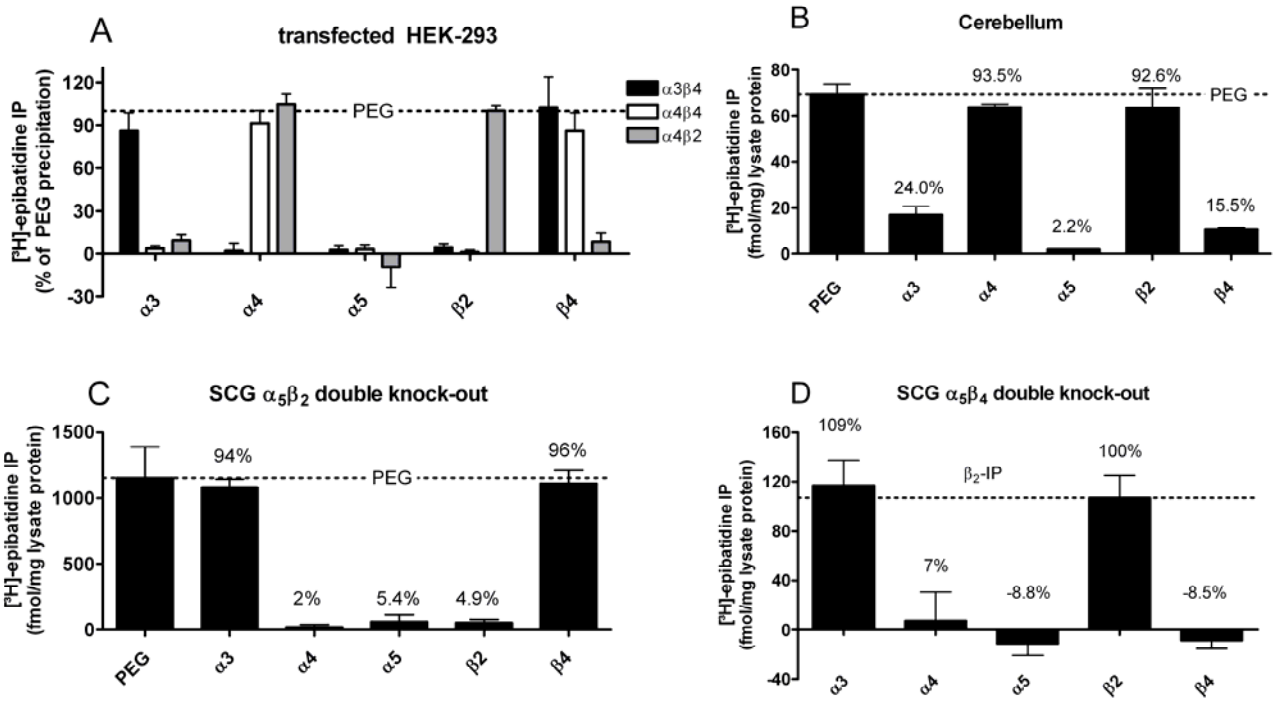
Supplemental material

<u>Supplemental Fig. 1:</u> Confirmation of the subunit specificity of antibodies by recombinant and native nAChRs



Antibodies used for immunoprecipitating solubilized nAChRs are indicated at the abscissa. The dotted lines in panels A, B, and C indicate specific [³H]-epibatidine binding as measured by polyethyleneglycol (PEG) precipitation. PEG precipitates all proteins in solution and thus serves as a reference for 100 % precipitated, radioligand-labeled receptors (Jechlinger *et al.*, 1998). In panel D, immunoprecipitation by indicated antibodies was compared with results obtained by the β2 antibody (dotted line). Nonspecific binding was measured in the presence of 300 µM nicotine and subtracted from overall to obtain specific binding. Data are means ± SEM of specific [³H]-epibatidine binding of 4-5 independent experiments, each performed in triplicate (panels A, B and C) or duplicate (panel D).

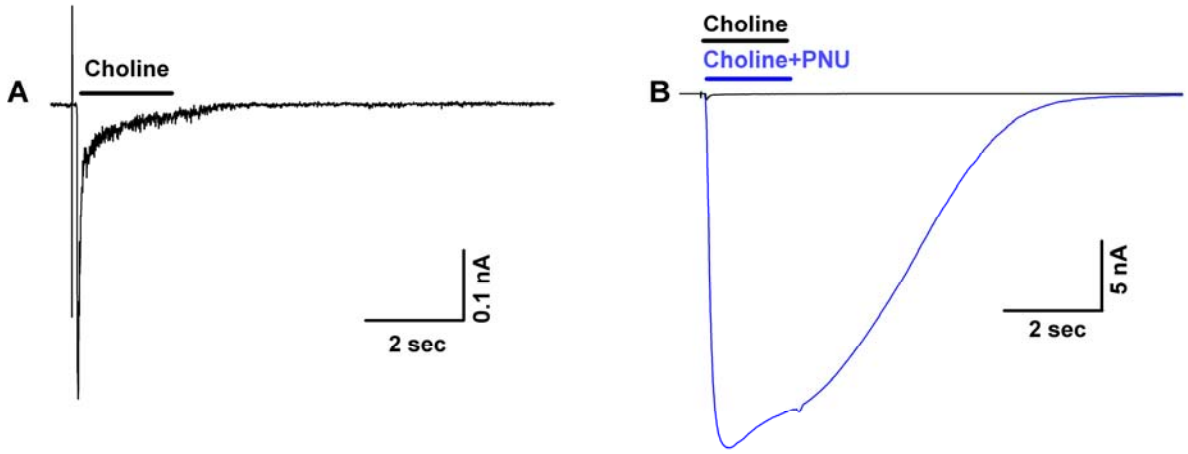
Methods

Receptors were solubilized from membrane preparations from transfected HEK-293 cells (panel A), cerebellum (panel B), α5β2 double knock out mice (panel C), or α5β4 double knock out mice (panel D) as described in Methods (*"Immunoprecipitation of [³H]-epibatidine labeled receptors"*). Total

[³H]-epibatidine binding in panels A, B, and C was measured using a polyethyleneglycol (PEG) precipitation assay (Polson *et al.*, 1964) modified as described (Jechlinger *et al.*, 1998). 150 µl of solubilized receptors were incubated with 1nM [³H]-epibatidine in a buffer containing 50 mM Tris/HCl pH = 7.4, 0.05 % bovine serum albumin, and 15 % PEG-6000 at a final volume of 500 µl for 2 hours at room temperature. Nonspecific binding was determined by the presence of 300 µM nicotine and subtracted from overall to obtain specific binding. Precipitated receptors were separated from free ligand by vacuum filtration over GF/B glass-microfiber filters and subjected to liquid scintillation counting. Additional details on techniques for the immunoprecipitation of receptors with our subunit-specific antibodies are described in Methods.

Transfection protocol: Human embryonic kidney 293 (HEK-293) cells from American Type culture collection (Rockville, MD) were maintained in Dulbecco's Modified Eagle Medium (D-MEM, high glucose including GlutaMAX) supplemented with 100 units/ml penicillin and 100 µg/ml streptomycin, non-essential amino acids (all components from Invitrogen-Gibco) and 10% FCS at 5% CO_2 and 36.5° C. 1.8 x 10^6 cells were seeded onto a 10 cm tissue culture dish (Nunc) and transfected with 20 µg plasmid DNA via the calcium phosphate precipitation technique (Chen & Okayama, 1987). Cells were harvested 44 hr after transfection. The generation of nAChR constructs (mouse subunit cDNA in pCI expression vector) has been described previously (Putz *et al.*, 2008).

Supplemental Fig. 2: Effect of PNU-120596 on the current induced by 10 mM choline in a freshly dissociated E14 chick ciliary ganglion neuron



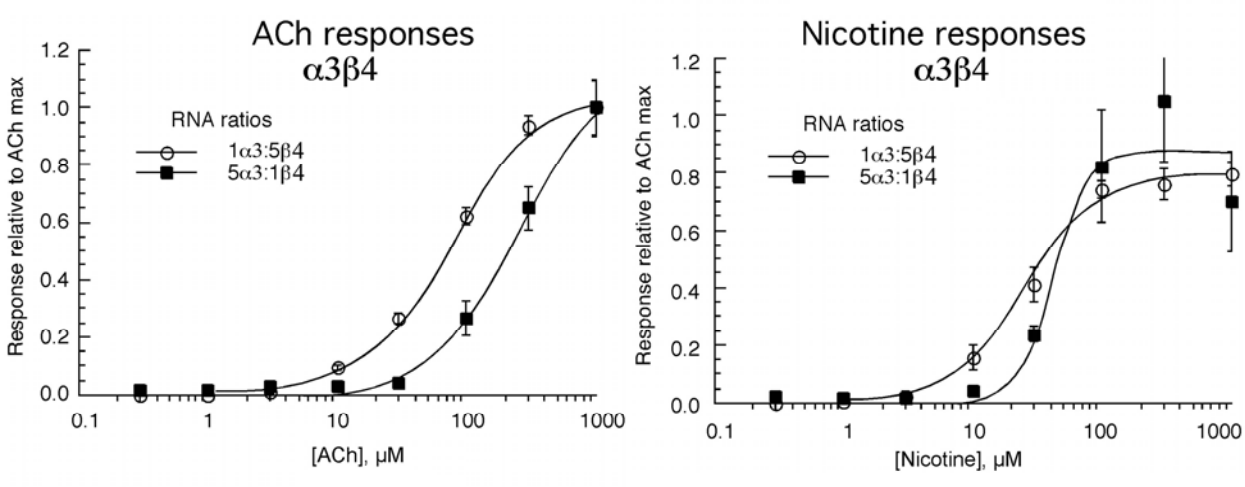
A. Application of 10 mM choline (bar) induces a fast desensitizing current, typical for the activation of α7-1 receptors

B. Pre-treatment for 10 sec with 10 μM PNU-120596, followed by the co-application of 10 mM choline and 10 μM PNU-120596 (bar, choline + PNU, blue current trace) resulted in a dramatic enlargement of the current induced by 10 mM choline alone (trace taken from panel A, with calibration adjusted to fit the size of the enlargement of the current by PNU-120596.

Methods:

Ciliary ganglia were dissected from E14 White Leghorn chick embryos sacrificed by decapitation. The enzymatic treatment and trituration of ganglia, and the plating of cells were according to (Conroy *et al.*, 2003). Whole cell patch clamp recordings were done the same day, with external (bathing) and internal (pipette) solution as described in Conroy et al., 2003. Substances were dissolved in bathing solution and applied via a DAD12 superfusion system (ALA Scientific Instruments).

<u>Supplemental Fig 3:</u> The pharmacological properties of α3β4 receptors are affected by their stoichiometry



RNA transcripts were prepared from human nAChR receptor clones (gift of Dr. Jon Lindstrom, University of Pennsylvania) in vitro using the appropriate mMessage mMachine kit from Ambion (Austin TX). Stage 5 oocytes were isolated and injected with α3 and β4 cRNA in an either 1:5 (β4 excess) or 5:1 ratio (α3 excess). The electrophysiological recordings were conducted 2 to 5 days after injection. For experimental details see (Williams *et al.*, 2009). Note that potencies of both ACh and nicotine decreased by enhancing the presence of α3 at the expense of β4 (EC_{50} ACh 1α3:5β4: 71μM; EC_{50} ACh 5α3:1β4: 263μM; EC_{50} Nic 1α3:5β4: 28μM; EC_{50} Nic 5α3:1β4: 41μM; n = $\geq$ 4 oocytes for each concentration response curve). When applied to our observations in the SCG, the first (indicated by arrows) and the second peak in Fig. 6A2 could thus be due to 2(α3)3(β4) and 3(α3)2(β4), respectively. Note that in the mouse SCG, α3 levels exceed β4 mRNA by about a factor of 2 (Putz *et al.*, 2008).

Reference List to the Supplemental Material

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