the antibody blocks collapsin-induced currents (Fig. 4*A*). Trituration of DRG neurons with anti-CRMP-62(30–48) blocks subsequent collapsin-induced growth cone collapse (Fig. 4*B*, *C*). Rabbit IgG and preabsorbed anti-CRMP-62(30–48) antibody have no effect. Addition of anti-CRMP-62(30–48) after trituration does not prevent collapsin-induced collapse. The Fab fragment of anti-CRMP-62(30–48) is as effective as intact immunoglobulin (Fig. 4*C*), indicating that cross-linking and aggregation of antigen do not account for these effects. Trituration with anti-CRMP-62(30–48) has no effect on neuronal survival, neurite formation or extension in the absence of collapsin (Fig. 4*E*).

We also triturated cells with anti-CRMP-62(475–491). This portion of CRMP-62 is not conserved with UNC-33 or between hCRMP-1 and hCRMP-2, and is not included in the original truncated clone isolated by oocyte expression. The anti-CRMP-62(475–491) antibody does not block collapse induced by collapsin or mastoparan (Fig. 4*D*). This verifies that the 475–491 region is not critical for CRMP-62 function, and that blockade of collapsin action by anti-CRMP-62(30–48) cannot be explained as a nonspecific effect of antibody colocalization with CRMP-62. Mastoparan stimulates PTX-sensitive G proteins and induces growth cone collapse in control cultures[6,8] and those triturated with anti-CRMP-62(30–48) antibody (Fig. 4*D*). Thus, those elements of the collapse pathway which are downstream of G protein activation are not disrupted by the anti-CRMP-62(30–48) antibody.

The hypothesis that collapsin activates a G protein signalling cascade is based on several findings: pertussis holotoxin blocks collapsin effects; collapsin activates an oocyte Ca^{2+}-sensitive chloride channel; and G protein activation collapses growth cones. CRMP-62 is likely to mediate or facilitate an interaction between a collapsin-binding transmembrane receptor and intracellular G proteins. In such a model, CRMP-62 may be an intracellular component of a multisubunit receptor, or may act as an intermediary between receptor and G protein. CRMP-62 cannot act downstream of the G protein because the response to ligands for other G protein-coupled receptors is not altered by CRMP-62, and because mastoparan-induced collapse is not blocked by anti-CRMP-62(30–48).

Generally, G protein-coupled receptors contain seven membrane-spanning domains and couple directly to G proteins. A collapsin-binding protein has not yet been identified, but its apparent requirement for CRMP-62 and its ability to respond to a macromolecular ligand which can exist in membrane-bound forms[2–4] imply that it has unique characteristics. Whether CRMP-62 and/or a collapsin-binding protein interact directly or indirectly with a G protein remains to be determined. It is clear that CRMP-62 is expressed selectively in developing nervous system, is capable of reconstituting a collapsin-regulated G protein cascade in oocytes, and is essential for collapsin-induced DRG growth cone collapse. Further analysis of the protein–protein interactions of CRMP-62 should lead to a more complete understanding of the molecular mechanism whereby repulsive axon guidance clues are transduced into changes in growth cone motility. □

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Channel gating governed symmetrically by conserved leucine residues in the M2 domain of nicotinic receptors

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In nicotinic acetylcholine receptors (nAChR), as well as glycine, $GABA_A$ (γ-aminobutyric acid), serotonin ($5\text{-}HT_3$), and GluCl glutamate receptors, a leucine residue at the approximate midpoint of the M2 transmembrane domain (the 9′ position[1]) is conserved across most known subunits[2]. Structural data for the nAChR suggest that the Leu 9′ residues occupy a 'kink' in each of the five M2 helices and point into the closed channel; in the opening step, the M2 helices rotate so that Leu 9′ side chains no longer occlude the conduction pathway[3]. Mutation of Leu 9′ to one of several other residues slows desensitization and increases sensitivity to agonist[4–6]. We have exploited the $\alpha_2\beta\gamma\delta$ stoichiometry of muscle nAChR to express receptors with $m_s^* = 0$ to 5 Leu 9′Ser mutated subunits. Strikingly, each Leu 9′Ser mutation shifts the dose–response relation for ACh to the left by ~10-fold; a nAChR with $m_s^* = 4$ is 10^4-fold more sensitive than the wild type. The results suggest that each of the five Leu 9′ residues participates independently and symmetrically in a key step in the structural transition between the closed and open states.

Oocytes were injected with mRNA for wild-type subunits or for subunits containing the Leu 9′Ser mutation; the latter are denoted here with an asterisk. Figure 1 shows raw traces and dose–response data for nAChRs that exemplify combinations for $m_s^* = 1$, 2, 3 and 4 Leu 9′Ser mutations, respectively. The clear trend is that receptors with increasing m_s^* require decreased ACh concentrations for half-maximum response (EC_{50}). We analysed these dose–response relations for all four possible hetero-oligomeric combinations with $m_s^* = 1$ and for all but one of the combinations yielding $m_s^* = 2$, 3, 4 and 5 that contain either 2α or $2\alpha^*$ subunits (Table 1; the $\alpha_2^*\beta^*\gamma^*\delta$ combination gave no detectable expression). The EC_{50} for ACh decreases by ~10-fold for each additional mutated subunit (Fig. 1*c*). Thus the wild-type nAChR has an EC_{50} of 24 μM, and the two combinations tested with $m_s^* = 4$ have EC_{50} of 2.0 and 2.3 nM.

There is a range of up to fourfold among the EC_{50} values within some groups with equal m_s^*. These extrema were not consistently associated with a particular mutated subunit.

Dose–response relations for the $\alpha^*\alpha\beta\gamma\delta$ receptor (in the $m_s^* =$ 1 group) were determined by injecting oocytes with mixtures of α and α* messenger RNA, along with β, γ and δ (for example,

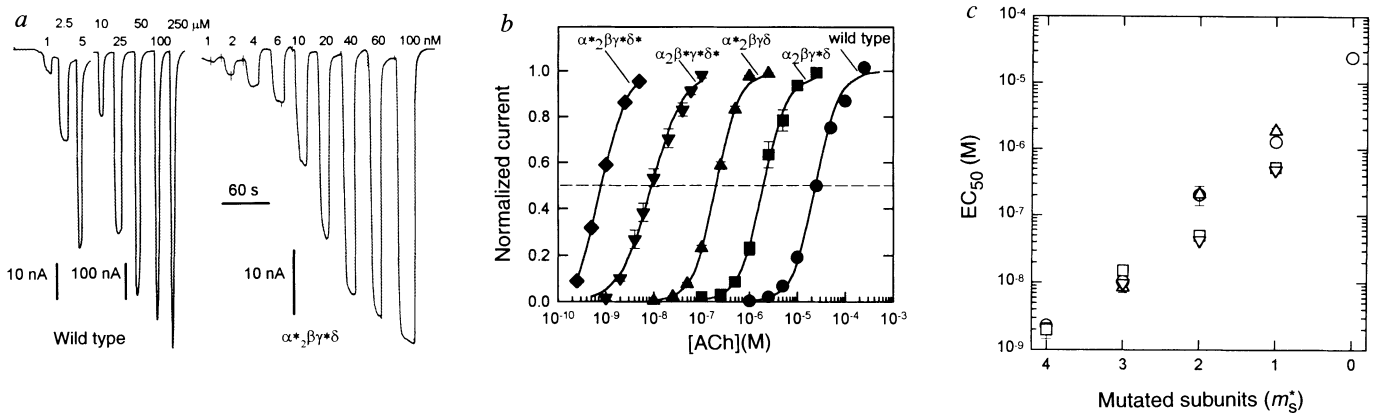

FIG. 1 *a*, Voltage-clamp currents from oocytes expressing mouse muscle acetylcholine receptors (AChR). Left, wild type; right, a subunit combination with 3 Leu 9′Ser mutations. ACh concentrations for each application are shown above the traces. *b*, Normalized average dose–response relations for exemplary combinations containing 0 (wild type) to 4 ($m_s^*=4$) Leu 9′Ser mutations: $\alpha_2\beta\gamma\delta$ (wild type), $\alpha\beta\gamma^*\delta$, $\alpha_2\beta^*\gamma^*\delta$, $\alpha_2^*\beta^*\gamma\delta$ and $\alpha_2^*\beta\gamma^*\delta^*$. The horizontal line at 0.5 represents the EC_{50} for each combination. The receptors with $m_s^*=4$ expressed rather low maximal current levels (20–100 nA). Each dose–response relation represents at least 5 oocytes from at least 2 batches. *c*, Relationship between number of mutated subunits and EC_{50} (logarithmic axis) for all the combinations measured (Table 1). Each symbol represents a distinct combination. S.e.ms are shown where they exceed the size of the symbols.

METHODS. Leucine-to-serine mutations were generated by site-directed mutagenesis using the Clontech Transformer site-directed mutagenesis kit (Palo Alto, CA) and confirmed by sequencing. mRNA was synthesized *in vitro* using the Megascript kit (Ambion, Austin, TX). pBluescript plasmids containing the AChR subunits were linearized and run-off transcripts prepared with T7 RNA polymerase Stage V–VI *Xenopus* oocytes were isolated and injected with 10–50 ng of mRNA in a stoichiometric ratio for $\alpha:\beta:\gamma:\delta$ of 2:1:1:1 (ref. 12). Before recording, oocytes were incubated at 18 °C in a modified Barth's solution supplemented with 50 μg ml^{-1} Gentamicin, 2.5 mM pyruvate and 0.6 mM theophylline. Electrophysiological recordings were carried out 2–4 days after injection. Membrane potential was held at −80 mV with a 2-electrode voltage-clamp circuit. Bath solutions contained 96 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$ and 5 mM HEPES, pH 7.5. Ca^{2+} was omitted from the bath solution and atropine (1 μM) was included to prevent activation of endogenous Ca^{2+}-activated Cl^- channels via muscarinic receptors. Individual dose–response relations were fitted to the Hill equation, $I/I_{max}=1/(1+\{EC_{50}/[A]\}^{n_H})$, where $[A]$ is the ACh concentration, EC_{50} is the ACh concentration giving half-maximal response, I_{max} is the maximal response, and n_H is the Hill coefficient.

Fig. 2). We analysed the responses assuming that (1) currents summed from independent populations of $\alpha_2^*\beta\gamma\delta$, $\alpha_2\beta\gamma\delta$ and $\alpha^*\alpha\beta\gamma\delta$ receptors, (2) receptors assembled equally well with α and with α*, and (3) the two possible subunit arrangements for $\alpha^*\alpha\beta\gamma\delta$ receptors had identical responses. The calculated EC_{50} values for $\alpha^*\alpha\beta\gamma\delta$ ranged from 1 to 2 μM, within the range observed for the other receptors with $m_s^*=1$. These results, and the observation that the $\alpha_2^*\beta\gamma\delta$ receptor has an EC_{50} in the range of other receptors with $m_s^*=2$, show that the αLeu 9′ residues do not occupy a privileged position in the gating process, despite the fact that agonist binds at least partly to the α-subunit.

TABLE 1 Dose–response relations for mouse muscle ACh receptors containing various numbers of mutated Leu 9′Ser subunits (m_s^*)

m_s^*	mRNA injections	EC_{50} (nM)	Hill coefficient
0	$\alpha_2\beta\gamma\delta$	24,010	1.68
1	$\alpha^*\alpha\beta\gamma\delta$	1,290	2.15 ± 0.22
	$\alpha_2\beta^*\gamma\delta$	531	2.03
	$\alpha_2\beta\gamma^*\delta$	1,910	1.82 ± 0.14
	$\alpha_2\beta\gamma\delta^*$	486	1.98
2	$\alpha_2^*\beta\gamma\delta$	202	1.81
	$\alpha_2\beta^*\gamma^*\delta$	49.7	1.64
	$\alpha_2\beta^*\gamma\delta^*$	208 ± 69	1.34
	$\alpha_2\beta\gamma^*\delta^*$	42.7	1.89
3	$\alpha_2^*\beta^*\gamma\delta$	10.3	1.44
	$\alpha_2^*\beta\gamma^*\delta$	15.1	1.61
	$\alpha_2^*\beta\gamma\delta^*$	8.4 ± 1.3	1.45
	$\alpha_2\beta^*\gamma^*\delta^*$	9.8 ± 1.3	1.30
4	$\alpha_2^*\beta^*\gamma\delta^*$	2.3	0.96
	$\alpha_2^*\beta\gamma^*\delta^*$	2.0 ± 0.6	1.02 ± 0.26
5	$\alpha_2^*\beta^*\gamma^*\delta^*$	<1	—

S.e.ms for EC_{50} were less than 10% of the mean, except where given; s.e.ms for Hill coefficient were less than 0.07, except where given. Responses for the $\alpha_2^*\beta^*\gamma^*\delta^*$ combination were too small for reliable measurements of EC_{50} or Hill coefficient.

In single-channel studies with the $\alpha_2^*\beta\gamma\delta$ receptor (Fig. 3), ACh evoked bursts of openings lasting hundreds of milliseconds, similar to records with the αLeu 9′Cys AChR (ref. 7) and much longer than bursts in the wild type at the same ACh concentration (ACh). Within a burst, however, there were many brief (time constant ~0.15 ms) closings, so that the longest compo-

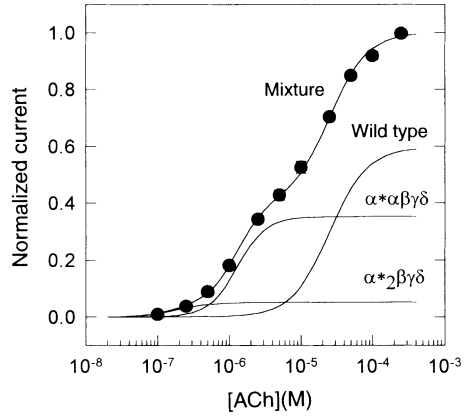

FIG. 2 Dose–response relation for $\alpha^*\alpha\beta\gamma\delta$, determined by injecting oocytes with a mixture of mRNA for α*, α, β, δ and δ subunits. The injected mRNA mole fraction $\alpha^*/(\alpha^*+\alpha)$ was 0.5. The dose–response relations were measured independently for $\alpha_2^*\beta\gamma\delta$ and for $\alpha_2\beta\gamma\delta$ in other oocytes from the same batch. The normalized currents for the mixture were expressed as the sum of dose–response relations for these 2 combinations plus a third relation for $\alpha\alpha^*\beta\gamma\delta$. With the assumptions given in the text, if the mole fraction of expressed α* subunit is a*, the proportion of receptors of each species is $(a^*)^2$, $(1-a^*)^2$, and $2a^*(1-a^*)$, respectively. The results fit best for $a^*=0.25$. The calculated dose–response relation for $\alpha^*\alpha\beta\gamma\delta$ is characterized by $EC_{50}=1.29$ μM and $n_H=2.15$.

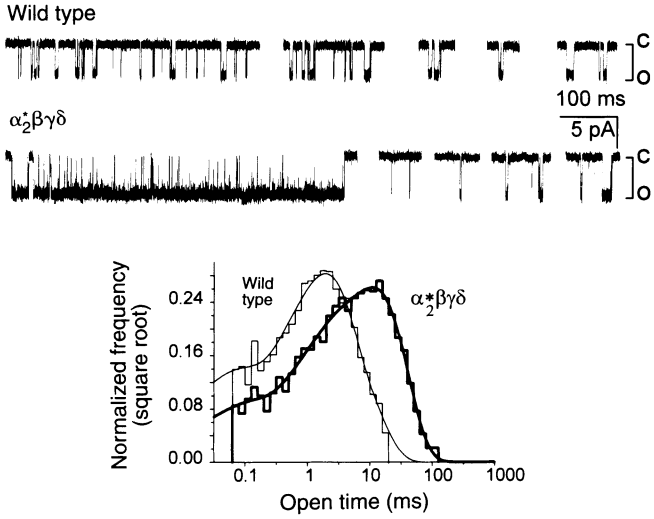

FIG. 3 Single-channel records from wild-type and $\alpha_2^*\beta\gamma\delta$ receptors. Outside-out patches were exposed to 25 μM and 200 nM ACh, respectively (~EC_{50} for both receptors) at −100 mV. The displayed traces were filtered at 5 kHz; gaps denote long closed times. The bottom panel shows open-time distributions. Smooth curves are fits to the time constants and normalized frequencies, as follows. Wild-type: 0.05 ms, 12%; 1.7 ms, 70%; 4.4 ms, 18%; $\alpha_2^*\beta\gamma\delta$: 0.08 ms, 5%; 1.9 ms, 18%; 11.4 ms, 77%. Bins have logarithmically increasing durations, and frequencies are plotted as square roots[13]. These procedures resolved event durations>60 μs.

METHODS. mRNA was generated from complementary DNA clones in pAMV (ref. 14). Bath and pipette solutions contained 100 mM KCl, 2 mM $MgCl_2$, 2 mM $BaCl_2$, 10 mM HEPES, 10 mM EGTA, 1 μM atropine, pH 7.4. Recordings were made with a GeneClamp 500/CV-5 instrument and analysed with pCLAMP software (Axon Instruments, Foster City, CA).

nent of open time was less than threefold longer for the $\alpha_2^*\beta\gamma\delta$ receptor than for the wild-type receptor. In a simple structural explanation of these data, mutant channels with two bound ACh molecules would remain open roughly as long as normal channels, then close, then rapidly reopen because the axial cluster of side chains that closes the channel[3] is less stable with Ser than with Leu. Other interpretations are possible[5]. Single-channel current amplitudes (~6 pA at −100 mV in symmetrical KCl solutions) differed by <10% among the receptors tested ($\alpha_2\beta\gamma\delta$, $\alpha_2^*\beta\gamma\delta$, $\alpha_2\beta\gamma^*\delta$, $\alpha_2^*\beta\gamma\delta^*$ and $\alpha_2\beta^*\gamma\delta$).

Average Hill coefficients for the groups with $m_s^* = 1$, 2 and 3 were 2.00 ± 0.07, 1.67 ± 0.12 and 1.45 ± 0.06 respectively (mean ±s.e.m.; $n > 20$; the Hill coefficients obtained for $m_s^* = 0$ and for $m_s^* = 4$ were not compared in this series because they could be unreliable owing to desensitization and to very small responses, respectively). This trend suggests that, for the mutated channels as for the wild type, the open state is more likely to be associated with the presence of two bound ACh molecules, but perhaps with an increasing contribution from unliganded or singly liganded receptors as m_s^* increases. Consistent with this idea, single-channel recordings showed a higher frequency of openings in the absence of ACh[8] for the $\alpha_2^*\beta\gamma\delta$ and $\alpha_2^*\beta\gamma\delta^*$ receptors than for the wild-type receptor (results not shown).

Receptors with $m_s^* = 0$ and 1 desensitized in the presence of ACh concentrations $\geq 0.2 \times EC_{50}$ and $\geq 1 \times EC_{50}$, respectively. Combinations with $m_s^* \geq 2$ showed no macroscopic desensitization for [ACh] at concentrations up to $5 \times EC_{50}$, with the exception of $\alpha_2^*\beta\gamma\delta^*$, which desensitized at [ACh] $\geq 3 \times EC_{50}$. These data agree with previous observations that Leu 9′ mutations of homooligomeric ACh and 5-HT_3 receptors reduce the ratio of desensitized to activated receptors[4,6].

That Leu 9′Ser mutations are nearly independent, equivalent, and multiplicative in their effect on the dose–response relations reinforces the general view that the five M2 domains move independently and symmetrically during AChR gating[3], although the energy for channel gating derives from contacts at only two binding sites. More specifically, our data support the suggestion that contacts involving each of the five Leu residues under consideration—one in each M2 region—play a key role in gating[3]. The 10-fold difference in EC_{50} for each mutation suggests that each contact contributes a free energy difference of ~1.4 kcal mol^{-1} to the ensemble of steps between binding of the receptor and opening of the channel.

Mutations at many M2 positions affect gating[4,5,9,10,15]. The suggestion that Leu 9′ plays a unique role in receptor function[3] led us to examine the consequences of mutating Leu 16′, which is also well conserved among AChR subunits and would lie on the same face of an α-helix as the 9′ position. $(\alpha\text{Leu } 16'\text{Ser})_2\beta\gamma\delta$ receptors had EC_{50} values of 320 nM, near that for the corresponding Leu 9′Ser mutation. However, Leu 16′Ser mutations in β- and γ-subunits had little effect by themselves on dose–response relations, and none of the non-αLeu 16′Ser subunits produced further shifts in the dose–response relation when combined with the αLeu 16′Ser subunit. Thus the symmetrical interactions involving the M2 domains do not extend to the 16′ position.

These data imply that the AChR and homologous receptors for other transmitters have not evolved to maximize either the affinity for transmitter or the open-state lifetime. Responses to low transmitter concentrations are inappropriate, given the high concentration of transmitter in the synaptic cleft at the start of transmission[11]; and long bursts of openings would vitiate rapid signal processing. Chemical synapses involving ligand-gated channels apparently evolved as exquisite electrochemical machines, specialized to function on a timescale of milliseconds and a distance scale of micrometres; and this optimization is evident even at the level of individual side chains on receptor proteins.

Note added in proof: Similar data have been obtained for Leu9′Thr mutations (G. Filatov and M. M. White *Molec. Pharmac.*, in the press). □

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