Selective Influence on Contextual Memory: Physiochemical Properties Associated with Selectivity of Benzodiazepine Ligands at $GABA_A$ Receptors Containing the α5 Subunit

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Ligands that bind to the benzodiazepine binding site on the $GABA_A$ receptor can attenuate or potentiate cognition. To investigate this property, the chemical determinants favoring selective binding or selective activation of the α5β2γ2 and α1β2γ2 $GABA_A$ receptor isoforms were examined. A 3D-pharmacophore, developed from a diverse set of BDZR ligands, was used as an initial basis for multivariate discriminant, fragment, and 3D-quantitative structure−activity relationship analyses, which formed the criteria for selection of additional compounds for study. We found that the electrostatic potential near the ligands' terminal substituent correlated with its binding selectivity toward the α5β2γ2 versus α1β2γ2 isoform; while the fragment length and frontier molecular orbital energetics correlated with a compounds influence on electrophysiological activity. Compounds with promising α5 profiles were further assessed for their ability to attenuate scopolamine-induced contextual memory impairment in mice. Surprisingly, both weak inverse agonist and antagonists that display binding selectivity toward the α5β2γ2 isoform were able to attenuate contextual memory impairment.

Introduction

Senile dementia of the Alzheimer's type (SDAT)[a] and age-related memory decline arise from progressive failure of the cholinergic system.[1,2] Although most neurotransmitter receptor systems are degenerating in the brains of individuals with SDAT, the GABAergic infrastructure remains relatively intact.[3–5] Reducing GABAergic inhibition in brain regions where the weakened cholinergic neurons project could potentially augment the functional impact of the residual ACh released.[6,7]

Mammalian γ-aminobutyric $acid_A$ ($GABA_A$) receptors are composed of a combination of multiple transmembrane protein subunit subtypes α_{1-6}, β_{1-3}, γ_{1-3}, δ, ϵ, π, θ).[8–11] These subunits assemble to form heteropentameric Cl^- channels that are regulated by the neurotransmitter GABA. Ligands that bind to the benzodiazepine receptor (BDZR) binding site on $GABA_A$ receptors are capable of modulating the influence of GABA on the $GABA_A$ receptor. In addition to BDZR ligands therapeutic value as sedatives, muscle relaxants, anticonvulsants, and anxiolytics,[12] they are also capable of influencing vigilance and cognition.[13–15] The pharmacology associated with a particular BDZR ligand is dependent in part on its ability to bind preferentially to a particular $GABA_A$ receptor combination. Therefore, targeting a specific receptor isoform that is largely restricted to certain brain regions could provide a means to selectively influence particular behaviors while limiting undesirable side effects. BDZR agonists often cause cognitive impairment in both animals and humans.[16] This impairment is thought to occur by preventing the induction of long-term potentiation (LTP) in hippocampal neurons.[17,18] Conversely, BDZR ligands that are inverse agonists consequently attenuate GABA-mediated Cl^- passage into neurons, thereby potentiating LTP in hippocampal neurons,[18,19] resulting in the facilitation of learning and memory[20] in both animal models[14,21] and in human studies.[15,22]

The association of $GABA_A$ receptor composition with the physiology it mediates has been advanced through the use of transgenic mice.[23–27] From such efforts, associations have been put forward for the involvement of the α_1 subunit in sedative, anticonvulsant, and cognitive effects: the α_2 and α_3 subunits in anxiolytic and myorelaxant effects[28,29] and the α_5 subunit with temporal and spatial memory.[30–32] The prominent expression of the α4 subunit in the thalamus and dentate gyrus favors a role in sensory processing and cognition, while the more restricted expression of the α6 subunit to the cerebellum favors its involvement in motor function. However, as our understanding of the role of the cerebellum in nonmotor functions continues to expand, the role of the α6 subunit will likely expand as well.

The α5 subunit is a component of about 20% of the $GABA_A$ receptors found in hippocampus, which are primarily extrasynaptically expressed. Subsequently, animal studies have demonstrated that compounds that either display binding selectivity[33] or efficacy selectivity[24,34,35] and attenuate GABA's effect on $GABA_A$ receptor that contain the α5 subunit can enhance cognition. An example of what is meant by efficacy selective activation is demonstrated with the compound α5IA, an imidazoquinoxaline which lacks binding selectivity among the different $GABA_A$ receptor isoforms but exhibits robust attenuation of GABA mediated currents through the α5 containing $GABA_A$ receptor isoform with only slight effects on currents via the other isoforms.[34,35] Until recently, only modest progress

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[a] Abbreviations: GABA, γ-aminobutyric acid; BDZR, benzodiazepine binding site on the $GABA_A$ receptor; SDAT, senile dementia of the Alzheimer's type; LTP, long-term potentiation; QSAR, quantitative structure−activity relationship; COMFA, comparative molecular field analysis; COMSIA, comparative molecular similarity indices analysis; HOMO, highest occupied molecular orbital; LUMO, Lowest unoccupied molecular orbital; HYD, hydrophobicity.

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had been made in the design of receptor-subtype selective ligands. However, this is changing, with a variety of reports detailing success in achieving selectivity in the synthesis of BDZ ligands that exhibit 100-fold selectivity toward particular $GABA_A$ receptor isoforms.[36–39] Unfortunately, BDZ ligands that exhibit true specificity to a single $GABA_A$ receptor isoform (affinities 1000-fold higher for one $GABA_A$ receptor isoform over all other isoforms) are to date rare.

To obtain meaningful correlations linking structural variation with changes in pharmacology, multivariate statistical approaches embodied in quantitative structure−activity relationships (QSAR) and chemometrics are required. This affords one the possibility of detecting subtle patterns that modulate either binding/nonbinding and/or the nature of activation through a particular receptor isoform. To date, several groups have investigated the structural/chemical basis for binding or activational selectivity of particular $GABA_A$ receptor isoforms that affect memory.[19,24,35,37] Both structure based design[40,41] and indirect design[42–44] approaches have been employed in guiding chemical transformation of lead compounds that were initially identified via mass-screening efforts. From such approaches, there have been numerous instances of success,[45,46] including the development of selective COX-2 inhibitors[47] and selective serotonin reuptake inhibitors.[48]

Considerable effort has been made in order to develop pharmacophores that characterize the binding of BDZR ligands to their respective binding site between the α and γ subunits of the various $GABA_A$ receptor isoforms.[26,37,49–52] While our past validation efforts involved using these pharmacophores to extract compounds with binding affinity and activity at the behavioral end point of interest, the pharmacophores themselves are for the most part "nonspecific"[43] in that compounds extracted from chemical database searches bound to and activated multiple $GABA_A$ receptor isoforms. Consequently, 3D-QSAR (comparative molecular field analysis (COMFA) and comparative molecular similarity indices analysis (COMSIA)) based on these overlap rules resulted in equivalent statistics for binding models of the ligands regardless of the $GABA_A$ receptor composition (i.e., $\alpha1\beta x\gamma2$, $\alpha2\beta x\gamma2$, $\alpha5\beta x\gamma2$, $\alpha6\beta x\gamma2$; where x = 2, 3). To the point of the present study, several groups of compounds synthesized by Cook's group[26] have proven to be a fruitful testing ground to elucidate global features that are important for binding selectivity to $\alpha5$-subunit-containing $GABA_A$ receptors as well as scrutinizing the activational influence these compounds exert upon cognition.

The core aim of the present study is to address the structural/chemical basis for selectively influencing contextual memory by modulating $\alpha5$ subunit containing $GABA_A$ receptors. The first step in this process is to understand the structural constraints necessary to elicit both potent and selective binding to $GABA_A$ receptors that contain the $\alpha5$ subunit, closely followed by developing an understanding of how the structural features influence the resulting electrophysiology of $\alpha5$ subunit containing $GABA_A$ receptors. Compounds displaying favorable profiles were further evaluated for cognitive influence in a mouse model of contextual memory impairment.

Results and Discussion

Organization of Study. We have previously assessed a set of nonselective BDZR ligands for their ability to influence spontaneous locomotor activity and/or contextual memory in adult C57BL/6 mice.[51,53] Initial computational work on this set of BDZR ligands (Figure 1A) suggested that particular stereoelectronic descriptors (e.g Sterimol L parameter) were discriminants of whether a compound that exhibits reasonable binding affinity to the $\alpha5\beta2\gamma2$ isoform could attenuate scopolamine-induced contextual memory impairment. This served as the initial criteria for selecting additional compounds to be used in the present study. The current efforts involved assessing in vitro binding and electrophysiological activation of the newly assembled compound test set in cells expressing single $GABA_A$ receptor variants. Subsequently, this information was used in conjunction with computational methods[42,52,54] in order to correlate structural, electrostatic, thermodynamic, and electronic properties of compounds of interest with their in vitro binding affinities and electrophysiological responses. Our goal was to discover chemical discriminants that favor selective influence (selective binding or efficacy selective activation) over α_5 subunit containing $GABA_A$ receptors to test the hypothesis that one can selectively influence contextual memory via the $\alpha5$ isoform. For practical purposes, our current computational focus was primarily restricted to the assessment of BDZR ligand binding affinities associated with the $\alpha1\beta x\gamma2$ and $\alpha5\beta x\gamma2$ $GABA_A$ receptor isoforms (where βx can be either the $\beta2$ or $\beta3$ subunit, which has little impact on binding affinities of BDZR ligands) because the $\alpha1$ subunit containing isoforms constitute a significant contributory factor toward undesirable psychomotor and convulsive effects. 3D pharmacophores were validated by searching chemical databases to find additional compounds that were consistent with the pharmacophore metrics. These "hits" were then tested for binding to, and subsequent influence on, GABA-induced activation of the $\alpha1\beta2\gamma2$ and/or $\alpha5\beta2\gamma2$ $GABA_A$ receptor isoforms, expressed separately in a cell expression system. Compounds found to display appropriate profiles were further assessed for their ability to attenuate contextual memory impairment in our previously characterized mouse model.[55]

Validation of the 3D Pharmacophore for Sedation and Contextual Memory End Points by Searching and Testing within 3D Databases. The initial pharmacophore development for the contextual memory and sedation end points centered on diverse sets of nonselective ligands based on 1,4-benzodiazepines, imidazobenzodiazepines, imidazopyridines, β-carbolines, pyrazoloquinolines, imidazoquinolines, imidazopyrimidines, and imidazothienodiazepines classes, which have been characterized in relation to sedation (reduction of spontaneous locomotor activity) and the attenuation of scopolamine-induced contextual memory impairment.[51,53] This set was dominantly comprised of BDZR ligands that lack selectivity between the benzodiazepine binding sites found on the various $GABA_A$ receptor isoforms. Using such a ligand set allowed us to first determine core features common to numerous classes of BDZR ligands that mediate their behavioral effects through multiple $GABA_A$ receptors isoforms. Figure 1A displays this diverse set of compounds along with their binding affinities (in nM) at the $\alpha1\beta2\gamma2$ and $\alpha5\beta2\gamma2$ $GABA_A$ receptor isoforms. As is typical for most benzodiazepines, subtle structural changes to pendant groups attached to particular scaffolds/templates result in significant variations in binding profiles relative to the different $GABA_A$ receptor isoforms. Moreover, as demonstrated in Figure 1B, small structural/chemical variations can drastically alter the observed behavioral pharmacology for a given compound template, e.g., the CGS series in this figure illustrates how permutation of a −H to a −OCH3 or a −Cl group dramatically alters the pharmacological profile of the pyrizoquinolines (CGS8216, CGS9896, and CGS9895). Likewise small changes in substituents on the imidazobenzodiazepine template common to Ro15-1788 and Ro16-6028 (Figure 1B) lead to quite different pharmacological profiles.[60–63]

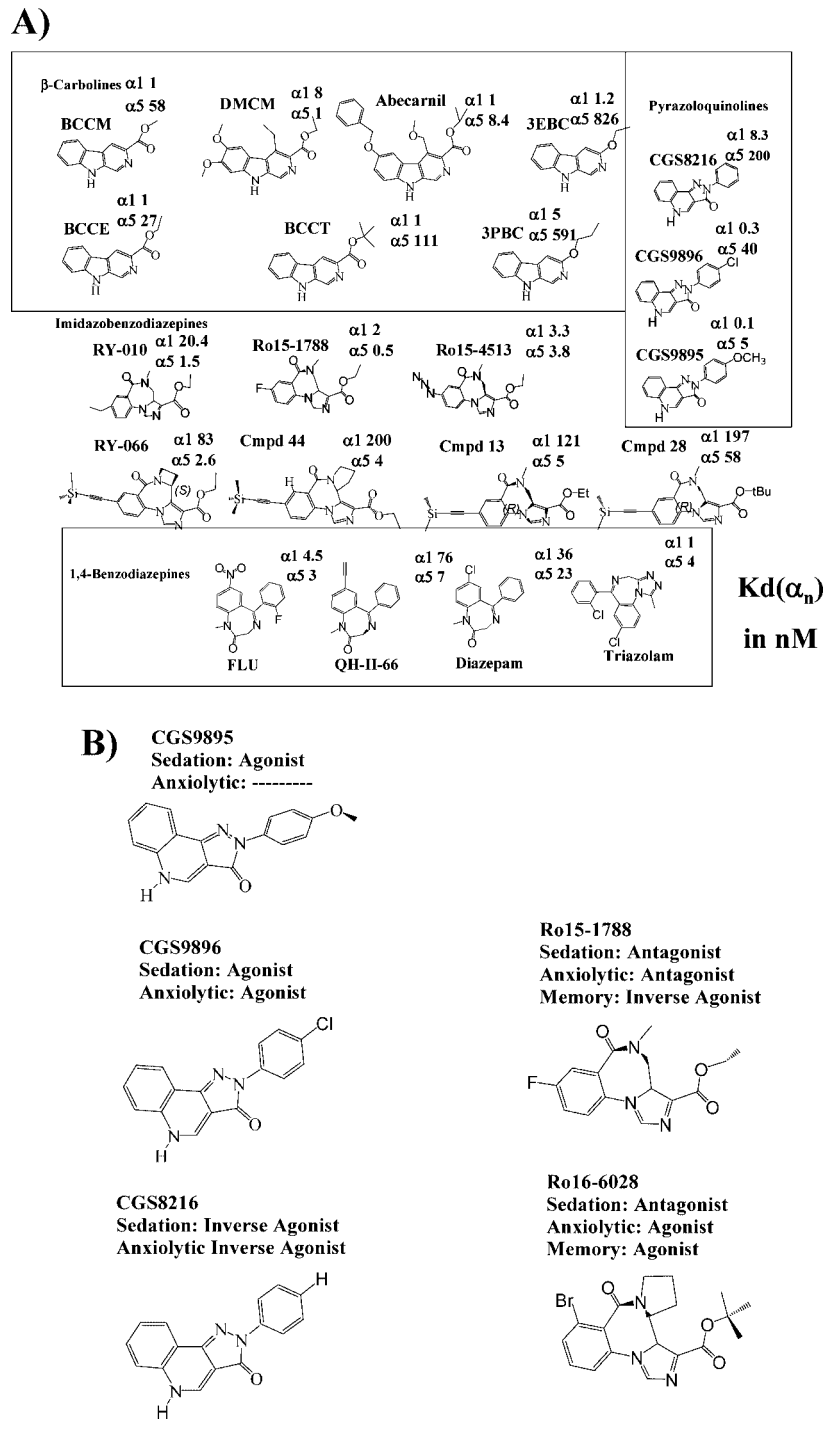
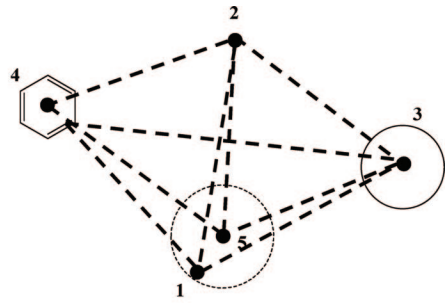


Figure 1. (A) Subset of the diverse training set used to develop the initial pharmacophores (overlap rules) for binding to the $\alpha1\beta2\gamma2$ and $\alpha5\beta2\gamma2$ $GABA_A$ receptor subtypes. (B) Examples illustrating subtle variations in scaffold substitutions that modulate the behavioral profiles of each of the selected benzodiazepine ligands.

Not surprisingly, pharmacophores derived from nonselective compounds that were active at both the sedation and contextual memory end points have similar distance (pharmacophore) metrics. The pharmacophores depicted in Figure 2 were derived by: (1) development of conformational libraries based on nested rotation of all rotatable bonds in compounds previously examined for influence on sedation or contextual memory[51,53] and (2) examining whether a common set of interpharmacophore point distances are present for at least a 1-conformation that was within 10 kcal/mol of the minimum energy conformer of each ligand, employing a 2 Å distance tolerance. The left upper panel of Figure 2 shows the interpharmacophore point distances corresponding to the 3D pharmacophore, shown in the upper right panel, that subsumes both the commonalities found in the training set compounds that influence sedation (overlapped in the lower left panel of Figure 2) and those that influence memory (overlapped in the lower right panel of Figure 2).

Figure 3 shows a small subset of compounds that were extracted from the Maybridge Chemical Database using the memory pharmacophore (Figure 2) as the basis for the search. Several of these compounds were found to exhibit submicromolar binding affinities to $\alpha1\beta2\gamma2$ and/or $\alpha5\beta2\gamma2$ $GABA_A$ receptor isoforms and could be considered lead compounds for further refinement. Furthermore, two of these compounds were

Distance No's	Sedation	Memory
1-2	4.9±1.1	3.2±1.7
1-3	5.6±1.8	3.5±1.0
1-4	4.4±1.1	6.9±0.7
1-5	2.3±0.9	
2-3	4.5±1.2	5.2±1.2
2-4	4.7±1.2	8.9±1.4
2-5	3.1±1.3	
3-4	5.1±1.1	4.9±0.0
3-5	3.0±0.8	
4-5	7.8±1.3	



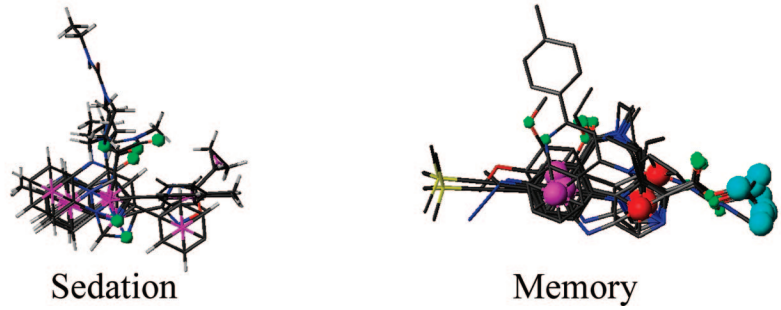


Figure 2. Inset table displays the distances between pharmacophore points (1, 2 = hydrogen bond acceptors, 3 = hydrophobic terminus, 4 = aliphatic/aromatic centroid with LUMO, 5 = ring system containing hydrogen bond acceptor) identified in benzodiazepines binding to and activating $GABA_A$ receptor subtypes associated with sedation and memory. Upper right panel is a graphical depiction of 3D pharmacophore points for compounds having behavioral effects at the sedation and memory end points corresponding to the table (upper left). Lower left and right panels show the overlap of several compounds complying with the sedation and memory pharmacophores, respectively, illustrating some of the commonalties: green spheres represent hydrogen bond-acceptors, red spheres represent the polar core ring system, purple centroid represents the aromatic/ring, LUMO center, blue sphere represents the hydrophobic group at one end of the ligands.

able to influence spontaneous locomotor activity at ≤30 mg/kg. These results illustrate that the contextual memory pharmacophore can serve to increase the likelihood of identifying compounds from large chemical databases that are capable of binding to $\alpha1\beta2\gamma2$ and/or $\alpha5\beta2\gamma2$ $GABA_A$ receptor isoforms. However, it does not encode sufficient information by itself to identify selective compounds from a binding or activational perspective. Rather, the development of this pharmacophore serves as a basis for overlapping structural features prior to performing 3D-QSAR (COMSIA) computations and serves to select a particular set of conformations, complying with the 3D-pharmacophore, to be used in computing properties for fragment QSAR analysis. It is these QSARs, rather than the initial 3D pharmacophore (overlap rule), that will actually provide us with the necessary insights toward understanding what is required to achieve the desired selectivity.

3D-QSAR (COMSIA) Models of Binding to $\alpha1\beta2\gamma2$ and $\alpha5\beta2\gamma2$ Receptor Isoforms. Figure 4 shows 3D-QSAR models for binding to the diverse set of compounds shown in Figure 1A derived by overlapping these compounds at the pharmacophore points shown in Figure 2 and performing a COMSIA analysis in terms of hydrophobicity, electrostatic, and steric similarity indices. Figure 4A shows that a predictive COMSIA for binding to $\alpha1\beta2\gamma2$ receptor subtypes is obtained with a $q^2 = 0.43$, a noncross- validated $r^2 = 0.99$, and a standard error of 0.50. Figure 4B shows a COMSIA model for binding to $\alpha5\beta2\gamma2$ isoforms with a $q^2 = 0.41$, $r^2 = 0.99$, and a standard error of 0.52. The order of magnitude of q^2 (the predictive r^2) values are reflective of the typical predictivity of the 3D-QSAR models. The expected value of q^2 for such models derived from diverse training sets is typically on the order of 0.3. The lower panel of Figure 4A reveals that this QSAR is capable of predicting the order of magnitude of binding affinities, toward the $\alpha1\beta2\gamma2$ isoform, of compounds not included in the training set of the COMSIA model. The 3D-QSAR (COMSIA) for compounds that bind nonspecifically to $\alpha1\beta2\gamma2$ and $\alpha5\beta2\gamma2$ would indicate comparable contributions of hydrophobic, electrostatic, and steric COMSIA fields as indicated by the magnitude of the fractions, which reflect the relative contributions to the COMSIA regression equation, as reported in Table 1A. The inset colored molecule in Figure 4B is of compound **44** (see Figure 1 A), indicating the areas that variations in the negative electrostatic (increases−red/decreases−blue) charge densities correlate with increases in the ligands binding affinity. The ester functional group is included in the region where increases in negative charge, affecting electrostatics, would increase the binding affinity.

While the use of a diverse training set is useful for developing a model capable of providing predictions of binding affinities of diverse compounds, one loses information as to what features are most important to binding selectivity when employing such an approach. We, therefore, took a subset of 10 imidazobenzodiazepines that were more homogeneous, to analyze what characteristics lead to more selective binding in regards to the $\alpha5\beta2\gamma2$ receptor isoform (see Table 1). The resultant analysis gave a $q^2 = 0.94$, $r^2 = 0.99$, and a standard error of 0.09.

While an analysis based on a narrow compound class such as this has limited predictivity for templates other than imidazobenzodiazepines, it does provide a means to examine the relative importance of particular components with regard to the influence on the modulation of binding affinities. For example, the data in the lower portion of Table 1B provides an analysis of the importance of the contribution (fraction) of the electrostatic component in explaining the variations in the experimental binding affinities for this more restricted set. For $\alpha5\beta2\gamma2$, the value of the electrostatic component was found to be 0.041, which is minor compared to the contributions of the hydrophobic and steric components, which were 0.61 and 0.35, respectively. In contrast, the relative contribution of the electrostatic com-

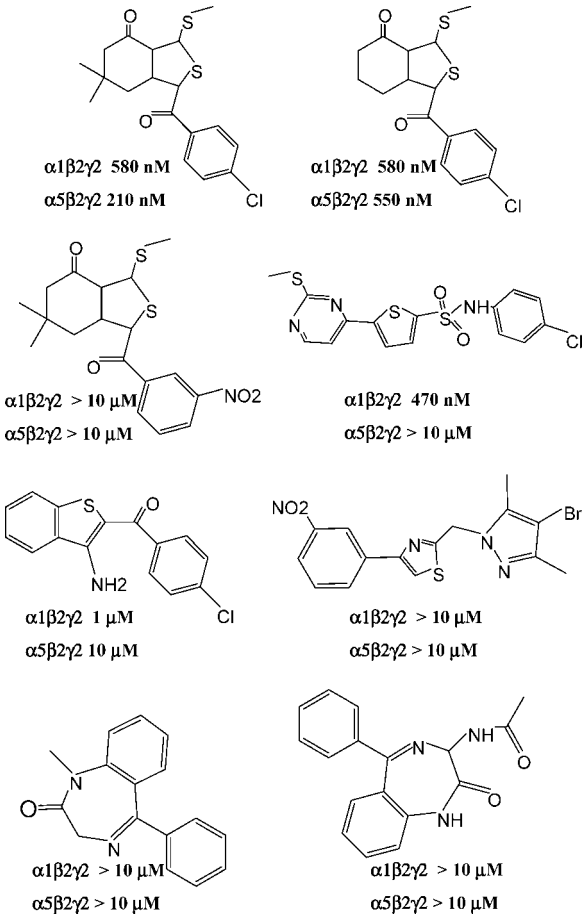


Figure 3. Selected structures retrieved from pharmacophore directed searches of the Maybridge Chemical Database with indicated binding affinities (IC_{50}, [H^3]Ro15-4513 competition in a cell based binding assay, see Experimental Section) to $GABA_A$ receptors with either the $\alpha1\beta2\gamma2$ or $\alpha5\beta2\gamma2$ stoichiometry.

ponent is much greater for the COMSIA analysis for $\alpha1\beta2\gamma2$. The more restrictive COMSIA model is suggestive of an electrostatic discriminating factor for selective binding to $\alpha1\beta2\gamma2$ versus $\alpha5\beta2\gamma2$. We therefore undertook a quantum mechanical study of this series to probe the underlying electrostatic potential surfaces and correlated the differences with differential binding selectivity to $\alpha5\beta2\gamma2$ and $\alpha1\beta2\gamma2$. This electrostatic information along with the volumes of the substituents was used in a fragment QSAR for binding, thereby providing an independent crosscheck on the information gleaned from the COMSIA analysis.

Fragment QSAR: Facets Effecting Differential Binding between $\alpha1\beta3\gamma2$ and $\alpha5\beta3\gamma2$ $GABA_A$ Receptor Isoforms. To scrutinize the robustness of the conclusions deduced from the COMSIA analysis, we performed an analysis by the fragment QSAR approach. While 3D-QSAR establishes the dependence of variations of binding affinities or pharmacological activity upon variations in molecular field components (electrostatics, hydrophobicity, steric, etc.), it is often quite sensitive to the superposition of compounds in the analysis. It is always comforting if the COMSIA analysis results, based on analysis of variations in fields surrounding the whole ligands, are chemically intuitive or if one obtains similar conclusions as to what properties are important from a fragment QSAR analysis based on substituent variations on a shared template (Figure 5A). The idea behind this additional analysis is illustrated in the four panels depicted in Figure 5B that display the computed

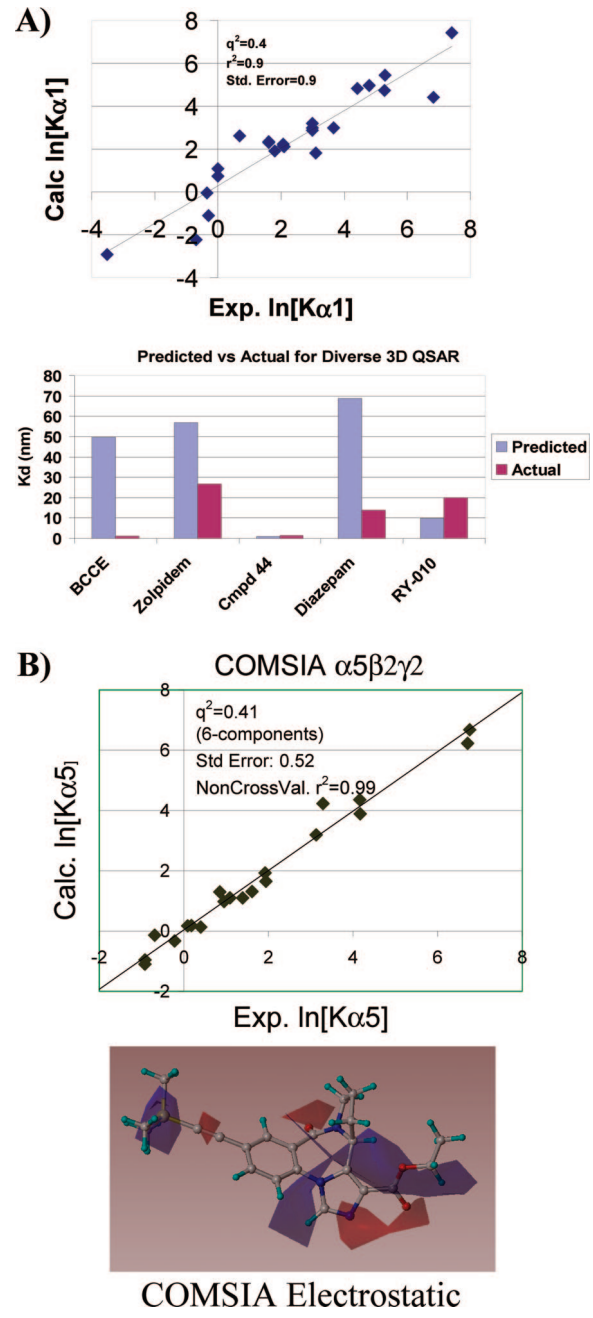


Figure 4. (A) COMSIA (comparison of calculated and experimental values) based on the overlap rules shown in Figure 2 and the observed binding affinities of the BDZR training set ligands at the $\alpha1\beta2\gamma2$ isoform. The q^2 for the COMSIA analysis of the diverse training set shows that the QSAR is modestly predictive. This is borne out by order of magnitude agreement of the predicted and observed $\alpha1\beta2\gamma2$ binding affinities (lower panel). (B) COMSIA model predictive of compounds binding to the $\alpha5\beta2\gamma2$ $GABA_A$ receptor isoform; lower panel indicates regions where variation in ligand negative electrostatic charge increases (red) or decreases (blue) binding affinity.

molecular electrostatic potential (MEP) surfaces computed on the van der Waals surface of the ligands. The surfaces in Figure 5B are colored by the magnitude and sign of the electrostatic potential with red regions being the most favorable regions for interaction with a $GABA_A$ receptor hydrogen bond donor and blue being the least favorable. A legend key, to the side of each surface, provides the correspondence between the magnitude (sign) of the electrostatic potential and coloration of the surface.

Table 1. Normalization Coefficients and Fractions of the Components in the COMSIA Analyses for A, a Diverse Training Set of Benzodiazepines (from Figure *1*A) and B, a Homogenous Set Consisting of Imidazobenzodiazepines[a]

A	α5β2γ2		α1β2γ2	
component	norm coeff	fraction	norm coeff	fraction
electrostatic	1.48	0.35	1.14	0.29
steric	1.13	0.27	0.99	0.26
hydrophobic	1.63	0.38	1.63	0.45

B	homogenous set of imidazobenzodiazepines[a]			
component	norm coeff	fraction	norm coeff	fraction
electrostatic	0.12	0.041	1.14	0.256
steric	1.04	0.35	1.15	0.29
hydrophobic	1.81	0.61	1.73	0.44

[a] RY-010, RY-024, RY-066, RY-080, Ro15-1788, Ro15-4513, PWZ-029, PWZ-031, PWZ-035A, and cmpd 44.

If we examine these surfaces, we first note that in cases where the terminal substituent has polar heteroatoms, there is a shallow second minimum (with red/orange coloration) in the electrostatic potential opposite the terminal substituent (fragment) polar atoms. As one goes from an ester terminal substituent ($-C(C{=}O)OCH_2CH_3$) in Ro15-1310, to an ether ($\rightarrow CH_2OCH_3$) in PWZ-029, to a keto functionality ($-(C{=}O)CH_2CH_2CH_2CH_3$) in compound **32** and finally to a mere alkyl substituent ($-CH_2CH_2CH_2CH_2CH_3$) in compound **40**, the potential minimum decreases in depth until it is absent in compound **40**. These MEP surface changes are reflected in the color changing from red/orange to blue/green in the region of the varied substituent polar atoms, mirroring a loss in favorable hydrogen acceptor capability as a result of terminal substituent (fragment) substitution.

Below each panel in Figure 5B are listed the binding affinities at the α1β3γ2 and α5β3γ2 $GABA_A$ receptor isoforms. In the present example, we have computed the MEP for the whole molecule rather than just the fragments/substitutions to the core template in a manner analogous to Huang and co-workers,[64] who examined 3D-QSAR models of flavanoids and found that MEP and frontier orbitals were discriminants of binding to $GABA_A$ receptors. From the depiction of the molecular surfaces colored by electrostatic potentials, one can deduce that the electrostatic potential surface minimum, close to the benzodiazepine ring −N−, is only slightly altered by the changes in the fragment a few angstroms away. The changes in the second electrostatic minimum, closest to the fragment itself, are more pronounced with changes in the terminal substituent (fragment). Note that as one proceeds clockwise from Ro15-1310 (Figure 5B) to PWZ-029 to compounds **32** and **40**, the binding affinities to α5β3γ2 and α1β3γ2 both drop, with the binding affinity to α1β3γ2 dropping more rapidly than for α5β3γ2. The ligand binding affinities appear to be correlated with decreases in the depth of the second minimum in the electrostatic potential near the fragment varied in the set.

While there appears to be a correlation of binding selectivity of compounds to α5β3γ2 (versus α1β3γ2) with the magnitude of the electrostatic potential near the terminal substituent, the substituent (fragment) changes, in fact, are simultaneously varying many facets: e.g., molecular volume, hydrophobicity, and electronic properties. We therefore performed a fragment QSAR analysis so as to determine the relative importance of these factors considered simultaneously. The fragment QSAR analysis results shown in Figure 5C,D correlate experimental binding affinities with values of the fragment electrostatic potential minima, volume, and hydrophobicity. This analysis resulted in r^2 values of 0.84 and 0.94 for binding models for α5β3γ2 and α1β3γ2, respectively. The fragment QSAR equations highlighted in the figure indicate, analogous to the COMSIA analyses, that the electrostatic contributions to binding are more important to the α1β3γ2 isoform rather than the α5β3γ2 isoform. The magnitude of the coefficient of the electrostatic term is significantly less in the α5β3γ2 QSAR analysis compared to the α1β3γ2 analysis (i.e., −81.1 for α5β3γ2 versus −109 α1β3γ2). The QSAR equations in Figure 5C,D indicate the contributions of the hydrophobic (coefficients of 6−7) and steric (volume) terms (coefficients of ∼0.12) are roughly equivalent between the α5β3γ2 and α1β3γ2 isoforms and are less important to the binding affinity than the electrostatic contribution (EL), which influences the α1β3γ2 isoform more than the α5β3γ2 isoform. Therefore, the depiction in Figure 5B and the QSAR equation/analysis both reveal that while the reduction in the polar components in the fragment results in reductions in binding affinity to α1β3γ2 and α5β3γ2 isoforms, it also substantially increases binding selectivity to α5β3γ2. The fragment QSAR analysis for binding to the α5β3γ2 and α1β3γ2 isoform are statistically significant with $F_{3,7}$ values for the analysis of 12.0 and 36.1, respectively, indicating that the regressions are significant at the 99% confidence level.

The original pharmacophore depicted in Figure 2, created from information derived from diverse compounds exhibiting activity at the contextual memory end point, revealed commonalities in core features and similar distance relationships[51] of ligands capable of binding to $GABA_A$ receptors with α1βxγ2 compositions. Most of the training set compounds in Figure 1 have an ester moiety at one end of the ligand that appears to be essential for eliciting substantial binding affinities to $GABA_A$ receptors associated with sedation and contextual memory effects. However, the results discussed above reveal that the elimination of one or more of the hydrogen bond acceptors in the region of the ester moiety in many of the training set compounds (corresponding to pharmacophore point 2) nevertheless preserves the substantial binding affinity to α5βxγ2 isoforms while lessening the binding affinities to the other isoforms. This suggests that one of the pharmacophore points indicated in Figure 2, while being a commonality in many of the training set compounds studied, is nonessential to appreciable binding to α5βxγ2 isoforms. It is clear, however, that inclusion of such a polar hydrogen bond accepting group at this location might increase the binding affinity an order of magnitude. Compounds PWZ-029, PWZ-031A, and PWZ-035A in Figure 5A, were originally reported as having binding affinities of greater than 300 nM in regards to the α1β3γ2 isoform.[37] We reassessed the binding of PWZ-029 to the BDZR binding site in regards to the α1β3γ2 isoform in order to establish a more accurate value and found its binding affinity to be 920 nM to the α1β3γ2 isoform compared to the binding affinity of this compound at the α5β3γ2 isoform of 39 nM.[26,37] By all indications, the elimination of the ester moiety from the 1,4-benzodiazpine template offers discrimination between α1βxγ2 and α5βxγ2 receptor compositions. The extra hydrogen bond acceptor is virtually a requirement for binding to α1βxγ2 but not α5βxγ2. We further address the pharmacological activities of these compounds below.

$GABA_A$ receptors containing α1βxγ2 and α5βxγ2 subunit compositions are believed to be associated with sedative and memory effects, respectively.[65] Given the 70% sequence identity between α subunits, this implies that small differences in the BZDR ligand binding site, situated at the interface between the α and γ subunits, are responsible for large changes in binding

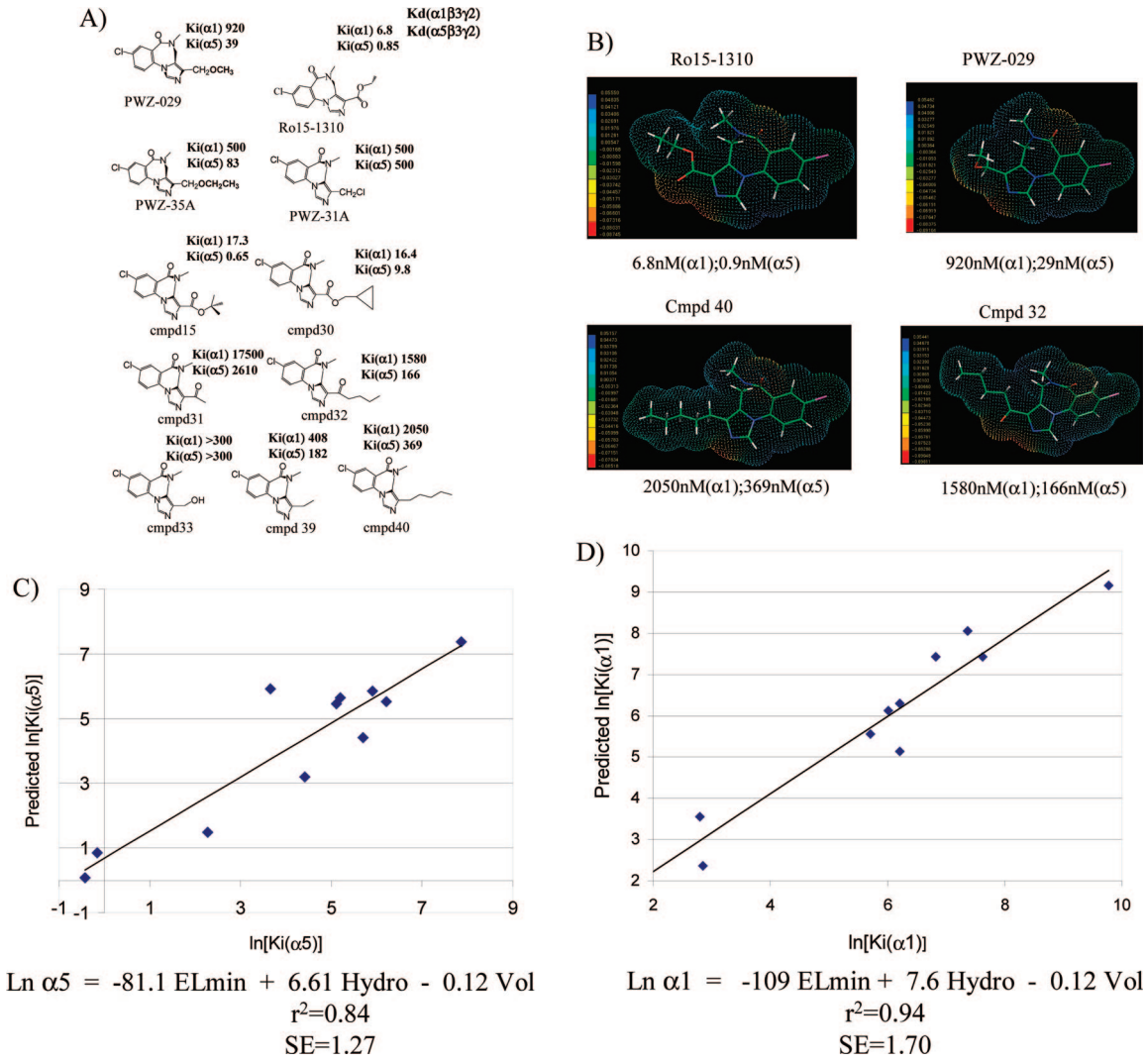


Figure 5. (A) Compounds employed in the fragment QSAR analyses for $\alpha1\beta3\gamma2$ and $\alpha5\beta3\gamma2$ binding (shown in panels C and D). The binding affinities to these two receptor subtypes are shown in insets. (B) Computed molecular electrostatic potential (computed using density function theory (B3LYP/6-31G**)) evaluated on the van der Waals surfaces of compounds showing selectivity of binding to $\alpha5\beta3\gamma2$ versus $\alpha1\beta3\gamma2$. (C) Fragment QSAR for binding of compounds in panel (A) to $\alpha5\beta3\gamma2$ and (D) to $\alpha1\beta3\gamma2$ receptor isoforms, illustrating, via the derived QSAR relations at the bottom of the figures, that the electrostatic contributions (EL min) are more important for ligand binding to the $\alpha5\beta3\gamma2$ receptor isoform than to the $\alpha1\beta3\gamma2$ isoform, as indicated by the larger magnitude of the coefficient of the electrostatic term for the $\alpha5\beta3\gamma2$ isoform compared to that for the $\alpha1\beta3\gamma2$ isoform. SE = standard error.

and activation. Renard and co-workers illustrated this principle in site directed mutagenesis studies on $\alpha5\beta2\gamma2$ subunits wherein they introduced α5-P162T, α5-E200G, and α5-T204S mutations.[66] The authors of that study argued that the first two mutations appeared to alter the binding pocket conformation, whereas α5-T204S appeared to better allow the formation of a hydrogen bond with a proton accepting group on zolpidem. The α5-T204S mutation itself seemed to confer α1-like binding properties to a receptor subtype that was essentially α5 in character, causing a change in the binding affinity of zolpidem from >10000 nM to ~300 nM. Clearly fine-tuning of the hydrogen bonding complementarity of ligand to receptor is a significant modulator of ligand binding selectivity toward $\alpha5\beta x\gamma2$ isoforms.

Computational Assessment of Molecular Properties Correlated with Electrophysiological Response. After examining the molecular properties that provide insight into binding discrimination between $\alpha5\beta x\gamma2$ and $\alpha1\beta x\gamma2$ receptor isoforms we sought to also determine what molecular properties (see Supporting Information, Table 1) were correlated with electrophysiological response. Our early assessment of fear conditioned contextual memory response used sterimol descriptors,[67] polar and nonpolar volumes, hydrophobicities, free energies of solvation, electronic properties (highest occupied molecular orbital (HOMO), lowest unoccupied molecular orbital (LUMO) energetics), and solvent accessible surface areas in a discriminant variable test of the ability to affect fear conditioned contextual memory results.[53] These preliminary analyses indicated that the longest dimension of the ligands (sterimol L parameter[67]) was able to discriminate between compounds that were able to attenuate scopolamine-induced contextual memory impairment from those that were not. This provided the working rationale to select an initial set of compounds for electrophysiological evaluation with regard to $\alpha5\beta3\gamma2$ activation. Information was also supplemented from data on other compounds reported in the literature. We then applied COMSIA and fragment QSAR analyses to the electrophysiological data on the compounds to determine molecular descriptors that correlate with the electrophysiological response at the $\alpha5\beta3\gamma2$ subtype to see to what

extent these descriptors correlate with descriptors derived from the analysis of the behavioral data set.

COMSIA Analyses of Electrophysiological Features. Figure 6A (right upper panel) displays compounds overlapped using the pharmacophore from Figure 2 along with the results of a COMSIA analysis (lower right panel) applied to electrophysiological data and employing steric, hydrophobic, and electrostatic COMSIA fields surrounding the overlapped ligands (see Experimental Section). In this type of analysis, each of the physiochemical descriptors are given a Gaussian weighted functional form and similarity indices computed for atoms in each of the training set compounds and a virtual atom used to probe each of the fields.[68–70] Figure 6A (left) displays the structures of the ligands as well as their electrophysiological response at the $\alpha5\beta3\gamma2$ $GABA_A$ receptor isoform when the ligand is applied at a concentration of 1 μM (see Experimental Section). This analysis provided a modestly predictive result with a $q^2 = 0.35$ and an $r^2 = 0.99$ and a standard error of 0.01. The fractions listed in the COMSIA analysis plot shown in the lower right-hand panel indicate, by virtue of their magnitudes, that variations in the steric and hydrophobic COMSIA field components of the ligands are key factors in explaining the differences in their electrophysiological activities at the $\alpha5\beta3\gamma2$ $GABA_A$ receptor isoform. These initial results suggest a correlation between substitutions altering the length of the ligands and the ligands activity analogous to that deduced from multivariate discriminant analysis of earlier contextual memory results.

COMSIA analyses are quite sensitive to the user overlap of compounds within the test set, whether using a computed or manual overlap at pharmacophore points. Therefore, we sought to independently verify that length variations of ligands in the training set truly correlate with the electrophysiological response seen in the $\alpha5\beta3\gamma2$ receptor isoform by performing an independent fragment QSAR analysis. In this step, we examined whether properties of the substituents (see Table 1 of the Supporting Information), on a common template shown in the left panel of Figure 6A, correlate with the electrophysiological response. Figure 6B shows the results of the fragment QSAR analysis in which variations in the electrophysiological response were modeled in terms of the length (Sterimol L) of the "fragment" substitutions at either end of the ligand template along with a second variable: either the hydrophobicity (HYD) or the HOMO−LUMO energy differences (HL) of the substituent. Both results indicate a reasonable correlation, $r^2 = 0.53$ for Sterimol L/HOMO−LUMO[67] and $r^2 = 0.63$ for Sterimol L/hydrophobicity with the electrophysiological response. The statistical significance, however, was not as great as with the binding fragment QSAR in which the significance was in the 99% confidence interval compared to an 80% confidence interval seen with the electrophysiological analysis. This is typical, in our experience of the greater challenge of modeling "activation data" compared to binding phenomenology.

Validation of the Pharmacophore/SAR Model Using a Distinct Training Set from the Published Literature (cf. Supporting Information). The validity of the conclusions derived from pharmacophore and QSAR analysis of ligands binding to and activating $\alpha5\beta x\gamma2$ receptor isoforms should, in principle, be independent of the training set. We therefore tested the importance of the variables identified from within our test set by examining a larger published test set based on a different core template (see Figure 1A of the Supporting Information,). For this purpose, we chose a ligand test set derived from studies by Chambers et al.[23,24]

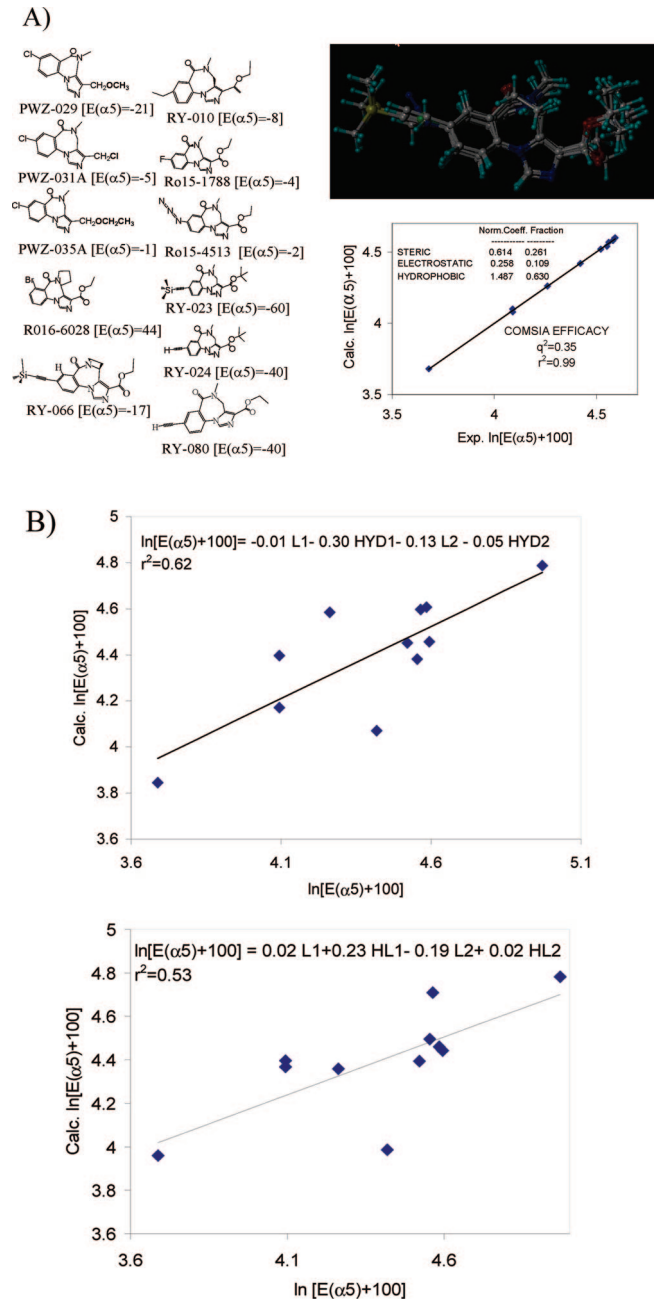


Figure 6. (A) COMSIA analyses (lower right panel) for the electrophysiological responses (ln[$E(\alpha5)$ + 100]) of the depicted compounds relative to the $\alpha5\beta3\gamma2$ $GABA_A$ receptor isoform. The electrophysiological response is indicated in parentheses below each of the structures. The molecular superposition used to perform the COMSIA analyses is shown in the upper right. This COMSIA analyses highlights the importance of steric and hydrophobic features in explaining variations in the electrophysiological response: (ln[$E(\alpha5)$ + 100]) at $\alpha5\beta3\gamma2$ in that these coefficients (0.3 steric/0.6 hydrophobic) are significantly larger than the electrostatic components (0.1 electrostatic). (B) Fragment QSAR analyses of selected properties of the substituents on the common imidazobenzodiazepine template in (A). The coefficients of the terms in the QSAR equations indicate the correlations of the magnitude of the fragment Sterimol L (L1 and L2), HOMO−LUMO (HL1 and HL2), and hydrophobicities (HYD1 and HYD2) of the terminal substituents with the resulting electrophysiological response. The finding of significant QSAR coefficients for the fragment Sterimol L values of the second substituents (L2, cf. Table 1 of the Supporting Information) is consistent with the initial notion derived from COMSIA analyses (see A) that ligand length is correlated with electrophysiological response.

Materials (tables, figures, and discussion) deposited in Supporting Information establish that both our training set and that of Chambers and co-workers are adequately described by a common pharmacophore (see Figure 1B of the Supporting Information) and that a fragment QSAR for the Chambers training set highlights the importance of the substituent/molecular length (Sterimol L) and the energy difference between the frontier orbitals on the substituents (HOMO−LUMO) (see Table 2 and Figure 1C,D of the Supporting Information). Compounds **43** and **44** (see Figure 1A of the Supporting Information) of the Chambers test set, differing only in the insertion of an extra −(CH2)−/methylenic fragment, display differences in electrophysiological response at α5 subunit containing $GABA_A$ receptor isoforms, changing from attenuation of GABA-induced Cl^- flux to potentiation of GABA-induced Cl^- flux (−38 to +25, respectively). This change from robust inverse agonism to agonism by the insertion of the single −CH2− into the parent compound lends additional support to our conclusions that the "length" of the substituents (affecting the ligand dimension) are linked to the electrophysiological response (Figure 6B) in α5β3γ2.

Behavioral Influence of Binding and/or Activational Selectivity. In the discussion above, we have established correlations between particular ligand physiochemical properties with their ability to selectively bind to and activate either $GABA_A$ receptors with α5βxγ2 or α1βxγ2 (where x = 2, 3) subunit compositions. Having established these connections, we sought to determine whether these principles can be applied to efficaciously attenuate memory deficits present in our animal model of contextual memory impairment. In addition, it is worthwhile to learn whether this selectivity reduces adverse behavioral side effects involving locomotion or convulsive properties.

The BDZR ligands RY-080, RY-023, and RY-024 (Figure 7A) while exhibiting high affinity binding toward the BDZR binding site on the α5 isoform also exhibit relatively high affinities toward isoforms containing other α subunits.[38] Moreover, these compounds elicit convulsive behavior at relatively low doses, thereby limiting their usefulness in examining cognitive influence. To dissect the nature of this problem, we examined the electrophysiological profile of RY-024 on the various α subunit isoforms (in association with the β3γ2 subunits, Figure 7B). RY-024 (1 μM) was observed to robustly attenuate GABA-mediated currents through $GABA_A$ receptor isoforms containing either the α1, α2, or α5 subunits (−31.0 ± 2.5%, −20.7 ± 1.2%, and −40.4 ± 0.8%, respectively). In addition, the binding affinity of RY-024 is relatively high at the α1, α2, and α5 isoforms[37] and the α1 and α2 isoforms make up a large percentage of all $GABA_A$ receptors found in brain. Consequently, systemic administration of RY-024 is likely to cause a general increase in excitatory tone of numerous brain regions, thereby favoring convulsive behavior. RY-024's convulsive effect is unlikely to be mediated through the α3, α4, or α6 isoforms as this compound is virtually without electrophysiological effect (−3.3 ± 2.1%) at the α3 isoform and potentiates GABA-elicited currents in α4 or α6 subunit isoforms (+43.0 ± 15.9 and +35.2 ± 1.5%, respectively) (Figure 7B and Table 3 of the Supporting Information).

Compound PWZ-029, a compound of particular interest in our joint computational−experimental investigations, exhibits modest ~20 fold binding selectivity toward the α5 isoform over other α isoforms[26] (Figure 7A). PWZ-029 (1 μM) was able to attenuate GABA-induced control currents (−20.6 ± 3.4%) through the α5 isoform. In contrast, PWZ-029 at the same

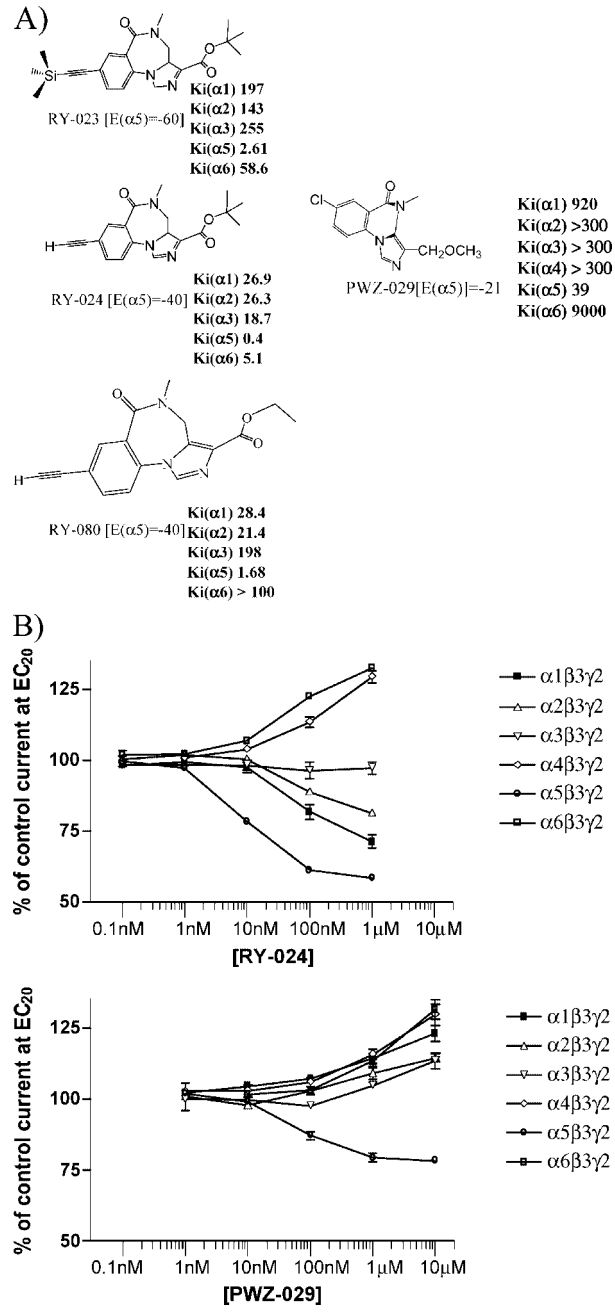


Figure 7. (A) Structures of RY-023, RY-024, RY-080, and PWZ-029 along with binding affinities (K_i in nM) and electrophysiological efficacies at the α5 isoform for the various α subunits in association with the β3γ2 subunits. (B) Electrophysiological characterization of RY-024 and PWZ-029. Dose response curves for RY-024 and PWZ-029 in oocytes expressing different $GABA_A$ receptors isoforms, as indicated in the legends. cRNA-injected *Xenopus* oocytes were held at −60 mV under two-electrode voltage clamp. Increasing concentrations of RY-24 or PWZ-029 were superfused together with a GABA concentration eliciting approx 20% of the maximal current amplitude. RY-024 and PWZ-029 were each preapplied for 30 s before the addition of GABA, which was coapplied with the drugs until a peak response was observed. Data were normalized for each curve assuming 100% for the response in the absence of drug. RY-024 and PWZ-029 were made up and diluted as stock solution in DMSO. Final concentrations of DMSO perfusing the oocyte were 0.1%. Values are presented as mean ± SD of at least four oocytes from at least two batches. Using the two-electrode voltage clamp method, currents in the μA range were measured for all subunit combinations in response to application of a saturating concentration of GABA (10 mM). In the absence of GABA, RY-024, and PWZ-029 at concentrations up to 1 μM were not able to trigger chloride currents in any of the tested subtypes of the $GABA_A$ receptor.

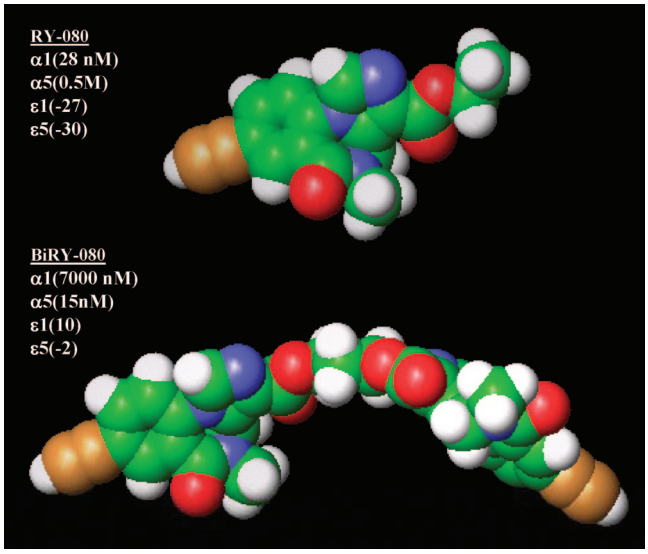


Figure 8. CPK representations of compounds RY-080 and the bivalent compound BiRY-080. Insets provide the binding affinities at the $GABA_A$ receptor isoforms that contain either the α1 or α5 subunits in combination with $\beta 3$ and $\gamma 2$ subunits. The bottom two numbers (i.e., ϵ) represent the electrophysiological values determined in the presence of the EC_{20} of GABA at a 1 μM concentration of the ligand being tested.

concentration potentiated the currents (4− 16%) through isoforms containing the α1, α2, α3, α4, or α6 subunit (Figure 7B), similar to the magnitude of modulations elicited by the prototypic benzodiazepine antagonist flumazenil (Ro15-1788) at similar concentrations on similar receptor isoforms.[56,57] Therefore, PWZ-029 can be said to exhibit both selective affinity and efficacy toward α5 subunit containing $GABA_A$ receptor isoforms. In addition, PWZ-029 administered systemically to mice was not convulsive up to the highest dose tested (30 mg/kg). These results support the notion that moderate attenuation of GABA mediated Cl^- flux through $GABA_A$ receptors containing the α5 subunit is not sufficient to induce convulsive behavior on its own. Therefore, convulsive behavior either requires a stronger attenuation of the GABA mediated effect through the α5 isoform, is mediated through another $GABA_A$ receptor isoform, or requires the attenuation of Cl^- flux through a combination of $GABA_A$ receptor isoforms as has been suggested by Atack et al.,[58] in the study of RY-080. Any of these scenarios bolsters the concept of exploiting α5 isoforms for potential therapeutic utilization, whether through highly selective binding and/or selective efficacy mediated through α5 subunit containing $GABA_A$ receptors.

In the course of our studies, we came across another interesting compound, the bivalent molecule BiRY-080 (created by linking together two molecules of RY-080 via their ester groups) (Figure 8), which in marked contrast to RY-80 exhibits a 60−130 fold higher degree of binding selectively toward the $\alpha 5\beta 3\gamma 2$ isoform over other α subunit containing $GABA_A$ receptor isoforms[59] and exhibits low convulsive potential. However, the robust 30% attenuation of GABA-induced Cl^- currents through the α5 subunit containing $GABA_A$ receptor isoform elicited by 1 μM RY-080 was diminished to about 4% attenuation when 1 μM of BiRY-080 was applied. At this concentration, BiRY-080 also fails to significantly effect GABA-induced Cl^- currents through $GABA_A$ receptor isoforms containing the α1, α2, or α3 subunits (in association with $\beta 3\gamma 2$).[59] At the same concentration, BiRY-080 potentiates GABA-mediated currents through the α4 and α6 subunit containing isoforms, by 38% and 17%, respectively. However, if one considers the low affinity binding (>1.5 μM) of BiRY-080 toward the α4 and α6 isoforms compared to the high affinity binding (15 nM) at α5 isoforms,[59] it is unlikely that the α4 and α6 isoforms are contributing significantly to the behavioral effects of this compound when administered in low doses.

We found several of the compounds in this study to display promising in vitro profiles as previously outlined were able to significantly attenuate scopolamine-induced contextual memory impairment in mice. However, few surpassed the level we had set as our threshold for more rigorous investigation. But there were three exceptions, PWZ-029, RY-010, and BiRY-080, all of which exhibited reasonable binding selectivity toward α5 subunit containing $GABA_A$ receptor isoforms.[26,59] For example, compound PWZ-029 at a dose of 10 mg/kg was able to robustly attenuate scopolamine-induced impairment of contextual memory in mice (Figure 9A). In addition, RY-010, a compound that displays a 13−117 fold selectivity toward α5 subunit containing $GABA_A$ receptor isoforms over other α isoforms at a concentration of 1 μM displayed modest attenuation (−8 ± 2%) of GABA-induced Cl^- flux on HEK cells expressing the $\alpha 5\beta 2\gamma 2$ isoform. RY-010 was also able to significantly attenuate scopolamine-induced contextual memory impairment at a dose of 10 mg/kg while lacking locomotor or convulsive effects.[53] Lastly, BiRY-080, a compound which displays a 60−130 fold selectivity toward a α5 subunit containing $GABA_A$ receptor isoforms over other α subunit isoforms and a marginal attenuation (−4 ± 2% at 1 μM) of GABA-induced Cl^- currents in the α5 isoform, was expected to lack effect on contextual memory in our mouse model. Surprisingly, this was not the case; BiRY-080 at a dose of 10 mg/kg was also able to significantly attenuate scopolamine-induced contextual memory impairment (Figure 9B). Influence of PWZ-029, RY-010, and BiRY-080 on cognition via interactions with other receptor classes was ruled out through the NIH Case Western Reserve Drug Screening Program, which found these compounds to lack appreciable binding to other major classes of receptors (B. Roth et al., NIMH Psychoactive Drug Screening Program, UNC, unpublished results, available at https://kidbdev.med.unc.edu/pdsp). BiRY-080's influence on contextual memory while exhibiting only minimal effects on GABA-induced Cl^- currents, was a little perplexing, as is the notion that selective antagonism of α5 subunit containing $GABA_A$ receptor isoforms is sufficient to elicit cognitive influence. However, this observation in itself is not entirely novel as the classic BZDR antagonist Ro15-1788 (flumazenil), which displays high affinity binding toward most $GABA_A$ receptor isoforms and a weak ability to alter GABA's efficacy, still possesses some intrinsic effects[71] both clinically[72] and in animal behavioral studies.[53] Over the years, investigators have hypothesized the existence of an endogenous ligand for the BDZR binding site that could modulate anxiolysis, muscle relaxation, vigilance, and/or memory[72–74] and that the administration of an antagonist, such as flumazenil, could inhibit the effects of these endogenous ligands, however, to date such evidence is controversial. An alternative explanation could involve the δ subunit, which can substitute for the $\gamma 2$ subunit in a $GABA_A$ receptor. $GABA_A$ receptor isoforms containing the δ subunit have recently been reported to bind Ro15-1788 and Ro15-4513 with high affinity, contrary to the widely held belief that the δ subunit containing $GABA_A$ receptor isoforms are insensitive to BDZR ligands.[75] However, as the current study did not involve the use of δ subunit containing $GABA_A$ receptors, we can only speculate as to its potential contribution to the observed behavioral effects. Providing a mechanistic

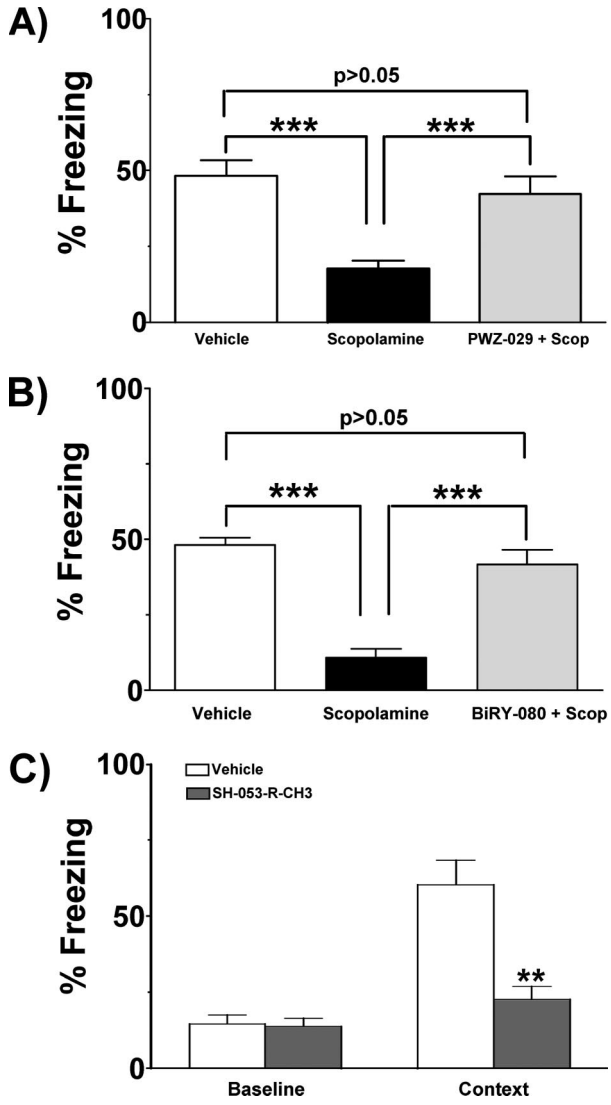


Figure 9. Pavlovian fear conditioned contextual memory. Each mouse was injected ip with either vehicle (0.9% saline containing 2.5% encapsin), 1.5 mg/kg scopolamine, or 1.5 mg/kg scopolamine + drug (A) 10 mg/kg PWZ-029 or (B) 10 mg/kg BiRY-080. Twenty min after injection, mice were fear conditioned to the context (see Experimental Section) and tested 24 h later, well after the drugs and scopolamine have cleared. A reduction in freezing, as observed in mice that received scopolamine alone, is reflective of a low level of contextual memory. Each drug, tested in the absence of scopolamine, was found not to differ significantly from vehicle administered alone ($p > 0.05$, data not shown). (C) 30 mg/kg SH-053-R-CH3 in the absence of scopolamine and tested as above; *$p < 0.05$, ***$p < 0.001$. $N = 12$ male C57BL/6J mice per vehicle or drug.

answer as to why antagonism of $GABA_A$ receptors influences behavior is beyond the scope of this paper.

Last, the selective α5 isoform agonist, SH-053-R-CH3,[76] was also assessed in mice in order to ascertain whether it is able to impair contextual memory in the absence of scopolamine. SH-053-R-CH3 (30 mg/kg) was indeed able to impair contextual memory in the absence of scopolamine (Figure 9C), thereby providing further "proof of principle" that compounds displaying selective influence on α5 isoforms, whether by potentiation or attenuation of GABA's effect on α5 isoforms, lead to significant effects on contextual memory. In contrast to the findings of Savic et al.,[76] we did not observe a significant reduction ($p = 0.284$) in locomotion between mice given either 30 mg/kg SH-053-R-CH3 or vehicle during a 40 min post injection observation period, as measured by automated assessment of spontaneous locomotor activity (1026 ± 57 versus 770 ± 210, respectively). In addition, we also assessed chamber circumnavigation during the initial 4 min exploration phase of the fear conditioning protocol prior to shock delivery and found no significant difference ($p = 0.63$) in this locomotor parameter between mice receiving SH-053-R-CH3 or vehicle (data not shown). In a similar fashion, Savic et al.,[76] did not observe a change in distance traveled between mice receiving SH-053-R-CH3 or vehicle when evaluated in the elevated plus maze protocol. It was beyond the scope of this study to systematically investigate the affects of structural substitutions on the imidazobenzodiazepine template that would lead to robust electrophysiological agonism at α5βxγ2 $GABA_A$ receptor isoforms. However, if one were to juxtapose the structures of the majority of imidazobenzodiazepines in this study (e.g., see Figure 1) with SH-053-R-CH3 and Ro16-6028, both robust electrophysiological agonists of α5 isoforms, one quickly discerns that both compounds involve structural changes in a region outside of our present pharmacophore, which was primarily designed to aid in predicting inverse agonism.

Summary and Conclusions

Numerous reports find benzodiazepine ligands capable of either attenuating or potentiating cognition. However, proconvulsive, psychomotor, and anxiogenic effects associated with this class of compound has limited their therapeutic development. As more subtype selective BZDR ligands become available, one can now ask whether a compound that interacts selectively with a particular $GABA_A$ receptor isoform(s) could influence cognition separate of other behavioral influences (i.e., side effects). With this goal in mind, pharmacophores were initially developed based on a set of nonselective high affinity BDZR ligands. Subsequently, these pharmacophores were used to search chemical database and "hits" tested for binding toward $GABA_A$ receptors having either the α1β2γ2 or α5β2γ2 stoichiometry in order to validate the pharmacophore definitions. Several of these "hits" demonstrated submicromolar binding affinities (ranging from 200 nM to 1μM), suggesting the pharmacophore indeed captures chemometric features important for BDZR binding site recognition. Subsequently, a set of imidazobenzodiazepines that display a higher degree of selectivity toward α5 isoforms over α1 isoforms were analyzed in order to glean principles underlying binding selectivity toward α5βxγ2 relative to α1βxγ2. Examination of computed molecular electrostatic potential surfaces, fragment QSAR, and COMSIA analyses for this series, independently revealed the greater importance of one of the hydrogen bond acceptor pharmacophore points for binding to the α1βxγ2 isoform rather than to the α5βxγ2 isoform. Our early chemometric analysis of the effects of benzodiazepine ligands on the reversal of scopolamine-induced memory deficits indicated that ligands in our training set, having a sterimol L parameter of less than 14, were unable to reverse the effects of scopolamine on contextual memory. In a similar vein, COMSIA and fragment QSAR further revealed a correlation between the size of the terminal substituents on the imidazobenzodiazepine template and HOMO−LUMO (fragment) energy differences with the electrophysiological response in α5βxγ2. To verify whether these activation discriminants are meaningful, we examined a separate compound set published by Chambers and co-workers[23,24] and found that the same fragment/substituent properties correlate with the electro-

physiological responses at the $\alpha5\beta3\gamma2$ isoform, as reported in that study.

Our investigations further found that compound PWZ-029, which exhibits reasonable binding selectivity toward $GABA_A$ receptors containing the α5 subunit and possesses a favorable electrophysiological profile, was able to attenuate scopolamine-induced contextual memory impairment in mice. In a similar fashion, Dawson et al., [35] demonstrated that compound α5IA, which also selectively lessens GABA-induced activation of α5 isoforms was able to enhance rat performance in the Morris water maze, a spatial memory task sharing similarities with contextual memory tasks. Therefore, we are left with the conclusion that selective attenuation of the activational influence of GABA (either by binding or efficacy) on $GABA_A$ receptors that contain the α5 subunit may prove valuable in addressing conditions that result in contextual/spatial memory impairment. The additional observation that compound SH-053-R-CH3, which selectively potentiates GABA's influence on $GABA_A$ receptors containing the α5 subunit, impairs contextual memory further supports the "proof of principle" that α5 subunit containing $GABA_A$ receptor isoforms can be selectively exploited in order to influence processes involved in contextual memory.

Last, the electrophysiological profiles of the bivalent compound BiRY-080, an α5 selective antagonist, when juxtaposed with its ability to attenuate scopolamine-induced contextual memory impairment, raises intriguing questions into the nature of the molecular mechanism by which a compound such as this is able to influence contextual memory. We observed that the ability of α5 selective antagonists and weak inverse agonists to affect memory was dependent on ligand size (Sterimol L parameter). These results are not unlike other nonclassical inhibition patterns, where the ability to inhibit appears to correlate better with ligand size rather than affinity.[77,78] While beyond the scope of this paper, these results suggest that either (1) the earlier hypotheses of endogenous ligand displacement by an antagonist, in this case only requiring antagonism of α5 subunit containing $GABA_A$ receptor isoforms, is sufficient for behavioral alteration; (2) alternatively, an additional $GABA_A$ receptor isoform (i.e., $\alpha4\beta3\delta$),[75] not originally expected to have an effect, is mitigating the observed effects. Our results lend support toward the notion that compounds that selectively influence $\alpha5\beta x\gamma2$ $GABA_A$ receptors, whether by inverse agonism, antagonism, or agonism, are each capable of modulating contextual memory.

Experimental Section

Binding. Sf-9 insect cell lines were obtained from Life Technologies, Grand Island, NY. Sf-9 cells were grown as suspension cultures at 27 °C in Sf-900 II SFM medium (Life Technologies, Grand Island, NY) supplemented with 10 units/mL penicillin and 10 mg/mL streptomycin. Baculovirus construct combinations containing $GABA_A$ receptor subunits were used to infect Sf-9 cells at a concentration of two viral particles per cell (2 MOI). Equimolar ratios of each of the three subunits (AcNPV-a1/AcNPV-b2/AcNPV-g2 or AcNPV-a5AcNPV-b2/AcNPVg2) were used for each infection. Exponentially growing Sf-9 cells were used for viral infection. Sixty hours postinfection, Sf-9 cells were harvested by centrifugation at 750*g* and cell pellet washed in ice-cold phosphate buffered saline. The washed pellet was suspended in 50 mM Tris-HCl (pH 7.7) at 4 °C and cell homogenate prepared with a polytron homogenizer. The homogenate was frozen in aliquots and stored at −86 °C until use.[79] All other chemicals were from standard commercial sources. All drugs were made as 10 mM stock solutions in 100% ethanol. The dilutions of the drugs were made in reaction buffer. The ethanol concentration in the assay tube was less than 0.1%. The range of the total ligand concentration was from 40 pM to 10 μM. Competition binding assays were incubated with 0.5 ± 1.0 nM of [^{3}H]-Ro15-4513 and increasing concentrations of unlabeled ligand in a total of 1 mL reaction volume containing 200 ± 300 mg of Sf-9 cell homogenate per assay tube, carried out in triplicates for each concentration of ligand. Incubation was at 0 °C for 90 min. The assay was terminated by rapid filtration through Whatman GF/B filters using a FilterMate cell harvester (Packard Instruments, Downers, Grove, IL) followed by three washes, 4 mL each, with ice-cold buffer. Radioactivity retained on the filters was measured using Microscint 0 in a TopCount liquid scintillation counter (Packard Instruments, Downers, Grove, IL). All binding data were analyzed using Affinity Analysis System software as described previously.[80]

Electrophysiology. Preparation of cloned mRNA. Cloning of $GABA_A$ receptor subunits α1, $\beta3$, and $\gamma2$ into pCDM8 expression vectors (Invitrogen, CA) has been described elsewhere.[81] $GABA_A$ receptor subunit α4 was cloned in an analogous way. cDNAs for subunits α2, α3, and α5 were gifts from P. Malherbe and were subcloned into the pCI vector. cDNA for subunit α6 was a gift from P. Seeburg and was subcloned into the vector pGEM-3Z (Promega). After linearizing the cDNA vectors with appropriate restriction endonucleases, capped transcripts were produced using the mMessage mMachine T7 transcription kit (Ambion, TX). The capped transcripts were polyadenylated using yeast poly(A) polymerase (USB, OH) and were diluted and stored in diethylpyrocarbonate-treated water at −70 °C.

Functional Expression of $GABA_A$ Receptors. The methods used for isolating, culturing, injecting, and defolliculating of the oocytes were identical with those described by E. Sigel.[82,83] Oocytes with follicle cell layers still around them were injected with 50 nL of an aqueous solution of cRNA. This solution contained the transcripts for the different α subunits and the $\beta3$ subunit at a concentration of 0.0065 ng/nL as well as the transcript for the $\gamma2$ subunit at 0.032 ng/nL. After injection of cRNA, oocytes were incubated for at least 36 h before the enveloping follicle cell layers were removed. After removing of the follicle cell layer, oocytes were allowed to recover for at least four hours before being used in electrophysiological experiments.

Electrophysiological Experiments. For electrophysiological recordings, oocytes were placed on a nylon-grid in a bath of *Xenopus* Ringer's solution (XR, containing 90 mM NaCl, 5 mM HEPES-NaOH (pH 7.4), 1 mM $MgCl_2$, 1 mM KCl, and 1 mM $CaCl_2$) The oocytes were constantly washed by a flow of 6 mL/min XR that could be switched to XR containing GABA and/or drugs. Drugs were diluted into XR from DMSO solutions, resulting in a final concentration of 0.1% DMSO perfusing the oocytes. Drugs were preapplied for 30 s before the addition of GABA, which was coapplied with the drugs until a peak response was observed. Between two applications, oocytes were washed in XR for up to 15 min to ensure full recovery from desensitization. For current measurements, the oocytes were impaled with two microelectrodes (2−3 MΩ), which were filled with 2 mM KCl. All recordings were performed at room temperature at a holding potential of −60 mV using a Warner OC-725C two-electrode voltage clamp (Warner Instruments, Hamden, CT) or a Dagan CA-1B oocyte clamp (Dagan Corporation, Minneapolis, MN). Data were digitized, recorded, and measured using a Digidata 1322A data acquisition system (Axon Instruments, Union City, CA). Results of concentration−response experiments were fitted using GraphPad Prism 3.0 (GraphPad Software, San Diego, CA). The equation used for fitting concentration−response curves was $Y = \text{Bottom} + (\text{Top}-\text{Bottom})/(1 + 10\text{^}(X - \text{Log EC}_{50}))$; X represents the logarithm of concentration, Y represents the response; Y starts at Bottom and goes to Top with a sigmoid shape.

In Vivo Assessments. Animals. Male C57Bl/6 mice were obtained from Charles Rivers Laboratories (Holister, CA) at 6 weeks of age. Mice used in fear conditioning were between 7 and 12 weeks of age. Animals were housed eight to a cage in rooms with a normal 12 h light/12 h dark cycle lights on 700−1900 h with free access to food and water. Tests were conducted during the light phase

between 1300 and 1700 h. All animal protocols used in this study conform to the guidelines determined by the National Institutes of Health, Office for Protection from Research Risks and are approved by the Animal Care and Use Committee of the Palo Alto Veterans Administration Health Care System, Palo Alto, CA.

Pavlovian Fear Conditioning. Before testing each day, the mice were moved to a holding room and allowed to acclimate for at least 30 min. Each mouse received an ip injection of one of the following: vehicle, BDZR ligand (2−30 mg/kg), scopolamine 1.5 mg/kg, or scopolamine 1.5 mg/kg combined with one of the BDZR ligands (2−30 mg/kg). The dose level chosen for each compound was one that neither elicited convulsions nor impaired locomotion. Twenty minutes after injection, the mice were placed individually into one of four identical experimental chambers (Med Associates, St. Albans, VT) that had been scented with 0.3% ammonium hydroxide solution before testing. Chambers were backlit with fluorescent light with a white noise generator providing 70 dB of background noise. After 4 min in the chamber, mice were exposed to a loud tone 85 dB, 2.9 kHz, for 33 s, with the last 3 s coupled with a 0.75 mA scrambled footshock. This procedure was repeated for a total of three episodes with a 1 min period separating each episode. One minute after the final footshock, the mice were returned to their home cages. Twenty-four hours later, contextual memory was assessed by placing the mice back into the freshly rescented (0.3% ammonium hydroxide) conditioning chambers in which they were trained for a 4 min test period in the absence of footshock. Conditioned fear to the context was assessed by measuring the freezing response according to the methods of Fanselow and Bolles.[84] Scoring was done using the FreezeScan (Clever Systems Inc., Reston, VA). These data were transformed to a percentage of total observations. Data were analyzed by one-way analysis of variance ANOVA using GraphPad PRISM 4.0 (GraphPad Software). Separate treatment effects between groups were analyzed post hoc using Dunnett's or Bonferroni's multiple comparisons.

Computational Methods. Conformational Libraries for Pharmacophore/Overlap Rule Development. Conformational libraries were developed using a nested rotation approach wherein the energy was evaluated by varying each of the rotatable bonds by 30°, constraining their values at this geometry, and then energy minimizing (using Conjugate Gradients) the remainder of the structure until a the rms changes in the gradient were smaller than 0.01. All force field parameters used were from the Quanta/CHARMM (Chemistry at Harvard Macromolecular Mechanics) force field. The net atomic charges used, based on parametrization in this program, were also consistent with the fact that all of the compounds are neutral. The initial structures were then energy minimized using 200 steps of steepest descents followed by 2000−3000 steps of conjugate gradients or until the rms changes in the gradient were smaller than 0.01 Å. A long 90 Å potential truncation was used to minimize effects on structure due to potential truncation via a switching function.

Initial Pharmacophore Development. MOLMOD developed in our laboratory[51,52] was used to develop 3D pharmacophores from input of conformational libraries for each of the ligands, independent distance criteria for conformational clustering, and pharmacophore distance criteria, a trial pharmacophore definition in terms of a set of ligand or receptor based pharmacophore points (including hydrogen bond donor/acceptor, hydrophobic centers, centroids of ring systems or functional groups, and/or user-defined classes based on user-defined property definitions) and an energy window criteria for consideration of ligand conformers. The program first performs conformational clustering of the input conformational libraries. It then determines whether there is at least one conformer, of each ligand in the training set, which has the same 3D distance metrics between pharmacophore points within the energy-windows and distance "tolerances" specified by the user. The program allows for different "tolerances" for each pharmacophore point to allow for conformational/sterically allowed variation in particular regions. If the 3D distrance metrics between pharmacophore points is the same at least at one conformation of each ligand, the pharmacophore distance metrics are reported and the ligands superimposed via a quaternion/least-squares procedure for those conformers complying with the pharmacophore. The latter superposition(s) may then be used in 3D-QSAR applications and properties evaluated for those conformers that comply with the pharmacophore for use in multivariate statistical analysis to ascertain the determinants of recognition or activation.

Validation of Overlap Rules/Pharmacophores via Database Searches. The overlap rules were input to SYBYL/UNITY as sets of hydrogen bond acceptors and centroids. Databases were searched using a distance window of ± 2 Å. In particular, Unity, Chapmann & Hall, and Maybridge databases were searched for two distinct purposes: 1) to ensure compounds with known $GABA_A$ receptor binding and activation were retrieved,[51,52] and 2) to extract new compounds for binding, electrophysiological, and behavioral assessment.

Quantum Chemical Evaluation of Properties in Conformations Complying with Pharmacophores for Use in QSAR. Following development of the initial pharmacophores, the properties of all training set ligands were computed in conformations fulfilling the distance criteria in the pharmacophore. The properties were evaluated using a combination of semiempirical quantum mechanics and a MOPAC-7AM1 Hamiltonian[85] as well as density functional theory as incorporated in Gaussian[86] and Jaguar (Schrödinger, Portland, OR). This included both whole molecule properties as well as those of "fragments" corresponding to substituent replacements on particular templates. In an effort to understand the SAR of these substitutions we computed: (i) frontier orbital energetics (HOMO/LUMO/HOMO−LUMO), (ii) Sterimol parameters, (iii) group hydrophobicities, (iv) volumes, (v) areas, (vi) solvent-accessible surface areas, (vii) polar and nonpolar volumes, (viii) globularities, (ix) electrostatic potentials on the van der Waals surface using MOPAC-AM1-derived properties developed by the in-house program GRAPHA, and (x) solvation energies. In the case of fragment properties, the substituents were "capped" with H's prior to calculation of properties.

Fragment and 3D-QSAR analyses. Both the TRIPOS QSAR module as well as MS-EXCEL were used to form multivariate QSAR analyses for both binding and electrophysiological response as a function of substitutions. The TRIPOS COMSIA module (Tripos QSAR (St. Louis, MO)) was used to perform molecular similarity index analysis (CoMSIA) employing field descriptors around ligands superimposed using MOLMOD.

Multivariate Discriminant Analysis. R-PLUS was used to generate multivariate discriminant analysis of properties for the purpose of finding properties correlated with activation biodata and post evaluation of significance based on Wilk's Lambda values.

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Supporting Information Available: Tables of computed properties essential to Fragment QSAR analyses of training sets, supplemental analyses of data published by Chambers et al., (references 23−24), and a table of concentration to electrophysiological response for the modulation of GABA by RY024 at different $GABA_A$ isoforms is provided. This material is available free of charge at http://www.acs.org.

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