British Journal of Pharmacology (2005) 144, 502–509
www.nature.com/bjp

The composition of the GABA receptor at the *Caenorhabditis elegans* neuromuscular junction

[1,3]Bruce A. Bamber, [1,4]Janet E. Richmond, [2]James F. Otto & *,[1]Erik M. Jorgensen

[1]Department of Biology, University of Utah, 257 South 1400 East, Salt Lake City, UT 84112-0840, U.S.A. and
[2]Department of Pharmacology and Toxicology, University of Utah, 30 South 2000 East, Salt Lake City, UT 84112-0840, U.S.A.

1 The *unc-49* gene of the nematode *Caenorhabditis elegans* encodes three γ-aminobutyric acid type A ($GABA_A$) receptor subunits. Two of these, UNC-49B and UNC-49C, are expressed at high abundance and co-localize at the neuromuscular junction.

2 The UNC-49B subunit is sufficient to form a $GABA_A$ receptor *in vitro* and *in vivo*. Furthermore, all loss-of-function *unc-49* alleles lack functional UNC-49B. No mutations specifically inactivate UNC-49C. Thus, UNC-49C appears to be dispensable for receptor function; however, UNC-49C has been conserved among different nematode species, suggesting it plays a necessary role.

3 To ascertain whether UNC-49C is part of the $GABA_A$ receptor *in vivo*, we performed patch-clamp electrophysiology on *C. elegans* muscle cells. Sensitivity to GABA, and to the antagonists picrotoxin and pregnenolone sulfate, matched the UNC-49B/C heteromer rather than the UNC-49B homomer, for both exogenous and synaptically-released GABA.

4 The synaptic localization of UNC-49C requires the presence of UNC-49B, indicative of a physical association between the two subunits *in vivo*. Thus, the *in vivo* receptor is an UNC-49B/C heteromer.

5 UNC-49C plays a negative modulatory role. Using the rapid ligand-exchange technique *in vitro*, we determined that UNC-49C causes accelerated receptor desensitization. Previously, UNC-49C was shown to reduce single-channel conductance in UNC-49B/C heteromers. Thus, the function of UNC-49B is to provide GABA responsiveness and localization to synapses, while the function of UNC-49C is to negatively modulate receptor function and precisely shape inhibitory postsynaptic currents.
British Journal of Pharmacology (2005) **144,** 502–509. doi:10.1038/sj.bjp.0706052
Published online 17 January 2005

Keywords: *C. elegans*; nematode; neuromuscular junction; $GABA_A$ receptor; *unc-49*; subunit composition; synaptic receptor structure; desensitization kinetics

Abbreviations: GABA, γ-aminobutyric acid; $GABA_A$, γ-aminobutyric acid type A; GFP, green fluorescent protein; HEK, human embryonic kidney; n_H, Hill coefficient; PS, pregnenolone sulfate; PTX, picrotoxin; τ_f, fast time constant; τ_s, slow time constant; τ_w, weighted time constant

Introduction

The major inhibitory neurotransmitter in the brain is GABA, which acts primarily through $GABA_A$ receptors (Macdonald & Olsen, 1994). Defective GABA neurotransmission causes neurological disease such as anxiety disorders and epilepsy. $GABA_A$ receptor agonists and antagonists have potent effects on neural circuit function, and allosteric enhancers of the $GABA_A$ receptor are effective against anxiety, insomnia, and epilepsy (Wong *et al*., 2003). To better understand the basic molecular mechanisms of GABA neurotransmission, we are studying the GABA neuromuscular junction of *Caenorhabditis elegans* (Schuske *et al*., 2004).

A single $GABA_A$ receptor, encoded by the *unc-49* gene, functions at the inhibitory neuromuscular junction in *C. elegans* (Bamber *et al*., 1999; Richmond & Jorgensen, 1999). *unc-49* is an unusual gene because it encodes three $GABA_A$ receptor subunits under the control of a single promoter. This gene encodes a single amino-terminal domain, which is spliced to three different sets of GABA-binding and transmembrane domains. Through alternative splicing of the *unc-49* mRNA, three subunits can be generated: UNC-49A, UNC-49B, and UNC-49C. However, only UNC-49B and UNC-49C are expressed at high abundance, while UNC-49A is almost undetectable. In *C. elegans* muscle cells, translational green fluorescent protein (GFP) fusions of UNC-49B and UNC-49C both localize to the neuromuscular junction (Bamber *et al*., 1999).

The UNC-49B subunit appears to be necessary and sufficient to form the neuromuscular junction $GABA_A$ receptor. All mutant alleles of *unc-49* contain mutations that inactivate UNC-49B, while none specifically inactivate UNC-49C. UNC-49B is capable of forming a functional homomeric $GABA_A$ receptor in both heterologous cells and worm muscle cells (Bamber *et al*., 1999). Thus, UNC-49C appears to be unnecessary. However, when both subunits are expressed in heterologous cells, UNC-49B preferentially co-assembles with UNC-49C (Bamber *et al*., 1999). Furthermore, UNC-49C

*Author for correspondence; E-mail: jorgensen@biology.utah.edu
[3]Current address: Department of Pharmacology and Toxicology, University of Utah, 30S, 2000E, Rm 201, Salt Lake City, UT 84112-5820, U.S.A.
[4]Current address: Department of Biological Sciences, University of Illinois at Chicago, Room 4309, MC 067, 840 West Taylor Street, Chicago, IL 60607, U.S.A.
Published online 17 January 2005

coding sequences are present in both *C. elegans* and the nematode *Caenorhabditis briggsae*, despite an estimated 100 million years of evolutionary separation (Stein *et al*., 2003), suggesting an important conserved function. We have determined the role of UNC-49C by investigating the pharmacological and kinetic properties of the UNC-49B homomeric receptor, the UNC-49B/C heteromeric receptor, and the native $GABA_A$ receptor in the body muscle. Furthermore, we determined the role of the UNC-49B and UNC-49C subunits in receptor localization. Our data indicate that UNC-49B and UNC-49C co-assemble to form the neuromuscular GABA receptor *in vivo*. UNC-49B is essential for synaptic localization and gating by GABA, while UNC-49C reduces GABA sensitivity and confers rapid desensitization to the receptor.

Methods

C. elegans *strains*

The wild-type N2 strain was obtained from the *C. elegans* Genetics Center (St Paul, MN, U.S.A.). Transgenic worm strains expressing various UNC-49 subunits were generated by standard microinjection techniques (Mello & Fire, 1995), using the parental strain EG1892 *unc-49(e407)III; lin-15(n765ts)X*. *unc-49(e407)* contains a stop codon in the common amino-terminal region, and is therefore a null allele. To express UNC-49B and UNC-49C together, we co-injected the two wild-type *unc-49* rescuing fragments as described previously (Bamber *et al*., 1999), resulting in the strain EG2460 *oxEx310(UNC-49BC)*. We refer to this strain as the UNC-49BC transgenic. To express UNC-49B in the absence of UNC-49C, we deleted a small *Nru*I fragment in the UNC-49C portion of the *unc-49* rescuing DNA, which removed the first transmembrane and part of the second transmembrane domains, and introduced a frameshift, resulting in the strain EG2459 *oxEx309(UNC-49B)*. We refer to this strain as the UNC-49B transgenic. To express subunits tagged with the GFP, we modified the constructs with GFP inserted into the cytoplasmic loops of UNC-49B and UNC-49C described previously (Bamber *et al*., 1999). To express UNC-49B-GFP in the absence of UNC-49C, we inactivated UNC-49C in the UNC-49B-GFP genomic construct by Klenow filling a *Bgl*II site to produce a frameshift mutation, resulting in the strains EG2316 *oxEx543(UNC-49B-GFPΔC)* and EG2317 *oxEx542(UNC-49B-GFPΔC*). To express UNC-49C-GFP in the absence of UNC-49B, we inactivated UNC-49B in the UNC-49C-GFP genomic construct by Klenow filling a *Bsi*WI site to produce a frameshift mutation. This construct was injected into MT1642, *lin-15(n765ts)X* and subsequently crossed into EG1366 *unc-49(e407)III; lin-15(n765ts)X,* resulting in the strain EG2306 *oxEx544(UNC-49C-GFPΔB)*.

C. elegans *electrophysiology*

Electrophysiological methods were essentially as described previously (Richmond *et al*., 1999). Briefly, adult worms were immobilized in saline with a cyanoacrylic glue (Histoacryl Blue, B. Braun, Melsungen, Germany). A lateral incision was made to expose the neuromuscular junctions along the ventral nerve cord. The basement membrane overlying the muscles was enzymatically removed using 0.23 mg ml^{-1} protease (type XXIV, Sigma) and 0.62 mg ml^{-1} collagenase (type-A, Boehringer-Mannheim) in preparation for recording. Electrophysiological recordings were made in the whole-cell voltage-clamp configuration (holding potential −60 mV) at room temperature (21°C) using an EPC-9 patch-clamp amplifier (HEKA, Lambrecht, Germany) and digitized at 2.9 kHz *via* an ITC-16 interface (Instrutech, Great Neck, NY, U.S.A.). Data were acquired by Pulse software (HEKA) run on a Power Mac 6500/225. The bath solution contained 140 mM NaCl, 5 mM KCl, 3 mM $CaCl_2$, 5 mM $MgCl_2$, 11 mM glucose, and 5 mM HEPES, pH 7.2, ∼330 mOsm. Pressure ejection of GABA (Sigma, St Louis, MO, U.S.A.) from pipettes of 2–4 μm tip diameter was computer triggered. Pregnenolone sulfate (PS) and picrotoxin (PTX) (Sigma) were bath-applied to the preparation by gravity flow at a rate of 2 ml min^{-1}. Presented values are the average of three to 15 replicates, with the exception of 3 and 100 μM PS values, which are the average of two replicates. GABA-dependent synaptic currents were isolated from cholinergic synaptic currents by including the cholinergic inhibitor, D-tubocurare (5×10^{-4} M) in the bath solution (Richmond & Jorgensen, 1999). The pipette solution contained: 120 mM KCl, 20 mM KOH, 4 mM $MgCl_2$, 5 mM *N*-tris[hydroxymethyl]methyl-2-aminoethane-sulfonic acid, 0.25 mM $CaCl_2$, 4 mM NaATP, 36 mM sucrose, 5 mM EGTA, pH 7.2, ∼315 mOsm. Subsequent analysis and graphing were performed using Pulsefit (HEKA), Mini Analysis (Synaptosoft Inc.), and Igor Pro (Wavemetrics, Lake Oswego, OR, U.S.A.).

The GABA dose–response curve was fitted using the Hill equation:

$$I = I_{max}/\{1 + (EC_{50}/[agonist])^{n_H}\}$$

where I is the current at a given GABA concentration, I_{max} is the current at saturation, EC_{50} is the GABA concentration required to produce half-maximal current, and n_H is the Hill coefficient. Dose–response curves for PTX and PS were fitted using the equation:

$$I_{inh+}/I_{Inh-} = 1/\{([inh]/IC_{50})^{n_H} + 1\}$$

where I_{inh+}/I_{inh-} is the current in the presence of the inhibitor (inh, PTX or PS) relative to GABA alone, IC_{50} is the concentration of inhibitor required to block 50% of the current in the absence of PTX, and n_H is the Hill coefficient. The program NFIT (Island Products, Galveston, TX, U.S.A.) was used for nonlinear curve-fitting. *P*-values were calculated using a two-tailed Student's *t*-test.

Plasmids and transfection

UNC-49B and UNC-49C cDNAs, contained on the plasmids pBGR4 and pBGR9, respectively (Bamber *et al*., 1999), were subcloned into pCDNA3.1 (Invitrogen). The β-globin 5′ UTR was added 5′ of the UNC-49 sequences, and the β-globin 3′ UTR along with a sequence containing 30 consecutive A residues and 30 consecutive C residues (Krieg & Melton, 1984) was added 3′ of the UNC-49 sequences. These plasmids were designated pCDNA-UNC-49B and pCDNA-UNC-49C. Human embryonic kidney cells (HEK 293 cell line, American Type Culture Collection CRL 1573) were plated on coverslips coated with poly-L-lysine and rat tail collagen (0.1 mg ml^{-1}), and maintained at 37°C with 5% CO_2 in minimal essential medium (MEM) with glutamine, glucose, and 10% fetal calf serum. A modified calcium phosphate precipitation technique

was used to transfect the HEK 293 cells. Once cells reached 40% confluence, a DNA mixture (3 μg), containing a plasmid encoding soluble GFP as a co-transformation marker, pCDNA-UNC-49B, and pCDNA-UNC-49C at a 2 : 1 : 1 ratio was added. After incubation at 37°C with 5% CO_2 for 10 h, the culture media was replaced and cells were transferred to an incubator maintained at 30°C with 8% CO_2. Recordings were performed at least 36 h after transfection.

Rapid application technique

The rapid application electrophysiological technique was used to record macroscopic GABA currents from outside-out pulled patches as described previously (McClellan & Twyman, 1999). Briefly, a double-lumen glass 'theta' tube was used to provide adjacent streams of external salt solution, with and without GABA, with a small (4–6 μm) interface between solutions. The external salt solution contained: 142 mM NaCl, 1.5 mM KCl, 10 mM HEPES, 10 mM glucose, 20 mM sucrose, 1 mM $CaCl_2$, and 1 mM $MgCl_2$, pH 7.4, adjusted to 310 mOsm. Saturating GABA concentrations of 1–10 mM were used, and data were pooled. Variations in GABA concentration within this range did not produce kinetic differences. Outside-out multichannel patches were pulled from GFP-expressing cells using borosilicate glass recording electrodes (2.5–3.5 MΩ), filled with a salt solution containing: 145 mM $CsCl_2$, 1 mM $MgCl_2$, 10 mM HEPES, 5 mM EGTA, and 3 mM MgATP, pH 7.3, adjusted to 290 mOsm. Patches were positioned in the stream of an external salt solution without GABA emanating from the theta tubing. Using a piezoelectric translator (Burleigh Instruments), the theta tubing was moved such that patch became rapidly exposed to the GABA-containing external salt solution, then moved back again 500 ms later. At the conclusion of each recording, patches were discarded, and open electrode tip junction potentials were measured in a parallel fashion using solutions of different molalities. By this method, solution exchange rates were found to be less than 1 ms. All recordings were performed at room temperature (20–23°C).

Kinetic analysis

The desensitization phase of the GABA receptor response was fit with a biexponential equation using Clampfit 8.2 (Axon Instruments, Foster City, CA, U.S.A.) as described previously (Otto *et al*., 2002). Kinetic fits for the desensitization phase were then separated into discrete components: weighted time constant (τ), slow τ, fast τ, and % fast τ. The effect of UNC-49C subunit inclusion on kinetic components was analyzed by ANOVA, followed by *post hoc* Student's *t*-tests (Excel).

Results

Strategy to evaluate subunit composition

The *unc-49* gene expresses three $GABA_A$ receptor subunits, UNC-49A, UNC-49B, and UNC-49C, by alternative splicing (Figure 1; Bamber *et al*., 1999). Of these, there is only evidence to definitively support a role for UNC-49B. The UNC-49A mRNA is not detected in adult hermaphrodites by Northern analysis, so UNC-49A is not a component of the native receptor. Similarly, the UNC-49C subunit is not required for receptor function *in vivo* or *in vitro* (Bamber *et al*., 1999). Nevertheless, UNC-49C could be part of the native receptor because it is expressed and localizes to synapses, and can efficiently co-assemble with UNC-49B in *Xenopus* oocytes to form an UNC-49B/C heteromer with distinct pharmacological properties (Bamber *et al*., 1999; 2003). We used the pharmacological signature of UNC-49C as the basis for determining whether it is present in the worm muscle $GABA_A$ receptor *in vivo*. We characterized the pharmacology of the native receptor in muscles of wild-type worms compared to worms expressing UNC-49B and UNC-49C together, or UNC-49B alone. To express UNC-49B and UNC-49C together, we injected the intact *unc-49* rescue construct into *unc-49* null mutant worms ('UNC-49BC transgenic'). To express UNC-49B alone, we injected an *unc-49* rescue construct lacking UNC-49C into *unc-49* null mutant worms ('UNC-49B

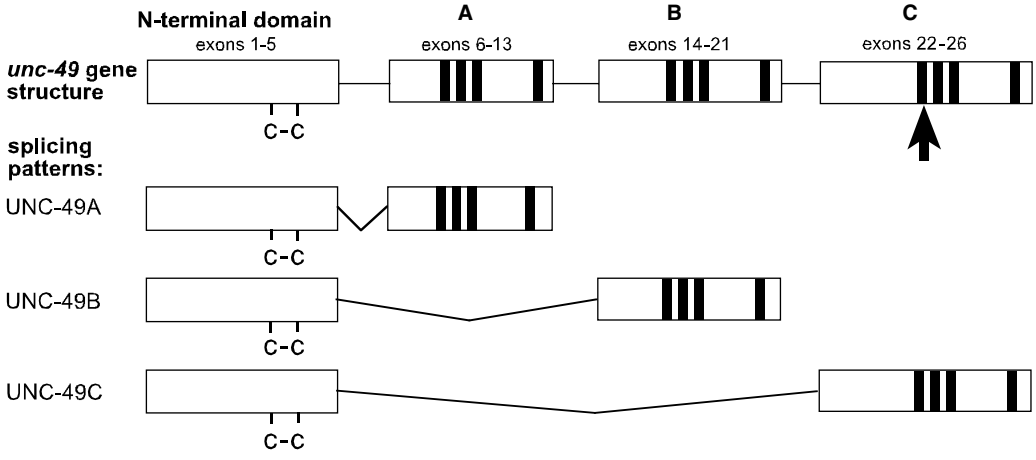

Figure 1 Summary of *unc-49* gene structure and mRNA splicing. 'c–c' refers to the conserved cysteine loop. Vertical bars represent transmembrane domains. Arrow indicates location of the deletion and frameshift mutations that eliminate the UNC-49C subunit in the *unc-49* rescuing transgene, used to construct the UNC-49B transgenic strain and the UNC-49B-GFPΔC strain. Exons encoding the N-terminal domain are spliced to one of three alternative sets of exons (A, B, and C) encoding carboxy-terminal GABA binding and transmembrane domains, to produce the UNC-49A, UNC-49B, and UNC-49C cDNAs.

transgenic'). In these strains, the null mutation in the endogenous *unc-49* locus prevents the synthesis of any endogenous subunit (Bamber *et al*., 1999), so the only UNC-49 subunits present are those encoded by the transgene that we introduced (see Methods).

Pharmacological evidence for an UNC-49B/C heteromer in vivo

UNC-49C reduces GABA sensitivity. In *Xenopus* oocytes, the GABA EC_{50} is increased and the Hill coefficient is reduced for UNC-49B/C heteromers, compared to UNC-49B homomers (Bamber *et al*., 1999). To investigate GABA sensitivity *in vivo*, we generated GABA dose–response curves for wild-type worms, and for the two transgenic strains. In wild type, the GABA EC_{50} was 25.9 μM, and the Hill coefficient was 1.2. Similarly, for UNC-49BC transgenics, the GABA EC_{50} was 19.6 μM and the Hill coefficient was 1.3. However, for UNC-49B transgenics, the GABA EC_{50} was reduced to 8.6 μM and the Hill number was increased to 2.0 (Figure 2a). This result suggests that native *C. elegans* $GABA_A$ receptor is an UNC-49B/C heteromer because its GABA sensitivity was much closer to the UNC-49BC transgenic than the UNC-49B transgenic.

PS is a neurosteroid that noncompetitively inhibits $GABA_A$ receptors (Majewska *et al*., 1988). UNC-49B homomers and UNC-49B/C heteromers are differentially sensitive to PS inhibition. In *Xenopus* oocytes, the presence of UNC-49C confers PS sensitivity to the receptor (IC_{50} values of 2.3 μM for UNC-49B/C heteromers and >100 μM for UNC-49B homomers, respectively; B.A. Bamber, E.M. Jorgensen, unpublished observations). We tested PS inhibition of currents in muscle cells evoked by 300 μM GABA, a near-saturating concentration (Figure 2b). PS produced strong, dose-dependent inhibition in wild-type worms ($IC_{50} = 21.7$ μM), and in the UNC-49BC transgenic ($IC_{50} = 22.9$ μM). By contrast, PS sensitivity was greatly reduced in the UNC-49B transgenic ($IC_{50} > 300$ μM).

Similar results were obtained using the $GABA_A$ receptor antagonist PTX. PTX inhibits $GABA_A$ receptors with both noncompetitive and competitive components (Wang *et al*., 1995). In *Xenopus* oocytes, the UNC-49C subunit confers PTX resistance (Bamber *et al*., 2003). In wild-type muscles, GABA currents evoked by 30 μM GABA (roughly the EC_{50}) were PTX insensitive ($IC_{50} = 418.5$ μM; Figure 2c). Muscles in UNC-49BC transgenic worms were also PTX insensitive ($IC_{50} > 1000$ μM). By contrast, muscles in UNC-49B transgenics were PTX sensitive ($IC_{50} = 11.8$ μM). Thus, for both PS and PTX antagonism, the native *C. elegans* $GABA_A$ receptor behaves like the UNC-49B/C heteromer rather than the UNC-49B homomer, consistent with the presence of UNC-49C.

The synaptic $GABA_A$ receptor is a UNC-49B/C heteromer

The above data indicate that UNC-49C is present in the muscle $GABA_A$ receptor *in vivo*; however, they do not prove that the *synaptic* $GABA_A$ receptor contains UNC-49C, because the measured currents were evoked by bathing the whole postsynaptic cell in GABA. To rule out the possible confounding effects of nonsynaptic $GABA_A$ receptors, we characterized synaptic currents. These are miniature postsynaptic currents caused by the release of GABA from synaptic vesicles, more simply called 'minis' (Richmond & Jorgensen, 1999). Acetylcholine-induced minis were eliminated by bath

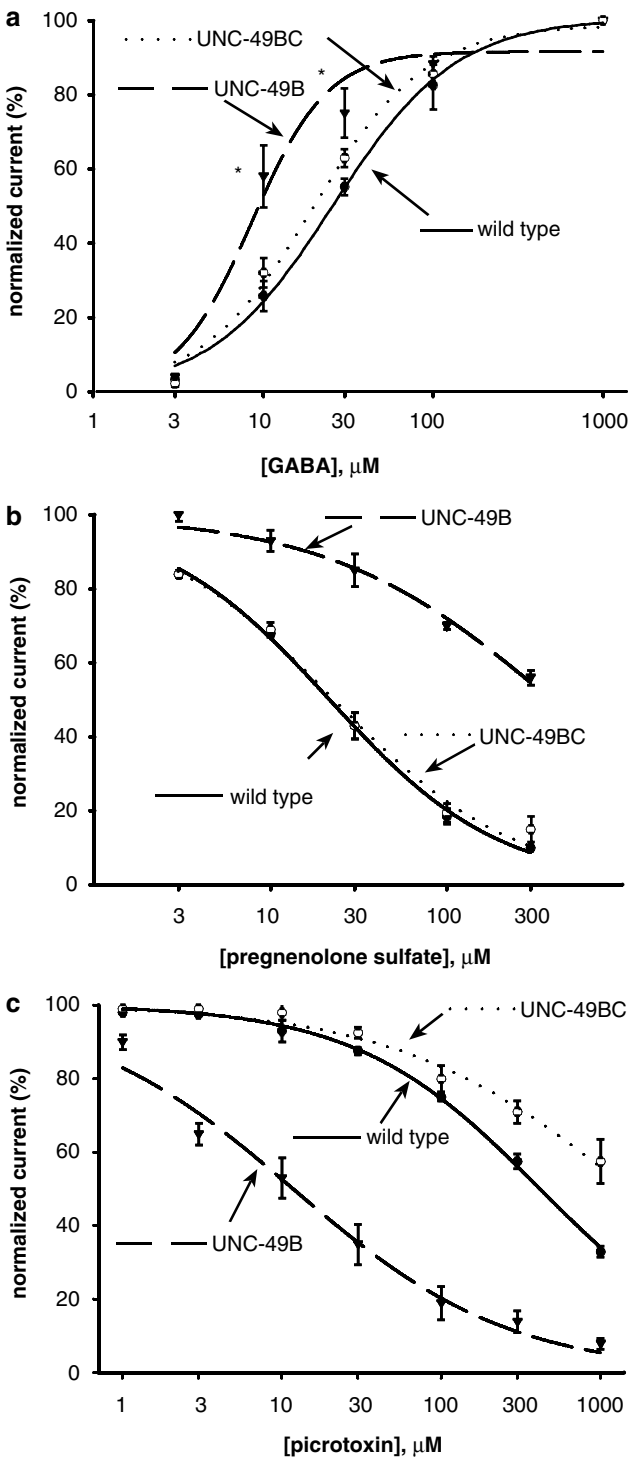

Figure 2 Agonist and antagonist sensitivity of *C. elegans* muscle cells. (a) GABA concentration–response curves for wild-type worms, UNC-49BC transgenic worms, and UNC-49B transgenic worms. Asterisks mark data points that are significantly different ($P<0.05$) between UNC-49B, and both the wild type and UNC-49BC. (b) Concentration–response curves for PS inhibition of currents evoked by 300 μM GABA. (c) Concentration–response curves for PTX inhibition of currents evoked by 30 μM GABA. Error bars are s.e.m.

application of 500 μM D-tubocurare. Presence of UNC-49C was determined in the remaining GABA minis by testing their sensitivity to PS and PTX.

The effects of PS and PTX on GABA minis indicated that UNC-49C is present in the synaptic $GABA_A$ receptor (Figure 3). PS (300 μM) eliminated 97 and 99% of GABA minis in wild-type worms and UNC-49BC transgenics, respectively, but only eliminated 60% of GABA minis in UNC-49B transgenics, consistent with the greater degree of PS sensitivity of the UNC-49B/C heteromer (Figure 3a). Likewise, 1000 μM PTX eliminated only about 50% of minis in wild-type worms and UNC-49BC transgenics, but eliminated 90% of minis in UNC-49B transgenics, consistent with the lower PTX sensitivity of the UNC-49B/C heteromer (Figure 3b). These results are consistent with the presence of UNC-49C in the native synaptic $GABA_A$ receptor in *C. elegans*.

The formal possibility exists that PS or PTX could be acting on presynaptic proteins to alter neurotransmitter release. Two lines of evidence make this possibility unlikely. First, if PS or PTX were inhibiting presynaptic GABA receptors, then their application would disinhibit the nerve terminal and increase mini frequency. We observed the opposite effect. Second, if PS and PTX were acting on presynaptic proteins other than $GABA_A$ receptors, we would observe the same postsynaptic effect irrespective of which UNC-49 subunits were present. We observed strong dependence on UNC-49 subunit expression. Therefore, the effects of $GABA_A$ receptor antagonists on mini frequency indicate that UNC-49C is present in the native synaptic $GABA_A$ receptor in *C. elegans*.

UNC-49B and UNC-49C physically interact at the neuromuscular junction

For mammalian $GABA_A$ and glycine receptors, the synaptic localization of the entire heteropentameric receptor complex sometimes depends on just one of its subunits (Meyer *et al*., 1995; Essrich *et al*., 1998). This dependence provides evidence for physical interaction between subunits. Such a function for UNC-49B provides further evidence that UNC-49C is incorporated into the synaptic receptor. We tested whether the subcellular localization of the UNC-49 subunits was interdependent by determining how the loss of one UNC-49 subunit affected localization of the other (Figure 4). We used a similar transgenic strategy as described above. Starting with the *unc-49(e407)* null mutant, we constructed *unc-49* extrachromosomal arrays in which one subunit was tagged with GFP and the other was eliminated by the introduction of a premature stop codon (see Methods). Thus, we could express UNC-49B-GFP in the absence of UNC-49C, or UNC-49C-GFP in the absence of UNC-49B. Previous work demonstrated that either UNC-49B-GFP or UNC-49C-GFP localize to neuromuscular junctions when expressed in the presence of the other (Bamber *et al*., 1999). Similarly, UNC-49B-GFP expressed in the absence of UNC-49C localizes to puncta in

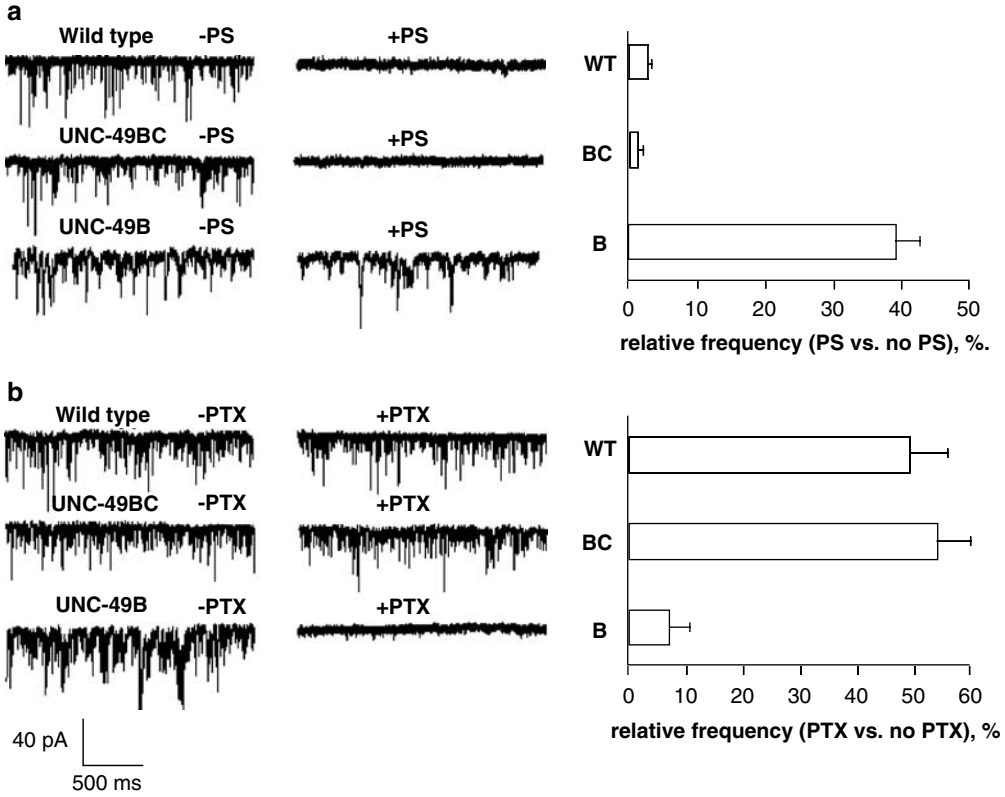

Figure 3 Antagonist pharmacology of the synaptic $GABA_A$ receptor in *C. elegans* muscle. Acetylcholine minis were eliminated by bath application of D-tubocurare (500 μM). (a) Traces of GABA minis recorded in the absence (left panel) and presence (middle panel) of 300 μM PS. Plot of relative frequencies of GABA minis in the absence and presence of PS (right panel). (b) GABA minis recorded in the absence (left panel) and presence (middle panel) of 1000 μM PTX. Plot of relative frequencies of GABA minis in the absence and presence of PTX (right panel). The genotype of the worm strain is indicated above each trace. PS is pregnenolone sulfate, PTX is picrotoxin. Error bars are s.e.m.

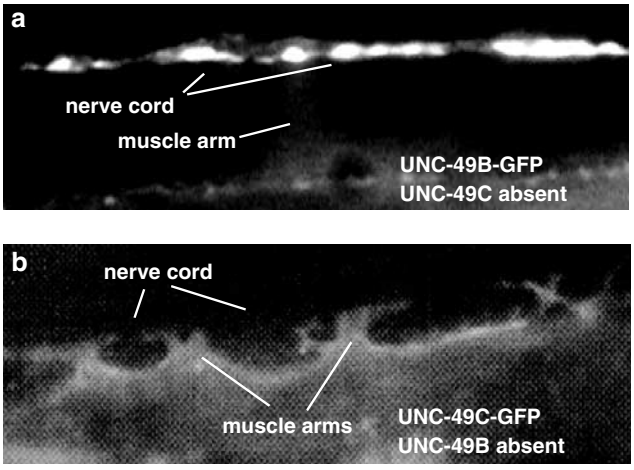

Figure 4 Synaptic localization of UNC-49C-GFP depends on UNC-49B. (a) Punctate nerve cord fluorescence is observed in worms expressing UNC-49B-GFP in the absence of UNC-49C. (b) No nerve cord-associated fluorescence is observed in worms expressing UNC-49C-GFP in the absence of UNC-49B. Fluorescence is observed on muscle arms and muscle cell membranes. The UNC-49C-GFP construct is localized to synapses in the wild type, which expresses UNC-49B (Bamber *et al*., 1999).

the ventral and dorsal nerve cords, indicating correct subcellular localization (Figure 4a). This subunit also rescues the uncoordinated phenotype of *unc-49(e407)*, which demonstrates that the tagged receptor is correctly localized to synapses (not shown). Thus, synaptic localization of UNC-49B does not depend on UNC-49C. By contrast, when UNC-49C-GFP was overexpressed in the absence of UNC-49B, synaptic fluorescence was not observed. Instead, fluorescence was visible on muscle membranes and muscle arms, and no enrichment of fluorescence was observed in the vicinity of the nerve cord (Figure 4b). Thus, UNC-49B is required to target UNC-49C to the synapse, which provides independent evidence of physical association between UNC-49B and UNC-49C *in vivo*.

A negative modulatory role for UNC-49C

The above data provide strong evidence that UNC-49C is part of the synaptic GABA receptor. What is its role? UNC-49B/C heteromers are less sensitive to GABA than UNC-49B homomers, as described above. Moreover, the single-channel conductance of UNC-49B/C heteromers in HEK cells was reduced by 20% compared to receptors composed solely of the UNC-49B subunit (Bamber *et al*., 1999). Thus, UNC-49C appears to negatively modulate receptor function. Comparing the kinetics of UNC-49B homomers and UNC-49B/C heteromers provides further evidence for a negative modulatory role for UNC-49C. We expressed UNC-49B homomers and UNC-49B/C heteromers in HEK 293 cells and analyzed desensitization using rapid ligand-exchange electrophysiology. Saturating GABA was applied for 500 ms to outside-out patches from HEK cells expressing UNC-49B homomers or UNC-49B/C heteromers (Figure 5a). Biexponential desensitization kinetics were observed for both receptors. Time constants for the fast component of desensitization were not significantly different for UNC-49B and UNC-49B/C receptors. However, the time

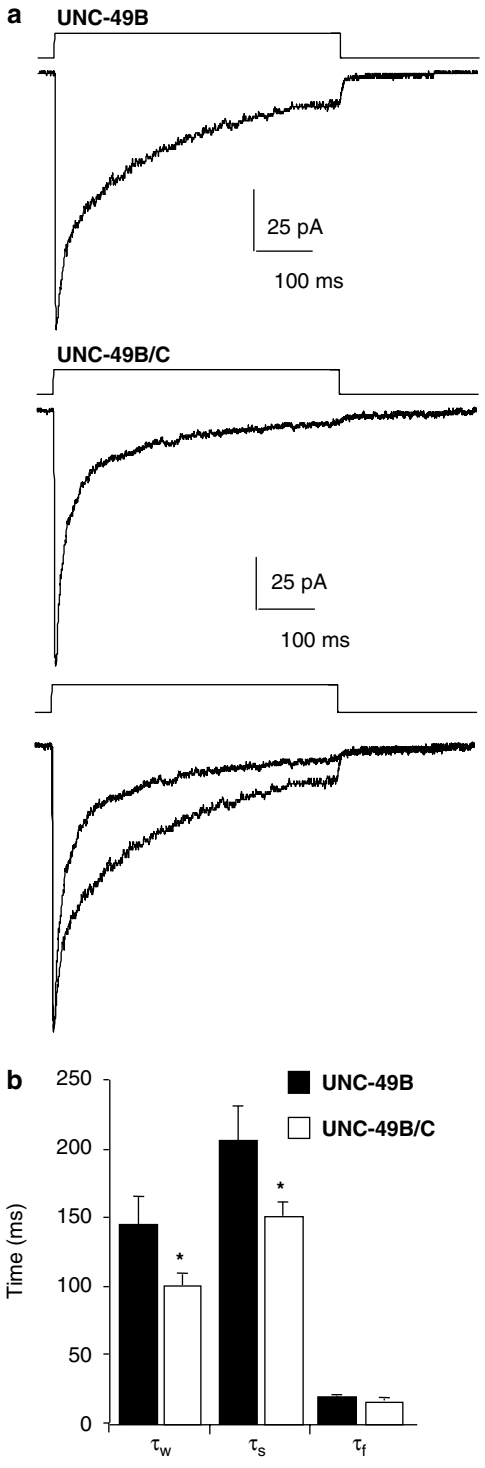

Figure 5 UNC-49C accelerates desensitization. (a) Outside-out patches pulled from HEK 293 cells expressing UNC-49B homomers (top trace) or UNC-49B/C heteromers (middle trace) were exposed to 10 mM GABA for 500 ms using the rapid ligand exchange technique. The line above each trace is the corresponding open tip junction potential, indicating when GABA was applied. Accelerated desensitization of UNC-49B/C heteromers is evident when the two traces are normalized and superimposed (lower panel). (b) Desensitization time constants for UNC-49B homomers and UNC-49B/C heteromers $n=8$ and 10, for UNC-49B homomers and UNC-49B/C heteromers, respectively. τ_w, τ_s, and τ_f are the weighted, slow, and fast decay time constants, respectively. Error bars are s.e.m., asterisk indicates $P<0.05$ (Student's *t*-test).

constant for the slow component of desensitization was larger for UNC-49B (206±26 ms, mean±s.e.m.) than for UNC-49B/C (149±10 ms, $P<0.05$, Student's *t*-test). Similar differences were observed between the weighted time constants (Figure 5b). The slow component accounted for most of the weighted time constant (95 and 91% for UNC-49B and UNC-49B/C, respectively). These data indicate that UNC-49C significantly increases receptor desensitization and therefore is likely to reduce overall charge transfer during GABA neurotransmission *in vivo*.

Discussion

The $GABA_A$ receptor at the *C. elegans* neuromuscular junction is encoded by the *unc-49* gene. *unc-49* encodes three subunits, UNC-49A, UNC-49B, and UNC-49C. Which of these subunits contributes to the function of the $GABA_A$ receptor *in vivo*? UNC-49A is not expressed in adult hermaphrodites, and therefore cannot play a role. UNC-49B is expressed at high levels, and localizes to the neuromuscular junction. All *unc-49* mutant alleles lack UNC-49B, indicating that UNC-49B is essential for receptor function. UNC-49C is also expressed at a high level and localizes to neuromuscular junctions, but none of the *unc-49* mutants specifically lack UNC-49C, calling into question whether it plays a role in receptor function. UNC-49B forms a homomeric receptor in heterologous cells. UNC-49C does not, but can co-assemble with UNC-49B to produce a heteromeric receptor with distinct pharmacological properties. We have shown that the native $GABA_A$ receptor at the neuromuscular junction *in vivo* has the pharmacological properties of the UNC-49B/C heteromer rather than the UNC-49B homomer. We demonstrate that to reconstitute a receptor with those properties in an *unc-49* null background requires expression of both UNC-49B and UNC-49C subunits. Further, we demonstrate that UNC-49B controls the synaptic localization of UNC-49C. These data indicate that UNC-49B and UNC-49C co-assemble to form a heteromeric receptor *in vivo*.

These data provide an opportunity to compare the behaviors of a receptor *in vivo* and *in vitro*. Inferences about receptor function in *in vivo* neural circuits are often drawn from *in vitro* experiments. We observed good agreement between the properties of the UNC-49 receptors in heterologous cells and *in vivo*. In every case, the UNC-49C subunit shifted drug sensitivity in the same manner. However, absolute values for drug sensitivity were not the same. For both UNC-49B homomers and UNC-49B/C heteromers, GABA sensitivity was always higher *in vivo*, while PS and PTX sensitivity were always lower *in vivo* (Bamber *et al*., 1999; 2003). The reason for these shifts is not clear. Cell type-specific variability in the properties of recombinant GABA receptors is well documented (Horne *et al*., 1993; White *et al*., 1995; Belelli *et al*., 1996; Lin *et al*., 1996; Fisher *et al*., 1997; Halliwell *et al*., 1999; Pistis *et al*., 1999). Relative subunit expression levels in heterologous cells might also influence receptor function by altering the stoichiometry or arrangement of the five subunits that co-assemble to form the functional receptor. Indeed, we observed that overexpression had a small but significant effect on the GABA sensitivity of the heteromer in the UNC-49BC transgenic, compared to the native wild-type receptor. We cannot rule out the possibility that another subunit expressed in *C. elegans* muscle cells co-assembles with UNC-49B and UNC-49C and alters the pharmacology of the native receptor, although the genetic evidence clearly indicates that no additional subunits are absolutely required for receptor function (McIntire *et al*., 1993). It is more likely that other proteins at the synapse modulate receptor responses. UNC-49B and UNC-49C subunits are highly enriched at the synapse (Bamber *et al*., 1999; Gally & Bessereau, 2003), and therefore most of the current we recorded from *C. elegans* muscle cells was due to synaptic GABA receptors. Increased receptor density, and interaction with synaptic tethering proteins, are known to alter the apparent agonist affinity and desensitization of the glycine receptor, a close relative of UNC-49 (Taleb & Betz, 1994; Legendre *et al*., 2002). Furthermore, the functional properties of synaptic $GABA_A$ receptors are known to be affected by phosphorylation (Jones & Westbrook, 1997). Therefore, the functional differences that we observed may arise from the fact that the UNC-49 receptors *in vivo* are integrated into a functioning synapse and are subject to synapse-specific post-translational modifications and protein–protein interactions.

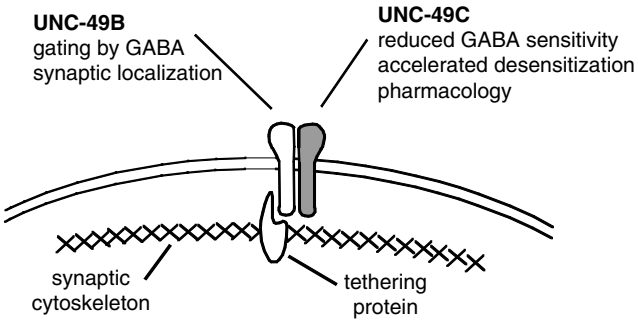

Figure 6 Model of interactions at the synapse. UNC-49B is linked to the cytoskeleton by a putative receptor tethering protein that binds to the subunit intracellular domain, and UNC-49C becomes localized to the synapse by physically associating with UNC-49B. The functional role of each subunit is indicated.

The findings in this study lead to a simple model for the interactions taking place at the *C. elegans* GABA neuromuscular junction (Figure 6). In this model, UNC-49B and UNC-49C form a heteromultimeric $GABA_A$ receptor. This receptor interacts with the synaptic cytoskeleton *via* the UNC-49B subunit. These sequences are very likely to be intracellular, since most of the extracellular sequences of UNC-49B and UNC-49C are identical. The two subunits of the UNC-49B/C heteromultimer subserve different functions. UNC-49B confers synaptic localization and gates the channel. By contrast, UNC-49C serves a modulatory role. It reduces GABA sensitivity and increases desensitization, and therefore negatively regulates receptor function. As UNC-49C is evolutionarily conserved, this negative modulation likely confers a selective advantage, perhaps by fine-tuning muscle cells for locomotion and posture.

The first two authors contributed equally to this work (B.A.B. and J.E.R.). This work was supported by NIH grants NS35307 (E.M.J.), NS041477 (J.E.R.), and NS43345 (B.A.B.), and the Klingenstein Fund. We wish to thank A. Rowland for the photograph shown in Figure 4a.

References

BAMBER, B.A., BEG, A.A., TWYMAN, R.E. & JORGENSEN, E.M. (1999). The *Caenorhabditis elegans unc-49* locus encodes multiple subunits of a heteromultimeric GABA receptor. *J. Neurosci.,* **19,** 5348–5359.

BAMBER, B.A., TWYMAN, R.E. & JORGENSEN, E.M. (2003). Pharmacological characterization of the homomeric and heteromeric UNC-49 GABA receptors in *C. elegans*. *Br. J. Pharmacol.,* **138,** 883–893.

BELELLI, D., CALLACHAN, H., HILL-VENNING, C., PETERS, J.A. & LAMBERT, J.J. (1996). Interaction of positive allosteric modulators with human and *Drosophila* recombinant GABA receptors expressed in *Xenopus laevis* oocytes. *Br. J. Pharmacol.,* **118,** 563–576.

ESSRICH, C., LOREZ, M., BENSON, J.A., FRITSCHY, J.M. & LUSCHER, B. (1998). Postsynaptic clustering of major $GABA_A$ receptor subtypes requires the gamma 2 subunit and gephyrin. *Nat. Neurosci.,* **1,** 563–571.

FISHER, J.L., ZHANG, J. & MACDONALD, R.L. (1997). The role of alpha1 and alpha6 subtype amino-terminal domains in allosteric regulation of gamma-aminobutyric acid A receptors. *Mol. Pharmacol.,* **52,** 714–724.

GALLY, C. & BESSEREAU, J.L. (2003). GABA is dispensable for the formation of junctional GABA receptor clusters in *Caenorhabditis elegans*. *J. Neurosci.,* **23,** 2591–2599.

HALLIWELL, R.F., THOMAS, P., PATTEN, D., JAMES, C.H., MARTINEZ-TORRES, A., MILEDI, R. & SMART, T.G. (1999). Subunit-selective modulation of $GABA_A$ receptors by the non-steroidal anti-inflammatory agent, mefenamic acid. *Eur. J. Neurosci.,* **11,** 2897–2905.

HORNE, A.L., HARKNESS, P.C., HADINGHAM, K.L., WHITING, P. & KEMP, J.A. (1993). The influence of the gamma 2L subunit on the modulation of responses to $GABA_A$ receptor activation. *Br. J. Pharmacol.,* **108,** 711–716.

JONES, M.V. & WESTBROOK, G.L. (1997). Shaping of IPSCs by endogenous calcineurin activity. *J. Neurosci.,* **17,** 7626–7633.

KRIEG, P.A. & MELTON, D.A. (1984). Functional messenger RNAs are produced by SP6 *in vitro* transcription of cloned cDNAs. *Nucleic Acids Res.,* **12,** 7057–7070.

LEGENDRE, P., MULLER, E., BADIU, C.I., MEIER, J., VANNIER, C. & TRILLER, A. (2002). Desensitization of homomeric alpha1 glycine receptor increases with receptor density. *Mol. Pharmacol.,* **62,** 817–827.

LIN, Y.F., ANGELOTTI, T.P., DUDEK, E.M., BROWNING, M.D. & MACDONALD, R.L. (1996). Enhancement of recombinant alpha 1 beta 1 gamma 2L gamma-aminobutyric acidA receptor whole-cell currents by protein kinase C is mediated through phosphorylation of both beta 1 and gamma 2L subunits. *Mol. Pharmacol.,* **50,** 185–195.

MACDONALD, R.L. & OLSEN, R.W. (1994). $GABA_A$ receptor channels. *Ann. Rev. Neurosci.,* **17,** 569–602.

MAJEWSKA, M.D., MIENVILLE, J.M. & VICINI, S. (1988). Neurosteroid pregnenolone sulfate antagonizes electrophysiological responses to GABA in neurons. *Neurosci. Lett.,* **90,** 279–284.

MCCLELLAN, A.M. & TWYMAN, R.E. (1999). Receptor system response kinetics reveal functional subtypes of native murine and recombinant human $GABA_A$ receptors. *J. Physiol.,* **515,** 711–727.

MCINTIRE, S.L., JORGENSEN, E. & HORVITZ, H.R. (1993). Genes required for GABA function in *Caenorhabditis elegans*. *Nature,* **364,** 334–337.

MELLO, C. & FIRE, A. (1995). DNA transformation. *Methods Cell Biol.,* **48,** 451–482.

MEYER, G., KIRSCH, J., BETZ, H. & LANGOSCH, D. (1995). Identification of a gephyrin binding motif on the glycine receptor beta subunit. *Neuron,* **15,** 563–572.

OTTO, J.F., KIMBALL, M.M. & WILCOX, K.S. (2002). Effects of the anticonvulsant retigabine on cultured cortical neurons: changes in electroresponsive properties and synaptic transmission. *Mol. Pharmacol.,* **61,** 921–927.

PISTIS, M., BELELLI, D., MCGURK, K., PETERS, J.A. & LAMBERT, J.J. (1999). Complementary regulation of anaesthetic activation of human ($\alpha_6\beta_3\gamma_{2L}$) and *Drosophila* (RDL) GABA receptors by a single amino acid residue. *J. Physiol. (London),* **515,** 3–18.

RICHMOND, J.E., DAVIS, W.S. & JORGENSEN, E.M. (1999). UNC-13 is required for synaptic vesicle fusion in *C. elegans*. *Nat. Neurosci.,* **2,** 959–964.

RICHMOND, J.E. & JORGENSEN, E.M. (1999). One GABA and two acetylcholine receptors function at the *C. elegans* neuromuscular junction. *Nat. Neurosci.,* **2,** 791–797.

SCHUSKE, K., BEG, A.A. & JORGENSEN, E.M. (2004). The GABA nervous system in *Caenorhabditis elegans*. *Trends Neurosci.,* **27,** 407–414.

STEIN, L.D., BAO, Z., BLASIAR, D., BLUMENTHAL, T., BRENT, M.R., CHEN, N., CHINWALLA, A., CLARKE, L., CLEE, C., COGHLAN, A., COULSON, A., D'EUSTACHIO, P., FITCH, D.H., FULTON, L.A., FULTON, R.E., GRIFFITHS-JONES, S., HARRIS, T.W., HILLIER, L.W., KAMATH, R., KUWABARA, P.E., MARDIS, E.R., MARRA, M.A., MINER, T.L., MINX, P., MULLIKIN, J.C., PLUMB, R.W., ROGERS, J., SCHEIN, J.E., SOHRMANN, M., SPIETH, J., STAJICH, J.E., WEI, C., WILLEY, D., WILSON, R.K., DURBIN, R. & WATERSTON, R.H. (2003). The genome sequence of *Caenorhabditis briggsae*: a platform for comparative genomics. *PLoS Biol.,* **1,** 166–192.

TALEB, O. & BETZ, H. (1994). Expression of the human glycine receptor alpha 1 subunit in *Xenopus* oocytes: apparent affinities of agonists increase at high receptor density. *EMBO J.,* **13,** 1318–1324.

WANG, T.L., HACKAM, A.S., GUGGINO, W.B. & CUTTING, G.R. (1995). A single amino acid in gamma-aminobutyric acid rho 1 receptors affects competitive and noncompetitive components of picrotoxin inhibition. *Proc. Natl. Acad. Sci. U.S.A.,* **92,** 11751–11755.

WHITE, G., GURLEY, D., HARTNETT, C., STIRLING, V. & GREGORY, J. (1995). Human alpha and beta subunits contribute to the EC_{50} for GABA at the $GABA_A$ receptor expressed in *Xenopus* oocytes. *Receptors Channels,* **3,** 1–5.

WONG, C.G., BOTTIGLIERI, T. & SNEAD III, O.C. (2003). GABA, gamma-hydroxybutyric acid, and neurological disease. *Ann. Neurol.,* **54** (Suppl 6), S3–S12.

(Received June 26, 2004
Revised October 1, 2004
Accepted October 13, 2004)