

# Valium without dependence? Individual GABA<sub>A</sub> receptor subtype contribution toward benzodiazepine addiction, tolerance, and therapeutic effects

Tianze Cheng<sup>1</sup>  
Dominique Marie Wallace<sup>2</sup>  
Benjamin Ponteri<sup>1</sup>  
Mahir Tuli<sup>3</sup>

<sup>1</sup>Pitzer College, Claremont, CA, USA;

<sup>2</sup>Portland State University, Portland,

OR, USA; <sup>3</sup>University of British Columbia, Vancouver, BC, Canada

**Abstract:** Benzodiazepines are one of the most prescribed medications as first-line treatment of anxiety, insomnia, and epilepsy around the world. Over the past two decades, advances in the neuropharmacological understanding of gamma aminobutyric acid (GABA)<sub>A</sub> receptors revealed distinct contributions from each subtype and produced effects. Recent findings have highlighted the importance of  $\alpha_1$  containing GABA<sub>A</sub> receptors in the mechanisms of addiction and tolerance in benzodiazepine treatments. This has shown promise in the development of tranquilizers with minimal side effects such as cognitive impairment, dependence, and tolerance. A valium-like drug without its side effects, as repeatedly demonstrated in animals, is achievable.

**Keywords:** benzodiazepines, subtype, tolerance, dependence, anxiolytic, GABA<sub>A</sub> receptor

## Introduction

Benzodiazepines are a class of tranquilizers that enhance gamma aminobutyric acid (GABA)ergic transmission. They are seen ubiquitously in the modern health care system as >5% of the total adults in USA are prescribed benzodiazepines each year.<sup>1</sup> The major behavioral and psychoactive effects of benzodiazepines include anticonvulsive, sedative, muscle relaxant, and anxiolytic effects.<sup>2</sup> They are readily prescribed by physicians and are regarded as a frontline treatment for many common psychiatric disorders such as anxiety, obsessive-compulsive disorder, seizures, as well as a number of sleep disorders.<sup>3</sup>

Benzodiazepines were discovered in 1955 by chemist Leo Sternbach and, when first introduced, were proposed as a promising replacement for barbiturates, another similar class of tranquilizers that also act on GABA.<sup>4,5</sup>

The medical use of barbiturates was prominent until the 1950s when serious side effects, such as high incidence of abuse, dependence, and overdose finally started to surface.<sup>6</sup>

When benzodiazepines hit the market in the 1960s, they were thought to be the successor to barbiturates due to lower toxicity and side effects. Despite having a lower abuse profile, benzodiazepines still cause dependence after repeated use, which was not widely recognized until the 1980s.<sup>7,8</sup>

Many attempts to produce dependence and tolerance-free benzodiazepine drugs have been investigated in the past. The selective agonist zolpidem was marketed, as promising data showed reduced abuse potential.<sup>9</sup> However, these results did not

Correspondence: Tianze Cheng  
221-10, 59th Avenue, Flushing,  
NY 11364, USA  
Tel +1 646 897 1322  
Email tcheng@pitzer.edu

translate in the clinic since zolpidem causes dependence after repeated use.<sup>10</sup>

Studies have also attempted to investigate neuropharmacological mechanisms of benzodiazepines. At first, the benzodiazepine site was categorized into benzodiazepine subtype I and benzodiazepine subtype II, where traditional benzodiazepines bind to both, but triazolopyridazines (TPZs) have high affinity for only type I.<sup>11</sup> It was later found that TPZ was actually just a selective agonist for one of many subtypes that exist within the GABA<sub>A</sub> receptor family.<sup>12</sup>

Progress in neuropharmacology has revealed various subtypes within the GABA<sub>A</sub> receptor family, as well as anatomical and pharmacological differences between them. Investigations have also been directed toward the addiction and tolerance mechanisms of benzodiazepines; their relationships with specific subtypes within the GABA<sub>A</sub> receptor family will be discussed in further depth.

## Pharmacological targets of benzodiazepine

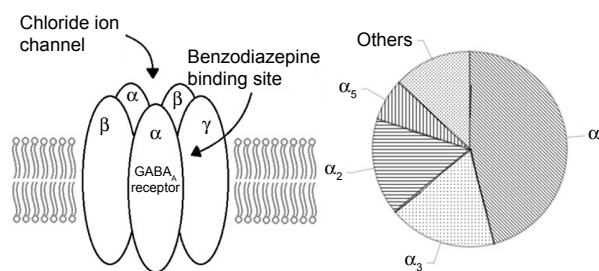
Benzodiazepines, although referred to as a positive allosteric modulator (PAM) of the GABA<sub>A</sub> receptor, does not actually enhance GABA's binding to the receptor, like conventional PAMs. Benzodiazepines increase the frequency of chloride channel influx which hyperpolarizes the GABA receptor, resulting in increased inhibitory postsynaptic potential.<sup>13,14</sup>

$\alpha_{1-6}$ ,  $\beta_{1-3}$ ,  $\gamma_{1-3}$ ,  $\delta$ ,  $\epsilon$ ,  $\theta$ , and  $\pi$  make up the currently defined GABA<sub>A</sub> subunits in the human brain.<sup>15</sup> Classic benzodiazepines such as diazepam binds to  $\alpha_1$ ,  $\alpha_2$ ,  $\alpha_3$ , and  $\alpha_5$  containing GABA<sub>A</sub> receptors.<sup>16</sup>  $\alpha_1$  containing GABA<sub>A</sub> receptors are the most abundant subtype and can be found throughout the brain, while  $\alpha_2$ ,  $\alpha_3$ , and  $\alpha_5$  subtypes are more region specific.<sup>17</sup>

Although numerous combinations exist, most GABA<sub>A</sub> receptors structurally contain two  $\alpha$ , two  $\beta$ , and a single  $\gamma$  subunit surrounding a chloride ion channel as shown in Figure 1.<sup>18</sup> The benzodiazepine site is located between the  $\alpha$  and  $\gamma$  subunit.

## Roles of GABA<sub>A</sub> receptor $\alpha_1$ , $\alpha_2$ , $\alpha_3$ , and $\alpha_5$ subunits in benzodiazepine pharmacology

GABA<sub>A</sub> subtype selective compounds and rodent models of subunit point mutation have provided promising data for identifying different subunit contributions toward each clinical effect. We will discuss clinical effects of sedation first, as it impairs the cognitive performance of benzodiazepine-prescribed patients.<sup>20</sup>



**Figure 1** The GABA<sub>A</sub> receptor and its approximate subtype composition (adapted from Wafford's study).

**Note:** Data from Wafford.<sup>19</sup>

**Abbreviation:** GABA, gamma aminobutyric acid.

We know that the  $\alpha_1$  subtype plays a major role in sedation because  $\alpha_1$ (H101R) point mutated mice were resilient to sedative effects of benzodiazepines.<sup>21</sup> Benzodiazepines that possess sparing efficacy at  $\alpha_1$  subtype such as L-838,417 act as a sedation-free anxiolytic in animal models.<sup>21,22</sup> L-838,417 has seen popular use in research and is a partial agonist of  $\alpha_2$ ,  $\alpha_3$ , and  $\alpha_5$  containing GABA<sub>A</sub> receptors.<sup>22</sup> Other compounds with low or absent  $\alpha_1$  subtype efficacy such as imidazenil, TPA123, and TPA023, are also shown to be sedation-free anxiolytics, revealing the importance of the  $\alpha_1$  subtype in the mediation of sedation.<sup>23,24</sup>

Moreover,  $\alpha_1$  subtype selective agonists such as zolpidem are unable to produce anxiolytic effects other than sedation.<sup>25</sup> The  $\alpha_5$  inverse agonist,  $\alpha 5$ IA, improves memory without producing anxiety, increased awakeness, or proconvulsant effects in animals.<sup>26</sup> So far, we could conclude that anxiety is mediated by either  $\alpha_2$  or  $\alpha_3$  subtypes or both, while sedation is mediated by  $\alpha_1$  containing GABA<sub>A</sub> receptors.

Because  $\alpha 5$ IA reversed alcohol-induced memory deficit when given prior to alcohol administration in humans,  $\alpha_5$  subtype is thought to contribute toward amnesic effects.<sup>26</sup> This was further confirmed when the  $\alpha_5$  subtype was found to be responsible for amnesic effects of GABA<sub>A</sub> receptor PAM etomidate, which correspond with earlier studies on the role of the  $\alpha_5$  subtype in memory and its anatomical presence in the hippocampus.<sup>27</sup>

However, it seems  $\alpha_5$  is not the sole subtype that contributes to amnesic effects.  $\alpha_1$  subtype full agonist zolpidem seemed to produce more memory and cognitive impairment compared to an equivalent dose of triazolam, an agonist of all benzodiazepine sites.<sup>28</sup> Given that zolpidem produces almost no efficacy at the  $\alpha_5$  subtype, we can conclude that  $\alpha_1$  subtype is also involved in amnesic effects of benzodiazepines.<sup>28</sup>

In 2000, it was thought that  $\alpha_2$  subtype solely mediated anxiolytic actions because only  $\alpha_2$ (H101R) point mutated mice were still anxious after diazepam treatment.<sup>29</sup> On the contrary, two studies 5 years later, the first study using

TP003, an  $\alpha_3$  subtype selective agonist, demonstrated that TP003 produced strong anxiolytic effects.<sup>30</sup> The second study used an  $\alpha_3$  subtype inverse agonist  $\alpha 3IA$  to produce anxiogenic effects in mice, further confirming the role of  $\alpha_3$  subtype in mediating anxiety.<sup>31</sup>

Although  $\alpha 3IA$ 's weak inverse efficacy at  $\alpha_2$  subtype could be argued to be anxiogenic, novel non-benzodiazepine compounds such as adiplon (NG2-73) and SB-205,384 produced anxiolytic effects without any efficacy at the  $\alpha_2$  subtype.<sup>32</sup>

Moreover, it was shown by data that various novel benzodiazepine compounds with stronger efficacy at  $\alpha_3$  than  $\alpha_2$  subtype showed more anxiolytic effects. Löw et al's study received several responses suggesting that the data from elevated plus maze in  $\alpha_2$ (H101R) mice could be more related and influenced by the motor activity mediated through  $\alpha_2$  subtype than anxiolysis.<sup>33,34</sup>

This is important because almost all past models and reviews have categorized the anxiolytic effects of benzodiazepines solely from efficacy at the  $\alpha_2$  subtype, some further suggesting novel benzodiazepine anxiolytics should be  $\alpha_2$  selective.<sup>35-39</sup> However, the most recent investigations on this matter suggest that both  $\alpha_2$  and  $\alpha_3$  subtypes mediate anxiety.<sup>40,41</sup> Therefore, the design of subtype selective benzodiazepine anxiolytics should not be limited to  $\alpha_2$  containing GABA<sub>A</sub> receptors. This paper will present a revised and comprehensive model of benzodiazepine pharmacology.

$\alpha_5$  subtype has also been shown to play a role in mediating anxiety and especially conditioned fear memory, as shown by  $\alpha_5$  subtype inverse agonists, RY024, and  $\alpha_5$ (H105R) point mutated mice.<sup>42,43</sup> However, the  $\alpha_5$  subtype does not seem to play a major role in mediating anxiety as another  $\alpha_3$  subtype inverse agonist,  $\alpha 5IA$ , does not produce anxiety as assessed by elevated plus maze.<sup>44</sup>  $\alpha_5$  subtype also appears to play a minor role in anticonvulsive actions as proconvulsive as  $\alpha 5IA$  did not increase seizure thresholds as assessed by pentylenetetrazol.<sup>44</sup> As a result, the  $\alpha_5$  subtype most probably mediates a narrow category of anxiety, such as conditioned fear, but could be clinically relevant.

Moreover, several models of GABA<sub>A</sub> subunit contribution in benzodiazepine pharmacology suggest anticonvulsive action only at  $\alpha_1$  subtype.<sup>36</sup> TPA023 devoid of activity at the  $\alpha_1$  subtype was still able to produce anticonvulsive effects, through  $\alpha_2$  and possibly  $\alpha_3$  subtype.<sup>24</sup>

Diazepam was anticonvulsive in both  $\alpha_2$ (H101R) and  $\alpha_3$ (H126R) mice; however, more  $\alpha_2$ (H101R) mice convulsed after treatment toward pentylenetetrazol, revealing that the  $\alpha_2$  subtype contributes more than  $\alpha_3$  subtype in the anticonvulsant properties of benzodiazepines.<sup>29</sup>

Most anticonvulsive actions of benzodiazepines are mediated by  $\alpha_1$  as diazepam only mildly attenuated pentylenetetrazol-induced seizures in  $\alpha_1$ (H101R) mice. However, this data should not discourage anticonvulsant drug discovery that has no efficacy at  $\alpha_1$  subtype. Recent investigations actually showed that the  $\alpha_1$  subtype antagonist,  $\alpha_2$ ,  $\alpha_3$ , and  $\alpha_5$  subtype partial agonist imidazenil was actually more potent than diazepam at attenuating diisopropyl fluorophosphate-induced convulsions and neuronal damage.<sup>46</sup>

Myorelaxation has been identified to involve  $\alpha_2/\alpha_3$  subtype using respective point mutated mice; however, the action appears to be primarily mediated through  $\alpha_2$  subunit because high doses were needed to induce myorelaxation in the  $\alpha_2$ (H101R) mice.<sup>47</sup>  $\alpha_1$  and  $\alpha_5$  subtypes might also contribute minorly toward myorelaxation because its respective selective antagonists are able to bluntly alleviate diazepam-induced myorelaxation.<sup>48,49</sup>

Not only does insufficient information support that  $\alpha_2$  subtype exclusively mediates anxiety, other than a single study, current evidence for the anxiolytic effects of benzodiazepines also points toward a dual contribution from  $\alpha_2$  and  $\alpha_3$  subtypes.<sup>29-41</sup> Therefore, in Table 1, anxiolytic effects were assigned to both  $\alpha_2/\alpha_3$  containing GABA<sub>A</sub> receptors.

In 2010, in a disappointing human trial on MRK-409, a GABA<sub>A</sub> receptor  $\alpha_2$ ,  $\alpha_3$ , and  $\alpha_5$  subtype partial agonist that promised to be a sedation-free anxiolytic in animals, demonstrated that its minor agonism on  $\alpha_1$  subtype produced sedation in humans.<sup>54</sup> However, the question of whether other subunits were involved in sedation was quickly overthrown as the clinical results of TPA023B, published shortly after, as an  $\alpha_1$  subtype antagonist and  $\alpha_2/\alpha_3$  subtype partial agonist, produced a sedation-free anxiety suppressing profile,<sup>50</sup> demonstrating that any efficacy at  $\alpha_1$  subtype could cause sedation in humans.

$\alpha_5$  subtype might be differentially distributed in different sexes. The selective  $\alpha_5$  subtype allosteric modulator, SH-053-2'F-R-CH3, seemed to particularly impact female but not male mice at alleviating stress.<sup>55</sup> In humans, long-term benzodiazepine use inducing alterations in long-term memory was only significant in women.<sup>56</sup>

Recent advances in novel clinical applications of benzodiazepines revealed densely populated  $\alpha_2$  containing GABA<sub>A</sub> receptors within the dorsal root involved in relieving pain, in which its inhibitory currents are believed to contribute to nociception.<sup>21</sup> Furthermore, although  $\alpha_1$  subtype agonists such as zolpidem and diazepam are efficacious for pain and neuropathy,  $\alpha_2$ ,  $\alpha_3$  and  $\alpha_5$  subtype partial agonists seem to produce similar results without the sedative, amnesic, and addictive properties of  $\alpha_1$  subtype agonists.<sup>57,58</sup>

**Table 1** Model of GABA<sub>A</sub> receptor subtypes and their contribution toward benzodiazepine's psychopharmacological effects

Reference GABA <sub>A</sub> Receptor	25, 28, 35–39, 49–52 $\alpha 1$	28, 35–39, 49–52, 64 $\alpha 2$	29–41, 50–53, 65 $\alpha 3$	26–39, 42–49 $\alpha 5$
Sedation				
Addiction				
Anxiolysis				
Myorelaxation				
Anticonvulsive				
Amnesia				
Key: contribution toward clinical effect	 Negligible	 Minor	 Moderate	 Significant

**Abbreviation:** GABA, gamma aminobutyric acid.

## Mechanism of benzodiazepine addiction

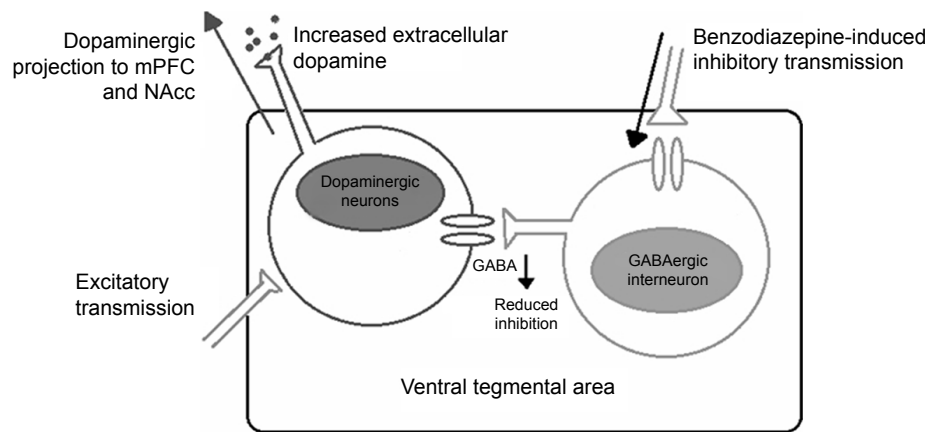
Benzodiazepine-induced activation of mesolimbic dopamine pathway was observed for the first time in 2009. Benzodiazepine indirectly acts upon the dopaminergic neurons in the ventral tegmental area (VTA), a brain region that plays a major role in addiction and reward.<sup>59</sup>

Both the selective  $\alpha_1$  subtype agonist zolpidem as well as diazepam were able to modulate glutamatergic transmission upon dopamine neurons in the VTA. This is groundbreaking because zolpidem has a significantly higher affinity toward  $\alpha_1$  ( $K_i=20$  nM) containing GABA<sub>A</sub> subtypes than  $\alpha_2$  and  $\alpha_3$  ( $K_i=400$  nM) subtypes, and is almost inactive at  $\alpha_5$ .<sup>60</sup> This suggests that  $\alpha_1$  containing GABA<sub>A</sub> receptors unquestionably play a role in addiction.

However, in the same study, diazepam had a significantly higher effect than zolpidem upon VTA; could there be more subtypes than just  $\alpha_1$  subtype in the role of addiction? The role of  $\alpha_3$  subtype in addiction was ruled out because  $\alpha_5$  subtype

inverse agonists were unable to prevent self-administration of ethanol.<sup>61</sup> Another study demonstrated that while  $\alpha_2$ ,  $\alpha_3$ , and  $\alpha_5$  point mutated mice showed clear preference toward drinking water contaminated with midazolam,  $\alpha_1$ (H101R) mice did not show any bias between water and midazolam solution,<sup>62</sup> implying that  $\alpha_1$  containing GABA<sub>A</sub> receptors are required for addictive behaviors associated with benzodiazepines. Furthermore,  $\alpha_1$  subtype inactive compounds such as TPA023 show almost no abuse properties.<sup>60</sup>

Recently, opposing evidence has shown that  $\alpha_2$  and  $\alpha_3$  subtypes might also be implicated in the addiction of benzodiazepines. After  $\alpha_2$  subtype within the nucleus accumbens (NAcc) are knockdown in mice, midazolam were no longer reinforcing. Implying that the  $\alpha_2$  subtype is at least in part involved in the reinforcing effects of benzodiazepines.<sup>63</sup> Rhesus monkeys with a history of benzodiazepine use, but not cocaine use, have been shown to self-administer  $\alpha_1$  subtype inactive compound L-838,417 and the  $\alpha_3$  subtype selective agonist TP003.<sup>64</sup> These findings hint that the  $\alpha_3$  containing



**Figure 2** Mechanism of benzodiazepines at VTA.

**Note:** Data adapted from Rudolph et al.<sup>57</sup> and Heikkinen et al.<sup>59</sup>

**Abbreviations:** VTA, ventral tegmental area; GABA, gamma aminobutyric acid; NAcc, nucleus accumbens; mPFC, medial prefrontal cortex.

GABA<sub>A</sub> receptors could be reinforcing in experienced benzodiazepine users. The experienced rhesus monkeys had been previously treated with numerous different benzodiazepines for around 6 months.<sup>64</sup>

The  $\alpha_1$  containing GABA<sub>A</sub> receptors expressed within the VTA favor its inhibitory projection toward GABA interneurons.<sup>62,65</sup> With interneurons being responsible for the inhibitory control over dopaminergic neurons of the VTA, inhibiting the inhibition of dopaminergic neurons results in free firing of the dopaminergic neurons. This results in increased dopamine levels as shown in Figure 2.<sup>62,65</sup> This finding is in congruence with the lack of midazolam self-administration in  $\alpha_1$ (H101R) mice.<sup>62</sup> It seems that  $\alpha_1$  subtype should be completely avoided since even partial agonists at this site could still produce sedation and addiction as seen in bretazenil and MRK-409.<sup>54,66</sup>

The increased AMPA/NMDA ratio in VTA has been shown in another GABA<sub>A</sub> PAM gaboxadol; however, it does not produce reinforcing effects.<sup>67</sup> Whether gaboxadol has another mechanism to avert addiction is unclear, but more regions than VTA could be involved. Selective  $\alpha_1$  subtype antagonist  $\beta$ CCt and 3-PBC were shown to be able to prevent the reinforcing effects of alcohol.<sup>68,69</sup>  $\alpha_1$  subtype antagonists effectively blocking addictive behaviors in mice indicate that  $\alpha_1$  containing GABA<sub>A</sub> receptors are highly involved and most likely contribute most or all of the reinforcing effects of GABAergic and benzodiazepine site acting substances. Furthermore, infusions of  $\alpha_1$  subtype antagonist into the ventral pallidum and extended amygdala, which accommodates a large amount of  $\alpha_1$  containing GABA<sub>A</sub> receptors, resulted in a reduction in ethanol-induced addictive behaviors.<sup>68,70</sup>

Opioid mechanisms are associated with reward pathways and a study has shown the competitive opioid receptor antagonist naloxone attenuating the addictive properties of benzodiazepines.<sup>71</sup> However, more studies opposing

the above study have been published. Interestingly, naloxone is able to block anxiolytic and sedative properties of benzodiazepines.<sup>72</sup> More recent studies showed that opioid peptides are involved in benzodiazepine's anxiolytic rather than addictive effects.<sup>73</sup>

The role of nicotinic acetylcholine receptor (nAChR), especially the  $\alpha 4\beta 2$  subtype, has been highlighted in drug addiction.<sup>74</sup> Since nAChR is a major modulator of GABA release in regions such as the thalamus, hippocampus, and VTA,<sup>68,75</sup> there is surprisingly less research in this direction. Is there really no link between acetylcholine and benzodiazepine addiction?

nAChRs, especially the  $\alpha 4\beta 2$  subtype, are upregulated after chronic exposure to drugs such as nicotine.<sup>76</sup> This phenomenon is also seen in naloxone-induced morphine withdrawal, alcohol withdrawal, and what we're interested in: flumazenil-induced benzodiazepine withdrawal.<sup>77,78</sup>

Most interestingly, this acetylcholine release was not seen in the partial agonists imidazenil and abecarnil, which has no efficacy at the  $\alpha_1$  subtype.<sup>79</sup> The fact that benzodiazepine withdrawal is marked by an acetylcholine increase in the nucleus accumbens, which is also seen in other drug withdrawals, further begs clarification of the benzodiazepine–acetylcholine affiliation, and possibly of subunit-specific involvement, which has been overlooked.<sup>78,79</sup>

Although a recent primate study showed that  $\alpha_3$  subtype might be reinforcing in those who are already dependent,  $\alpha_1$  containing GABA<sub>A</sub> receptors contribute to most if not all of the reinforcing effects of benzodiazepines.

## Mechanisms of benzodiazepine tolerance

Efficacy of benzodiazepine progressively reduces after long-term exposure; not only is a higher dosage of the drug required



to experience the same therapeutic effects, but also discontinuation of prolonged treatment induces withdrawal.<sup>80</sup>

A simple way to explain tolerance to any drug is downregulation of the receptor as the aftermath of neuroplasticity. However, it is demonstrated that even after chronic administration of benzodiazepines, the number of benzodiazepine sites is not reduced, neither is the sensitivity of the benzodiazepine site.<sup>81,82</sup>

Benzodiazepine site downregulation does not seem to happen unless astronomical doses are given, in many studies over 100 mg/kg in rats, which translated into human doses that are far above the therapeutic range.<sup>83,84</sup> Not to mention, only inconclusive and inconsistent results have been presented regarding possible changes in subtype composition and their mRNA expression.<sup>85,86</sup> So, if its not downregulation, what is the underlying mechanism?

While tolerance to sedative and anticonvulsant effects seems to build quickly in both humans and animal models, a lack of tolerance regarding the anxiolytic and amnesic effects of long-term benzodiazepine use has been consistently demonstrated in clinical trials.<sup>87-89</sup>

Diazepam and alprazolam for the treatment of panic attacks, social phobia, and other anxiety-related disorders are effective even after chronic use.<sup>90,91</sup> Could this mean  $\alpha_2$  and  $\alpha_3$  containing GABA<sub>A</sub> receptors, which mediate anxiety, have less significance in the tolerance building mechanism of benzodiazepines?

Other than putting the blame of addiction on  $\alpha_1$  subtype,  $\alpha_5$  subtype might be required for tolerance toward sedative effects of benzodiazepines. With the guidance of  $\alpha_1$ ,  $\alpha_2$ ,  $\alpha_3$ , and  $\alpha_5$  subtype point mutated mice, repetitive dosing of diazepam showed that  $\alpha_5$  containing GABA<sub>A</sub> receptors of the dentate gyrus lead the adaptive changes associated with sedative tolerance to benzodiazepines.<sup>92</sup> After chronic benzodiazepine administration, mice of the  $\alpha_5$  point mutated species retained most of the motor-depressant effects of benzodiazepines, whereas  $\alpha_1$ ,  $\alpha_2$ , and  $\alpha_3$  point mutated mice started habituating from the sedative effects of benzodiazepines.<sup>92</sup>

Now one might ask, how would you then explain tolerance of zolpidem since it has no  $\alpha_5$  subtype efficacy? Well, in the same study,  $\alpha_1$ (H101R) mice showed downregulation of  $\alpha_5$  containing GABA<sub>A</sub> receptors in the dentate gyrus of the hippocampus, but not in  $\alpha_2$  and  $\alpha_3$  point mutated mice.<sup>92</sup> Therefore, a hypothesis that the involvement of  $\alpha_5$  as well as  $\alpha_1$  subtypes is required to induce tolerance in benzodiazepine use was put forward.

Although long-term benzodiazepine treatment did not reduce the number of benzodiazepine sites or alter the binding affinity as assessed by <sup>3</sup>H-flumazenil,<sup>93</sup> it did downregulate adenosine receptors in the striatum by almost half in mice

of the same investigation. It has been consistently shown that benzodiazepine use is associated with downregulation of adenosine A<sub>1</sub> and A<sub>2</sub> receptors in animals.<sup>94,95</sup> Although the mechanism of how benzodiazepines can influence adenosine receptors is unclear, a possible explanation for the downregulation of adenosine is for the attenuation of benzodiazepine-induced sedation.

It has been proven that benzodiazepines can indirectly increase adenosine after acute administration, through inhibition of adenosine reuptake. The adenosine reuptake inhibitor dipyrindamole and the adenosine deaminase EHNA were able to reverse the sedative effects of benzodiazepines, as measured by excitatory currents within the hippocampus.<sup>96</sup> This mechanism was recently further validated when the sedative effects of benzodiazepines, barbiturates, and propofol all appeared to be mediated by the adenosine system.<sup>97</sup> Downregulation of adenosine receptors as discussed could in part explain the tolerance to the sedative effects of benzodiazepines.

It came forth that not only adenosine is involved in the tolerance toward benzodiazepines. NDMA and AMPA receptor upregulation was observed in the cerebral cortex of mice after abrupt ending of chronic diazepam administration.<sup>98,99</sup> More recent inspections of this mechanism showed comparable results especially in the rat hippocampus.<sup>100,101</sup>

It appears that NMDA upregulation happens acutely after benzodiazepine administration, because NMDA antagonists dizocilpine (MK-801) and CPP were able to prevent tolerance to sedative and withdrawal effects after benzodiazepine administration.<sup>102,103</sup> While the NMDA antagonist can suppress withdrawal symptoms in acute benzodiazepine abstinence, the AMPA antagonist prevented withdrawal during the long-term phase in mice.<sup>104,105</sup> The upregulation and changes of glutamatergic system are most likely a compensatory mechanism.

Numerous investigations have observed uncoupling between allosteric linkage of GABA and the benzodiazepine site. Uncoupling is a mechanism wherein the benzodiazepine site loses its allosteric modulatory effects over GABAergic activities.<sup>18,89</sup> It explains the reduced efficacy of benzodiazepines after chronic use and is further verified in numerous in vivo demonstrations.<sup>89</sup> Benzodiazepine site uncoupling is associated with negative modulation of GABAergic transmission and is likely a result of compensation to suppress repeated benzodiazepine-induced GABAergic enhancement.<sup>106</sup>

In rats continuously exposed to either full agonist, partial agonist, or the antagonist flumazenil, the benzodiazepine efficacy correlates to the degree of uncoupling. Full agonists resulted in the highest percentage of benzodiazepine site

uncoupling, especially in benzodiazepine sites that regulated anticonvulsant actions.<sup>107</sup> This not only explains the rapid tolerance building toward anticonvulsant effects but also indicates that partial agonists may be able to produce less tolerance.

Interestingly, a single flumazenil dose can reverse the anticonvulsant tolerance after chronic benzodiazepine exposure.<sup>108,109</sup> The mechanisms of how flumazenil completely reverses the allosteric uncoupling of benzodiazepine and GABA are unclear. Flumazenil appears to be capable of yielding opposite downstream mechanisms of benzodiazepine agonists, and actually upregulate benzodiazepine binding and GABAergic chloride uptake.<sup>110</sup> Similar results have been observed in clinics, where flumazenil was able to reverse tolerance of daily clonazepam in users with partial seizures, who have been taking the medication for over a year.<sup>111,112</sup>

Benzodiazepine uncoupling recovers after 2 days of abstinence in rats, and only happens after repeated administration.<sup>113</sup> This correlates with previously mentioned human clinical data in terms of the quick adaptations to benzodiazepine's anticonvulsant tolerance. Because about one third of all postsynaptic GABA<sub>A</sub> receptors are continuously activated, uncoupling might impact continual GABA transmission, which could also contribute to withdrawal.<sup>114</sup>

In chick cortical neurons, benzodiazepine treatment leads to mild reductions of GABA<sub>A</sub> receptors on membrane surface; these missing receptors are discovered intracellularly and contribute around 7% of all GABA<sub>A</sub> receptors.<sup>115</sup> Tehrani et al also demonstrated that these intracellular GABA<sub>A</sub> receptors located on clathrin-bound vesicles are uncoupled as benzodiazepine sites with reduced affinity and allosteric modulatory control over the receptor.<sup>116–118</sup>

Long-term benzodiazepine treatment caused a significant 83% increase in the number of GABA<sub>A</sub> receptors on clathrin-coated vesicles versus control. Although benzodiazepines are still able to bind at these sites, they produce no modulatory effect. The internalization also meant a 12% reduction of GABA<sub>A</sub> receptors located on synaptic membranes, as assessed by <sup>3</sup>H-flunitrazepam after a 7-day benzodiazepine treatment.<sup>118</sup>

This internalization of GABA<sub>A</sub> receptors at the synaptic membrane possibly contributes to the tolerance of benzodiazepines. Although benzodiazepine agonists are able to bind to intracellular GABA receptors, they produce little or no allosteric modulation. A recent examination of benzodiazepine allosteric uncoupling has shown that benzodiazepine site internalization is part of the uncoupling mechanism.<sup>114</sup> This finding is likely the observation of benzodiazepine sites located on clathrin vesicles.

Considerable evidence has been compiled recently underlying a complete and complex molecular mechanism of phosphorylation and posttranslational modifications of GABA<sub>A</sub> as a response to benzodiazepines that possibly contribute to the observed uncoupling and tolerance building mechanism.<sup>119</sup> These complex molecular mechanisms of posttranslational modifications of the GABA<sub>A</sub> receptor resulting from palmitoylation, ubiquitination, and especially phosphorylation, are believed to dictate the role in regulating the recycling of GABA<sub>A</sub> receptors through different protein kinases and ultimately impact inhibitory currents.<sup>18,84,119</sup>

cAMP-dependent protein kinase A (PKA) has been identified to lead the changes and alterations of GABA<sub>A</sub> receptor functioning of CA1 pyramidal cells within the hippocampus after long-term flurazepam administration.<sup>120</sup> Further evidence accumulated over the importance of PKA in the formation of benzodiazepine tolerance showed that mutations to a single PKA phosphorylation site prevented uncoupling, even after chronic diazepam treatment.<sup>121</sup>

As compared to wild-type mice, a wide range of transcripts that are thought to contribute to the neuroplastic mechanisms of tolerance remained unchanged in  $\alpha_1$ (H101R) point mutated mice after diazepam administration.<sup>122</sup> Transcripts changes such as brain-derived neurotrophic factor and calcium/calmodulin-dependent kinase II play important roles in synaptic plasticity;  $\alpha_1$ (H101R) mice did not produce transcript changes after diazepam treatment.<sup>122</sup> This is direct evidence regarding the involvement of  $\alpha_1$  containing GABA<sub>A</sub> receptors in the role of benzodiazepine tolerance. It agrees with earlier studies wherein  $\alpha_1$  subtype inactive benzodiazepines imidazenil and TPA023 consistently failed to show anticonvulsant tolerance in chronic dosing in mice and monkeys.<sup>123–129</sup>

Interestingly, there seems to be almost no downregulation of GABA<sub>A</sub> receptors despite long-term heavy administration of imidazenil as compared to diazepam.<sup>83</sup> Future studies could compare the uncoupling and posttranslational modification differences between traditional benzodiazepine agonists such as diazepam and  $\alpha_1$  subtype ineffective compounds like TPA023 and imidazenil.

## Conclusion

Although partial agonism at  $\alpha_1$  containing GABA<sub>A</sub> receptors appears to reduce abuse potential, it appears insufficient. Bretazenil and etizolam which showed reduced abuse potential in animal models do not keep their promise.<sup>129,130</sup> When the bretazenil dose increased from 2 to 4 mg in humans, users reported liking the drug. While etizolam has seen popular

recreational use, it is sold online as a research chemical capable of inducing euphoria.<sup>131</sup> As a result, if clinical effects can be achieved without the involvement of  $\alpha_1$  subtype, it should be avoided in future drug design of benzodiazepines and similar compounds.

As already discussed, both  $\alpha_2$  and  $\alpha_3$  containing GABA<sub>A</sub> receptors and their involvement in anxiety suppressing effects of benzodiazepines are critical. This is because most current models have characterized  $\alpha_2$  containing GABA<sub>A</sub> receptors as the sole mediator of anxiety in benzodiazepines.<sup>35–39</sup> As highlighted in the model presented in this review, the search for a selective anxiolytic should not be constrained to  $\alpha_2$  selective.<sup>29–41</sup>

Benzodiazepine tolerance is complicated and appears to result from a combination of various factors. Complex mechanisms involving uncoupling, intracellular trafficking, post-translational modifications of GABA<sub>A</sub> receptors all appear to contribute toward benzodiazepine tolerance.<sup>89,119</sup> Various studies have also suggested the involvement of other neurotransmitters, especially adenosine and glutamate, during the tolerance and withdrawal effects of benzodiazepines.<sup>92–105</sup>

The efficacy of benzodiazepines may also play a role in tolerance as full agonists produce more tolerance than partial agonists.<sup>107</sup>

In the light of current evidence,  $\alpha_1$  dormant,  $\alpha_2$ ,  $\alpha_3$ , and  $\alpha_5$  subtype partial agonists not only possess low abuse potential, but are also low or devoid of tolerance building. There is evidence that  $\alpha_1$  containing GABA<sub>A</sub> receptors directly contribute to the downstream effects of tolerance, because  $\alpha_1$ (H101R) mice have been shown to maintain expressions in neuroplasticity-coding transcripts after diazepam administration.<sup>120</sup> This perfectly agrees with data from animal studies regarding the lack of tolerance in  $\alpha_1$  subtype inactive compounds such as TPA023B and imidazenil.<sup>121–126</sup> Future drug discovery involving tranquilizers should look for partial agonists of  $\alpha_2$ ,  $\alpha_3$ , and  $\alpha_5$  containing GABA<sub>A</sub> receptors; Valium without its side effects is potentially achievable.

## Disclosure

The authors report no conflicts of interest in this work.

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