Evidence for Inhibition Mediated by Coassembly of GABA_A and GABA_C Receptor Subunits in Native Central Neurons

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Fast inhibition in the nervous system is commonly mediated by GABA_A receptors comprised of 2 α /2 β /1 γ subunits. In contrast, GABA_C receptors containing only ρ subunits (ρ 1– ρ 3) have been predominantly detected in the retina. However, here using reverse transcription-PCR and *in situ* hybridization we show that mRNA encoding the ρ 1 subunit is highly expressed in brainstem neurons. Immunohistochemistry localized the ρ 1 subunit to neurons at light and electron microscopic levels, where it was detected at synaptic junctions. Application of the GABA_C receptor agonist *cis*-4-aminocrotonic acid (100–800 μ M) requires the ρ 1 subunit to elicit responses, which surprisingly are blocked independently by antagonists to GABA_A (bicuculline, 10 μ M) and GABA_C [(1,2,5,6-tetrahydropyridin-4-yl)methylphosphinic acid (TPMPA); 40–160 μ M] receptors. Responses to GABA_C agonists were also enhanced by the GABA_A receptor modulator pentobarbitone (300 μ M). Spontaneous and evoked IPSPs were reduced in amplitude but never abolished by TPMPA, but were completely blocked by bicuculline. We therefore tested the hypothesis that GABA_A and GABA_C subunits formed a heteromeric receptor. Immunohistochemistry indicated that ρ 1 and α 1 subunits were colocalized at light and electron microscopic levels. Electrophysiology revealed that responses to GABA_C receptor agonists were enhanced by the GABA_A receptor modulator zolpidem (500 nM), which acts on the α 1 subunit when the γ 2 subunit is also present. Finally, coimmunoprecipitation indicated that the ρ 1 subunit formed complexes that also contained α 1 and γ 2 subunits. Taken together these separate lines of evidence suggest that the effects of GABA in central neurons can be mediated by heteromeric complexes of GABA_A and GABA_C receptor subunits.

Key words: autonomic; bicuculline; brainstem; GABA; IPSP; preganglionic

Introduction

Fast inhibition in the CNS is principally mediated by the neurotransmitter GABA acting on GABA_A and GABA_C ionotropic receptors (Macdonald and Olsen, 1994). GABA_A receptors mediating CNS inhibition most commonly comprise 2 α /2 β /1 γ subunits, but the subunit composition is highly variable (Whiting et al., 1999) and in many cases may still to be determined in native neurons (Fritschy and Brunig, 2003). Differences in subunit composition profoundly influence receptor characteristics, both functional and pharmacological (Rudolph et al., 2001), with significant physiological consequences (Sundstrom-Poromaa et al., 2002).

GABA_C receptors comprise exclusively ρ subunits, of which there are three (Chebib and Johnston, 1999; Bormann, 2000). GABA_C responses were first observed in the spinal cord (Johnston et al., 1975), and a “GABA_C” binding site was later

discovered in the cerebellum that did not bind GABA_A or GABA_B receptor compounds (Drew et al., 1984). Subsequently, GABA responses attributed to GABA_C receptors were obtained when retinal mRNA was expressed in *Xenopus* oocytes (Polenzani et al., 1991). The ρ 1 receptor subunit was cloned from a retinal library (Cutting et al., 1991), and recombinant ρ subunits exhibited GABA_C properties (Zhang et al., 1995; Qian et al., 1997). Because responses in retina to GABA were highly similar to activation of these recombinant receptors, they were attributed to GABA_C receptors (Feigenspan et al., 1993; Qian and Dowling, 1993; Zhang et al., 2001).

Whereas ρ subunits are expressed in the CNS, the characteristics of GABA receptors in regions containing ρ subunits often do not correspond to GABA_C receptors (Arakawa and Okada, 1988; Park et al., 1999; Delaney and Sah, 1999; Zhu and Lo, 1999). One explanation could be that ρ subunits assemble with subunits from other receptor subfamilies, but this hypothesis is contentious. Immunohistochemistry in the retina using an antibody recognizing all three ρ receptor subunits revealed staining that did not overlap with GABA_A α , β , or glycine receptor subunits (Koulen et al., 1998). In addition, coinjection of ρ 1 with GABA_A α 1, β 1, and γ 2s subunits in oocyte expression systems revealed no change in GABA_C responses (Shimada et al., 1992). In contrast, coexpression of ρ 1 with GABA_A γ 2 subunits has produced responses consistent with heteromeric formation (Qian and

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Ripps, 1999; Pan et al., 2000; Ekema et al., 2002; Pan and Qian, 2003). Some of this contention may be attributable to the fact that the properties and pharmacology of GABA receptor channels in expression systems may differ to those in native tissue because of lack of associated intracellular proteins (Everitt et al., 2004). Indeed, such differences have also been observed between native and expressed nicotinic receptors because some nicotinic receptors assemble appropriately only in cells of neuronal origin (Wanamaker et al., 2003).

In this study we provide evidence that $\rho 1$ subunits are expressed in native brainstem neurons *in situ*, where they may assemble with GABA_A receptor subunits. This suggests that the GABA_A and GABA_C receptor subtypes are not always distinct molecular and functional entities and provides a new substrate for inhibition in the CNS.

Materials and Methods

RNA isolation and reverse transcription-PCR. Total RNA was extracted from freshly dissected brainstem using TRI reagent (Sigma, Poole, UK) according to the manufacturer's instructions. Two micrograms of RNA were reverse-transcribed as described (Deuchars et al., 2001a). Oligonucleotide primers used to amplify the $\rho 1$ –3 genes were as follows: $\rho 1.71s$, 5'-GTTGGCTGTCCGGAATATGA-3'; $\rho 1.273a$, 5'-TTGGTGAGAGGCGACTTGGT-3' (numbering according to GenBank accession number U21070); $\rho 2.62s$, 5'-CAAGAAGCCACATTCTTCCA-3'; $\rho 2.175a$, 5'-TTCTGGAAGATATAGAGTCC-3' (GenBank accession number S79784); $\rho 3.1258s$, 5'-GAAGAGTGAAGCAGCTCAA-3'; and $\rho 3.1505a$, 5'-AGTAGGTATCAATGACGTGGT-3' (GenBank accession number D50671). Cycling conditions were 95°C for 5 min followed by 35 cycles of 95°C for 30 sec, 60°C for 30 sec, and 72°C for 1 min followed by a final extension step of 72°C for 10 min. Amplification products were subcloned into pGEM-T Easy (Promega, Southampton, UK) and verified by sequencing. The specificity of the amplified sequences to the ρ subunits was confirmed by a search of the BLAST database. Cerebellar tissue was used as a positive control because $\rho 1$ has been previously reported within this region (Boue-Grabot et al., 1998).

In situ hybridization. Single-stranded digoxigenin (DIG)-UTP-labeled (Roche Diagnostics, Lewes, UK) sense (control) and antisense riboprobes were transcribed from a 202 bp $\rho 1$ DNA template, a 113 bp $\rho 2$ DNA template, or from a 247 bp $\rho 3$ DNA template inserted in opposite orientations into pGEM-T Easy (Promega) using linearized templates and T₇ or SP₆ RNA polymerases (Ambion, Austin, TX). The BLAST database was used to verify the specificity of these probes to their targets, and these were further verified by sequencing. Probe concentrations were determined by dot-immunoblotting. *In situ* hybridization was performed on coronal brainstem sections as described (Deuchars et al., 2001a), with the alteration that 30 μ m free-floating sections were used here.

Western blotting and immunohistochemistry. The specificity of the rabbit antisera to GABA_C (Enz et al., 1996) and the $\rho 1$ subunit (Boue-Grabot et al., 1998) has been described previously. The $\rho 1$ (1:1000), goat anti- $\alpha 1$ antisera (1:2500; Santa Cruz Biotechnology, Santa Cruz, CA) and guinea pig anti- $\gamma 2$ (1:2500; a gift from Dr. Jean Marc Fritschy, Institute of Pharmacology, University of Zurich, Zurich, Switzerland) were verified to detect proteins of appropriate molecular weights in brainstem by Western blotting as described previously (Deuchars et al., 2001a). Western blots were also performed on non-transfected JTC-19 cells using the above antisera. After electrophoresis of JTC-19 cell membranes, the gel was fixed and stained successively with 0.1% Coomassie Brilliant Blue R250 (Sigma) in 10% acetic acid. The gel was initially stained with Coomassie Blue solution for 1 hr and destained gradually with several changes of destaining solution (25% methanol and 10% acetic acid) for ~60 min until bands showed the appropriate color.

For immunohistochemistry, rats were anesthetized with Sagatal (60 mg/kg, i.p.; Rhône Merieux, Harlow, Essex, UK), perfusion-fixed with 0.05–0.25% glutaraldehyde and/or 4% paraformaldehyde, and 50 μ m brainstem sections were prepared as described previously (Deuchars et al., 2001a). For immunofluorescence, primary antibodies (GABA_C-1:

100; $\rho 1$ –1:200, $\alpha 1$ –1:300) were detected with donkey anti-rabbit Cy3 (1:1000; Stratech Scientific, Luton, UK) and donkey anti-goat Cy2 (1:1000; Stratech Scientific) as appropriate. For immunoelectron microscopy, anti-GABA_C (1:100) and anti- $\rho 1$ (1:200) were localized with biotinylated donkey anti-rabbit (1:500; Stratech Scientific), detected with extravidin-peroxidase (1:1500; Sigma) and visualized with diaminobenzidine. In some cases anti- $\rho 1$ was localized with a 1 nm gold-conjugated secondary antibody raised in donkey (1:200; British Biocell International, Cardiff, UK) and visualized by silver intensification. For dual-labeling electron microscopic procedures, anti- $\rho 1$ was first visualized using the peroxidase procedure and anti- $\alpha 1$ subsequently with the pre-embedding gold procedure. Control sections were incubated with omission of one or both primary antisera. Tissue processing for electron microscopy is described elsewhere (Deuchars et al., 2001a).

Immunoprecipitation. Brainstem dissected from male Wistar rats was homogenized in radioimmunoprecipitation assay buffer containing 50 mM Tris HCl, pH 8.0, 150 mM NaCl, 5% sodium deoxycholate, 0.1% Nonidet P-40, and protease inhibitor with 10 strokes of a glass–glass homogenizer. Twenty microliters of this sample were used as an input lane to verify the presence of $\rho 1$, $\alpha 1$, or $\gamma 2$ subunits in the lysate. Pre-cleared supernatant was incubated in $\rho 1$ antibody (1:200), $\alpha 1$ antibody (1:200), or $\gamma 2$ antibody [1:250; guinea pig, a gift from Dr. Jean-Marc Fritschy, specificity published in Somogyi et al. (1996)] for 4 hr at 4°C. After incubation, 20 μ l of protein G-Sepharose beads (Sigma) was added, and samples were tumbled for 4 hr at 4°C. The resin was collected by centrifugation and washed four times in buffer. For control purposes, brainstem lysates were incubated in IgG from the appropriate species. The bound proteins were eluted by boiling the resin in 20 μ l of 2× SDS-PAGE loading buffer and analyzed by SDS-PAGE and Western blotting using enhanced chemiluminescence.

Electrophysiology. Rats aged 17–21 d were anesthetized with Sagatal (60 mg/kg, i.p.) and perfused with ice-cold sucrose artificial CSF (aCSF) containing (in mM): sucrose (217); NaHCO₃ (26); KCl (3); MgSO₄ (2); NaH₂PO₄ (2.5); CaCl₂ (1); and glucose (10). The brain was removed, the brainstem was isolated, and the dura was removed. Slices of 250 μ m were cut on a Vibroslice and superfused at a rate of 3–5 ml/min with aCSF at room temperature [composition in mM: NaCl (124); NaHCO₃ (26); KCl (3); MgSO₄ (2); NaH₂PO₄ (2.5); CaCl₂ (2); glucose (10)]. Low Ca²⁺–high Mg²⁺ solutions contained 0.3 mM CaCl₂ and 4 mM MgSO₄ and were applied in place of normal aCSF until responses to electrical stimulation were abolished. Patch electrodes (tip diameter 3 μ m, resistance 4–6 M Ω) were filled with (in mM) K-gluconate (110); EGTA (11); MgCl₂ (2); CaCl₂ (0.1); HEPES (10), and Na₂ATP. Neurobiotin (0.5%; Vector Laboratories) and Lucifer yellow (dipotassium salt, 2%; Sigma) were also included and diffused into the neuron during recording. Whole-cell patch clamp recordings were performed in current-clamp mode using an Axopatch 1D. Electrophysiological characterization of neurons was performed using methods as before (Deuchars et al., 2001b), and responses of neurons to drugs applied to the bathing medium were determined. Antagonists and modulators of GABA receptor activities were preincubated for 5–10 min before reapplications of the agonists. IPSPs were elicited in dorsal vagal nucleus (DVN) neurons by stimulation (2 × threshold) of the NTS and isolated by applications of the excitatory amino acid antagonists, 1,2,3,4-tetrahydro-6-nitro-2,3-dioxobenzo-[f]quinoxaline-7-sulfonamide disodium (NBQX) and D(-)-2-amino-5-phosphopentanoic acid (AP-5). Spontaneous IPSPs were recorded at holding potentials of –10–0 mV, and bath applications of (1,2,5,6-tetrahydropyridin-4-yl)methylphosphonic acid (TPMPA) and bicuculline determined which receptors were involved in spontaneous synaptic transmission.

Drugs used were GABA, muscimol, the GABA_C agonist *cis*-4-aminocrotonic acid (CACA; Sigma and Tocris Cookson, Bristol, UK), the GABA_C antagonist TPMPA, the GABA_A antagonist bicuculline methiodide, and the GABA_B antagonist (2S)-3-[[[(1S)-1-(3,4-dichlorophenyl)ethyl]amino-2-hydroxypropyl](phenylmethyl)phosphonic acid (CGP55845; dissolved in DMSO, used to counteract any effects of TPMPA on GABA_B receptors). The GABA_A modulators sodium pentobarbitone, N,N,6-trimethyl-2-(4-methylphenyl)imidazol[1,2-a]pyridine-3-acetamide (zolpidem, dissolved in ethanol) and 5-[aminosulfonyl]-4-

Figure 1. Expression and localization of the $\rho 1$ subunit in brainstem. $\rho 1$ (A), $\rho 2$ (Ai), $\rho 3$ (Aii) subunit PCR products in brainstem (lane 1) and the positive control cerebellum (lane 3). RNA (lane 2) and water controls (lane 4) are negative. Bands correspond to the calculated size for all PCR products, and their veracity was confirmed by sequencing. The positive control hprt gene indicates the integrity of cDNA (Aiii, brainstem lane 5, cerebellum lane 6, and water control lane 7). B, C, *In situ* hybridization revealed that $\rho 1$ subunit mRNA was highly expressed in neurons throughout the dorsal vagal nucleus (DVN) and nucleus tractus solitarius (NTS) using antisense (B) but not sense (C) digoxigenin riboprobes. D, E, Immunohistochemistry confirmed neuronal localization of the $\rho 1$ subunits because antibodies to GABA_C subunits (D, arrows indicate immunoreaction product on the membrane of a neuron) or specific to the $\rho 1$ subunit (E) labeled neurons throughout the DVN and NTS. F, At the electron microscopic level, peroxidase reaction product indicating immunoreactivity (closed arrow) using the antibody to $\rho 1$, 2, and 3 subunits (termed GABA_C), was observed on the postsynaptic side of synaptic junctions apposed to a presynaptic terminal (t). G–I, Immunoreactivity using the antibody specific to $\rho 1$ subunit detected using pre-embedding peroxidase procedures (closed arrows) was also observed postsynaptic to terminals (t) at synaptic junctions (open arrows).

chloro-2-[(2-furanylmethyl)amino]-benzoic acid (furosemide; Sigma, dissolved in DMSO) were also used. Tetrodotoxin (TTX), NBQX, and AP-5 were used to isolate postsynaptic effects. All drugs were obtained from Tocris Cookson and dissolved in water unless otherwise stated. When drugs were dissolved in DMSO, the final concentration of DMSO in the bath was 0.1%.

Microinjection studies were performed using a pneumatic picopump (PV800; World Precision Instruments, Hertfordshire, UK) and electrodes with a tip diameter of 10 μ m. Electrodes were filled with GABA and positioned visually over the cell soma close to the recording electrode so that a response was elicited with a pulse of 100 msec duration.

Responses to drug applications were measured as the peak amplitude of the depolarization. The IPSP amplitude was the peak change from the holding potential averaged over 10–20 consecutive sweeps for control and drug responses. The effects of TPMPA and bicuculline on spontaneous IPSPs were analyzed off-line using the Synaptosoft (Decatur, GA) package. Histograms of IPSP amplitude distributions were plotted for each neuron by measuring IPSP amplitude for 100–200 IPSPs in control, TPMPA, and bicuculline. Effects of antagonists and modulators were determined using the paired *t* test with significance at *p* < 0.05.

To determine whether CACA-responsive neurons contained the $\rho 1$ subunit, slices containing Lucifer yellow-filled neurons were fixed in 4% paraformaldehyde for 12 hr, resectioned at 50 μ m, and incubated to detect $\rho 1$ immunoreactivity using Cy3 immunofluorescence as described above. Lucifer yellow was visualized with an FITC filter set.

Results

Neuronal expression and synaptic localization of $\rho 1$ subunits

To test for expression of ρ subunits in the brainstem, we performed reverse transcription (RT)-PCR with primers specific to cDNA to $\rho 1$, $\rho 2$, and $\rho 3$ (Fig. 1A), which revealed that all three ρ subunits are expressed in the rat brainstem. However, *in situ* hybridization with riboprobes complementary to and selective for the mRNA encoding each subunit revealed mRNA for the $\rho 1$ subunit (Fig. 1B) but not $\rho 2$ and $\rho 3$, suggesting that the latter two subunits are expressed at much lower levels. The control sense probe (identical to the $\rho 1$ cellular mRNA) did not result in hybridization, indicating that staining was specific to the antisense probe (Fig. 1C). A BLAST database search indicated that the sequence detected with the antisense probe was specific to the $\rho 1$ subunit. In the DVN, 100% of neurons contained $\rho 1$ mRNA, but in the nucleus tractus solitarius (NTS) ~70% of neurons were labeled (Fig. 1B). mRNA expression is consistent with the presence of protein, because similar populations of cells were labeled using antiserum directed to an epitope common to all three ρ

subunits (Fig. 1D) and also with an anti-serum selective for the $\rho 1$ subunit (Fig. 1E).

To determine whether the $\rho 1$ subunit was present at synaptic junctions, we performed immunoelectron microscopy. Because antibodies to GABA receptors do not readily penetrate synaptic junctions during pre-embedding immunohistochemical procedures (Nusser et al., 1995), we tried a post-embedding approach to localize the $\rho 1$ subunit. However, the $\rho 1$ antigen did not survive the embedding procedures, resulting in absence of immunoreactivity. We therefore examined tissue prepared with pre-embedding techniques, which limited us to a more qualitative analysis. However, we reasoned that if we could detect immunoreactivity at synaptic junctions even with this reduced probability, it would provide an indication of potential for synaptic localization of the $\rho 1$ subunit. In nine blocks from the dorsal vagal complex, three each from three rats, 240 synapses were identified that had $\rho 1$ immunoreactivity adjacent to the postsynaptic membrane (Fig. 1G–I, see Fig. 7A–D). Similar ultrastructural examination of tissue reacted with the GABA_C antibody also revealed reaction product adjacent to the postsynaptic junction (Fig. 1F) ($n = 30$ terminals). Because the ultrastructural localization with both antibodies was identical and *in situ* hybridization could detect only the $\rho 1$ subunit, $\rho 1$ appears to be the predominant ρ subunit present in the brainstem. Although ρ subunits have been localized within cell groups in the CNS, these first ultrastructural observations of postsynaptic $\rho 1$ subunits indicate a possible involvement in central synaptic transmission.

Atypical GABA receptor properties

We investigated whether these $\rho 1$ -containing receptors are functional using whole-cell current clamp recordings from both DVN and NTS neurons in rat brainstem slices. Current clamp was the preferred mode of recording because the DVN cells in particular have large dendritic trees that are maintained in the slice, hindering effective space clamp. Bath application of the GABA_C-selective agonist CACA (Chebib and Johnston, 1999; Zhang et al., 2001) at concentrations of 100–800 μM depolarized all DVN neurons (10.5 ± 0.9 mV; mean $\pm$ SEM; $n = 41$) (Figs. 2A, 3A,B) and 56% of NTS neurons (8.6 ± 1.7 mV; $n = 9/16$) (see Fig. 5B). To reduce the possibility of CACA contamination, we used two sources and several batch numbers, with identical results. CACA acted directly on the postsynaptic membrane of the recorded neuron because responses were maintained in tetrodotoxin (1 μM ; $n = 3$) (Fig. 2A) and/or blockade of excitatory synaptic transmission with NBQX (20 μM) and AP-5 (50 μM ; data not shown). Furthermore, responses to CACA ($n = 5$) were maintained in low Ca^{2+} –high Mg^{2+} , which totally blocked synaptic responses to electrical stimulation (Fig. 2B). Responses to GABA (500 μM ; $n = 5$) (Fig. 2C, see Fig. 5C) and muscimol (0.5–2 μM ; $n = 5$) (Fig. 3C) were also depolarizing, consistent with the responses to CACA arising from activation of GABA receptors on the recorded neuron. Such depolarizations are commonly observed after intense activation of GABA receptors (Staley and Proctor, 1999), which may induce a bicarbonate efflux, especially with intracellular chloride levels as in our experiments (Kaila and

Figure 2. CACA elicits depolarizing responses by a direct action on postsynaptic receptors. *A*, Responses to CACA (400 μM) were maintained in tetrodotoxin (1 μM , which blocked action potentials, inset), indicating a direct action on the postsynaptic membrane. *B*, Responses to CACA (400 μM) were maintained in low Ca^{2+} –high Mg^{2+} (which blocked synaptic responses to electrical stimulation, inset), which also supports a direct action on the postsynaptic membrane. *C*, Responses to GABA were also depolarizing, suggesting that the receptors were being intensively activated. *D*, In support of this, microinjections of high concentrations of GABA over the cell soma of a DVN neuron held at -50 mV elicited an initial hyperpolarization which became depolarizing with time. In the same neuron held at -80 mV, microinjection of GABA elicited a sustained depolarization similar to that observed with bath applications.

Voipio, 1987). To test this we made microinjections of GABA ($n = 7$) near the cell soma at -50 mV, and these elicited a biphasic response comprised of a transient hyperpolarization followed by a depolarization ($n = 7$) (Fig. 2D). At more hyperpolarized potentials a sustained depolarization similar to that of bath application of agonists was observed (Fig. 2D). In the same DVN neurons that were depolarized by bath application of CACA, monosynaptic responses to electrical stimulation of the NTS were hyperpolarizing ($n = 7$) (Fig. 3B). Furthermore, spontaneous IPSPs recorded at more depolarized potentials were also hyperpolarizing ($n = 5$) (Fig. 4B), confirming that depolarizations are likely caused by prolonged activation of GABA receptors (Staley and Proctor, 1999).

CACA-mediated responses were significantly reduced in amplitude by TPMPA (40–160 μM), a GABA_C antagonist (Bormann, 2000; Zhang et al., 2001) (13.3 ± 2.5 to 3.5 ± 0.2 mV; $n = 14$) (Fig. 3A) and in keeping with the ρ subunits forming a GABA_C receptor. However, responses to CACA were also significantly reduced by the classical GABA_A receptor antagonist bicuculline (10 μM ; 12.6 ± 1.7 to 1.6 ± 0.6 mV; $n = 10$) (Fig. 3A). This was unexpected because, in immature hippocampal (Strata and Cherubini, 1994) and pelvic ganglion (Akasu et al., 1999) neurons, CACA at concentrations of up to 1 mM could elicit responses that were resistant to very high bicuculline concentrations (100 μM) and were mediated by GABA_C receptors. Nevertheless, we observed a similar profile of responses with low concentrations of GABA (5–20 μM ; $n = 4$) and muscimol (0.5–2 μM ; $n = 5$) (Fig. 3C), which preferentially activate GABA_C receptors (Schmidt et al., 2001; Zhang et al., 2001). Although the highest concentration of TPMPA used could abolish CACA responses (Fig. 3B1), monosynaptic IPSPs were only reduced in amplitude by TPMPA (-8.7 ± 1.9 to -4 ± 0.5 mV; $n = 7$) (Figs. 3B2, 4A). After recovery from TPMPA, bicuculline abolished the entire IPSP (-7.9 ± 1.8 to -0.3 ± 0.1 mV; $n = 7$) (Figs. 3B2, 4A). This indicated that TPMPA at the concentrations used did not affect all GABA receptors in a nonselective manner. More importantly, these data show that IPSPs elicited in DVN neurons by stimulation of the NTS were likely to be mediated not only by activation of GABA_A receptors but also by pharmacologically distinct GABA receptors that contain ρ subunits.

Figure 3. CACA responses in brainstem neurons display unusual pharmacology. *A*, *A1*, CACA-mediated responses in this DVN neuron were reduced by preincubation of the GABA_C antagonist TPMPA. After recovery from TPMPA, bicuculline also antagonized the CACA response. Furthermore, after partial recovery from bicuculline, application of picrotoxin also abolished the response to CACA. *A2*, Pooled data: CACA responses were decreased to a similar degree by TPMPA and bicuculline, antagonized by picrotoxin, and enhanced by sodium pentobarbital, but not affected by furosemide. *B*, In this DVN neuron, 160 μ M TPMPA abolished CACA-mediated responses (*B1*) but only partially decreased the amplitude of IPSPs elicited by NTS stimulation, whereas bicuculline (10 μ M) abolished the IPSP (*B2*). IPSPs were elicited by double pulse stimulation (arrows) of the NTS and responses shown are averages of 10 sweeps for each condition. *C*, Responses to low doses of muscimol (1 μ M) were also reduced by preincubation of TPMPA at 80 μ M.

Further evidence for an unusual receptor was evident because responses to CACA were significantly increased by pentobarbital (300 μ M; 7.6 ± 1.4 to 16.4 ± 2.5 mV; $n = 4$) (Fig. 3*A2*), which does not modulate GABA_C receptors but enhances effects of GABA on GABA_A receptors (Bormann, 2000; Zhang et al., 2001). In the cerebellum, CACA-elicited responses were mediated by activation of $\alpha 6$ -containing GABA receptors (Wall, 2001), and $\alpha 6$ subunit transcripts can be detected using RT-PCR in the brainstem (T. F. C. Batten, personal communication). However, furosemide (100 μ M), a selective modulator of $\alpha 6$ -

Figure 4. Evoked and spontaneous IPSPs are reduced in amplitude but never blocked by TPMPA. *A*, IPSPs elicited by stimulation of the NTS region of the brainstem were reduced in amplitude by TPMPA at 40 μ M. Full recovery was observed on washout. This IPSP was then abolished by bicuculline. *B*, Spontaneous IPSPs recorded at 0 mV were reduced in amplitude by TPMPA, indicated by the raw data and the shift to the left of the histogram. After washout of TPMPA, these IPSPs were abolished by bicuculline.

containing heteromers (Korpi and Luddens, 1997), had no effect on CACA-mediated responses (Fig. 3*A2*) ($n = 5$). Although the pharmacology of putative receptors that may contain $\alpha 6$ and ρ subunits is not known, these data suggest that the $\rho 1$ -containing receptor is unlikely to contain $\alpha 6$ subunits. Responses to CACA were also abolished by picrotoxin (100 μ M; $n = 3$) (Fig. 3*A*), showing that they were mediated by activation of GABA-gated chloride channels.

Because electrical stimulation may result in neurotransmitter release sufficient to activate extrasynaptic receptors, we tested whether $\rho 1$ -containing receptors become activated during spontaneous transmitter release. Spontaneous IPSPs were recorded in DVN neurons at depolarized potentials (-10 – 0 mV) to enhance their amplitude by increasing the chloride driving force. These spontaneous IPSPs were significantly reduced in amplitude by TPMPA (40–80 μ M; 5.7 ± 0.5 to 3.7 ± 0.5 mV; $n = 5$) (Fig. 4*B*). This indicates a likely participation of $\rho 1$ -containing receptors in synaptic transmission. Once more, application of bicuculline then abolished the spontaneous IPSPs ($n = 5$) (Fig. 4), indicating that spontaneous and evoked IPSPs were mediated by GABA receptors with a similar profile. The TPMPA-sensitive component of IPSPs, elicited by ρ -containing receptors, could not be studied in isolation because bicuculline completely abolished responses. Therefore we were clearly unable to determine whether this TPMPA-sensitive component was influenced by other GABA_A receptor modulators because any change in IPSP ampli-

Figure 5. CACA responses require the presence of the $\rho 1$ subunit. *A*, CACA depolarizes this DVN neuron (A1). The recorded neuron was filled with Lucifer yellow (A2) and also shown to be $\rho 1$ immunopositive (A3). *B*, *C*, These NTS neurons were recorded from opposite sides of the same slice so were processed identically for $\rho 1$ immunohistochemistry. The NTS neuron responding to CACA (B1) was Lucifer yellow filled (B2) and $\rho 1$ immunopositive (B3). The second NTS neuron did not depolarize to a higher concentration of CACA, but did respond to GABA (C1). This Lucifer yellow-filled neuron (C2) was $\rho 1$ immunonegative (C3).

tude could have been caused by an effect on either or both of the components of the IPSPs.

Responses to GABA_C agonists require $\rho 1$ subunits

Although responses to CACA at concentrations of up to 1 mM have been shown to be resistant to bicuculline and therefore not mediated by GABA_A receptors (Strata and Cherubini, 1994; Akasu et al., 1999), we further tested whether CACA only has an effect when ρ subunits are present. During electrophysiological recordings, neurons were filled with Lucifer yellow contained within the patch pipette and subsequently processed for $\rho 1$ immunofluorescence. In slices that were successfully stained for the $\rho 1$ subunit, 100% of DVN neurons responding to CACA were $\rho 1$ -immunopositive ($n = 16$) (Fig. 5*A*). In contrast, only NTS neurons that responded robustly to CACA were $\rho 1$ -immunopositive ($n = 3$) (Fig. 5*B*). NTS neurons that were not affected by CACA but responded to GABA did not contain $\rho 1$ ($n = 4$) (Fig. 5*C*). These data, together with the selective blockade of CACA responses with TPMPA, show that the $\rho 1$ subunit is necessary for the action of CACA in brainstem neurons, and therefore it is unlikely that CACA is activating GABA_A receptors.

GABA receptors contain $\rho 1$ and $\alpha 1$ subunits

Because CACA-mediated responses were dependent on the presence of the $\rho 1$ subunit yet were affected by both GABA_A and GABA_C receptor modulators, we hypothesized that the $\rho 1$ subunit combines with GABA_A receptor subunits. We focused on DVN neurons because our immunohistochemistry and *in situ* hybridization indicated that they all contain $\rho 1$ subunits and they all responded to CACA. In electrophysiological experiments, CACA-mediated responses were significantly enhanced by zolpidem (500 nM; 7.8 ± 1.2 to 14.7 ± 1.7 mV; $n = 5$) (Fig. 6*A,B*), a GABA receptor modulator that binds *in vitro* with high affinity to GABA_A receptors expressing $\alpha 1$ subunits but with relatively low affinity to receptors expressing $\alpha 2$, $\alpha 3$, and $\alpha 5$ subunits (Pritchett and Seeburg, 1991; Kralic et al., 2002). These data also suggest the presence of $\gamma 2$ subunits in the receptor because GABA receptors with mutant $\gamma 2$ subunits are not responsive to zolpidem (Cope et al., 2003).

We therefore tested for the possibility of coexpression of $\rho 1$ with $\alpha 1$ or $\gamma 2$ subunits in DVN neurons. Immunofluorescence was conducted using antibodies verified with Western blotting as

Figure 6. Enhancement of CACA responses by zolpidem correlates with immunoreactivity for $\alpha 1$ and $\gamma 2$ subunits in $\rho 1$ -positive DVN neurons. *A*, The CACA-mediated response in a DVN neuron (top) is enhanced by zolpidem (bottom). *B*, Pooled data showing the degree of enhancement by zolpidem in five neurons tested. *C*, *D*, Micrographs of the same section of brainstem that was processed for both $\rho 1$ and $\alpha 1$ immunohistochemistry. All DVN neurons are immunoreactive for both $\rho 1$ (*C*) and $\alpha 1$ (*D*) subunits. *E*, *F*, $\rho 1$ -positive neurons in the DVN (*E*, arrows) also contain $\gamma 2$ immunoreactivity (*F*, arrows).

Figure 7. Ultrastructural colocalization of $\rho 1$ subunits with $\alpha 1$ subunits. *A–D*, Examples of synapses where immunoperoxidase for $\rho 1$ (closed head arrows) and $\alpha 1$ (silver, open head arrows) were colocalized adjacent to the postsynaptic membrane. Asterisks in *B* and *D* indicate presynaptic terminals at synapses where the postsynaptic membrane is not labeled.

detecting proteins of the appropriate molecular weights in the brainstem (see Fig. 8*A–C*) ($n = 3$), but not in cultured JTC-19 cells (see Fig. 8*D*). The $\rho 1$ subunit was detected in DVN neurons that were also immunoreactive for $\alpha 1$ (Fig. 6*C,D*) or $\gamma 2$ (Fig. 6*E,F*). To determine whether the $\rho 1$ and $\alpha 1$ subunits could be colocalized ultrastructurally, we performed dual labeling pre-embedding immunohistochemistry. In tissue from the dorsal vagal complex of three rats, immunoreactivity for $\rho 1$ and $\alpha 1$ was colocalized postsynaptically (Fig. 7*A–D*). Examination of 60 synaptic junctions that contained labeling for either subunit postsynaptically revealed that 12% were immunoreactive solely for $\rho 1$ and 30% only for $\alpha 1$. The remaining 58% were immunoreactive for both $\rho 1$ and $\alpha 1$ which were located at an identical site on the postsynaptic membrane. Although such calculations indicate that colocalization of $\rho 1$ and $\alpha 1$ subunits is not a chance occurrence, the absolute values may be affected by factors such as unequal access of primary or secondary antibodies to different antigens or restriction by conjugation to gold particles. These data therefore indicate that $\rho 1$ GABA receptor subunits can be coexpressed in individual neurons with $\alpha 1$ and $\gamma 2$ subunits. Furthermore, we have shown that $\rho 1$ and $\alpha 1$ subunits may be present at an identical site at synapses.

Our electrophysiological and anatomical data are consistent with the formation of heteromers of $\rho 1$ with $\alpha 1$ subunits, with involvement of $\gamma 2$ subunits. To test for possible association of $\rho 1$ with $\alpha 1$ or $\gamma 2$ subunits in native tissue, we performed coimmunoprecipitation studies from brainstem. We first verified that $\rho 1$, $\alpha 1$, and $\gamma 2$ were present in nonprecipitated supernatant (Fig. 8*E–G*, input lanes). Protein complexes precipitated from brainstem cell lysates using the $\rho 1$ antibody contained $\alpha 1$ (Fig. 8*E*) ($n = 5$), and $\gamma 2$ (Fig. 8*H*) ($n = 3$), whereas $\rho 1$ was detected in complexes precipitated with $\alpha 1$ (Fig. 8*F*) ($n = 3$) or $\gamma 2$ (Fig. 8*G*) ($n = 3$) antibodies. Such association of $\rho 1$ with $\gamma 2$ subunits is consistent with recent observations (Ekema et al., 2002; Pan and Qian, 2003). Thus, when considered in conjunction with anatomy and pharmacological findings, these data suggest that in native brainstem neurons $\rho 1$ subunits may form heteromers that also contain at least one $\alpha 1$ and a $\gamma 2$ subunit.

Figure 8. Western blotting analysis of brainstem with $\rho 1$, $\alpha 1$, and $\gamma 2$ antibodies and coimmunoprecipitation of $\rho 1$ with $\alpha 1$ and $\gamma 2$ subunits. *A–C*, Western blots indicate that antisera to $\rho 1$ (*A*), $\alpha 1$ (*B*), and $\gamma 2$ (*C*) specifically recognize appropriate 55, 52, and 43 kDa proteins, respectively, in brainstem. No bands were detected when the $\rho 1$ antibody was replaced with rabbit IgG, when the $\alpha 1$ antibody was replaced with goat IgG, or when $\gamma 2$ antibody was replaced with guinea pig IgG (each is representative of five blots). *D*, Western blot analysis of JTC-19 cells. Membranes were subjected to SDS-PAGE and Western blotting with $\rho 1$, $\alpha 1$, and $\gamma 2$ antiserum. Molecular weight marker (lane M) and membranes from JTC-19 cells (lane P) stained with Coomassie Brilliant Blue indicates the presence of protein. *E*, Protein complexes precipitated from brainstem lysates using the $\rho 1$ antibody and probed with the $\alpha 1$ antibody resulted in a band at the molecular weight appropriate to the $\alpha 1$ subunit. No bands were detected when the $\rho 1$ antibody was replaced with rabbit IgG. *F*, $\rho 1$ subunits were detected in protein complexes precipitated using the $\alpha 1$ antibody, but not when brainstem lysates were precipitated with goat IgG. *G*, $\rho 1$ subunits were detected in protein complexes precipitated using the $\gamma 2$ antibody, but not when brainstem lysates were precipitated with guinea pig IgG. *H*, $\gamma 2$ subunits were detected in protein complexes precipitated using the $\rho 1$ antibody, but not when brainstem lysates were precipitated with rabbit IgG. Nonprecipitated proteins are in the supernatant lanes marked “input” (*E–G*). Each coimmunoprecipitation is representative of three results.

Discussion

Inotropic GABA receptors are critical mediators of synaptic inhibition in the brain. Each pentameric GABA-gated ion channel may be comprised of different subunits, and the exact composition defines the properties of the receptor (McKernan and Whiting, 1996; Rudolph et al., 2001). Whereas subunits from the GABA_A and GABA_C receptor subfamilies are widely regarded as forming separate receptors, the data from this current study strongly support the formation of heteromeric GABA receptors that contain subunits from the GABA_A and GABA_C receptor subfamilies.

First, we show by RT-PCR that all three ρ subunits are expressed in the brainstem. However, the only ρ subunit that we could detect with *in situ* hybridization was the $\rho 1$ subunit. Because RT-PCR can detect lower mRNA levels than *in situ* hybridization, this implies that $\rho 1$ is expressed at higher levels than $\rho 2$ and $\rho 3$ in the brainstem. This suggestion is also supported by immunohistochemical labeling because labeling using an anti-serum to an epitope common to all three ρ subunits was similar to that with a $\rho 1$ -specific antibody. This suggests that the receptors labeled with the pan ρ antisera were likely to contain $\rho 1$ subunits. Because $\rho 1$ was the only subunit detectable with *in situ* hybridization, it seems likely that $\rho 1$ is the predominant ρ subunit in the brainstem.

Although presynaptic GABA receptors with pharmacology consistent with homomeric GABA_C receptors have been described in the retina (Matthews et al., 1994; Shields et al., 2000) and superior colliculus (Boller and Schmidt, 2001; Kirischuk et al., 2003), our evidence favors direct activation of ρ -containing receptors in the postsynaptic membrane for several reasons: (1) the presence of the $\rho 1$ subunit is necessary to elicit responses in DVN and NTS neurons with CACA, consistent with the view that this agonist acts preferentially on ρ subunit containing GABA receptors (Chebib and Johnston, 1999; Zhang et al., 2001), because only neurons that were $\rho 1$ -immunopositive responded to this compound, (2) the actions of CACA persist in the presence of TTX and blockade of excitatory amino acid receptors, as well as when synaptic transmission is blocked in a low Ca^{2+} –high Mg^{2+} solution, (3) ρ subunits were also localized postsynaptically using two different antibodies at the ultrastructural level. However, these responses were highly unusual because they were blocked by low concentrations of bicuculline (10 μM) as well as the GABA_C receptor antagonist TPMPA. This contrasts strikingly with previous studies in which responses to CACA at concentrations of up to 1 mM were resistant to very high concentrations of bicuculline (up to 100 μM) and were therefore regarded as mediated by GABA_C (comprising only ρ subunits) and not GABA_A receptors (Strata and Cherubini, 1994; Akasu et al., 1999). Because the CACA responses we observed were reliant on the $\rho 1$ subunit, but were not caused by activation of a receptor comprising only ρ subunits, as indicated by bicuculline sensitivity, we tested the hypothesis that the $\rho 1$ subunit was present in a receptor that also contained GABA_A subunits.

Several lines of evidence supported this hypothesis. First, $\rho 1$ and $\alpha 1$ subunits were colocalized at light and electron microscopic levels. Second, although CACA responses require the $\rho 1$ subunit, they were also enhanced by zolpidem and sodium pentobarbitone, which do not affect receptors comprising only ρ subunits (Bormann, 2000; Zhang et al., 2001), but which do modulate receptors with GABA_A subunits (Pritchett and Seeburg, 1991; Kralic et al., 2002). Third, $\rho 1$ is associated with $\alpha 1$ and $\gamma 2$ subunits, as revealed by coimmunoprecipitation. The association of $\rho 1$ and $\gamma 2$ subunits has recently been confirmed in yeast two hybrid studies (Ekema et al., 2002), coimmunoprecipitation from rat brain tissue (Ekema et al., 2002), and from expression systems (Ekema et al., 2002; Pan and Qian, 2003), as well as by observations that coexpression of $\gamma 2$ with $\rho 1$ subunits results in trafficking of $\gamma 2$ to the cell surface, whereas $\gamma 2$ subunits expressed alone remain intracellularly (Pan and Qian, 2003). Taking these results together supports the hypothesis that the $\rho 1$ subunit in these brainstem neurons is part of a GABA receptor that also contains subunits traditionally from the GABA_A subfamily.

Possible stoichiometry of these GABA receptors

Based on the stoichiometry of other GABA receptors and our current data, the GABA receptor reported here is likely to contain at least one $\rho 1$, one $\alpha 1$, and one $\gamma 2$ subunit. Evidence that $\alpha 1$ is involved is the enhancement of GABA_C agonist responses by zolpidem, a benzodiazepine that acts with high affinity on GABA receptors containing $\alpha 1$ subunits (Pritchett and Seeburg, 1991). Furthermore, $\rho 1$ and $\alpha 1$ are present in the receptor complex because they coimmunoprecipitate and are colocalized within neurons. $\gamma 2$ subunits are also likely to be present because they also precipitate with $\rho 1$ subunits (Ekema et al., 2002) and are necessary for the action of zolpidem on $\alpha 1$ -containing receptors (Pritchett and Seeburg, 1991). Thus, in native neurons, $\rho 1$ subunits can form heteromers that also contain at least one $\alpha 1$ and a $\gamma 2$ subunit. The ρ subunits have the potential to function like β subunits because they share highest sequence homology with β subunits (Whiting et al., 1999), which in some cases can form homomeric channels similarly to ρ subunits (Moss and Smart, 2001). The remaining subunits are likely to be an $\alpha 1$ and either another $\rho 1$ or a β subunit if the subunit composition is similar to the majority of GABA_A receptors. Such heteromeric GABA receptors may account for some of the GABA receptors throughout the CNS that exhibit atypical characteristics (Arakawa and Okada, 1988; Delaney and Sah, 1999; Park et al., 1999; Zhu and Lo, 1999; Semyanov and Kullmann, 2002; Dean et al., 2003) and so provide a new substrate for inhibitory neurotransmission.

Localization of $\rho 1$ subunits

GABA_C receptors comprising only ρ subunits have a higher sensitivity to GABA than GABA_A receptors (Chebib and Johnston, 1999; Bormann, 2000; Zhang et al., 2001). Interestingly, GABA_A subunits with relatively higher GABA sensitivities have been located extrasynaptically where they mediate tonic inhibition, such as the δ subunit in the cerebellum (Wisden and Farrant, 2002). Because bath applications of compounds may activate both synaptic and extrasynaptic receptors on individual neurons, we considered whether the ρ subunits were located at synapses. Three lines of evidence support this possibility. First, electron microscopy revealed $\rho 1$ immunoreactivity at synaptic junctions. Second, spontaneous IPSPs were partially antagonized by TPMPA, suggesting that they were mediated in part by $\rho 1$ -containing receptors because the presence of the $\rho 1$ subunit is essential for TPMPA-mediated reductions of GABA responses in mouse spinal cord (Zheng et al., 2003). Third, $\rho 1$ and $\gamma 2$ subunits coimmunoprecipitate (Fig. 7) (Ekema et al., 2002; Pan and Qian, 2003) and $\gamma 2$ subunits are targeted to synaptic receptors (Somogyi et al., 1996), where they may be necessary for synaptic clustering of GABA receptors (Schweizer et al., 2003). Together these data suggest that the $\rho 1$ -containing receptors may be located at the synaptic junction where they are involved in mediating inhibitory synaptic transmission.

The evidence we show here therefore favors the formation of GABA receptors that contain subunits from both GABA_A and GABA_C subfamilies, and these receptors display some unique properties. Because modulation of GABA-mediated neuronal inhibition is one of the most potent therapeutic approaches for the treatment of CNS diseases (Keverne, 1999), precise targeting of such treatments depends on identification and characterization of the different subunit complexes that exist. These heteromers of GABA_A and GABA_C subunits may therefore provide an important target for future therapeutic investigations.

References

- Akasu T, Munakata Y, Tsurusaki M, Hasuo H (1999) Role of GABA_A and GABA_C receptors in the biphasic GABA responses in neurons of the rat major pelvic ganglia. *J Neurophysiol* 82:1489–1496.
- Arakawa T, Okada Y (1988) Excitatory and inhibitory action of GABA on synaptic transmission in slices of guinea pig superior colliculus. *Eur J Pharmacol* 158:217–224.
- Boller M, Schmidt M (2001) Postnatal maturation of GABA_A and GABA_C receptor function in the mammalian superior colliculus. *Eur J Neurosci* 14:1185–1193.
- Bormann J (2000) The “ABC” of GABA receptors. *Trends Pharmacol Sci* 21:16–19.
- Boue-Grabot E, Roudbaraki M, Bascles L, Tramu G, Bloch B, Garret M (1998) Expression of GABA receptor rho subunits in rat brain. *J Neurochem* 70:899–907.
- Chebib M, Johnston GA (1999) The “ABC” of GABA receptors: a brief review. *Clin Exp Pharmacol Physiol* 26:937–940.
- Cope DW, Wulff P, Oberto A, Jones A, Hoeger JH, Capogna M, Ferraguti F, Sieghart W, Korpi ER, Wisden W, Somogyi P (2003) Abolition of zolpidem potentiation of GABA_A receptor mediated mIPSCs in neurons of gamma2 point mutant mice. *Soc Neurosci Abstr* 29:47.13.
- Cutting GR, Lu L, O'Hara BF, Kasch LM, Montrose-Rafizadeh C, Donovan DM, Shimada S, Antonarakis SE, Guggino WB, Uhl GR (1991) Cloning of the gamma-aminobutyric acid (GABA) rho 1 cDNA: a GABA receptor subunit highly expressed in the retina. *Proc Natl Acad Sci USA* 88:2673–2677.
- Dean I, Robertson SJ, Edwards FA (2003) Serotonin drives a novel GABAergic synaptic current recorded in rat cerebellar Purkinje cells: a Lugaro cell to Purkinje cell synapse. *J Neurosci* 23:4457–4469.
- Delaney AJ, Sah P (1999) GABA receptors inhibited by benzodiazepines mediate fast inhibitory transmission in the central amygdala. *J Neurosci* 19:9698–9704.
- Deuchars SA, Atkinson L, Brooke RE, Musa H, Milligan CJ, Batten TF, Buckley NJ, Parson SH, Deuchars J (2001a) Neuronal P2X7 receptors are targeted to presynaptic terminals in the central and peripheral nervous systems. *J Neurosci* 21:7143–7152.
- Deuchars SA, Brooke RE, Frater B, Deuchars J (2001b) Properties of interneurons in the intermediolateral cell column of the rat spinal cord: role of the potassium channel subunit Kv3.1. *Neuroscience* 106:433–446.
- Drew CA, Johnston GA, Weatherby RP (1984) Bicuculline-insensitive GABA receptors: studies on the binding of (–)-baclofen to rat cerebellar membranes. *Neurosci Lett* 52:317–321.
- Ekema GM, Zheng W, Lu L (2002) Interaction of GABA receptor/channel rho(1) and gamma(2) subunit. *Invest Ophthalmol Vis Sci* 43:2326–2333.
- Enz R, Brandstatter JH, Wasse H, Bormann J (1996) Immunocytochemical localization of the GABA_C receptor rho subunits in the mammalian retina. *J Neurosci* 16:4479–4490.
- Everitt AB, Luu T, Cromer B, Tierney ML, Birnir B, Olsen RW, Gage PW (2004) Conductance of recombinant GABA channels is increased in cells coexpressing GABA_A A receptor-associated protein. *J Biol Chem* 279:21701–21706.
- Feigenspan A, Wasse H, Bormann J (1993) Pharmacology of GABA receptor Cl[–] channels in rat retinal bipolar cells. *Nature* 361:159–162.
- Fritschy JM, Brunig I (2003) Formation and plasticity of GABAergic synapses: physiological mechanisms and pathophysiological implications. *Pharmacol Ther* 98:299–323.
- Johnston GA, Curtis DR, Beart PM, Game CJ, McCulloch RM, Twitchin B (1975) Cis- and trans-4-aminocrotonic acid as GABA analogues of restricted conformation. *J Neurochem* 24:157–160.
- Kaila K, Voipio J (1987) Postsynaptic fall in intracellular pH induced by GABA-activated bicarbonate conductance. *Nature* 330:163–165.
- Keverne EB (1999) GABA-ergic neurons and the neurobiology of schizophrenia and other psychoses. *Brain Res Bull* 48:467–473.
- Kirischuk S, Akyeli J, Iosub R, Grantyn R (2003) Pre- and postsynaptic contribution of GABA_C receptors to GABAergic synaptic transmission in rat collicular slices and cultures. *Eur J Neurosci* 18:752–758.
- Korpi ER, Luddens H (1997) Furosemide interactions with brain GABA_A receptors. *Br J Pharmacol* 120:741–748.
- Koulen P, Brandstatter JH, Enz R, Bormann J, Wasse H (1998) Synaptic clustering of GABA_C receptor rho-subunits in the rat retina. *Eur J Neurosci* 10:115–127.
- Kralic JE, O'Buckley TK, Khisti RT, Hodge CW, Homanics GE, Morrow AL (2002) GABA(A) receptor alpha-1 subunit deletion alters receptor sub-type assembly, pharmacological and behavioral responses to benzodiazepines and zolpidem. *Neuropharmacology* 43:685–694.
- Macdonald RL, Olsen RW (1994) GABA_A receptor channels. *Annu Rev Neurosci* 17:569–602.
- Matthews G, Ayoub GS, Heidelberger R (1994) Presynaptic inhibition by GABA is mediated via two distinct GABA receptors with novel pharmacology. *J Neurosci* 14:1079–1090.
- McKernan RM, Whiting PJ (1996) Which GABA_A-receptor subtypes really occur in the brain? *Trends Neurosci* 19:139–143.
- Moss SJ, Smart TG (2001) Constructing inhibitory synapses. *Nat Rev Neurosci* 2:240–250.
- Nusser Z, Roberts JD, Baude A, Richards JG, Sieghart W, Somogyi P (1995) Immunocytochemical localization of the alpha 1 and beta 2/3 subunits of the GABA_A receptor in relation to specific GABAergic synapses in the dentate gyrus. *Eur J Neurosci* 7:630–646.
- Pan YL, Qian H (2003) Biochemical determination of interactions among GABA rho and gamma2 subunits. *Soc Neurosci Abstr* 29:570.3.
- Pan ZH, Zhang D, Zhang X, Lipton SA (2000) Evidence for coassembly of mutant GABA_C rho1 with GABA_A gamma2S, glycine alpha1 and glycine alpha2 receptor subunits in vitro. *Eur J Neurosci* 12:3137–3145.
- Park JS, Higashi H, Nagata K, Yoshimura M (1999) Bicuculline-resistant, Cl[–]-dependent GABA response in the rat spinal dorsal horn. *Neurosci Res* 33:261–268.
- Polenzani L, Woodward RM, Miledi R (1991) Expression of mammalian gamma-aminobutyric acid receptors with distinct pharmacology in *Xenopus* oocytes. *Proc Natl Acad Sci USA* 88:4318–4322.
- Pritchett DB, Seeburg PH (1991) gamma-Aminobutyric acid type A receptor point mutation increases the affinity of compounds for the benzodiazepine site. *Proc Natl Acad Sci USA* 88:1421–1425.
- Qian H, Dowling JE (1993) Novel GABA responses from rod-driven retinal horizontal cells. *Nature* 361:162–164.
- Qian H, Ripps H (1999) Response kinetics and pharmacological properties of heteromeric receptors formed by coassembly of GABA rho- and gamma 2-subunits. *Proc R Soc Lond B Biol Sci* 266:2419–2425.
- Qian H, Hyatt G, Schanzer A, Hazra R, Hackam AS, Cutting GR, Dowling JE (1997) A comparison of GABA_C and rho subunit receptors from the white perch retina. *Vis Neurosci* 14:843–851.
- Rudolph U, Crestani F, Mohler H (2001) GABA(A) receptor subtypes: dissecting their pharmacological functions. *Trends Pharmacol Sci* 22:188–194.
- Schmidt M, Boller M, Ozen G, Hall WC (2001) Disinhibition in rat superior colliculus mediated by GABA_C receptors. *J Neurosci* 21:691–699.
- Schweizer C, Balsiger S, Bluethmann H, Mansuy IM, Fritschy JM, Mohler H, Luscher B (2003) The gamma2 subunit of GABA(A) receptors is required for maintenance of receptors at mature synapses. *Mol Cell Neurosci* 24:442–450.
- Semyanov A, Kullmann DM (2002) Relative picrotoxin insensitivity distinguishes ionotropic GABA receptor-mediated IPSCs in hippocampal interneurons. *Neuropharmacology* 43:726–736.
- Shields CR, Tran MN, Wong RO, Lukasiewicz PD (2000) Distinct ionotropic GABA receptors mediate presynaptic and postsynaptic inhibition in retinal bipolar cells. *J Neurosci* 20:2673–2682.
- Shimada S, Cutting G, Uhl GR (1992) gamma-Aminobutyric acid A or C receptor? gamma-Aminobutyric acid rho 1 receptor RNA induces bicuculline-, barbiturate-, and benzodiazepine-insensitive gamma-aminobutyric acid responses in *Xenopus* oocytes. *Mol Pharmacol* 41:683–687.
- Somogyi P, Fritschy JM, Benke D, Roberts JD, Sieghart W (1996) The gamma 2 subunit of the GABA_A receptor is concentrated in synaptic junctions containing the alpha 1 and beta 2/3 subunits in hippocampus, cerebellum and globus pallidus. *Neuropharmacology* 35:1425–1444.
- Staley KJ, Proctor WR (1999) Modulation of mammalian dendritic GABA(A) receptor function by the kinetics of Cl[–] and. *J Physiol (Lond)* 519:693–712.
- Strata F, Cherubini E (1994) Transient expression of a novel type of GABA response in rat CA3 hippocampal neurons during development. *J Physiol (Lond)* 480:493–503.
- Sundstrom-Poromaa I, Smith DH, Gong QH, Sabado TN, Li X, Light A, Wiedmann M, Williams K, Smith SS (2002) Hormonally regulated alpha(4)beta(2)delta GABA(A) receptors are a target for alcohol. *Nat Neurosci* 5:721–722.

- Wall MJ (2001) Cis-4-amino-crotonic acid activates alpha 6 subunit-containing GABA(A) but not GABA(C) receptors in granule cells of adult rat cerebellar slices. *Neurosci Lett* 316:37–40.
- Wanamaker CP, Christianson JC, Green WN (2003) Regulation of nicotinic acetylcholine receptor assembly. *Ann NY Acad Sci* 998:66–80.
- Whiting PJ, Bonnert TP, McKernan RM, Farrar S, le Bourdelles B, Heavens RP, Smith DW, Hewson L, Rigby MR, Sirinathsinghji DJ, Thompson SA, Wafford KA (1999) Molecular and functional diversity of the expanding GABA-A receptor gene family. *Ann NY Acad Sci* 868:645–653.
- Wisden W, Farrant M (2002) Insights into GABA_A receptor complexity from the study of cerebellar granule cells: synaptic and extrasynaptic receptors. In: *Glutamate and GABA receptors and transporters: structure, function and pharmacology*, pp 189–201. London: Taylor and Francis.
- Zhang D, Pan ZH, Zhang X, Brideau AD, Lipton SA (1995) Cloning of a gamma-aminobutyric acid type C receptor subunit in rat retina with a methionine residue critical for picrotoxinin channel block. *Proc Natl Acad Sci USA* 92:11756–11760.
- Zhang D, Pan ZH, Awobuluyi M, Lipton SA (2001) Structure and function of GABA(C) receptors: a comparison of native versus recombinant receptors. *Trends Pharmacol Sci* 22:121–132.
- Zheng W, Xie W, Zhang J, Strong JA, Wang L, Yu L, Xu M, Lu L (2003) Function of GABA receptor/channel rho 1 subunits in spinal cord. *J Biol Chem* 278:48321–48329.
- Zhu JJ, Lo FS (1999) Three GABA receptor-mediated postsynaptic potentials in interneurons in the rat lateral geniculate nucleus. *J Neurosci* 19:5721–5730.