Structural Determinants of Benzodiazepine Allosteric Regulation of GABA_A Receptor Currents

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Benzodiazepine enhancement of GABA_A receptor current requires a γ subunit, and replacement of the γ subunit by the δ subunit abolishes benzodiazepine enhancement. Although it has been demonstrated that benzodiazepines bind to GABA_A receptors at the junction between α and γ subunits, the structural basis for the coupling of benzodiazepine binding to allosteric enhancement of the GABA_A receptor current is unclear. To determine the structural basis for this coupling, the present study used a chimera strategy, using γ 2L- δ GABA_A receptor subunit chimeras coexpressed with α 1 and β 3 subunits in human embryonic kidney 293T cells. Different domains of the γ 2L subunit were replaced by δ subunit sequence, and diazepam sensitivity was determined. Chimeric subunits revealed two areas of interest: domain 1 in transmembrane domain 1 (M1) and domain 2 in the C-terminal portion of transmembrane domain 2 (M2) and the M2–M3 extracellular loop. In those domains, site-directed mutagenesis demonstrated that the following two groups of residues were involved in benzodiazepine transduction of current enhancement: residues Y235, F236, T237 in M1; and S280, T281, I282 in M2 as well as the entire M2–M3 loop. These results suggest that a pocket of residues may transduce benzodiazepine binding to increased gating. Benzodiazepine transduction involves a group of residues that connects the N terminus and M1, and another group of residues that may facilitate an interaction between the N terminus and the M2 and M2–M3 loop domains.

Key words: GABA_A receptors; benzodiazepines; ion channel structure–function; binding–gating transduction; coupling; receptor chimera

Introduction

Benzodiazepines are antiepileptic and anxiolytic drugs that act by binding to GABA_A receptors and enhancing GABA-evoked chloride current. GABA_A receptors, the primary mediators of fast inhibitory neurotransmission in the CNS (Macdonald and Olsen, 1994; Smith and Olsen, 1995), are members of the ligand-gated ion channel (LGIC) superfamily, which includes the acetylcholine receptor (AChR), serotonin receptor (5HT-3), and glycine receptor (GlyR). GABA_A receptors form pentamers composed of combinations of subunit types, α (1–6), β (1–3), γ (1–3), δ , ϵ , ρ , and π , each of which determine the pharmacological properties of the receptor. Most GABA_A receptors are composed of α , β , and γ subunits in a 2:2:1 ratio (Chang et al., 1996; Baumann et al., 2002), although the δ subunit may replace the γ subunit to form $\alpha\beta\delta$ receptors in a subpopulation of neurons (Korpi et al., 2002). GABA_A receptor subunits have a $\sim$ 200 aa extracellular N-terminal domain that contains the GABA and benzodiazepine binding sites, an extracellular loop (M2–M3 loop), a large cytoplasmic loop (M3–M4 loop), and four transmembrane domains

(M1–M4) (see Fig. 1). GABA binds at the interface of α and β subunits, whereas benzodiazepines bind at a homologous site at the interface of α and γ subunits (Sigel and Buhr, 1997).

Benzodiazepine binding is only the first step in enhancing GABA_A receptor current. The second step is a conformational change in the receptor (Boileau and Czajkowski, 1999) that couples benzodiazepine binding to an increase in GABA_A receptor single-channel opening frequency (Rogers et al., 1994). Using chimeric α - γ receptors, the N-terminal 161 aa of the γ 2S subunit were shown to bind benzodiazepines with wild-type affinity when coexpressed in *Xenopus* oocytes with α 1 and β 2 subunits (Boileau et al., 1998). However, although the chimeric receptor bound benzodiazepines normally, the N-terminal residues were not sufficient to couple binding to modulation of the receptor. The chimeric receptor had reduced GABA_A receptor current enhancement by benzodiazepines, suggesting a role for non-N-terminal domains in the coupling of benzodiazepine binding to enhancement of current.

To study the structural basis of benzodiazepine coupling, we used a chimera strategy, using γ 2L- δ chimeras coexpressed with α 1 and β 3 subunits. We chose γ 2L- δ chimeras because the δ subunit can replace the γ subunit in GABA_A receptors (Quirk et al., 1994) but does not contribute to either benzodiazepine binding (Quirk et al., 1995) or modulation (Saxena and Macdonald, 1994). We used γ 2L- δ chimeras that contained the γ 2L N terminus to preserve the benzodiazepine binding site and replaced each domain of the remaining γ 2L subunit with δ subunit se-

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quence. We revealed two γ 2L subunit domains, the distal portion of M1 (domain 1) and the distal portion of M2 and the M2–M3 loop (domain 2), that play critical roles in the coupling of benzodiazepine binding to potentiation of the GABA_A receptor current. Our findings, in addition to studies that demonstrate the involvement of these structural areas in gating (O'Shea and Harrison, 2000; Lynch et al., 2001; Bera et al., 2002; Kash et al., 2003) and allosteric regulation of LGICs (Kucken et al., 2000), suggest common transduction machinery in the LGIC superfamily.

Materials and Methods

Construction of GABA_A receptor subunits, chimeras, and mutants. Rat GABA_A receptor wild-type α 1, β 3, δ , γ 2L, and chimeric subunits were individually subcloned into the mammalian expression vector pCMV-neo through the *Bgl*III restriction site. Chimeras were constructed using restriction fragments at engineered sites, a PCR-based overlap-extension method, or by site-directed mutagenesis using the QuikChange kit (Stratagene, La Jolla, CA) in existing chimeric or wild-type subunits. The transition point of the subunit amino acid sequence is listed for each chimera as the N-terminal parent subunit with the last amino acid of that segment, followed by the C-terminal parent subunit with the first amino acid of that segment [γ - δ M1e (γ G234– δ V233), γ - δ M1 pre-iso (γ F236– δ I235), γ - δ M1q (γ Q239– δ S238), and γ - δ M1i (γ I257– δ S256)] (Fig. 1). The placement of an “e” at the end of the construct name represents a chimera splice site at the extracellular membrane interface (e.g., γ - δ M1e refers to a chimera with the γ subunit sequence until the extracellular membrane interface of M1, with δ subunit sequence after the extracellular membrane interface of M1), whereas “i” similarly refers to a chimera splice site at the intracellular membrane interface. Other letters used (e.g., “q” in M1q) refer to the given γ subunit amino acid in which the splice site occurred. The numbering refers to the mature peptide. Specifically, chimeras γ - δ M1e, γ - δ M2e, γ - δ M1q, and γ - δ M1i were generated progressively by replacing the wild-type rat γ 2L with the rat δ sequence or replacing the wild-type rat δ with the rat γ 2L sequence through site-directed mutagenesis by using existing chimera γ - δ M1 pre-iso as a template. Rat γ 2Ls with δ domain swaps (γ - δ ____) were also made progressively through site-directed mutagenesis by using the rat γ 2L subunit as a template and are as follows: γ - δ M1 (γ Y235– γ I257 was replaced by δ V233– δ I255), γ - δ M2 (γ A261– γ A283 was replaced by δ A259– δ A281), γ - δ M1–M2 (γ Y235– γ A283 was replaced by δ V233– δ A281), γ - δ M2–M3 loop (γ R284– γ T294 was replaced by δ R282– δ K292), γ - δ M1 to M2–M3 loop (γ Y235– γ T294 was replaced by δ V233– δ K292). Mutant constructs were designed using site-directed mutagenesis (QuikChange; Stratagene) in a γ 2L subunit backbone and contained a homologous δ subunit sequence in place of a γ 2L subunit sequence. Alignments between γ 2L and δ subunit sequences were obtained using the Align X program of the Vector NTI Suite 8.0 (Informax, Frederick, MD). Sequence validity was verified by sequencing the full-length coding sequence of the final constructs. In experiments in which chimeras or mutants were used, chimeric or mutant γ 2L subunits were transfected in place of wild-type γ 2L subunits at the same relative concentrations.

Expression of recombinant GABA_A receptors in cultured human embryonic kidney 293T cells. For electrophysiological recordings, human embryonic kidney (HEK) 293T fibroblast cells at a density of 200,000–400,000 cells/60 mm culture dish were maintained in DMEM (Invitrogen, Carlsbad, CA), supplemented with 10% fetal bovine serum and 100 IU/ml each of penicillin and streptomycin (Invitrogen) at 37°C in 5% CO₂/95% O₂. On day 1, cells were transfected with 4 μ g of each subunit plasmid (ratio, 1:1:1), along with 2 μ g of pHook-1 (Invitrogen) for immunomagnetic bead selection on day 2 (Greenfield et al., 1997), using a previously established calcium phosphate precipitation technique (Angelotti et al., 1993). After immunomagnetic bead selection, the cells were plated on 35 mm dishes, and recordings were made on day 3, ~18–32 h after selection.

For radioligand binding experiments, four 10 cm culture dishes of HEK293T cells were plated at a density of 500,000 cells/10 cm culture dish 3 d before transfection and maintained in DMEM supplemented with 10% fetal bovine serum and 100 IU/ml each of penicillin and strep-

Figure 1. Membrane topology of a single GABA_A receptor subunit and sequence homology between the GABA_A receptor γ 2L subunit and the GABA_A receptor δ subunit. Each GABA_A receptor subunit is composed of an extracellular N terminus containing a conserved cysteine loop motif, four transmembrane domains (M1–M4), an extracellular M2–M3 loop, a smaller intracellular M1–M2 loop, as well as a large intracellular M3–M4 loop that is the site of post-translational modulation of the receptor and an extracellular C terminus. The area between the two black bars is enlarged to show the amino acid sequence of individual subunits in that region. Sequence alignments between the γ 2L and δ subunits were obtained using the Align X program of the Vector NTI Suite 8.0 (Informax). Regions delineated by black bars indicate transmembrane regions as described previously based on hydrophobicity; the M2–M3 loop is indicated by a dotted bar. Shaded regions indicate nonconservation of the sequence between the γ 2L and δ subunits. Sequence validity was verified by sequencing the full-length coding sequence of the final constructs.

tomycin at 37°C in 5% CO₂/95% O₂. On the day of transfection, 10 cm dishes of cells were transfected with 5.6 μ g of each subunit cDNA plasmid using Fugene 6 (Roche Diagnostics, Indianapolis, IN) transfection reagent (at a ratio of 2.67 μ l of Fugene/ μ g of cDNA). Cells remained plated for ~48 h after transfection before use in the radioligand binding assay.

Radioligand binding. For benzodiazepine radioligand binding experiments, a solution consisting of (in mM) 142 NaCl, 1 CaCl₂, 8 KCl, 6 MgCl₂, 10 glucose, and 10 HEPES, pH 7.4, was used as a buffer throughout the duration of the experiment. Four milliliters of buffer solution were added to each 10 cm dish, and cells were scraped from the dishes.

Cells were manually homogenized and then sonicated for 15 s at an amplitude of 100 using a VibroCell ultrasonic processor (model VC-130; Sonics and Materials, Danbury, CT). The homogenates were then centrifuged at $30,000 \times g$ for 20 min at 4°C. The resulting pellet was resuspended in buffer followed by trituration to break up the pellet. After full homogenous resuspension of the pellet, duplicate membrane samples were incubated at room temperature with seven increasing concentrations of [^3H]flunitrazepam (74 Ci/mmol; PerkinElmer, Boston, MA) in the presence of either nonradioactive 100 μM flurazepam (flurazepam dihydrochloride; Sigma-Aldrich, St. Louis, MO) or an equivalent concentration of DMSO (final DMSO concentration, 0.1%; Sigma-Aldrich) to determine nonspecific and total binding, respectively. Samples were incubated at room temperature for ~ 2 h.

After incubation, the membrane suspensions were applied to glass-fiber filters (Whatman GF/B; Brandel, Gaithersburg, MD) that were pretreated with 0.3% polyethylenimine (Sigma-Aldrich), vacuum-filtered using a cell harvester (model MPR-24T; Brandel), and then washed with 2 ml of buffer. For each experiment, the nonspecific [^3H]flunitrazepam binding at each concentration point was fit using a least-squares method to a linear function. Average nonspecific binding was 22% of total binding at K_D concentrations of [^3H]flunitrazepam. Specific [^3H]flunitrazepam binding was calculated as the total [^3H]flunitrazepam bound in the absence of flurazepam minus the fit value of nonspecific binding. Specific binding was fit to the one-site competitive binding equation: $\text{Bound} = (B_{\text{max}} \times [\text{flunitrazepam}]) / (K_D + [\text{flunitrazepam}])$ (GraphPad Software, San Diego, CA). Relative maximal [^3H]flunitrazepam binding of the $\alpha 1\beta 3\gamma\delta\text{M1e}$ chimeric receptor was calculated by normalizing the specifically bound [^3H]flunitrazepam counts per minute obtained from the $\alpha 1\beta 3\gamma\delta\text{M1e}$ chimera at each [^3H]flunitrazepam concentration to specifically bound [^3H]flunitrazepam counts per minute obtained from the $\alpha 1\beta 3\gamma 2\text{L}$ receptor at the same [^3H]flunitrazepam concentration. The normalized values were then fit to the above one-site competitive binding equation.

Electrophysiological recording. Whole-cell voltage-clamp recordings were performed on transfected HEK293T fibroblast cells. All experiments were performed using at least two separate transfected batches of cells from at least two separate days of recording. Cells were bathed in an external solution consisting of (in mM) 142 NaCl, 1 CaCl_2 , 8 KCl, 6 MgCl_2 , 10 glucose, and 10 HEPES, pH 7.4, ~ 320 – 335 mOsm, throughout the duration of the experiment. Glass microelectrodes were formed from thin-walled borosilicate glass with a filament (World Precision Instruments, Sarasota, FL) using a P-87 Flaming-Brown or P2000 laser electrode puller (Sutter Instruments, San Rafael, CA) and fire polished with a microforge (Narishige, East Meadow, NY). Microelectrodes had resistances of 1–4 M Ω when filled with an internal solution consisting of the following (in mM): 153 KCl, 1 MgCl_2 , 10 HEPES, 5 EGTA, 2 Mg^{2+} , ATP, pH 7.3, ~ 300 – 310 mOsm. This combination of external and internal solutions produced a chloride equilibrium potential (E_{Cl}) of ~ 0 mV.

Membrane voltages were usually clamped at -10 to -75 mV using an EPC7 (List-Electronic, Darmstadt-Eberstadt, Germany) or an Axon 200B (Molecular Devices, Foster City, CA) amplifier. No voltage dependence of diazepam modulation was observed in this study.

The same concentration of GABA ($1 \mu\text{M}$) was applied to most chimeras. This was a concentration that was determined to correspond to an effective concentration (EC) of GABA of $\text{EC}_{15} \pm 10$ for each chimera, using a two-point concentration–response method. In this method, the response of a given construct to $1 \mu\text{M}$ GABA was compared with the maximal response of the given construct (1 mM GABA). The percentage of the maximal response elicited by $1 \mu\text{M}$ GABA was determined to be the EC_x value (supplemental table, available at www.jneurosci.org as supplemental material). Complete concentration–response curves were obtained for chimeras when the response to $1 \mu\text{M}$ GABA deviated from 20% of the maximal current evoked by 1 mM GABA by $>10\%$. The EC_{20} GABA concentration determined from the concentration–response curve was applied for those chimeras (e.g., for $\alpha 1\beta 3\gamma\delta\text{M1}$, $10 \mu\text{M}$ GABA was used, and for $\alpha 1\beta 3\gamma 2\text{V90A}$ plus Y292A plus V293I plus T294K , $3 \mu\text{M}$ GABA was used). GABA ($\text{EC}_{15} \pm 10$) and GABA ($\text{EC}_{15} \pm 10$) plus diazepam ($1 \mu\text{M}$) were applied to the cells via hand-pulled triple-barreled

square glass attached to the Warner SF-77B Perfusion Fast-Step (Warner Instruments, Hamden, CT), allowing for rapid solution changes. The application system provided for simultaneous flow of all solutions to which the cells were exposed through three parallel glass square barrels. All step protocols began with a cell positioned in the flow of external bath solution from which the multibarreled array was repositioned such that the unmoved cell and electrode were now exposed to a drug (e.g., GABA). The drug application was initiated by an analog pulse triggered by the pClamp 8.1 software, which caused the motor of the Warner Fast-Step to reposition the multibarrel array from one barrel to another (e.g., external solution to GABA). Exchange times were measured to be 1–5 ms at an open electrode tip. These exchange times may be slower around an intact cell, although this was not explicitly measured.

For generation of concentration–response relationships, peak GABA $_A$ receptor currents evoked by multiple increasing concentrations of GABA were fitted to a sigmoidal function using a four-parameter logistic equation (sigmoidal concentration–response) with a variable slope to generate concentration–response curves. The equation used to fit the concentration–response relationship was the following:

$$I = \frac{I_{\text{max}}}{1 + 10^{(\log \text{EC}_{50} - \log \text{drug}) \times \text{Hill slope}}}, \quad (1)$$

where I was the peak current at a given GABA concentration and I_{max} was the maximal peak current.

Signals were acquired simultaneously on a WR7400 chart recorder (Graphtec, Irvine, CA) and on a computer. Current amplitudes were measured on a computer using the Molecular Devices pClamp 8.1 software package.

Data analysis. Peak current was determined using Clampfit of the Axoclamp software suite (Molecular Devices). Percentage enhancement (control, 0%) was determined using Microsoft Excel 2000 for Windows. Percentage enhancement of control is defined as follows: $[(I_{\text{GABA}} + \text{DRUG} - I_{\text{GABA}}) / (I_{\text{GABA}})] \times 100 + 100$. Statistical significance was assessed with unpaired Student's t test: $*p < 0.05$, $**p < 0.01$, or $***p < 0.001$, using GraphPad Prism version 4.00 for Windows (GraphPad Software). The data for each construct were always distributed normally, although Welch's correction was applied to unpaired Student's t test when variances were found to be unequal.

Results

Subunit-dependent differences in diazepam modulation of GABA $_A$ receptor currents

GABA-evoked currents were recorded from all chimeric and mutant receptors expressed in HEK293T cells, confirming that they yielded functional receptor channels. $\alpha 1\beta 3\gamma 2\text{L}$ receptor currents evoked by $1 \mu\text{M}$ GABA were enhanced by a maximal ($1 \mu\text{M}$) diazepam concentration (mean, $139.3 \pm 22.9\%$; $n = 13$) (Fig. 2A). In contrast, $\alpha 1\beta 3\delta$ receptor currents evoked by $1 \mu\text{M}$ GABA were insensitive to $1 \mu\text{M}$ diazepam (mean, $-4.7 \pm 5.1\%$; $n = 9$) (Fig. 2A). A higher diazepam concentration ($5 \mu\text{M}$) was also tested on all receptors to confirm that the lack of effect of nonresponsive receptors was not concentration dependent (data not shown).

Functional characterization of chimeric subunits

Several studies have implicated amino acids within the N-terminal domains of α and γ subunits of the GABA $_A$ receptor in the binding of diazepam and other benzodiazepines (Mihic et al., 1994; Amin et al., 1997; Sigel and Buhr, 1997). Because N-terminal γ subunit residues are necessary for benzodiazepine binding, it was expected that replacement of the $\gamma 2\text{L}$ subunit N terminus with δ sequence would prevent benzodiazepine binding; thus, we used $\gamma\delta$ (rather than $\delta\gamma$) chimeras with the intact $\gamma 2\text{L}$ subunit N-terminal sequence. To determine the structural domains in the $\gamma 2\text{L}$ subunit that couple benzodiazepine binding to enhancement of GABA-evoked currents, we constructed a se-

Figure 2. N-terminal binding structures are not sufficient to elicit potentiation. **A**, Currents evoked by rapid application of 1 μM GABA (open circle) in an $\alpha 1\beta 3\gamma 2L$ -containing (white) GABA_A receptor subunit display robust potentiation in response to coapplication of 1 μM GABA and 1 μM diazepam (DZP) (closed circle) (mean, 139.3 $\pm$ 22.9%; n = 13), whereas currents from an $\alpha 1\beta 3\delta$ -containing (shaded) GABA_A receptor subunit do not respond to a similar application (mean, $-4.7 \pm 5.1\%$; n = 9). **B**, Replacement of the δ subunit N terminus with a γ subunit N terminus (white) does not confer 1 μM DZP enhancement of 1 μM GABA-evoked currents in a δ subunit-containing receptor (shaded). Extension of the $\gamma 2L$ subunit sequence through the end of M2 restores potentiation, although at a slightly reduced level from that of $\alpha 1\beta 3\gamma 2L$ receptors. The dotted lines indicate wild-type $\alpha 1\beta 3\gamma 2L$ receptor levels of enhancement. Asterisks indicate statistical significance between the wild-type $\alpha 1\beta 3\delta$ subunit or chimeric subunit and the $\alpha 1\beta 3\gamma 2L$ subunit, as determined by Student's t test. Welch's correction was applied to unpaired Student's t test when variances were found to be unequal. N-term, N-terminal. Error bars indicate SEM.

ries of $\gamma 2L$ - δ subunit chimeras (χ s). The wild-type $\gamma 2L$, δ , and γ - δ chimeric subunits were coexpressed with $\alpha 1$ and $\beta 3$ subunits to form $\alpha 1\beta 3\gamma 2L$, $\alpha 1\beta 3\delta$, and $\alpha 1\beta 3\chi$ receptors.

If binding of diazepam to the receptor was sufficient for current enhancement, it was possible that all γ subunit sequence distal to the N terminus could be replaced by δ subunit sequence without altering coupling. However, an alternative hypothesis is that the $\gamma 2L$ subunit has additional domains C-terminal to the N-terminal domains that are important for transduction of benzodiazepine binding to enhancement of current. If this is correct, it would allow the use of multiple γ - δ chimeras to identify these $\gamma 2L$ subunit domains. To test the hypothesis that the $\gamma 2L$ subunit has additional domains C-terminal to the N-terminal domain that are important for transduction, we constructed a γ - δ chimera that retained the $\gamma 2L$ subunit N-terminal sequence but replaced $\gamma 2L$ with δ subunit sequence from the extracellular end of the M1 domain to the C terminus (γ - δ M1e) (Fig. 2B). The γ - δ M1e chimera was insensitive to diazepam (mean, 4.3 $\pm$ 6.9%; n = 8; p < 0.0001), similar to the $\alpha 1\beta 3\delta$ receptor.

This result has two alternative interpretations. First, diazepam did not bind to the $\alpha 1\beta 3\gamma$ - δ M1e receptor, and second, diazepam bound to the receptor but was unable to allosterically alter

Figure 3. The $\alpha 1\beta 3\gamma$ - δ M1e chimera displays normal binding of benzodiazepines. Specific binding of [3H]flunitrazepam to membrane suspensions of HEK293T cells expressing $\alpha 1\beta 3\gamma 2L$ (wild type; closed squares) or $\alpha 1\beta 3\gamma$ - δ M1e (closed triangles) receptors was determined. The $\alpha 1\beta 3\gamma$ - δ M1e chimera K_D was 15.14 $\pm$ 4.47 nM, with a B_{max} of 1.1 $\pm$ 0.1, indicating binding of benzodiazepines to the receptor. The graph displays the normalized mean of three experiments (each performed in duplicate) $\pm$ SEM for each construct.

receptor-channel gating, suggesting the presence of additional structural requirements for diazepam allosteric regulation of the GABA_A receptor. If the first interpretation was correct, then it would be likely that no γ - δ receptor chimera would be modulated by diazepam. However, $\alpha 1\beta 3\gamma$ - δ M1e receptors were inhibited by DMCM (0.3 μM) (data not shown), an inverse agonist of the benzodiazepine binding site, suggesting that the benzodiazepine binding site was substantially intact. To further confirm that the $\alpha 1\beta 3\gamma$ - δ M1e receptor could bind benzodiazepines, $\alpha 1\beta 3\gamma 2L$ and $\alpha 1\beta 3\gamma$ - δ M1e receptors were expressed in HEK293T cells and specific binding of [3H]flunitrazepam was measured. Radioligand binding experiments demonstrated that the $\alpha 1\beta 3\gamma$ - δ M1e receptor bound benzodiazepines (K_D = 15.14 $\pm$ 4.466 nM; B_{max} = 1.098 $\pm$ 0.093; relative B_{max} = 0.12 $\pm$ 0.02) (Fig. 3), indicating that the lack of diazepam enhancement of the $\alpha 1\beta 3\gamma$ - δ M1e receptor was not the result of a lack of diazepam binding.

If the second interpretation was correct, then chimeric receptors containing the appropriate transmembrane $\gamma 2L$ subunit domains should respond to diazepam. To distinguish between these two alternative interpretations, we tested the diazepam sensitivity of a chimera that preserved the $\gamma 2L$ subunit sequence from the N terminus through M2, with δ subunit sequence C-terminal to M2 (γ - δ M2e) (Fig. 2B). The diazepam sensitivity of this chimera was slightly reduced (mean, 61.5 $\pm$ 32.3%; n = 6); however, the reduction was not significantly different from wild-type $\gamma 2L$ subunit-containing receptors (p = 0.07). This result confirms the importance of the $\gamma 2L$ N terminus in the binding of diazepam and suggests that the M1 and M2 domains participate in the coupling of benzodiazepine binding to enhancement of GABA-evoked currents. The slight reduction in enhancement further suggests a possible small contribution of structures distal to the M2 domain in the coupling of benzodiazepine binding to gating of the GABA_A receptor.

Functional characterization of chimeric subunits: importance of multiple domains

To identify the specific domains C-terminal to the N terminus involved in benzodiazepine transduction, we constructed a series of chimeras that replaced $\gamma 2L$ subunit domains with δ subunit sequence and expressed them with $\alpha 1$ and $\beta 3$ subunits. These

Figure 4. Multiple structural areas coordinately regulate transduction of benzodiazepine binding to GABA_A receptor gating. **A**, In the absence of the γ 2L N-terminal sequence, diazepam (DZP) is ineffective, presumably because of a lack of binding. Interchanging the γ 2L subunit sequence (white) in individual domains for δ subunit sequence (shaded) does not disrupt potentiation of EC_{equivalent} GABA-evoked currents by 1 μ M DZP. **B**, Interchanging the γ 2L subunit sequence (white) in the M1 and M2 domains for δ subunit sequence (γ - δ M1-M2; shaded) significantly reduces potentiation of 1 μ M GABA-evoked currents by 1 μ M DZP ($***p = 0.0007$). This reduction was also significantly different from α 1 β 3 δ levels of potentiation ($^{^^}p = 0.0002$). Interchanging the γ 2L subunit sequence (white) for δ subunit sequence (shaded) from the M1 through the M2-M3 loop domain (γ - δ M1-M2-3 loop) significantly reduces potentiation of 1 μ M GABA-evoked currents by 1 μ M DZP ($***p < 0.0001$) to wild-type α 1 β 3 δ levels of potentiation. Open circles indicate current evoked by GABA; closed circles indicate current evoked by GABA plus DZP. The dotted lines indicate wild-type α 1 β 3 γ 2L receptor levels of enhancement. $***$ Statistical significance between chimera and α 1 β 3 γ 2L subunits, as determined by Student's *t* test. $^{^^}$ Significance between chimera and α 1 β 3 δ subunits, as determined by Student's *t* test. Welch's correction was applied to unpaired Student's *t* test when variances were found to be unequal. n.s., Not significant; N-term, N-terminal. Error bars indicate SEM.

constructs substituted δ subunit sequence for the γ 2L subunit sequence in the N terminus (δ - γ M1e), the M1 domain (γ - δ M1), the M2 domain (γ - δ M2), and the M2-M3 loop (γ - δ M2-M3 loop) (Fig. 4A). As anticipated, the (δ - γ M1e) chimera, lacking the N-terminal γ 2L subunit residues necessary for benzodiazepine

binding, did not display enhancement, presumably because of a lack of binding (mean, $-4.5 \pm 1.5\%$; $n = 8$) (Fig. 4A, top row). Surprisingly, none of the other constructs significantly altered diazepam potentiation compared with wild-type α 1 β 3 γ 2L subunit-containing receptors (γ - δ M1: mean, $88.0 \pm 17.9\%$, $n = 5$; γ - δ M2: mean, $235.6 \pm 40.2\%$, $n = 6$; γ - δ M2-M3 loop: mean, $86.7 \pm 17.2\%$, $n = 6$) (Fig. 4A, bottom three rows). However, our previous results, showing that extension of the N-terminal γ 2L subunit sequence to include the M2 domain (γ - δ M2e) restored benzodiazepine sensitivity (see above) (Fig. 2B) to an insensitive subunit (γ - δ M1e), offer a possible explanation.

This result can be explained by the hypothesis that more than one structural domain is involved in benzodiazepine potentiation. For example, although a subunit may have δ subunit sequence in the M1 domain, the γ 2L subunit M2 domain would be sufficient for transduction, and vice versa. Therefore, it would be expected that replacement of both γ 2L domains by the δ subunit sequence would be necessary to abolish potentiation. To test this hypothesis, we replaced both M1 and M2 domains of the wild-type γ 2L subunit with δ subunit sequence (γ - δ M1-M2) (Fig. 4B, top row). Expression of the γ - δ M1-M2 chimera resulted in a significant reduction (mean, $35.9 \pm 5.9\%$; $n = 6$; $p = 0.0007$) in diazepam potentiation of GABA-evoked currents (Fig. 4B, top row), suggesting that M1 and M2 each contained structures that were sufficient to support benzodiazepine enhancement. Yet the presence of the two structural domains in concert was necessary for full wild-type α 1 β 3 γ 2L levels of potentiation. Nevertheless, the potentiation obtained with the γ - δ M1-M2 construct was also significantly different ($p = 0.0002$) from the lack of potentiation seen with wild-type δ subunit-containing receptors, suggesting the presence of additional structural areas that may contribute to subunit responsiveness to benzodiazepines.

The N terminus has previously been shown to interact with the M2-M3 loop to regulate the gating of LGICs (Akabas and Karlin, 1995; Kash et al., 2003). Given this finding and our observation that the γ - δ M2e chimera did not completely restore full benzodiazepine potentiation, we hypothesized that the M2-M3 loop might also be involved in the coupling of benzodiazepine binding to enhancement of GABA_A receptor currents. To determine whether the M2-M3 loop was important for and could augment enhancement by diazepam in the context of the M1-M2 swap, we created an additional construct that contained a δ sequence from the M1 domain through the M2-M3 loop in a γ 2L subunit (α - δ M1-M2-3 loop). This construct completely abolished benzodiazepine enhancement (mean, $4.9 \pm 2.3\%$; $n = 5$; $p < 0.0001$) (Fig. 4B, bottom row) and was indistinguishable from δ subunit enhancement levels ($p = 0.1170$). These data suggested that there were three domains in the γ 2L subunit that were necessary, although not individually sufficient, for benzodiazepine enhancement: M1, M2, and the M2-M3 loop.

M1 residues relevant for the coupling of benzodiazepine binding to current enhancement

In comparing the sequence of the γ 2L and δ subunits in the M1 region, several differences were noted (Fig. 5A). Based on these differences, we created two additional M1 domain chimeras in an attempt to discern the specific structural area in M1 responsible for benzodiazepine transduction. Chimeras with progressive extension of the γ 2L subunit sequence toward the intracellular end of M1 (γ - δ M1pre-iso and γ - δ M1q) (Fig. 5B, third and fourth rows, respectively) were compared with the wild-type γ 2L subunit. Extension of the γ 2L subunit sequence by two amino acids from γ - δ M1e (γ - δ M1pre-iso) significantly increased benzodiazepine

Figure 5. The N-terminal region of the M1 domain is important for transduction of benzodiazepine binding to GABA_A receptor gating. **A**, Sequence homology between the GABA_A receptor γ2L subunit and the GABA_A receptor δ subunit. Region delineated by black bar indicates M1 domain as previously described based on hydrophobicity. Shaded regions indicate nonconservation of sequence between the γ2L and δ subunits. Chimera splice sites are indicated by dotted lines. **B**, Progressive extension of the γ2L subunit sequence from the N terminus toward the intracellular end of the M1 domain (white) restores 1 μM diazepam (DZP) potentiation of 1 μM GABA in a δ subunit (shaded). Open circles indicate current evoked by GABA; closed circles indicate current evoked by GABA plus DZP. The dotted lines indicate wild-type α1β3γ2L receptor levels of enhancement. ***Significance between chimera and α1β3γ2L subunit. ^, ^^^Statistical significance between chimera and α1β3δ subunit, as determined by Student's *t* test. Welch's correction was applied to unpaired Student's *t* test when variances were found to be unequal. n.s., Not significant; N-term, N-terminal. Error bars indicate SEM.

azepine sensitivity compared with the δ subunit-containing receptors (mean, $23.4 \pm 2.7\%$; $n = 5$; $p = 0.0005$) (Fig. 5B, third row). However, diazepam potentiation of γ-δ M1pre-iso receptors was also significantly different from γ2L subunit levels of

Figure 6. γ2L subunit M1 YFT and γ2L subunit M2 STI sequences alone are sufficient but not necessary for enhancement of GABA-evoked currents by diazepam (DZP). **A**, Sequence homology between the GABA_A receptor γ2L subunit and the GABA_A receptor δ subunit. The region delineated by black bars indicates the M2 domain as previously described based on hydrophobicity. The shaded regions indicate nonconservation of sequence between the γ2L and δ subunits. Mutation locations are indicated by boxed A and B areas. **B**, Individual replacement of γ2L subunit residues in either the M1 and M2 domains for δ subunit sequence (dots), does not affect potentiation of 1 μM GABA-evoked currents by 1 μM DZP. Open circles indicate current evoked by GABA; closed circles indicate current evoked by GABA plus DZP. The dotted lines indicate wild-type α1β3γ2L receptor levels of enhancement. N-term, N-terminal. Error bars indicate SEM.

enhancement ($p = 0.0003$). Interestingly, subsequent extension of the γ2L sequence by one additional residue (γ-δ M1q) resulted in a receptor with diazepam enhancement that was not significantly different from that of the γ2L subunit-containing receptors (mean, $80.5 \pm 28.7\%$; $n = 8$; $p = 0.1267$) (Fig. 5B, fourth row). This suggested that a domain responsible for the benzodiazepine enhancement in γ2L subunit-containing receptors resides in the N-terminal region of the M1 domain. Our previous results, demonstrating a lack of enhancement in the γ-δ M1e chimera, suggested that this difference in enhancement was attributable to the structural differences between the γ-δ M1e and γ-δ M1q chimeras.

To test this hypothesis, we constructed a series of M1 muta-

A

M1											
γ	235	Y	F	T	I	Q	T	Y	I	P	C
δ	233	V	Y	I	I	Q	S	Y	M	P	S
M2											
γ	261	A	V	P	A	R	T	S	L	G	I
δ	259	A	V	P	A	R	V	S	L	G	I
M3											
γ	287	R	K	S	L	P	K	V	S	Y	V
δ	285	R	S	S	L	P	R	A	S	A	I
M2-3 loop											
γ	287	R	K	S	L	P	K	V	S	Y	V
δ	285	R	S	S	L	P	R	A	S	A	I

B

C

M1											
γ	235	Y	F	T	I	Q	T	Y	I	P	C
δ	233	V	Y	I	I	Q	S	Y	M	P	S
M2											
γ	261	A	V	P	A	R	T	S	L	G	I
δ	259	A	V	P	A	R	V	S	L	G	I
M3											
γ	296	T	A	M	D	L	F	V	S	V	C
δ	294	K	A	L	D	V	Y	F	W	I	C

Figure 7. Coordinate regulation by γ 2L subunit M1 YFT, M2 STI, and the M2–M3 loop are necessary for transduction of benzodiazepine binding to GABA_A receptor gating. **A**, Sequence homology between the GABA_A receptor γ 2L subunit and the GABA_A receptor δ subunit. Regions delineated by black bars indicate transmembrane regions as previously described based on hydrophobicity; the M2–M3 loop is indicated by a dotted bar. Shaded regions indicate nonconservation of the sequence between the γ 2L and δ subunits. Mutation locations are indicated by boxed A, B, C, and D areas. **B**, Replacement of γ 2L subunit residues in both the M1 and M2 domains for δ subunit sequence (dots) significantly reduces potentiation of

tions, substituting δ subunit M1 residues into the γ 2L subunit (Fig. 6). The aligned sequence in M1 revealed a triplet set of residues that differed between the γ - δ M1e and γ - δ M1q chimeras, namely, γ YFT- δ VYI (Fig. 6A, region A). However, because the M1 domain swap did not alter diazepam enhancement of GABA_A receptor current, it was unlikely that point mutations in the distal M1 domain identified above would alter current enhancement by diazepam. As expected, these residues did not attenuate benzodiazepine enhancement (γ T237I: mean, $165.4 \pm 30.2\%$, $n = 5$; γ Y235V plus F236Y: mean, $182.6 \pm 24.1\%$, $n = 5$; γ Y235V plus F236Y plus T237I: mean, $103.9 \pm 13.5\%$, $n = 6$), consistent with the hypothesis that this M1 sequence was necessary but not sufficient for enhancement of GABA-evoked currents by diazepam.

M2 residues relevant for the coupling of benzodiazepine binding to current enhancement

Similar point mutations were made in the M2 domain. There are only four amino acid differences between the γ 2L and δ subunits in M2 (Fig. 6A). Of these four differences, three (γ STI- δ MVS) (Fig. 6A, region B) were of particular interest because they have been implicated in allosteric coupling of both enhancement and inhibition of the GABA_A receptor (Boileau and Czajkowski, 1999; Nagaya and Macdonald, 2001; Hosie et al., 2003). Interestingly, two of the amino acids in this area, γ T281 and I282, have been previously identified as areas of interest in coupling benzodiazepine binding to GABA_A gating using γ - α chimeras (Boileau and Czajkowski, 1999). We therefore mutated the three γ 2L residues to the homologous δ residues in a γ 2L subunit (γ S280M plus T281V plus I282S). Because the M2 domain swap did not attenuate diazepam enhancement of GABA_A receptor current, we did not expect that isolated mutations in M2 would alter the enhancement. As expected, we found that these three mutations did not abolish potentiation; this mutant was not significantly different from γ 2L subunit levels of benzodiazepine enhancement (mean, $183.0 \pm 52.8\%$; $n = 5$; $p = 0.3840$) (Fig. 6B, bottom row).

Combined M1 and M2 residues relevant for the coupling of benzodiazepine binding to current enhancement

Based on our previous finding that substituting both the M1 and the M2 domain of a γ 2L subunit with δ subunit sequence resulted in a significant decrease in diazepam enhancement, we investigated a combination of M1 and M2 mutations. We combined the M1 and M2 mutants to create a double (triplet) mutant (γ Y235V plus F236Y plus T237I plus S280M plus T281V plus I282S) (Fig. 7A, regions A and B). The γ Y235V plus F236Y plus T237I plus S280M plus T281V plus I282S mutant displayed a significant

EC₅₀ equivalent GABA-evoked currents by 1 μ M diazepam (DZP); however, full abolishment of potentiation is achieved only when the γ 2L subunit M1 YFT, M2 STI, and the M2–M3 loop sequence is replaced with δ subunit sequence (mean, $-3.9 \pm 10.8\%$; $p < 0.0001$). Open circles indicate current evoked by GABA; closed circles indicate current evoked by GABA plus DZP. The dotted lines indicate wild-type α 1 β 3 γ 2L receptor levels of enhancement. *, **, ***Statistical significance between chimera and α 1 β 3 γ 2L subunit. ^, ^^^Significance between chimera and α 1 β 3 δ subunit, as determined by Student's *t* test. Welch's correction was applied to unpaired Student's *t* test when variances were found to be unequal. N-term, N-terminal. C, Sequence homology between the GABA_A receptor γ 2L subunit and the GABA_A receptor δ subunit based on sequence alignment and structural data. Regions delineated by gray bars indicate transmembrane regions based on sequence alignment and structural data; the M2–M3 loop is indicated by a dotted bar. Shaded regions indicate nonconservation of the sequence between the γ 2L and δ subunits. The black bars indicate extracellular regions of transmembrane domains. Error bars indicate SEM.

reduction in diazepam potentiation compared with wild-type $\alpha 1\beta 3\gamma 2L$ receptors (mean, $57.4 \pm 18.6\%$; $n = 6$; $p = 0.0375$) (Fig. 7B, top row). The potentiation exhibited by this mutant was not significantly different from that noted in the γ - δ M1–M2 swap construct (mean, $35.9 \pm 5.9\%$; $n = 6$; $p = 0.3121$) (Fig. 4B, top row). However, similar to the M1–M2 swap construct, diazepam potentiation of currents recorded from the double-mutant receptor construct was also significantly different from potentiation of currents from the $\alpha 1\beta 3\delta$ receptor ($p = 0.0233$) (Fig. 2A), further suggesting involvement of another structural domain in the enhancement of GABA-evoked currents by diazepam.

M2–M3 loop residues relevant for the coupling of benzodiazepine binding to current enhancement

Based on the similarity between our mutant data and the swap construct data, we speculated that the third area of interest would be the M2–M3 loop. We focused on the amino acid sequence of the M2–M3 loop to look for potential areas for mutagenesis (Fig. 7A, regions C and D). The M2–M3 loop varies at six amino acid positions between the $\gamma 2L$ and δ subunits. Five of these variations are nonconservative and were therefore of interest. We created mutant constructs in the context of the previously identified M1 and M2 residues, investigating the differences between the $\gamma 2L$ and δ subunits. We constructed two M2–M3 loop mutant constructs, $\gamma K285S$ (Fig. 7A, region C) and $\gamma V290A$ plus $Y292A$ plus $V293I$ plus $T294K$ (Fig. 7A, region D) combined with the $\gamma Y235V$ plus $F236Y$ plus $T237I$ plus $S280M$ plus $T281V$ plus $I282S$ mutations. Expression of either M2–M3 loop mutation ($\gamma K285S$ or $\gamma V290A$ plus $Y292A$ plus $V293I$ plus $T294K$) with the M1 and M2 triplet mutations ($\gamma Y235V$ plus $F236Y$ plus $T237I$ plus $S280M$ plus $T281V$ plus $I282S$) (Fig. 7B, second and third rows, respectively) did not further affect benzodiazepine sensitivity compared with the M1 and M2 triplet mutations alone ($\gamma Y235V$ plus $F236Y$ plus $T237I$ plus $S280M$ plus $T281V$ plus $I282S$ plus $K285S$: mean, $77.4 \pm 12.2\%$, $n = 6$; $\gamma Y235V$ plus $F236Y$ plus $T237I$ plus $S280M$ plus $T281V$ plus $I282S$ plus $V290A$ plus $Y292A$ plus $V293I$ plus $T294K$: mean, $62.9 \pm 9.4\%$, $n = 5$). However, when both M2–M3 loop regions were mutated (i.e., the entire M2–M3 loop of $\gamma 2L$ was replaced with δ subunit sequence) and combined with the M1 and M2 triplet mutations ($\gamma Y235V$ plus $F236Y$ plus $T237I$ plus $S280M$ plus $T281V$ plus $I282S$ plus M2–M3 loop) (Fig. 7B, bottom row), enhancement was completely abolished (mean, $-3.9 \pm 10.8\%$; $n = 5$), and currents obtained in the presence of diazepam were indistinguishable from $\alpha 1\beta 3\delta$ receptor currents ($p = 0.9341$).

The previously identified areas of the distal M2 domain and the M2–M3 loop are adjacent and continuous, suggesting that they form a single transduction domain (Bera et al., 2002; Miyazawa et al., 2003; Trudell and Bertaccini, 2004). Structurally, previous studies indicate that, although hydropathy analysis indicates that M2 ends as indicated in our study, sequence analysis and structural modeling indicate that the M2 α -helix extends further, to at least $\gamma P288$. These findings again suggest that our M2 and M2–M3 loop domains may be part of a single functional domain modulating benzodiazepine transduction. Our data suggest that there are two functional areas in the $\gamma 2L$ subunit that are responsible for enhancement of GABA_A receptor current in response to benzodiazepines: $\gamma 235$ – 237 in the N-terminal M1 domain (Fig. 7A, area A) and $\gamma 280$ – 294 (the C-terminal M2 domain and M2–M3 loop) (Fig. 7A, areas B–D).

Discussion

Agonist transduction is mediated by distinct structural domains

Positive allosteric regulation of LGICs consists of at least three components: ligand binding to the receptor (binding), a subsequent receptor conformational change (transduction or coupling), and enhancement of channel opening and ion flux (gating). It has previously been suggested that these processes are coupled together functionally and structurally (Boileau and Czajkowski, 1999; Thompson et al., 1999; Carlson et al., 2000; Kash et al., 2003; Miyazawa et al., 2003). In the nicotinic AChR (nAChR), as in all other members of the cys-loop superfamily of LGICs, the ligand-binding domain is located between two subunit interfaces (α/γ and α/δ for nAChRs) in the extracellular N termini, and gating occurs in the pore-lining M2 segment.

Crystallization of the AChBP (acetylcholine binding protein) and x-ray diffraction studies have advanced understanding of the structural basis of the coupling between binding and gating of the nAChR and, by extension, of other members of the LGIC superfamily. It is thought that opening of the nAChR works via a “pin-into-socket” mechanism, resulting in two transduction events. After agonist binding, the ligand-binding domain transduces a rotation via the N-terminal $\beta 1/\beta 2$ loop (pin) to the α subunit M2 helices (socket). The rotation of these α subunit inner sheets forces rotation of the extracellular end of M2, causing the gate to open (Miyazawa et al., 2003). Similarly, binding of GABA is thought to occur at the β/α subunit interfaces in the distal N terminus, whereas receptor gating involves movement of the pore-lining M2 segment. Kash et al. (2003) investigated two flexible loops in the GABA_A receptor (loops 2 and 7) that interact with the extracellular region of M2 and the M2–M3 loop. In their model, loop 7 of the $\beta 2$ subunit acts as the N-terminal pin that fits into the M2–M3 loop socket, allowing rotations caused by N-terminal agonist binding to be communicated to the pore. Electrostatic interactions among these regions may strengthen with activation, coupling binding and gating events for the receptor.

We show that there are two functional domains responsible for transduction of benzodiazepine binding to modulation of the GABA_A receptor. Each of these determinants, located in M1 (domain 1) and the M2 and M2–M3 loop regions (domain 2), has been previously implicated in the gating and modulation of LGICs; however, we implicate these domains as a functional unit regulating benzodiazepine modulation.

Benzodiazepine transduction mediated by domain 1

The pre-M1 area is important for transduction of benzodiazepine binding to the M2 gate. Given that it is physically connected to both the N terminus and the M2 gate region, M1 seemed a likely candidate for the transduction of binding to gating. Additional support comes from a GABA_A receptor model (Trudell and Bertaccini, 2004), indicating that the N-terminal region of M1 before the conserved P243 lines the receptor pore by intercalating between M2 channel-lining domains. The γ - δ M1q chimera, which extends the N-terminal $\gamma 2L$ subunit sequence through the extracellular end of M1 in a $\alpha 1\beta 3\delta$ receptor, exhibited robust diazepam potentiation of GABA-evoked currents, in contrast to the nonresponsive γ - δ M1e chimera, which contained only the $\gamma 2L$ subunit N-terminal binding domain. This result suggested that major structural determinants of benzodiazepine transduction lie in domain 1, the N-terminal region of M1, in which we identified three critical residues, $\gamma Y235$, $\gamma F236$, and $\gamma T237$.

Benzodiazepine transduction mediated by domain 2

Residues in M2 have also been shown to be involved in the modulation of the gating of the receptor by allosteric modulators. In particular, a residue located at the extracellular edge of M2, β 3H267, homologous to γ 2L282, is involved in zinc modulation (Nagaya and Macdonald, 2001; Hosie et al., 2003). Additionally, this residue and the residue preceding it, γ T281, have been implicated in coupling benzodiazepine binding to GABA_A receptor gating (Boileau and Czajkowski, 1999). Our results implicate these two residues as well as γ S280, also identified as part of the anesthetic binding pocket (Jenkins et al., 2001) in benzodiazepine transduction. Although these three residues alone were not required for benzodiazepine enhancement of GABA_A receptor current, when combined with the three M1 mutations (γ Y235V plus F236Y plus T237I), potentiation was significantly reduced. This suggested a mechanism of transduction involving the physically adjacent and intercalated extracellular M1 and M2 domains of the receptor, acting in concert to increase GABA_A receptor gating by benzodiazepines. Although either domain was sufficient to support diazepam potentiation, “full” enhancement required both domains.

Recently, the M2–M3 loop has been implicated in LGIC gating [nAChR (Bera et al., 2002); GABA_A receptor (O’Shea and Harrison, 2000; Kash et al., 2003, 2004; Trudell and Bertaccini, 2004); glycine receptor (Lynch et al., 2001)]. The N-terminal portion of the M2–M3 loop in GABA_A and glycine receptors has been shown to undergo a conformational change during gating [GABA (Bera et al., 2002); glycine (Lynch et al., 1997)], suggesting that it may interact with other receptor areas. In particular, it has been shown that coupling between N-terminal loops 2 and 7 and the M2–M3 loop results in efficient gating of LGICs. Furthermore, mutations of residues in the M2–M3 loop have been shown to alter agonist efficacy of LGICs (Campos-Caro et al., 1996; Lynch et al., 1997; O’Shea and Harrison, 2000; Davies et al., 2001; Kash et al., 2003) and have been implicated in human channelopathies (Gomez et al., 1997; Lewis and Schofield, 1999; Baulac et al., 2001).

Additionally, the M2–M3 loop has been implicated in the coupling of benzodiazepine binding to gating (Kucken et al., 2000), suggesting a similar transduction pathway for both agonists and allosteric modulators. In this study, replacement of the M2–M3 loop of the γ 2L subunit with δ subunit sequence resulted in a trend toward reduced benzodiazepine modulation, suggesting reduced coupling efficacy. Nevertheless, efficient benzodiazepine enhancement could be achieved via the intact M1 and M2 domains. The observation of reduced enhancement can be reconciled by considering that the M2 and M2–M3 loop regions act as one functional domain. This idea is supported by recent structural evidence that M2 is not confined to the membrane, as previously thought, but extends extracellularly into the M2–M3 loop region (Miyazawa et al., 2003; Trudell and Bertaccini, 2004) (Fig. 7C).

Coordinate regulation of benzodiazepine effects on GABA_A receptor gating by two domains

Williams and Akabas (2000) reported that diazepam induced a GABA_A receptor conformational change that was structurally different from closed, open, GABA-bound, or desensitized states. Furthermore, Bianchi and Macdonald (2001) reported that benzodiazepines enhanced spontaneous GABA_A receptor currents. These findings are difficult to reconcile with the classic mechanism of benzodiazepines increasing microscopic affinity of GABA binding, because GABA was not present. We reconcile

these findings by postulating that benzodiazepines facilitate gating using a mechanism similar to GABA. Using the gating models of Unwin et al. (2002) (AChR) and Kash et al. (2003) (GABA_A receptor), GABA binds at the β/α interfaces (supplemental figure, panel A, black star; available at www.jneurosci.org as supplemental material) and causes a conformational change in the N terminus of the β subunit (supplemental figure, panel A, arrow 1; available at www.jneurosci.org as supplemental material) that results in a rotational opening of the girdle of the pore (supplemental figure, panel A, arrow 2; available at www.jneurosci.org as supplemental material) via the M2–M3 loop, requiring a certain amount of energy. Structurally, these areas are positioned in a manner to promote communication between these domains, because N-terminal loop 7 is juxtaposed above the M2–M3 loop. Furthermore, both areas are reported to be mobile. On agonist binding, the N terminus undergoes a constriction during activation and the M2–M3 loop moves closer to loop 7 of the N terminus (Kash et al., 2003). We postulate that benzodiazepines bind at the α/γ interface (supplemental Fig. 2A, gray star; available at www.jneurosci.org as supplemental material) and use an analogous pin-into-socket pathway in the γ subunit, which, via its rotation, transduces from the N-terminal binding pocket (pin) through M2 and the proximal portion of the M2–M3 loop (socket), as well as a pathway from the N terminus through M1 (supplemental figure, panel A, gray arrow; available at www.jneurosci.org as supplemental material). Transduction via the γ 2 subunit could result in a decrease in the amount of energy required to activate the GABA_A receptor agonist binding sites by providing a certain amount of energy of its own that is not sufficient to gate the channel. In this model, benzodiazepine modulation of spontaneous current can be explained by envisioning spontaneous current as a low-probability baseline rotational movement of the receptor that can be enhanced by the additional energy provided by benzodiazepines. The effect at the pore of benzodiazepine binding would simply lower the energy barrier for GABA-mediated gating.

Our results provide evidence that there are two areas integral to the coupling of benzodiazepine binding to GABA_A receptor gating. The proximity of loops 2 and 7, the extracellular M1 domain, and the extracellular M2 domain/M2–M3 loop (Trudell and Bertaccini, 2004) (supplemental figure, panel B, available at www.jneurosci.org as supplemental material) suggests that these domains may form transduction elements that are physically positioned to communicate with the N-terminal binding pocket. The effect of benzodiazepine binding on channel gating via these domains may result in a decreased energy requirement for channel opening. Our findings, in addition to previous studies, suggest that allosteric regulation of the GABA_A receptor, although complicated, may proceed via common structural machinery that may be shared among other LGIC superfamily members and other allosteric modulators.

References

- Akabas MH, Karlin A (1995) Identification of acetylcholine receptor channel-lining residues in the M1 segment of the α -subunit. *Biochemistry* 34:12496–12500.
- Amin J, Brooks-Kayal A, Weiss DS (1997) Two tyrosine residues on the α subunit are crucial for benzodiazepine binding and allosteric modulation of γ -aminobutyric acid A receptors. *Mol Pharmacol* 51:833–841.
- Angelotti TP, Uhler MD, Macdonald RL (1993) Assembly of GABA_A receptor subunits: analysis of transient single-cell expression utilizing a fluorescent substrate/marker gene technique. *J Neurosci* 13:1418–1428.
- Baulac S, Huberfeld G, Gourfinkel-An I, Mitropoulou G, Beranger A, Prud’homme JF, Baulac M, Brice A, Bruzzone R, LeGuern E (2001) First

- genetic evidence of GABA_A receptor dysfunction in epilepsy: a mutation in the $\gamma 2$ -subunit gene. *Nat Genet* 28:46–48.
- Baumann SW, Baur R, Sigel E (2002) Forced subunit assembly in $\alpha 1\beta 2\gamma 2$ GABA_A receptors. Insight into the absolute arrangement. *J Biol Chem* 277:46020–46025.
- Bera AK, Chatav M, Akabas MH (2002) GABA_A receptor M2–M3 loop secondary structure and changes in accessibility during channel gating. *J Biol Chem* 277:43002–43010.
- Bianchi MT, Macdonald RL (2001) Agonist trapping by GABA_A receptor channels. *J Neurosci* 21:9083–9091.
- Boileau AJ, Czajkowski C (1999) Identification of transduction elements for benzodiazepine modulation of the GABA_A receptor: three residues are required for allosteric coupling. *J Neurosci* 19:10213–10220.
- Boileau AJ, Kucken AM, Evers AR, Czajkowski C (1998) Molecular dissection of benzodiazepine binding and allosteric coupling using chimeric γ -aminobutyric acid A receptor subunits. *Mol Pharmacol* 53:295–303.
- Campos-Caro A, Sala S, Ballesta JJ, Vicente-Agullo F, Criado M, Sala F (1996) A single residue in the M2–M3 loop is a major determinant of coupling between binding and gating in neuronal nicotinic receptors. *Proc Natl Acad Sci USA* 93:6118–6123.
- Carlson BX, Engblom AC, Kristiansen U, Schousboe A, Olsen RW (2000) A single glycine residue at the entrance to the first membrane-spanning domain of the γ -aminobutyric acid type A receptor $\beta 2$ subunit affects allosteric sensitivity to GABA and anesthetics. *Mol Pharmacol* 57:474–484.
- Chang Y, Wang R, Barot S, Weiss DS (1996) Stoichiometry of a recombinant GABA_A receptor. *J Neurosci* 16:5415–5424.
- Davies M, Newell JG, Dunn SM (2001) Mutagenesis of the GABA_A receptor $\alpha 1$ subunit reveals a domain that affects sensitivity to GABA and benzodiazepine-site ligands. *J Neurochem* 79:55–62.
- Gomez CM, Maselli R, Gundek JE, Chao M, Day JW, Tamamizu S, Lasalde JA, McNamee M, Wollmann RL (1997) Slow-channel transgenic mice: a model of postsynaptic organellar degeneration at the neuromuscular junction. *J Neurosci* 17:4170–4179.
- Greenfield Jr LJ, Sun F, Neelands TR, Burgard EC, Donnelly JL, Macdonald RL (1997) Expression of functional GABA_A receptors in transfected L929 cells isolated by immunomagnetic bead separation. *Neuropharmacology* 36:63–73.
- Hosie AM, Dunne EL, Harvey RJ, Smart TG (2003) Zinc-mediated inhibition of GABA_A receptors: discrete binding sites underlie subtype specificity. *Nat Neurosci* 6:362–369.
- Jenkins A, Greenblatt EP, Faulkner HJ, Bertaccini E, Light A, Lin A, Andreassen A, Viner A, Trudell JR, Harrison NL (2001) Evidence for a common binding cavity for three general anesthetics within the GABA_A receptor. *J Neurosci* 21:RC136(1–4).
- Kash TL, Jenkins A, Kelley JC, Trudell JR, Harrison NL (2003) Coupling of agonist binding to channel gating in the GABA_A receptor. *Nature* 421:272–275.
- Kash TL, Dixon MJ, Trudell JR, Harrison NL (2004) Charged residues in the $\beta 2$ subunit involved in GABA_A receptor activation. *J Biol Chem* 279:4887–4893.
- Korpi ER, Mihalek RM, Sinkkonen ST, Hauer B, Hevers W, Homanics GE, Sieghart W, Luddens H (2002) Altered receptor subtypes in the forebrain of GABA_A receptor δ subunit-deficient mice: recruitment of $\gamma 2$ subunits. *Neuroscience* 109:733–743.
- Kucken AM, Wagner DA, Ward PR, Teissere JA, Boileau AJ, Czajkowski C (2000) Identification of benzodiazepine binding site residues in the $\gamma 2$ subunit of the γ -aminobutyric acid A receptor. *Mol Pharmacol* 57:932–939.
- Lewis TM, Schofield PR (1999) Structure-function relationships of the human glycine receptor: insights from hyperekplexia mutations. *Ann NY Acad Sci* 868:681–684.
- Lynch JW, Rajendra S, Pierce KD, Handford CA, Barry PH, Schofield PR (1997) Identification of intracellular and extracellular domains mediating signal transduction in the inhibitory glycine receptor chloride channel. *EMBO J* 16:110–120.
- Lynch JW, Han NL, Haddrill J, Pierce KD, Schofield PR (2001) The surface accessibility of the glycine receptor M2–M3 loop is increased in the channel open state. *J Neurosci* 21:2589–2599.
- Macdonald RL, Olsen RW (1994) GABA_A receptor channels. *Annu Rev Neurosci* 17:569–602.
- Mihic SJ, Whiting PJ, Klein RL, Wafford KA, Harris RA (1994) A single amino acid of the human γ -aminobutyric acid type A receptor $\gamma 2$ subunit determines benzodiazepine efficacy. *J Biol Chem* 269:32768–32773.
- Miyazawa A, Fujiyoshi Y, Unwin N (2003) Structure and gating mechanism of the acetylcholine receptor pore. *Nature* 424:949–955.
- Nagaya N, Macdonald RL (2001) Two $\gamma 2$ L subunit domains confer low Zn²⁺ sensitivity to ternary GABA_A receptors. *J Physiol (Lond)* 532:17–30.
- O'Shea SM, Harrison NL (2000) Arg-274 and Leu-277 of the γ -aminobutyric acid type A receptor $\alpha 2$ subunit define agonist efficacy and potency. *J Biol Chem* 275:22764–22768.
- Quirk K, Gillard NP, Ragan CI, Whiting PJ, McKernan RM (1994) Model of subunit composition of γ -aminobutyric acid_A receptor subtypes expressed in rat cerebellum with respect to their α and γ/δ subunits. *J Biol Chem* 269:16020–16028.
- Quirk K, Whiting PJ, Ragan CI, McKernan RM (1995) Characterisation of delta-subunit containing GABA_A receptors from rat brain. *Eur J Pharmacol* 290:175–181.
- Rogers CJ, Twyman RE, Macdonald RL (1994) Benzodiazepine and β -carboline regulation of single GABA_A receptor channels of mouse spinal neurons in culture. *J Physiol (Lond)* 475:69–82.
- Saxena NC, Macdonald RL (1994) Assembly of GABA_A receptor subunits: role of the δ subunit. *J Neurosci* 14:7077–7086.
- Sigel E, Buhr A (1997) The benzodiazepine binding site of GABA_A receptors. *Trends Pharmacol Sci* 18:425–429.
- Smith GB, Olsen RW (1995) Functional domains of GABA_A receptors. *Trends Pharmacol Sci* 16:162–168.
- Thompson SA, Arden SA, Marshall G, Wingrove PB, Whiting PJ, Wafford KA (1999) Residues in transmembrane domains I and II determine γ -aminobutyric acid type A receptor subtype-selective antagonism by furosemide. *Mol Pharmacol* 55:993–999.
- Trudell JR, Bertaccini E (2004) Comparative modeling of a GABA_A $\alpha 1$ receptor using three crystal structures as templates. *J Mol Graph Model* 23:39–49.
- Unwin N, Miyazawa A, Li J, Fujiyoshi Y (2002) Activation of the nicotinic acetylcholine receptor involves a switch in conformation of the α subunits. *J Mol Biol* 319:1165–1176.
- Williams DB, Akabas MH (2000) Benzodiazepines induce a conformational change in the region of the γ -aminobutyric acid type A receptor $\alpha(1)$ subunit M3 membrane-spanning segment. *Mol Pharmacol* 58:1129–1136.