficacy at different GABA_A receptor subtypes. For example, methyl α -carboline-3-carboxylate can be a positive or a negative modulator depending upon which α or β subunits are present in the receptor complex [124,169]. Further, the classically defined antagonist flumazenil (Ro 15-1788) and partial agonist Ro 15-4513 have both been shown to be positive allosteric modulators at GABA_A receptors containing the β_6 subunit [199]. It therefore becomes important that benzodiazepine-site ligands be described by the appropriate terms, which should, when talking about their efficacy, include reference to the receptor subtype being examined wherever possible. In whole animal studies, however, where multiple receptor subtypes are present, researchers need to be aware that compounds like flumazenil, traditionally regarded as an antagonist at all receptor subtypes, actually has partial positive modulatory activity at certain receptor subtypes [199], actions which may explain some of the behavioural effects of such ligands.

MOLECULAR EFFECTS OF LONG-TERM BENZODIAZEPINE EXPOSURE

An explanation for the development of tolerance and physical dependence has been sought for a number of decades

(see [9]). Down-regulation in the number GABA_A receptors as consequence of prolonged exposure to benzodiazepine positive modulators is an obvious potential mechanism for the development of tolerance. Following termination of the drug treatment the reduced receptor levels could underlie the observed withdrawal phenomena thereby providing mechanisms to explain both tolerance and dependence. Studies aimed at testing this hypothesis by radioligand binding have met with mixed success. CNS benzodiazepine binding sites have been reported to be either unchanged [93,200-206,213] or decreased [89,207-209] in response to chronic benzodiazepine agonist treatment, although some of these studies used very high drug doses [208,209]. These studies were primarily conducted prior to the cloning studies that revealed the large GABA_A receptor gene family present in mammals. They therefore suffer from the drawbacks of using blunt pharmacological tools, i.e. radioligands with limited or unclear GABA_A receptor subtype specificity, to assess potential changes in a highly heterogeneous receptor population.

There is increasing experimental evidence, however, for a number of molecular processes being invoked by long-term exposure of animals or cultured cells to benzodiazepine-site ligands. These include uncoupling of the allosteric linkage between the GABA and benzodiazepine sites, changes in receptor subunit turnover, and changes in receptor gene expression. This has led to competing hypotheses as to which underlies the clinical phenomena of tolerance and dependence. Examples of the evidence for each of the main categories of molecular events are presented below.

Uncoupling

A number of early studies provided evidence to suggest that following chronic benzodiazepine administration to experimental animals the ability of benzodiazepine agonists to potentiate the action of GABA was diminished concomitant to a decrease in the ability of GABA to potentiate the binding of benzodiazepine-site ligands (GABA-shift). Chronic treatment of rats with diazepam caused a loss in the ability of benzodiazepines to potentiate GABA-stimulated chloride flux [210] or GABA to potentiate benzodiazepine radioligand binding [201] in cortex. Gallager's group went on to examine the effects of ligands that display different efficacy at the benzodiazepine site and found that the classical antagonist Ro 15-1788 produced no uncoupling following chronic administration, with other ligands producing degrees of uncoupling that correlated with their efficacy [211]. Further, and most importantly, they found that those ligands with the greatest potential for anticonvulsant tolerance produced the most uncoupling [211]. This correlation provided a sound hypothesis for the involvement of receptor uncoupling in tolerance and dependence.

Further evidence in support of this notion was produced from a study that found regional differences in uncoupling in rat brain following 4 weeks of flurazepam treatment [212]. Most importantly, they discovered that the allosteric coupling between the benzodiazepine and GABA recognition sites was compromised immediately after the 4 week

treatment period, but had returned to normal 2 days later. There was also no effect of acute treatment on GABA-shift as measured in rats given diazepam 30 minutes prior to sacrifice. These experiments indicated that uncoupling *in vivo* was a chronic effect. Moreover, the ability of this biochemical phenomenon to recover over a similar time-course to that of the recovery from tolerance and dependence supported the notion that uncoupling could underlie the development of these phenomena. The key question at this stage was whether uncoupling was due to changes in the expression of GABA_A receptors or changes in receptor function that did not involve altering subunit composition.

Primary cultured neurons have proven an effective tool for the analysis of the pharmacological properties of GABA_A receptors. Early studies on the effects of chronic treatment of cultured chick cortical neurons with benzodiazepine-site ligands demonstrated that flurazepam caused uncoupling of the benzodiazepine and GABA sites with a $t_{1/2}$ of about 18 hours [213]. This was notable as its kinetics were equivalent to the average of the fast (3.8 hours) and slow (32 hours) rates of GABA_A receptor turnover [214]. Uncoupling was also reported in similar paradigms using mouse [215,216] or chicken [217] cortical neurons, or rat cerebellar granule neurons [218]. No changes were found in the total number of benzodiazepine [213,215] or muscimol binding [218] sites. Thus, although the timescale was much more rapid than that seen in animals, the neuronal cultures appeared to represent a good model of the *in vivo* situation. Two laboratories showed that concomitant treatment with GABA-site antagonists did not prevent uncoupling, suggesting that receptor activation was not required [213,216], in contrast to another study in which flurazepam-induced uncoupling in chick cortical neurons was attenuated with bicuculline [217].

In order to isolate the post-transcriptional effects on GABA_A receptor uncoupling, a number of groups examined the effects of prolonged benzodiazepine exposure on the allosteric linkage between the GABA and benzodiazepine binding sites in cells that permanently express GABA_A receptor subunit cDNAs under the control of a heterologous promoters [219-222].

Harris and colleagues [219,220] found that exposure of cells expressing 1, 1- and 2L-subunit cDNAs to clonazepam or flunitrazepam caused a time- and dose-dependent decrease in the ability of GABA to potentiate benzodiazepine binding. For clonazepam, they determined the $t_{1/2}$ of the uncoupling to be 32 min, with an estimated $t_{1/2}$ for the recovery following washout of about 2 hours. Interestingly, they demonstrated that following 2 hours of clonazepam treatment a 10 min exposure to the antagonist Ro 15-1788 partially reversed the uncoupling. They provided evidence to suggest that neither phosphorylation nor receptor internalisation were involved, and showed that the effect was lost when receptors were partially purified, suggesting a role for interacting lipids or intracellular proteins. Klein et al. (1994) argued that since the expression of these receptors is under the control of a heterologous promoter the mechanism responsible for GABA-benzodiazepine site uncoupling is post-transcriptional and that this rules out a role for subunit switching [219]. While the former premise is sound the latter is brought into

question by their own observation that α -subunit protein levels decreased in response to flunitrazepam treatment, suggesting that changes in stoichiometry were possible, although the drug was applied to the cells for 4 days [220]. These authors further reasoned that GABA_A receptor turnover is slow and therefore not likely involved [219]. In support of this, however, they cited Borden and Farb [223] as determining the $t_{1/2}$ to be 18 hours. In fact, in that report Borden and Farb [223] confirmed the findings of their earlier study [214] that showed approximately 50% of receptors had a $t_{1/2}$ of 3.8 hours while the remainder underwent a slower turnover that had a $t_{1/2}$ of 32 hours. This highlights one of the problems with the study of Harris and colleagues [219,220] in that their treatment regimes were 2 hours for clonazepam but 4 days for flunitrazepam and flurazepam, making comparison of the effects of these benzodiazepine-site positive allosteric modulators difficult. The $t_{1/2}$ for flunitrazepam- and flurazepam-induced effects were not reported nor was it noted whether clonazepam treatment altered protein or mRNA levels. Consideration of other studies performed in neurons (see, for example, the studies from the Bristow laboratory discussed below) suggests that exposing cells to benzodiazepine-site ligands for days may produce multiple effects.

Skolnick's group used an engineered cell line that expresses 1, 2- and 2-subunit cDNAs [222] and reported essentially similar findings to those of Harris and colleagues [219,220]. They found, however, that the $t_{1/2}$ of the flurazepam-induced uncoupling to be 3 hours, which although still rapid, is considerably longer than that reported for clonazepam [219]. Interestingly, they also found a decrease in GABA-shift when these cells were chronically exposed to the classical benzodiazepine-site antagonist Ro 15-1788 or to the inverse agonist α -carboline DMCM.

Using a different expression system Gallagher and colleagues demonstrated that uncoupling occurs for other GABA_A receptor subtypes [221]. Expressing GABA_A receptor subunit cDNAs from baculovirus vectors in the insect cell line Sf9 results in large amounts of receptor protein in the plasma membrane that displays appropriate pharmacological properties [224]. These authors examined the effects of exposure to various benzodiazepine-site ligands on 1/2/2, 2/2/2 and 5/2/2 subunit combinations [221]. No effect was seen following 1 hour exposure to benzodiazepine positive allosteric modulators but uncoupling between the GABA and benzodiazepine sites was found following 24 hours exposure with an increase in this effect after 60 hours exposure. In contrast to Wong and colleagues [222] they found no effect of chronic treatment with Ro 15-1788. Further, ligands of lower efficacy than diazepam produced a smaller degree of uncoupling, and down regulation of total receptor number or receptor internalisation did not appear to occur. They also found that a 30 min exposure to Ro 15-1788 reversed the uncoupling induced by chronic diazepam exposure.

The findings of these various *in vivo* and *in vitro* studies on GABA_A receptor uncoupling are highly suggestive of a role for this phenomena in tolerance and dependence. The problem lies, however, in the rapidity of the effects seen in

cultured cells compared to the *in vivo* situation. The phenomenon of uncoupling *in vivo* has recently been reexamined demonstrating that in rats it occurs after a single dose of diazepam, reaching a peak attenuation of coupling between 4 and 12 hours later, returning to normal after 24 hours [225]. Further, 24 hours following a 14 day chronic treatment of rats with the same daily dose of diazepam (15 mg/kg/day) no uncoupling could be observed. These data are in accordance with the data derived from cell culture studies demonstrating relatively rapid uncoupling [213,216,218-222]. That a return to control GABA-shift values after 24 hours was observed [225] is likely the result of the removal of diazepam in the animal by metabolism, which is rapid [226] and which obviously does not occur in the cell culture systems. It seems likely, therefore, that the uncoupling observed immediately following 4 weeks diazepam treatment [212] is a consequence of the acute action of the final benzodiazepine treatment [225]. More importantly, these data indicate that there are not marked differences in the timescales of the development of uncoupling *in vitro* and *in vivo*. Thus, uncoupling, in and of itself, occurs too rapidly to be the mechanism that is the direct cause of benzodiazepine tolerance and dependence.

Regulation of Protein Internalisation, Degradation, Synthesis

GABA_A receptor proteins are subject to regulated synthesis and degradation [227]. In addition to synthesis of the subunit polypeptides, their assembly into receptor complexes and subsequent insertion into and removal from the plasma membrane and intracellular compartments are also subject to regulation. Modulation of these processes are obvious possible targets for the regulation of receptor number and composition in response to benzodiazepine-site ligand exposure [228].

As mentioned previously, one of the first studies to look at GABA_A receptor turnover found that in cultured chick cortical neurons two phases, slow and fast, could be detected [214,223]. It was also suggested at this time that a non-lysosomal pathway for receptor degradation existed in neurons [223]. Tehrani and Barnes demonstrated that a small proportion of GABA_A receptors (7 to 16%) are present intracellularly on clathrin-coated vesicles in both cultured chick cortical neurons [229] and rat brain [230]. Moss and colleagues confirmed the involvement of clathrin-mediated endocytosis in constitutive GABA_A receptor turnover [231,232] and showed that the adapter protein AP-2, which is necessary for recruitment of proteins to clathrin-coated pits, associates with GABA_A receptor and subunits [232]. They also showed that phosphorylation plays a role in regulating receptor recycling, probably via proteins other than the receptor itself [231]. Interestingly, the rapid recruitment of GABA_A receptors to the plasma membrane has been observed following acute exposure to insulin via a mechanism that likely involves the tyrosine kinase activity of the insulin receptor [233]. This suggests that GABA_A receptor cycling is subject to physiological regulation allowing the numbers of receptors on the cell surface to be rapidly controlled in response to external stimuli.

In order to explain the uncoupling observed in flurazepam-treated chick cortical neuronal cultures, Roca et al. (1990) first suggested that new receptor synthesis could be involved [213], but they could not definitively ascertain the mechanism. Tehrani and Barnes (1993) showed that the intracellular receptors on clathrin-coated vesicles displayed altered pharmacological properties [230], such that the affinity for flunitrazepam was markedly reduced, compared to that measured in synaptic membranes, and there was no allosteric coupling between the GABA and benzodiazepine binding sites on these receptors [234]. They further showed that a 7 day lorazepam treatment results in an 80% increase in the proportion of GABA_A receptors present on clathrin-coated vesicles in rat brain concomitant with an increase in the amount of $\alpha 1$ subunit protein present in these vesicles [235]. Interestingly, a recent report demonstrates that Rac1, a small GTPase which regulates cytoskeletal proteins, is necessary for full GABA_A receptor activity suggesting it has a role in receptor turnover and/or clustering [236].

A series of studies from the Bristow laboratory looked at the effects of chronic benzodiazepine exposure in more detail and showed that neurons respond in a complex fashion. Using cultured rat cerebellar granule cells they found that a 1 hour exposure to flunitrazepam was sufficient to produce a decrease in $\alpha 1$ and $\alpha 2/3$ subunit protein levels via a protein kinase C dependent mechanism [237]. This effect was still detectable following 2 days but was lost after 4 to 12 days treatment [218,238]. 2 and 7 day flunitrazepam treatment caused a reduction in flunitrazepam-simulated GABA function (as measured by microphysiometry) without causing a change in GABA EC₅₀ or total muscimol binding [218]. Interestingly, they showed that the degree of change in $\alpha 1$ -subunit protein level was dependent on the efficacy of the benzodiazepine-site ligand used for long-term exposure [239]. This is perhaps surprising, however, given that they had previously shown that GABA did not potentiate the effects of flunitrazepam exposure [238], a result more in keeping with the notion that GABA-site activation is not required for the induction of uncoupling [216,213], as discussed above. These results suggest that occupation of the benzodiazepine site, but not receptor activation, is required for the down regulation of certain GABA_A receptor subunit polypeptides [239].

If benzodiazepine-induced downregulation does not require GABA-site agonist binding it suggests that it operates via a different mechanism to the downregulation induced by GABA-site agonist binding alone [228,240,241]. Such an activity-independent downregulation may not be important *in vivo* where GABA binds to both synaptic and presynaptic receptors [189-191,193-196]. Further, benzodiazepines should be able to potentiate sub-threshold GABA-induced activity-independent downregulation, but this has not been tested.

While it is clear from these studies that the complex regulation of GABA_A receptor synthesis, turnover and recycling are likely modulated by benzodiazepine exposure, the rapidity of these effects questions whether they lead directly to tolerance and dependence. It should be borne in mind that the kinetics of receptor protein turnover appear to differ between neuronal and non-neuronal cells and that this

has been suggested to be attributable to the presence or absence, respectively, of post-synaptic scaffold proteins that interact directly with the receptor [227]. A further complication in assessing the role of receptor turnover in tolerance and dependence is the potential for different rates of turnover at synaptic sites compared to extrasynaptic sites [231,227]. What is certain is that surface GABA_A receptor protein levels are altered in response to benzodiazepine exposure. This may play a role in the initial response to chronic benzodiazepine exposure, possibly even tolerance, but not necessarily dependence.

Regulation of mRNA Steady-State Levels, Synthesis, Degradation

The long-term nature of the development of tolerance and dependence has led many authors to suggest that changes in gene expression resulting in the switching of subunits likely underlie these phenomena [150,151,213,225,242-254]. Thus, alterations in mRNA synthesis or degradation are hypothesised to give rise to long-lasting changes in the particular GABA_A receptor subtype expressed at specific synapses, with such receptors either being less sensitive to GABA or benzodiazepines, or displaying decreased coupling between the GABA and benzodiazepine sites, properties which are then manifest as tolerance. Dependence would be evinced as a consequence of "inappropriate" receptors being present at specific synapses upon removal of the drug. Given the differential pharmacological properties engendered by different subunits there are a number of possible subunit combinations that would meet the appropriate criteria for "tolerant" receptors.

Two major problems exist with our current ability to rigorously test this hypothesis. First, our understanding of which GABA_A receptor subunits form specific receptor subtypes *in vivo*, even under normal physiological conditions, is limited at best [124,136,181]. Second, the neural loci responsible for either the pathophysiology for which benzodiazepines are prescribed or the behavioural manifestations of tolerance and dependence are not known. Despite these problems, however, a significant body of literature exists to demonstrate that chronic benzodiazepine administration causes specific changes in the expression GABA_A receptor genes. Linking those changes to tolerance and dependence is the current focus of many groups working in this area.

Measuring changes in GABA_A receptor gene expression has for the most part been conducted by determining steady-state mRNA levels which suffers from the disadvantage that mRNA synthesis and degradation are not determined separately, only their sum effect. Steady-state mRNA levels have been determined by a variety of techniques, such as Northern blotting, nuclease protection or polymerase chain reaction. The first such report described a reduction in $\alpha 1$ -but not $\alpha 1$ -subunit mRNAs in rat cortex following chronic diazepam administration [244]. This group subsequently described a corresponding decrease in $\alpha 2$ -subunit mRNA in rat cortex under the same treatment regime [249]. Most interestingly, they also found that chronic exposure to the

inverse agonist FG 7142 caused an increase these two mRNA species in rat cortex [249].

Numerous studies have followed since from a number of different research groups. Unfortunately, direct comparison of these studies is difficult because of the differences in treatment paradigm used such as the benzodiazepine ligand, dose, period of treatment, and brain regions examined. It is clear that within a given study, however, chronic treatment with benzodiazepine positive modulators produces subunit-specific changes which differ temporally and between brain regions. Consequently, a few selective series of studies will be reviewed to illustrate major findings in this area.

Following on from their earlier studies on the behavioural [89,93,204] and neurochemical [89,93,204] effects of long-term lorazepam exposure in mice, Kang and Miller (1991) demonstrated that 1- and 2-subunit mRNA levels were decreased in mouse cortex. Importantly [247], these changes in expression occurred at 14 days treatment [247] which was after the onset of behavioural tolerance and altered GABA_A receptor function at day 7 [89]. This finding is interesting in that 1-subunit mRNA levels decrease in cultured chick cortical neurons in response to exposure to GABA but this occurs subsequent to a decrease in 1-subunit polypeptide [241,255]. This has led to the suggestion that changes in the levels of GABA_A receptor mRNAs may occur as a consequence of receptor subunit downregulation, with the latter somehow providing part of the signalling mechanism for the regulation of gene expression [227,228].

A more extensive study of GABA_A receptor mRNA steady-state levels was conducted by O'Donovan and colleagues in rat whole brain following treatment with flurazepam over a 32 day period [248,256]. They found no changes in the levels of 1-, 2-, 2- or all three -subunit mRNA species [248,256] whereas the 5-, 3-, 6-subunit mRNAs were sequentially increased over the treatment period [248]. Although this study was one of the first to examine a large number of GABA_A receptor subunit mRNA species simultaneously it did so in whole brain, thus only detecting average changes. Later studies demonstrated that GABA_A receptor expression is altered by chronic benzodiazepine treatment in a brain region-specific manner [245,246,250-253,257-259].

Tietz and colleagues developed a 7 day flurazepam treatment regime which appears to allow the development of tolerance without leading to dependence [102,103]. This treatment regime has the significant advantage that it should only produce molecular changes associated with tolerance rather than with both tolerance and dependence. Thus, these authors make no assumptions about the inter-relatedness of tolerance and dependence [251]. Using quantitative *in situ* hybridisation 1- and 3- subunit mRNAs were found to be decreased and 2-subunit mRNA increased in the hippocampus of tolerant animals [251]. Immunohistochemistry and quantitative western blot analysis showed a concomitant decrease in 1- and 3-subunit proteins [242].

Direct comparison of the effects of chronic exposure to benzodiazepine-site ligands with differing tolerance liabilities

has been conducted by two groups. Abecarnil is a γ -carboline that displays positive modulatory activity at a subset of GABA_A receptors. Classical benzodiazepines have a similar high affinity for GABA_A receptors that comprise X 2 2 subunits, where X equals subunit isoform 1, 2, 3 or 5 [161,162,260] whereas abecarnil binds with higher affinity to those containing the 1 subunit [261]. Further, abecarnil is partial modulator at receptors containing the 2 or 5 subunits but a full modulator at those containing 1 or 3 subunits [262,263]. A number of studies [264-269], but not all [270,271], have reported a reduced tolerance and dependence liability of abecarnil. Holt and colleagues [150] compared the changes produced by diazepam and abecarnil in steady state mRNA levels for 13 GABA_A receptor subunits in rat cortex. They used a dosing regime designed to give equivalently high receptor occupancies. Seven days diazepam treatment increased 4-, 1- and 3-subunit mRNA levels which were sustained at day 14 along with increases in 3- and 5-, and a decrease in 2-subunit mRNAs. Abecarnil only produced decreases in 2- and 2-subunit mRNA levels. These authors postulated that the mRNA increases seen in the diazepam-treated group could possibly account for tolerance. Receptors containing 4 subunits (for which they found the largest change in mRNA levels) do not bind diazepam [165], 5 subunit-containing receptors demonstrate a reduced ability for diazepam potentiation of GABA-gated currents [169], 3 subunit-containing receptors have a low affinity for GABA [272] and 3 subunit-containing receptors show a reduced affinity for classical benzodiazepine positive modulators [166,170,172,173]. Predictions of this nature are, however, obviously compromised by the assumption that changes in mRNA levels reflect changes in the corresponding subunit proteins and by our limited understanding of which subunits combine *in vivo* to form functional receptors.

In a similar study from Costa's group imidazenil was used, which is a partial allosteric modulator benzodiazepine that is anxiolytic and anticonvulsant but non-sedative [243,273] and does not produce tolerance to its anticonvulsant effects on long-term administration [243,246,259,274,275]. Costa and co-workers compared long-term to exposure of diazepam [246,258,259] with that of imidazenil [246] on specific GABA_A receptor mRNA and proteins in certain rat brain regions. No significant changes were found in the mRNA levels of those subunits examined between vehicle, diazepam and imidazenil treated groups in the frontoparietal somatosensory (FrPaSS) cortex, hippocampus or cerebellum, except for a 20% decrease in 1-subunit mRNA in hippocampus [246]. In contrast, a number of significant changes were observed in the frontoparietal motor (FrPaM) cortex of diazepam treated animals but not those exposed to imidazenil. 1- and 2-subunit mRNAs were decreased while 5-subunit mRNA was increased. Analysis of immunogold labelling of specific subunits in the same brain regions indicated that while changes in the 1- and 5-subunit proteins matched their cognate mRNAs in FrPaM, this was not true for the 2-subunit protein which increased in this region. Other changes in GABA_A receptor subunit proteins observed in FrPaSS also did not correlate with alterations in the corresponding mRNAs [246]. While this study had the advantages of examining both mRNA and protein levels, and

in a number of discrete brain regions, only five to ten GABA_A receptor mRNA species were examined, depending upon the brain region. Further, as these authors point out, it is not possible to directly compare the protein levels of two different subunits using immunogold procedures as the antibodies do not saturate their epitopes. Thus, comparisons can be made between the levels of a specific subunit following different treatment regimes, they cannot be made between different subunits. Despite these limitations these data provide strong support for the notion of subunit switching as a molecular mechanism for the development of tolerance.

Changes in steady-state mRNA levels can arise from alterations in the rates of transcription or mRNA degradation. Based on their data on the effects of diazepam and abecarnil on steady-state mRNA levels Holt and colleagues [150] suggested a role for transcription. GABA_A receptor genes are clustered on the mammalian genome: for example, in humans, the 1-, 6-, 2-, and 2-subunit genes are closely grouped on chromosome 5q32-5q33 [149,276-279]. The changes caused by diazepam and abecarnil of eleven GABA_A receptor subunit mRNA species in rat cortex were analysed according to gene cluster [150]. For a given gene cluster GABA_A receptor subunit mRNA levels responded to chronic diazepam treatment in a similar fashion, with a mean decrease at the 1/2/2 cluster and mean increases at the 5/3/3 and 2/4/1/1 clusters. Abecarnil also produced a decrease in the mean mRNA levels of the 1/2/2 cluster to the same degree as diazepam, but produced no mean change compared to vehicle at the 5/3/3 and 2/4/1/1 clusters. Not only does this highlight the differences in the changes in gene expression produced by diazepam and abecarnil but it also suggests that GABA_A receptor genes respond to chronic benzodiazepine-site ligand exposure in a gene cluster specific manner. The most parsimonious explanation of such a response is that gene transcription is involved and that the expression of genes within a given cluster are co-regulated [150]. Evidence for such co-regulation exists. In the pancreas [280] and in Bergmann glial cells of the cerebellum [136], the 2/1/1 subtype has been identified and the genes encoding these subunits colocalise to human chromosome 4p13-q11 [281]. Similarly, three of four genes found on human chromosome 5, the 1- 2- and 2-subunit genes [148-279], are predominantly (but not exclusively) expressed in the same brain regions and give rise to the major GABA_A receptor subtype [282-286]. Recently, evidence that the 3, and 3-subunit genes, which cluster on human chromosome Xq28 [134,287,288], has been presented to suggest that they form a subtype in some brain regions [152].

In order to directly test the hypothesis that chronic diazepam alters gene transcription *in vivo*, Holt and colleagues [245] examined the rate of GABA_A receptor 2-subunit mRNA synthesis in cortex and cerebellum using a nuclear run-off assay. This technique allows the quantitation of nascent mRNA *in vitro* from transcriptionally competent nuclei. A significant decrease in 2-subunit mRNA synthesis was found in cortex following 14 day diazepam treatment while in cerebellum an increase was found. These changes in transcription rate paralleled the changes in steady-

state 2-subunit mRNA levels [245]. Thus, at least for the 2-subunit gene, prolonged diazepam exposure alters transcription. In a different approach, Kang and colleagues [289] showed that the 5' end of the human GABA_A receptor 1-subunit gene conferred benzodiazepine-sensitive transcriptional activity in transiently transfected neurons. These data do not obviate a role for changes in mRNA degradation but certainly indicate a major role for transcription in the modulation of GABA_A receptor gene expression. Analysis of whether changes in GABA_A receptor subunit mRNA degradation rates occur in response to benzodiazepine exposure have not been reported.

A UNIFIED MODEL OF MOLECULAR MECHANISMS UNDERLYING BENZODIAZEPINE TOLERANCE AND DEPENDENCE

It is clear from the examples described in the preceding sections that chronic exposure to benzodiazepine-site positive allosteric modulators leads to changes in GABA_A receptor allosteric properties, turnover and expression. How can these multiple effects be reconciled with a single mechanism of tolerance and dependence? This may not be possible, given the evidence which shows that tolerance to different behavioural parameters develops at different rates and that different behavioural properties of the benzodiazepines appear to be mediated via different GABA_A receptor subtypes. Recent studies from two laboratories using transgenic mice have demonstrated that receptors which contain the 1 subunit mediate the sedative effects of classical benzodiazepines [197,290] whereas 2 subunit-containing receptors mediate anxiolytic properties [198]. A prediction from these studies is that because tolerance to differing behavioural effects develops along different timecourses, changes in GABA_A receptors caused by chronic exposure to benzodiazepine-site positive allosteric modulators would equally occur with different timecourses. This is clearly the case for changes in expression of GABA_A receptor subunit genes. Uncoupling at different GABA receptor subtypes appears, however, to occur with similar timecourses, at least in heterologous expression systems, but we currently do not have sufficient information to determine whether the same is true for receptor turnover and subunit protein synthesis and degradation.

It has been suggested recently that internalised receptors may provide the signal for subsequent changes in GABA_A receptor gene expression [227]. Taking this model further, it is possible that uncoupling may be a signal for changes in receptor turnover and degradation. Thus, exposure to benzodiazepine-site positive allosteric modulators would result in the following chain of events. Initial potentiation of the GABA response would be quickly followed by desensitisation. Prolonged desensitisation could be the signal for uncoupling to occur, which either happens as a consequence of the internalisation of receptors or is itself the signal for internalisation. Once internalised, preferential degradation of certain subunits occurs which provides some sort of signal to the nucleus for changes in GABA_A receptor gene transcription. These changes in gene transcription therefore provide a more stable long-term response to chronic

drug treatment. This pathway could operate on slightly different timescales depending upon the receptor subtype and/or brain region/neuronal cell type thereby giving rise to the differential temporal aspects of tolerance and dependence.

This model, while clearly not comprising a single mechanism to explain tolerance and dependence, draws together findings from a number of research groups, each of which has to a large extent been pursuing their individual hypotheses. Such a unified model should allow advancement in the field as it points to key research questions. For example, what is the signal to the nucleus? One approach to this question is the detailed analysis of GABA_A receptor promoters and the identification of the transcription factor involved in their regulation. At the level of the protein, the use of transgenic animals in which individual GABA_A receptor subunits are tagged with labels such as green fluorescent protein would provide a valuable tools to study the regulation of subunit proteins in real time. As the biochemical and molecular biological tools become more sophisticated we can expect a greater understanding of the mechanisms underlying the development of tolerance to and dependence on benzodiazepine-site ligands.

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