

## Online Methods

### *Whole-cell electrophysiological characterizations of the agonist-induced responses*

Rat  $\alpha 4$  and  $\beta 2$  mRNAs as well as mouse  $\alpha 1$ ,  $\beta 1$ (L9'S),  $\gamma$ , and  $\delta$  mRNAs were obtained from NotI linearizations of the expression vector pAMV, followed by *in vitro* transcription using the mMessage mMachine T7 kit (Ambion, Austin, TX). The mutations for each subunit were introduced according to the QuikChange mutagenesis protocol (Stratagene).

To express wild type neuronal ion channels,  $\alpha 4$ L9'A mRNA was co-injected with  $\beta 2$  mRNA at various ratios (total mRNA 10-25 ng/cell). Stage V-VI *Xenopus laevis* oocytes were injected and incubated at 18°C for 24-48 h (whole-cell recording) or 60-90 h (single-channel recording).

Agonist-induced currents were recorded in two-electrode voltage clamp mode using the OpusXpress 6000A (Molecular Devices Axon Instruments) at a holding potential of -60 mV. Agonists were prepared in  $\text{Ca}^{2+}$ -free ND96 solution and applied for 12 s followed by a 2 min wash with  $\text{Ca}^{2+}$ -free ND96 solution between each agonist application.

Acetylcholine chloride and (-)-nicotine tartrate were purchased from Sigma/Aldrich/RBI (St. Louis, MO). Dose-response data were obtained for  $\geq 6$  concentrations of agonist and for  $\geq 5$  oocytes. Mutants with  $I_{\text{max}}$  of  $\geq 100$  nA were defined as functional.  $\text{EC}_{50}$  and Hill coefficient were calculated by fitting the dose-response relation to the Hill equation. All data are reported as mean  $\pm$  SEM.

Voltage jump experiments were performed in the absence of ACh and also at  $\text{EC}_{50}$  concentration of ACh. The membrane potential was held at -60 mV, and stepped to 10 test potentials at 20 mV increments between +70 mV and -110 mV for 400 ms each. The voltage was then held for 600 ms at -60 mV holding potential between each episode. To isolate the ACh-induced currents, control traces ( $[\text{ACh}] = 0$ ) were subtracted from the steady-state amplitudes of the ACh-induced currents of the test pulses. Normalized I-V curves were generated using current amplitudes normalized to that at -110 mV. For each  $\alpha 4$ L9'A $\beta 2$  mutant, normalized  $I_{+70 \text{ mV}} \pm$  s.e.m. from  $\geq 5$  cells was reported.

### *Unnatural amino acid / $\alpha$ -hydroxy acid incorporation*

Nitroveratryloxycarbonyl (NVOC) protected cyanomethyl ester forms of unnatural amino acids and  $\alpha$ -hydroxythreonine cyanomethyl ester were synthesized, coupled to the dinucleotide dCA, and enzymatically ligated to 74-mer THG73 tRNA<sub>CUA</sub><sup>15</sup>. The unnatural amino acid-conjugated tRNA was deprotected by photolysis immediately prior to co-injection with mRNA containing the UAG mutation at the site of interest. Approximately 10-25 ng mRNA and 25 ng tRNA-amino acid or tRNA-hydroxy acid were injected into stage V–VI oocytes in a total volume of 70 nL. For unnatural amino acid mutagenesis experiments in the muscle-type receptor, the  $\alpha$ 1,  $\beta$ 1,  $\gamma$ , and  $\delta$  subunits were co-injected in a 10:1:1:1 ratio. All muscle-type receptors contained a L9'S mutation in the  $\beta$  subunit.

The fidelity of unnatural amino acid incorporation was confirmed at each site with a “wild type recovery” experiment and a “read-through/ reaminoacylation” test. In the “wild type recovery” experiment, UAG mutant mRNA was co-injected with tRNA charged with the amino acid that is present at this site in the wild type protein. Generation of receptors that were indistinguishable from the wild type protein indicated that the residue carried by the suppressor tRNA was successfully and exclusively integrated into the protein. In the “read-through/ reaminoacylation” test, the UAG mutant mRNA was introduced with (1) no tRNA, (2) tRNA THG73 that was not charged with any amino acid or (3) tRNA THG73 enzymatically ligated with dinucleotide dCA. Lack of currents in these experiments validated the reliability of the nonsense suppression experiments.

### *Single-channel characterization of $\alpha$ 4 $\beta$ 2*

Single-channel recording was performed in the cell-attached configuration on devitellinized *Xenopus laevis* oocytes at  $20 \pm 2^\circ\text{C}$  with a pipette potential of +100 mV, as described previously<sup>27</sup>. Pipettes were fabricated from thick-walled (I.D. = 0.80 mm, O.D. = 1.60 mm) KG-33 glass (Garner Glass Company, Claremont, CA) and coated with sylgard (World Precision Instruments, Sarasota, FL); they had resistances of 10-20 M $\Omega$ . The bath solution contained 120 mM KCl, 5 mM HEPES, 1 mM MgCl<sub>2</sub>, and 2 mM

CaCl<sub>2</sub>, pH = 7.4, so that the reversal potential for agonist-induced currents of devitellinized oocytes was ~0 mV, and the transmembrane potential of the patch was ~ -100mV. The pipette solution contained 100 mM KCl, 10 mM HEPES, 1 mM MgCl<sub>2</sub>, 10 mM K<sub>2</sub>EGTA, pH = 7.4 and was supplemented with the indicated concentrations of nicotine. Data were collected using a GeneClamp 500B amplifier (Axon Instruments, Union City, CA) at full bandwidth (50 kHz; 4-pole Bessel, -3 dB) with a CV-5 100 GU headstage. The signal was then low-pass filtered (Avens Signal Equipment, AP220, 8-pole Bessel, -3 dB 20 kHz) and sampled with a Digidata 1320A and Clampex 9.2 (Axon Instruments, Union City, CA) at 50 kHz. Only patches that showed no simultaneous activations were analyzed. For each mutant, this was  $\geq 3$  patches from oocytes from 2 different donor frogs. Data were filtered offline (Gaussian, -3 dB, 5 kHz) and electrical interference at harmonics of 60 Hz was removed if necessary. Event transitions were detected with Clampfit 9.2 (single-channel search). A dead time,  $t_d$ , of 100  $\mu$ s was applied to all events. Open and closed dwell time histograms were generated as described previously<sup>31</sup> and fitted using the predefined log-transformed exponential probability density function in Clampfit 9.2. To delineate clusters, a critical closed duration,  $\tau_{crit}$ , was defined using two separate methods (Supplementary Discussion); in both cases closed dwell times longer than  $\tau_{crit}$  were excluded from further analysis. Sojourns to a subconductance state (<85% of the full conductance level) were treated as closed and accounted for <10% of the total openings in all records. The time-average probability that the channel is open ( $P_{open}$ ) was calculated as the total open time divided by the revised total closed time.

31. McManus, O.B., Blatz, A.L. & Magleby, K.L. Sampling, Log Binning, Fitting, and Plotting Durations of Open-and-Shut Intervals from Single Channels and the Effects of Noise. *Pflugers Archiv-Eur. J. of Physiol.* **410**, 530-553 (1987).