

# The E Loop of the Transmitter Binding Site Is a Key Determinant of the Modulatory Effects of Physostigmine on Neuronal Nicotinic $\alpha 4\beta 2$ Receptors

Xiaochun Jin, Megan M. McCollum, Allison L. Germann, Gustav Akk, and Joe Henry Steinbach

Department of Anesthesiology (X.J., M.M.C., A.L.G., G.A., J.H.S.) and Taylor Family Institute for Innovative Psychiatric Research (G.A., J.H.S.), Washington University School of Medicine, St. Louis, Missouri

Received August 11, 2016; accepted November 23, 2016

## ABSTRACT

Physostigmine is a well known inhibitor of acetylcholinesterase, which can also activate, potentiate, and inhibit acetylcholine receptors, including neuronal nicotinic receptors comprising  $\alpha 4$  and  $\beta 2$  subunits. We have found that the two stoichiometric forms of this receptor differ in the effects of physostigmine. The form containing three copies of  $\alpha 4$  and two of  $\beta 2$  was potentiated at low concentrations of acetylcholine chloride (ACh) and physostigmine, whereas the form containing two copies of  $\alpha 4$  and three of  $\beta 2$  was inhibited. Chimeric constructs of subunits indicated that the presence of inhibition or potentiation depended on the source of the extracellular ligand binding domain of the subunit. Further sets of chimeric constructs demonstrated that a portion of the ACh binding domain, the E loop, is a key determinant. Transferring

the E loop from the  $\beta 2$  subunit to the  $\alpha 4$  subunit resulted in strong inhibition, whereas the reciprocal transfer reduced inhibition. To control the number and position of the incorporated chimeric subunits, we expressed chimeric constructs with subunit dimers. Surprisingly, incorporation of a subunit with an altered E loop had similar effects whether it contributed either to an intersubunit interface containing a canonical ACh binding site or to an alternative interface. The observation that the  $\alpha 4$  E loop is involved suggests that physostigmine interacts with regions of subunits that contribute to the ACh binding site, whereas the lack of interface specificity indicates that interaction with a particular ACh binding site is not the critical factor.

## Introduction

Physostigmine is best known as an inhibitor of the enzyme acetylcholinesterase (Triggle et al., 1998). In addition to its action on acetylcholinesterase, physostigmine was one of the first drugs characterized as an allosteric activator of neuronal nicotinic receptors (Storch et al., 1995) and as a potentiating drug for receptors containing the nicotinic  $\alpha 4$  subunit (Zwart et al., 2000; Smulders et al., 2005). There has been recent interest in the ability of physostigmine and other drugs to act as “allosteric potentiating ligands” for neuronal nicotinic receptors, due to possible therapeutic uses in, for example, treatment of dementias including Alzheimer disease (Triggle et al., 1998; Maelicke and Albuquerque, 2000). Physostigmine can also inhibit nicotinic receptors, an action thought to result from the block of open channels (Smulders et al., 2005).

We examined the actions of physostigmine on the neuronal nicotinic receptor containing the  $\alpha 4$  and  $\beta 2$  subunits. This is the most prevalent type of nicotinic receptor in the brain (Dani and Bertrand, 2007; Gotti et al., 2007), where nicotinic receptors are thought to play an important, if subtle, role to modulate the release of transmitters (McGehee and Role, 1995; Dani and

Bertrand, 2007). The  $\alpha 4\beta 2$  receptors can assemble in two functional stoichiometries, one containing three copies of  $\alpha 4$  and two copies of  $\beta 2$  and the other containing two copies of  $\alpha 4$  and three of  $\beta 2$ . These two stoichiometric forms have distinct pharmacological properties, including the potency of acetylcholine chloride (ACh) [2-acetoxy-*N,N,N*-trimethylethanaminium; Chemical Abstracts Service (CAS) number 51-84-3] at activating the receptor (Zwart and Vijverberg, 1998; Zhou et al., 2003; Moroni et al., 2006). Previous studies of the action of physostigmine had used the form containing three copies of the  $\alpha 4$  subunit (Zwart et al., 2000; Smulders et al., 2005) and found that low concentrations of physostigmine potentiated the response to low concentrations of acetylcholine. However, we reported that physostigmine potentiated the form containing three copies of  $\alpha 4$ , while inhibiting the form containing two copies (Jin et al., 2014). These results suggested that the actions of physostigmine may be more complex than appreciated.

To further examine the subunit dependence, we studied the effects of physostigmine on receptors containing various chimeric constructs, expressed with concatemeric constructs to allow us to control the number and position of the chimeric subunits in the assembled receptor. We found that the potentiating and inhibiting actions of physostigmine map to the amino-terminal, extracellular domain of the subunits, where the key determinant is the “E loop” that forms part of

This research was supported by the National Institutes of Health National Institute of Neurological Disorders and Stroke [Grant R01 NS22356].  
dx.doi.org/10.1124/mol.116.106484.

**ABBREVIATIONS:** ACh, acetylcholine chloride; CAS, Chemical Abstracts Service; cRNA, complementary RNA; TM, transmembrane.

the complementary side of the ACh binding site. When the E loop of the  $\beta 2$  subunit was transferred to the  $\alpha 4$  subunit, potentiation was lost and physostigmine inhibition was strong. Transferring the E loop from the  $\alpha 4$  to the  $\beta 2$  subunit reduced the extent of inhibition but did not confer potentiation. Surprisingly, the position of the transferred E loop does not appear to be critical: similar effects were seen when the transferred E loop was present at an interface formed by two  $\alpha 4$  subunits ( $\alpha 4/\alpha 4^*$ , where the asterisk indicates a subunit with altered sequence) or a  $\beta 2$  and an  $\alpha 4$  subunit ( $\beta 2/\alpha 4^*$ ).

## Materials and Methods

**Constructs and Expression.** We used human  $\alpha 4$  (NM000744) and  $\beta 2$  (NM000748) subunits kindly provided by Dr. J. Lindstrom (University of Pennsylvania, Philadelphia, PA). The generation of the dimeric constructs  $\alpha 4\text{-}\beta 2$  and  $\beta 2\text{-}\alpha 4$  was described previously (Jin and Steinbach, 2011). The pentameric constructs  $\beta 2\text{-}\alpha 4\text{-}\beta 2\text{-}\alpha 4\text{-}\alpha 4$  and  $\beta 2\text{-}\alpha 4\text{-}\beta 2\text{-}\alpha 4\text{-}\beta 2$  have also been described (Carbone et al., 2009). All constructs were fully sequenced through the subunit sequence. In the pentamers, subunits were excised using the appropriate restriction enzymes and sequenced independently to verify that each copy was intact. RNA was synthesized using the mMessage mMachine T7 kit (Ambion, Austin, TX). The concentration of RNA was estimated from the optical density at 260 nm.

*Xenopus* oocytes were prepared in Dr. C. Zorumski's laboratory (Washington University, St. Louis, MO) using an approved protocol. Oocytes were injected with 12–20 ng complementary RNA (cRNA) in a volume of 18–23 nL. Oocytes were maintained at 18°C for 2–7 days before physiologic study.

**Electrophysiology.** Standard methods were used for two-electrode voltage clamp of *Xenopus* oocytes (Jin and Steinbach, 2011), using an OC-725C voltage clamp (Warner Instruments, Hamden, CT). Oocytes were clamped at  $-50$  mV unless noted otherwise, and all recordings were made at room temperature (23–25°C). Currents were filtered at 20 Hz, then digitized at 50 Hz (Digidata 1200 interface; Molecular Devices, Sunnyvale, CA) and stored using pClamp 8.0 (Molecular Devices). Transients were analyzed with Clampfit (Molecular Devices). Oocyte recordings were performed in a small chamber that was continuously perfused with external solution. Drug applications were made using a manually controlled perfusion system. The external solution contained 96 mM NaCl, 2 mM KCl, 1.8 mM BaCl<sub>2</sub>, 1 mM MgCl<sub>2</sub>, and 10 mM HEPES, pH 7.3. External Ca<sup>2+</sup> was replaced with Ba<sup>2+</sup> to avoid activation of Ca<sup>2+</sup>-activated channels. We did not use atropine to block muscarinic receptors, because it potentiates  $\alpha 4\beta 2$  receptors (see *Results* and Zwart and Vijverberg, 1997). Occasional oocytes showed delayed responses to ACh; these oocytes were not studied.

The concentration-response relationship for activation by ACh was characterized for data from each cell using nonlinear regression in SigmaPlot (Systat Software, San Jose, CA) by fitting the following Hill equation:

$$\left( Y([\text{ACh}]) = Y_{\max} \left( \frac{1}{1 + (\text{EC}_{50}/[\text{ACh}])^n} \right) \right),$$

where  $Y$  is the response to a concentration of ACh,  $Y_{\max}$  is the maximal response,  $\text{EC}_{50}$  is the concentration producing half-maximal activation, and  $n$  is the Hill coefficient. Concentration-response data were collected for an individual cell, and data were normalized to the response to 1 mM ACh. The fit was rejected if the estimated error in any fit parameter was greater than 60% of the fit value, and all parameter estimates for that fit were discarded. The relationship was analyzed for each cell and then overall mean values were calculated for oocytes injected with that set of constructs.

Potentiation by physostigmine and other modulators is strongest for low concentrations of ACh (Smulders et al., 2005). Since the  $\text{EC}_{50}$

for activation by ACh depends on the subunit combinations expressed (see *Results*), a low concentration of ACh, chosen to be able to evoke less than 20% of the maximal current, was used. Each oocyte was tested with 1 mM ACh to estimate the maximal response. To examine the effects of modulators, the low concentration of ACh was applied. After the response to ACh had reached a stable level, the application was switched to ACh plus modulator. The application was then switched to bathing solution, followed by repeat of the control low concentration of ACh. The relative response in the presence of drug to that in the absence of drug was then calculated. Drug was not preapplied. ACh or ACh plus drug was applied for 10–20 seconds, until a response was stable, and applications were separated by 3 to 4 minutes to allow full washout.

Modulators were used at concentrations of 10–30  $\mu\text{M}$  physostigmine (most often 15  $\mu\text{M}$ ), 100  $\mu\text{M}$  atropine, 0.5 and 10  $\mu\text{M}$  galantamine, and 100  $\mu\text{M}$  Zn<sup>2+</sup> unless otherwise stated.

To examine the voltage dependence of inhibition by physostigmine, cells were held at  $-50$  mV, then the potential was changed to  $-100$  mV and a voltage ramp from  $-100$  to  $+40$  mV was applied over 4 seconds (approximately 35 mV/s). Ramps were collected in the absence of ACh or physostigmine (background), then in the presence of 0.3  $\mu\text{M}$  ACh alone and in the presence of 0.3  $\mu\text{M}$  ACh plus 15  $\mu\text{M}$  physostigmine. The ramp currents in the presence of drug were corrected by subtracting the background ramp current, then the ramp current with ACh plus physostigmine was normalized to the current with ACh alone to estimate the voltage dependence of inhibition. At potentials more positive than about  $-50$  mV, the responses were quite small and the ratio showed a marked increase in variability.

Values are presented as arithmetic means  $\pm$  S.E.M. (number of observations). The ability of a given receptor to be potentiated was assessed by a one-sample  $t$  test of the potentiation ratio to 1 (no effect). Comparison of potentiation between receptors was assessed by the  $t$  test (two-tailed with unequal variance). Comparisons among multiple types of receptors were done by analysis of variance with Dunnett or Bonferroni correction, as described in the *Results*. Fits of concentration-response relationships were performed using SigmaPlot (Systat Software, Inc.). Statistical tests were performed using Excel (Microsoft, Redmond, WA) or STATA (StataCorp LP, College Station, TX) software.

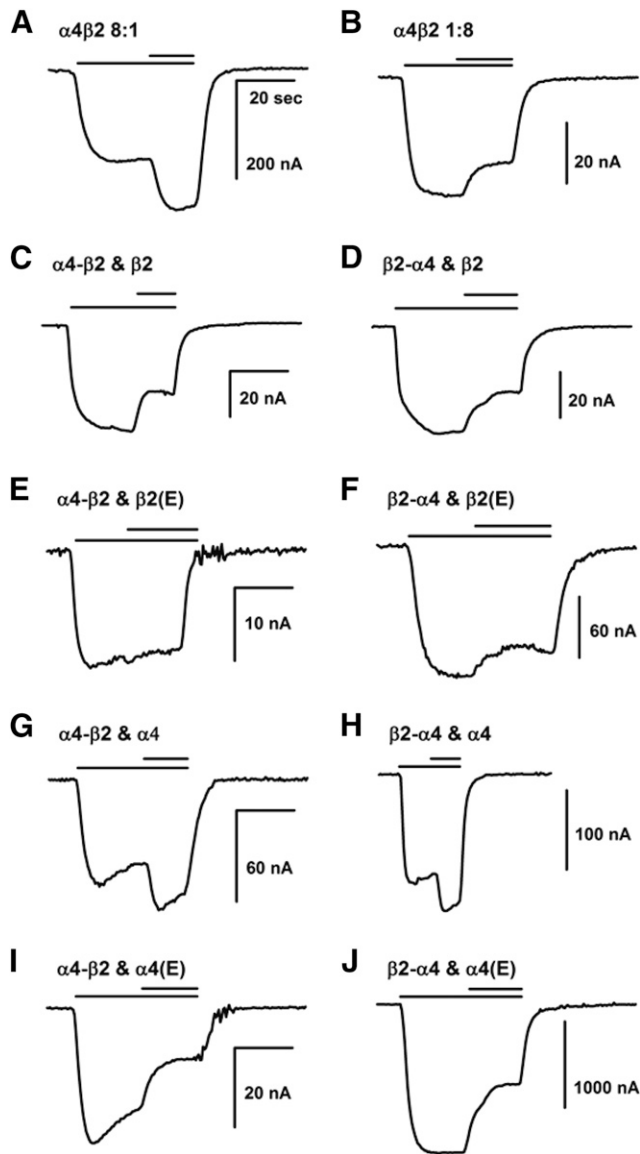
**Drugs.** ACh (CAS 60-31-1) was purchased from Sigma-Aldrich (St. Louis, MO). ACh was prepared as a 500-mM stock solution in bath solution and stored frozen at  $-20^\circ\text{C}$ . Physostigmine hemisulfate (physostigmine; CAS 64-47-1) was purchased from Tocris (Ellisville, MO). Physostigmine was prepared as a 10-mM stock in deionized water and stored frozen at  $-20^\circ\text{C}$ . Atropine (atropine sulfate monohydrate; CAS 5908-99-6) was obtained from Sigma-Aldrich and prepared as a 10-mM stock in deionized water, stored frozen at  $-20^\circ\text{C}$ . Galantamine hydrobromide (CAS 1953-04-4) was obtained from Tocris and prepared as a 10-mM stock in deionized water, stored frozen at  $-20^\circ\text{C}$ . Zinc chloride (CAS 7646-85-7) was obtained from Sigma-Aldrich and prepared as a 2-mM stock in bath solution, stored at 4°C. Working solutions were prepared on the day of experiments.

## Results

**Physostigmine Potentiates Receptors Containing Three Copies of  $\alpha 4$  and Inhibits Receptors Containing Three Copies of  $\beta 2$ .** Physostigmine can potentiate activation of nicotinic receptors containing the  $\alpha 4$  and  $\beta 2$  subunits when both ACh and physostigmine are applied at low concentrations, as reported previously (Zwart et al., 2000; Smulders et al., 2005). These studies were performed using expression conditions in which the majority of the receptor population was the stoichiometric subtype containing three copies of the  $\alpha 4$  subunit and two of the  $\beta 2$  subunit. We confirmed that responses of receptors containing three copies of  $\alpha 4$  were well potentiated by injecting oocytes with cRNA at a ratio of 8:1  $\alpha 4/\beta 2$  (Fig. 1A).

In contrast, when we repeated the applications on receptors largely of the subtype containing two copies of  $\alpha 4$  and three of  $\beta 2$ , we found that physostigmine was inhibitory to responses elicited by low concentrations of ACh (Fig. 1B). The data are summarized in Table 1.

The dependence of the effects on the concentration of physostigmine was flat between 10 and 30  $\mu\text{M}$  (Fig. 2), as



**Fig. 1.** Effect of transplanting the E loop between the  $\alpha 4$  and  $\beta 2$  subunits. Traces are shown from oocytes when ACh was initially applied alone and then the perfusion was switched to ACh + physostigmine (ACh alone application indicated by the lower line above the trace, and the time of the application of both ACh + physostigmine shown by the upper line). The constructs injected are shown above the trace (constructs are indicated with & separating the independent constructs, e.g.  $\alpha 4\beta 2$  8:1 indicates that free subunits were injected at a ratio of 8:1:: $\alpha 4\beta 2$ , while  $\alpha 4\text{-}\beta 2$  indicates that the  $\alpha 4\text{-}\beta 2$  dimer was injected with free  $\alpha 4$  subunit); Panel A:  $\alpha 4\beta 2$  8:1; B  $\alpha 4\beta 2$  1:8; C  $\alpha 4\text{-}\beta 2$  &  $\beta 2$ ; D  $\beta 2\text{-}\alpha 4$  &  $\beta 2$ ; E  $\alpha 4\text{-}\beta 2$  &  $\beta 2(\text{E})$ ; F  $\beta 2\text{-}\alpha 4$  &  $\beta 2(\text{E})$ ; G  $\alpha 4\text{-}\beta 2$  &  $\alpha 4$ ; H  $\beta 2\text{-}\alpha 4$  &  $\alpha 4$ ; I  $\alpha 4\text{-}\beta 2$  &  $\alpha 4(\text{E})$ ; J  $\beta 2\text{-}\alpha 4$  &  $\alpha 4(\text{E})$ . The horizontal bar shows 20 sec for all traces while the vertical bar shows the current calibration for each trace. The ACh concentration was adjusted to result in a response of less than 20% of the maximal response for that oocyte. Concentrations used were: A 1  $\mu\text{M}$  ACh; B 0.3  $\mu\text{M}$ ; C 0.3  $\mu\text{M}$ ; D 0.3  $\mu\text{M}$ ; E 0.3  $\mu\text{M}$ ; F 0.1  $\mu\text{M}$ ; G 1  $\mu\text{M}$ ; H 1  $\mu\text{M}$ ; I 0.3  $\mu\text{M}$ ; J 0.3  $\mu\text{M}$ . 15  $\mu\text{M}$  physostigmine was used for all traces.

reported previously for potentiation (Smulders et al., 2005). The responses of oocytes injected with  $\alpha 4$  and  $\beta 2$  cRNA at a 1:1 ratio were similar to that reported previously showing a broad plateau of potentiation between 10 and 30  $\mu\text{M}$  followed by a change to inhibition at 100  $\mu\text{M}$  physostigmine (Smulders et al., 2005), although the extent of potentiation was lower than reported. Potentiation was greater for oocytes injected at an 8:1 ratio. Inhibition of responses from oocytes injected at a 1:8 ratio appeared to be concentration independent in this concentration range, which suggests that inhibition is only partial even at saturating concentrations.

Previous work has shown that inhibition by higher concentrations of physostigmine is strongly voltage dependent (Zwart et al., 2000). However, the inhibition produced by 15  $\mu\text{M}$  physostigmine on responses from oocytes injected at a 1:8 ratio was only weakly voltage dependent between  $-50$  and  $-100$  mV (Fig. 2).

A residue in the  $\alpha 1$  subunit of *Torpedo* nicotinic receptors can be labeled by physostigmine ( $\alpha 1\text{K125}$ ; Schrattenholz et al., 1993) and has been proposed to form part of the physostigmine binding site. However, mutation of the homologous residue in the  $\alpha 4$  subunit had no effect on potentiation.  $\alpha 4\text{K130Q}$  expressed with  $\beta 2$  at an 8:1 ratio had an  $\text{EC}_{50}$  for ACh of  $93 \pm 15$   $\mu\text{M}$  ( $n = 5$  cells) and potentiation by physostigmine of  $1.4 \pm 0.1$  ( $n = 7$ ), which did not differ significantly from wild-type values (see Table 1). This agrees with a more recent photolabeling study that found no incorporation of physostigmine at  $\alpha 1\text{K125}$  (Hamouda et al., 2013), and the finding that  $\alpha 1\text{K125Q}$  and  $\alpha 1\text{K125E}$  mutations do not affect activation of the muscle-type receptor by physostigmine (Militante et al., 2008).

**Design of Studies of Chimeric Subunits.** We wanted to test the consequences of alterations in the sequences of particular subunits. To do this, we expressed chimeric subunits with dimeric constructs composed of an  $\alpha 4$  and a  $\beta 2$  linked together (Fig. 3). We wished to control the position of the free (chimeric) subunit in the pentameric receptor to obtain insights into the possible role for an effect at a particular interface in the receptor: canonical  $\alpha 4/\beta 2$  or alternative  $\alpha 4/\alpha 4$  or  $\beta 2/\alpha 4$  interfaces (Seo et al., 2009; Harpsøe et al., 2011; Mazzaferro et al., 2011; Lucero et al., 2016).

The dimers assemble in a clockwise fashion (Jin and Steinbach, 2011), so the  $\beta 2\text{-}\alpha 4$  dimer assembles to place an  $\alpha 4$  clockwise from the  $\beta 2$  in the pentamer (Fig. 3). In this case, the two subunits in the dimer generate an internal  $\alpha 4/\beta 2$  interface that forms a canonical ACh binding site, with the positive side contributed by  $\alpha 4$  and the negative side by  $\beta 2$ . This binding site is located within the dimer, and the fifth, free subunit occupies the nonbinding position in the pentamer and contributes to  $\beta 2/X$  and  $X/\alpha 4$  interfaces (where X indicates the surface contributed by the free subunit). In contrast, the  $\alpha 4\text{-}\beta 2$  dimer forms one canonical ACh binding site between the  $\alpha 4$  subunit of one dimer and the  $\beta 2$  of the adjacent dimer and the second canonical site is formed between a dimer and the free subunit (either  $\alpha 4/X$  or  $X/\beta 2$ ; see Fig. 3).

**Role of the Membrane-Spanning Region.** An initial thought was that inhibition by physostigmine might reflect an increased sensitivity to open-channel block due to the differences in sequence of the channel-lining regions contributed by the second transmembrane (TM) domains of the  $\alpha 4$  and  $\beta 2$  subunits. Accordingly, we constructed chimeras in which the regions forming the major portion of the ion channel (the first to third TM regions, TM1–TM3) were exchanged between the subunits. When the chimeras were expressed with the  $\beta 2\text{-}\alpha 4$

TABLE 1

The effect of physostigmine depends on the subunit stoichiometry

Subunits injected	Response to 1 mM ACh			ACh EC <sub>50</sub>			Response to physostigmine		
	Mean $\pm$ S.E. <i>nA</i>	No. of Cells	<i>P</i> value <sup>a</sup>	Mean $\pm$ S.E. $\mu$ M	No. of Cells	<i>P</i> value <sup>a</sup>	Mean $\pm$ S.E.	No. of Cells	<i>P</i> value <sup>b</sup>
$\alpha$ & $\beta$ 8:1	-13042 $\pm$ 1303	51	—	132 $\pm$ 10	33	—	1.42 $\pm$ 0.04	38	—, <0.001
$\alpha$ - $\beta$ & $\alpha$	-7238 $\pm$ 1179	68	0.004	78 $\pm$ 5	47	<0.001	1.51 $\pm$ 0.08	25	0.3, <0.001
$\beta$ - $\alpha$ & $\alpha$	-14421 $\pm$ 1081	94	0.6	121 $\pm$ 10	59	0.6	1.38 $\pm$ 0.03	51	0.7, <0.001
$\alpha$ & $\beta$ 1:8	-1029 $\pm$ 193	47	—	4.5 $\pm$ 0.6	20	—	0.69 $\pm$ 0.03	15	—, <0.001
$\alpha$ - $\beta$ & $\beta$	-341 $\pm$ 98	32	0.01	2.9 $\pm$ 0.5	14	0.3	0.78 $\pm$ 0.07	10	0.6, 0.01
$\beta$ - $\alpha$ & $\beta$	-1007 $\pm$ 116	91	1.0	3.7 $\pm$ 0.5	57	0.5	0.72 $\pm$ 0.05	28	0.9, <0.001
$\alpha$ - $\beta$	-761 $\pm$ 136	45	—	90 $\pm$ 11	30	—	1.02 $\pm$ 0.05	19	—, 0.7
$\beta$ - $\alpha$	-4837 $\pm$ 738	88	—	105 $\pm$ 12	55	—	1.00 $\pm$ 0.07	23	—, 1.0

Subunits are shown by the combination injected; for example,  $\alpha$ & $\beta$  8:1 indicates that free subunits were injected at a ratio of 8:1: $\alpha$ : $\beta$ 2, whereas  $\alpha$ - $\beta$ & $\alpha$  indicates that the  $\alpha$ 4- $\beta$ 2 dimer was injected with a free  $\alpha$ 4 subunit (1:1 ratio). Means  $\pm$  S.E. and the number of cells are presented for the response to 1 mM ACh, for the concentration of ACh producing a half-maximal response (EC<sub>50</sub>) and for the ratio of the response to a low concentration of ACh in the presence of physostigmine to the response of the same cell to ACh in the absence of physostigmine. Data for dimers injected in the absence of a free subunit are given in the last two rows, for comparison. Note that responses can be seen when dimers alone are injected, but the pharmacology of the responses differs from that seen when a free subunit is also injected demonstrating the presence of the free subunit in the pentamers (see also Jin and Steinbach, 2011). Dashes indicate that that particular subunit combination was the comparison value for the *P* computation.

<sup>a</sup>*P* values give the significance of the difference in value to that of the first entry in the set (one-way analysis of variance with Dunnett correction).

<sup>b</sup>The first *P* value is the significance of the difference to the first entry in the set, whereas the second value is the difference of the potentiation to a value of 1 (i.e., no effect; one-sample *t* test). Dashes indicate that that particular subunit combination was the comparison value for the *P* computation.

dimeric construct, activation by ACh showed the EC<sub>50</sub> values expected for the number of  $\beta$ 2 subunit extracellular domains present in the receptor (Table 2). Critically, inhibition by physostigmine did not depend on the number of copies of the  $\beta$ 2 TM1–TM3 region, but rather on the number of copies of the  $\beta$ 2 extracellular domain. A second set of chimeric subunits was made in which the region following TM3 comprising the main cytoplasmic loop and TM4 domain was swapped. Expression of these constructs indicated that this region did not contribute to the differential effects of physostigmine (Table 2). That is, none of the TM regions appeared to be critical for the difference in responses to physostigmine. We conclude from these results that key determinants underlying the difference in response for the two stoichiometric forms of the receptors are located in the N-terminal, extracellular domain of the subunits.

This finding is in agreement with the weak concentration and voltage dependencies seen (Fig. 2) and indicates that the inhibition we observed is unlikely to result from open-channel block. We note that voltage-dependent open-channel block has been reported for inhibition of both muscle and neuronal nicotinic receptors (Wachtel, 1993; Zwart et al., 2000; Militante et al., 2008). In general these experiments have used higher concentrations of ACh to activate and higher concentrations of physostigmine to inhibit, and so a contribution from open-channel block would be enhanced. We also note that Smulders et al. (2005) concluded that “a mechanism more complex than simple channel block appears to be required” for inhibition by physostigmine of receptors formed from  $\alpha$ 4 and  $\beta$ 2 subunits injected at a 1:1 ratio. It is likely that the inhibition we observed at lower concentrations reflects an additional action of physostigmine on the receptor form containing two copies of  $\alpha$ 4 and three of  $\beta$ 2, whereas the inhibition seen at higher concentrations of physostigmine and ACh may well result from open-channel block.

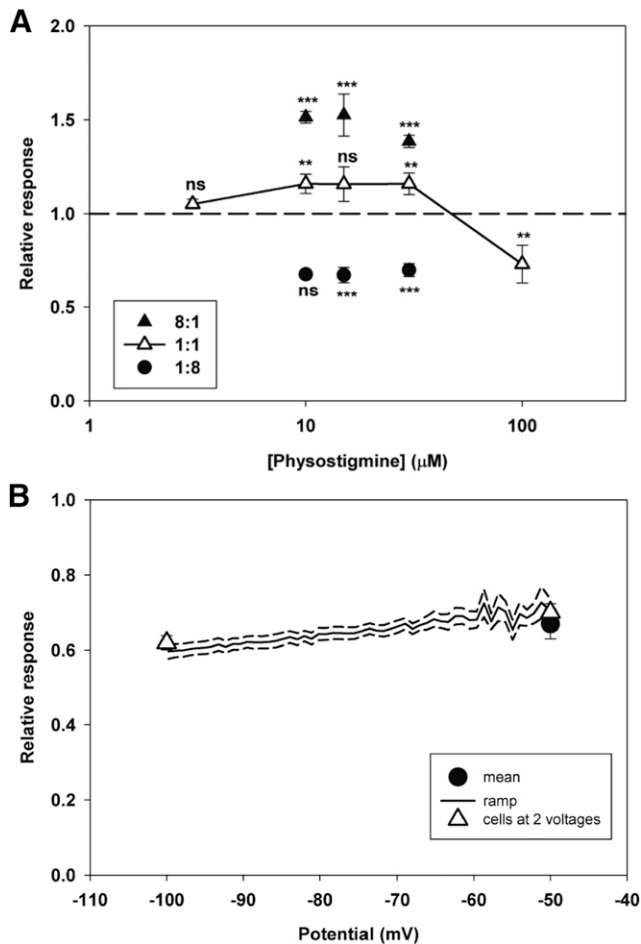
**The E Loop in the N-Terminal Extracellular Domain Plays a Key Role.** Since the difference in response to physostigmine appeared to lie in the amino-terminal extracellular domain, we constructed a series of chimeric subunits in which regions were exchanged (summarized in Fig. 3). Chimeric subunits were made by transferring the loops that form the canonical ACh binding sites between the  $\alpha$ 4 and  $\beta$ 2

subunits. In a canonical site, the  $\alpha$ 4 subunit contributes loops to the positive side of the interface (the A, B, and C loops) while the  $\beta$ 2 subunit contributes loops to the negative side (D, E, and F) (see Fig. 3). The chimeric subunit is named for the subunit contributing the major portion, followed by the transferred region in parentheses [e.g.,  $\beta$ 2(E) indicates a  $\beta$ 2 subunit containing the E loop from  $\alpha$ 4].

We first transferred loops from the  $\alpha$ 4 subunit to the  $\beta$ 2 subunit, in an effort to either remove inhibition or confer potentiation. These constructs were initially tested with the  $\beta$ 2- $\alpha$ 4 dimer, in which the free subunit will occupy a position that does not form a canonical ACh binding site. The chimeric construct will provide the minus side of the interface to a  $\beta$ 2/ $\beta$ 2 interface and the positive side to a  $\beta$ 2/ $\alpha$ 4 interface (see Fig. 3). None of the loops contributing to the positive side (the A, B, and C loops) had any effect on inhibition by physostigmine (Table 3). A construct in which all three loops were transferred also had no effect on inhibition. We also tested the  $\beta$ 2(ABC) chimera with the  $\alpha$ 4- $\beta$ 2 dimer, and we again saw no effect on physostigmine action (Table 3). These results indicated that the  $\beta$ 2 regions homologous to the regions contributed by  $\alpha$ 4 to the positive side of the ACh binding site were not involved in determining the actions of physostigmine, whether located at a  $\beta^*/\beta$  interface (when expressed with the  $\alpha$ 4- $\beta$ 2 dimer) or a  $\beta^*/\alpha$  interface (with the  $\beta$ 2- $\alpha$ 4 dimer). (Here,  $\beta^*$  indicates that the chimera affected the “plus” side of an interface contributed by a  $\beta$  subunit.)

We then constructed chimeras in which loops contributed by  $\beta$ 2 to the negative side were swapped (the D, E, and F loops). Transferring the D and F loops from  $\alpha$ 4 to  $\beta$ 2 did not affect any properties examined (Table 3). When the  $\alpha$ 4 E loop was transferred, however, the average maximal response was increased significantly and inhibition by physostigmine was reduced, albeit insignificantly (Fig. 1, C and E; Table 3). The  $\beta$ 2(E) construct was also tested with the  $\alpha$ 4- $\beta$ 2 dimer, and again inhibition by physostigmine was reduced (Fig. 1, D and F; Table 3). Again, the effect of the chimera was the same whether the affected interface was  $\alpha^*/\beta$  or  $\beta^*/\beta$ .

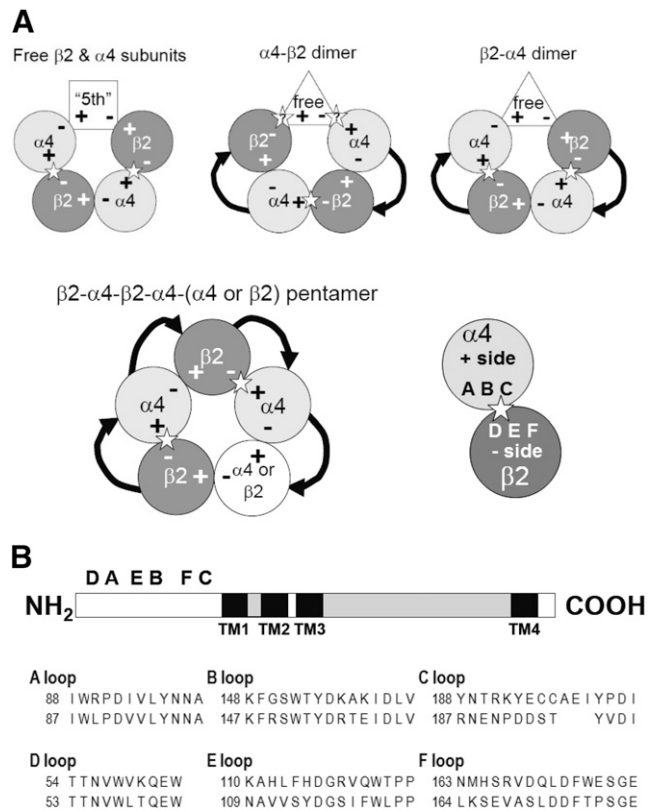
We did not test chimeras made in the  $\alpha$ 4 subunit by transferring the  $\beta$ 2 A, B, or C loops. However, since there appeared to be an effect when the  $\alpha$ 4 E loop was transferred into the  $\beta$ 2



**Fig. 2.** Concentration and voltage dependence of physostigmine actions. (A) The effect of different concentrations of physostigmine on responses from oocytes injected with free  $\alpha 4$  and  $\beta 2$  subunits at the indicated ratios (means  $\pm$  S.E.M.). Data are from 6–40 oocytes, except for the point at 10  $\mu$ M physostigmine on  $\alpha 4$ & $\beta 2$  1:8, which is a single experiment. The *P* value symbols indicate that the value differs from 1 (no effect) by chance [one-sample *t* test; ns indicates *P* > 0.05 (not significant); \*\**P* < 0.01, \*\*\**P* < 0.001]. (B) The inhibition of responses from oocytes injected with  $\alpha 4$ & $\beta 2$  1:8 (0.3  $\mu$ M ACh, 15  $\mu$ M physostigmine) at different voltages. Symbols show means  $\pm$  S.E.M. for four oocytes tested at both –50 and –100 mV (“cells at two voltages”) and for 10 eggs tested at –50 mV (“mean” including the four tested at both voltages). The heavy line shows the average relative response for four oocytes subjected to a voltage ramp (see the *Materials and Methods*), whereas the dashed lines show the standard error.

subunit, we examined the consequences of moving the  $\beta 2$  E loop to the  $\alpha 4$  subunit. This transfer had dramatic effects on receptor function, converting potentiation by physostigmine to inhibition and reducing the EC<sub>50</sub> for ACh (Fig. 1, G and H; Table 3). Indistinguishable effects occurred whether the chimera was expressed with the  $\alpha 4$ - $\beta 2$  ( $\alpha^*/\alpha$ ) or  $\beta 2$ - $\alpha 4$  ( $\beta^*/\alpha$ ) dimer (Fig. 1, I and J; Table 3).

Finally, we constructed three pentameric concatemers:  $\beta 2$ - $\alpha 4$ - $\beta 2$ - $\alpha 4$ - $\alpha 4$ (E),  $\beta 2$ - $\alpha 4$ - $\beta 2$ - $\alpha 4$ - $\beta 2$ (E), and  $\beta 2$ - $\alpha 4$ - $\beta 2$ (E)- $\alpha 4$ - $\beta 2$ . Previous work (Carbone et al., 2009; Mazzaferro et al., 2011) showed that pentameric concatemers assemble with subunits in clockwise order (Fig. 3), so the first would contain a  $\beta^*/\alpha$  interface with a transposed E loop, the second an altered  $\beta^*/\beta$  interface, and the third an altered  $\alpha^*/\beta$  interface. Only the third concatemer would have affected a canonical ACh binding site. The results are summarized in Table 3 and agree with the



**Fig. 3.** Cartoons of receptor structure. (A) The arrangement of subunits in the receptors formed after injections of various combinations of subunits. The diagrams are of the receptor viewed from the extracellular side. Stars indicate the locations of canonical ACh binding sites ( $\alpha 4$ / $\beta 2$  interface). The contributions of loops to a canonical ACh binding site are also shown. (B) A linear diagram of a generic nicotinic subunit with the relative positions of the binding site loops (A–F) and the TM domains (TM1–TM4) is shown. Extracellular portions are indicated by white shading, intracellular by gray shading and TM regions by black. The residues transferred in the chimeric constructs, together with the initial residue number in the mature subunit, are shown below the diagram.

results obtained with dimers. Altering the  $\alpha 4$  E loop at a  $\beta^*/\alpha$  interface resulted in reduced potentiation and a significant reduction in the EC<sub>50</sub> for ACh. Altering the  $\beta 2$  E loop at a  $\beta^*/\beta$  interface resulted in a small potentiation. Finally, altering the  $\beta 2$  E loop at a canonical ACh binding interface ( $\alpha^*/\beta$ ) removed block.

**Effects of E Loop Transfer on Actions of Other Potentiating Agents.** We performed initial studies of three additional potentiating agents: galantamine, atropine, and zinc ions. Galantamine was chosen because it photolabels the same residues in the *Torpedo* nicotinic receptor as physostigmine (Hamouda et al., 2013). Atropine is reported to be a strong potentiator of  $\alpha 4\beta 2$  receptors, but the effects of stoichiometry have not been examined (Smulders et al., 2005). Finally, Zn<sup>2+</sup> potentiates the form containing three copies of  $\alpha 4$  and inhibits the form containing two copies, but the residues involved do not lie in the E loop (Hsiao et al., 2006; Moroni et al., 2008). We tested selected concentrations of the drugs on receptors formed from free subunits at different ratios of injected cRNA and on chimeric  $\alpha 4$ (E) and  $\beta 2$ (E) subunits expressed with the  $\beta 2$ - $\alpha 4$  dimeric construct. The results are summarized in Table 4.

We tested 0.5  $\mu$ M galantamine, a concentration that has been reported to produce maximal potentiation for human  $\alpha 4\beta 2$  receptors expressed in human embryonic kidney cells (Samochocki et al., 2003). It produced minimal potentiation

TABLE 2

The effect of physostigmine does not depend on the TM domain

Subunits injected	Response to 1 mM ACh			ACh EC50			Response to physostigmine		
	Mean $\pm$ S.E. nA	No. of Cells	<i>P</i> value <sup>a</sup>	Mean $\pm$ S.E. $\mu$ M	No. of Cells	<i>P</i> value <sup>a</sup>	Mean $\pm$ S.E.	No. of Cells	<i>P</i> value <sup>b</sup>
$\beta$ - $\alpha$ & $\alpha$	-14421 $\pm$ 1081	94	–	121 $\pm$ 10	59	–	1.38 $\pm$ 0.03	51	–, <0.001
$\beta$ - $\alpha$ & $\alpha$ (TM1 to TM3)	-15034 $\pm$ 3105	12	0.98	316 $\pm$ 61	8	<0.001	1.28 $\pm$ 0.06	11	0.19, 0.001
$\beta$ - $\alpha$ & $\alpha$ (TM3 to C)	-27249 $\pm$ 3339	17	<0.001	82 $\pm$ 24	10	0.37	1.19 $\pm$ 0.04	7	0.03, 0.005
$\beta$ - $\alpha$ & $\beta$	-1007 $\pm$ 116	91	–	3.7 $\pm$ 0.5	57	–	0.72 $\pm$ 0.05	28	–, <0.001
$\beta$ - $\alpha$ & $\beta$ (TM1 to TM3)	-959 $\pm$ 162	28	0.97	2.3 $\pm$ 0.3	25	0.14	0.76 $\pm$ 0.05	14	0.82, 0.001
$\beta$ - $\alpha$ & $\beta$ (TM3 to C)	-1801 $\pm$ 543	6	0.14	4.6 $\pm$ 1.1	6	0.74	0.63 $\pm$ 0.06	3	0.80, 0.03

Chimeric subunits are named as subunit contributing the amino terminus followed by the region swapped between subunits (in parentheses), so  $\alpha$ (M1–M3) indicates an  $\alpha$ 4 subunit containing  $\beta$ 2 sequence for the TM1–TM3 domains. The joining points for the chimeras were as follows: start of TM1  $\alpha$ 4 V(208)IRR  $\beta$ 2 V(204)IRR, end of TM3  $\alpha$ 4N (344)VHH  $\beta$ 2 (N(340)VHH. Means  $\pm$  S.E. and the number of cells are presented for the response to 1 mM ACh, for the concentration of ACh producing a half-maximal response (EC<sub>50</sub>), and for the ratio of the response to a low concentration of ACh in the presence of physostigmine to the response of the same cell to ACh in the absence of physostigmine. Dashes indicate that that particular subunit combination was the comparison value for the *P* computation.

<sup>a</sup>*P* values give the significance of the difference in value to that of the first entry in the set (one-way analysis of variance with Dunnett correction).

<sup>b</sup>The first *P* value is the significance of the difference to the first entry in the set, whereas the second value is the difference of the potentiation to a value of 1 (i.e., no effect; one-sample *t* test). Dashes indicate that that particular subunit combination was the comparison value for the *P* computation.

or inhibition in our experiments, as was reported in a previous study of human  $\alpha$ 4 $\beta$ 2 receptors expressed in *Xenopus* oocytes (Smulders et al., 2005). We then used 10  $\mu$ M galantamine, which produces inhibition of responses for receptors expressed in oocytes (Smulders et al., 2005). At this concentration, galantamine strongly inhibited receptors containing three copies of  $\alpha$ 4 and less strongly inhibited receptors containing only two copies (Table 4) (*P* < 0.0001 for the difference in inhibition, using one-way analysis of variance on the four constructs with Bonferroni correction). Indeed, inhibition by galantamine of responses from receptors containing only two copies of  $\alpha$ 4 was less than inhibition by physostigmine (0.69 response ratio, Table 1). Replacing the E loop of the  $\alpha$ 4 subunit reduced inhibition (comparison with free subunits at an 8:1 ratio, *P* < 0.0001),

whereas replacing the E loop of the  $\beta$ 2 subunit increased inhibition (*P* = 0.001). These results show a similar pattern to the results obtained with physostigmine; however, in the case of galantamine, inhibition occurred rather than potentiation. This suggests that the inhibition produced by galantamine differs from that of physostigmine and might reflect an inverse agonist action at the site mediating potentiation by physostigmine.

Atropine (100  $\mu$ M) strongly potentiated receptors containing three copies of the  $\alpha$ 4 subunit and strongly inhibited receptors containing two copies (Table 4). Replacement of the E loop of the  $\alpha$ 4 subunit converted potentiation to inhibition, whereas replacement of the E loop in the  $\beta$ 2 subunit significantly reduced inhibition (compared with the 1:8 ratio injection, *P* = 0.02) but

TABLE 3

The E loop is important in determining the effect of physostigmine

Subunits injected	Response to 1 mM ACh			ACh EC50			Response to physostigmine		
	Mean $\pm$ S.E. nA	No. of Cells	<i>P</i> value <sup>a</sup>	Mean $\pm$ S.E. $\mu$ M	No. of Cells	<i>P</i> value <sup>a</sup>	Mean $\pm$ S.E.	No. of Cells	<i>P</i> value <sup>b</sup>
$\beta$ - $\alpha$ & $\beta$	-1007 $\pm$ 116	91	–	3.7 $\pm$ 0.5	57	–	0.72 $\pm$ 0.05	28	–, <0.001
$\beta$ - $\alpha$ & $\beta$ (ABC)	-511 $\pm$ 120	4	1.0	6.1 $\pm$ 1.1	4	1.0	0.52 $\pm$ 0.04	4	0.3, 0.001
$\beta$ - $\alpha$ & $\beta$ (A)	-479 $\pm$ 66	5	1.0	8.0 $\pm$ 3.2	5	0.7	0.71 $\pm$ 0.07	3	1.0, 0.06
$\beta$ - $\alpha$ & $\beta$ (B)	-323 $\pm$ 121	13	0.8	12.6 $\pm$ 2.5	3	<0.001	0.71 $\pm$ 0.05	6	1.0, 0.001
$\beta$ - $\alpha$ & $\beta$ (C)	-317 $\pm$ 88	6	1.0	9.3 $\pm$ 1.6	6	0.3	0.69 $\pm$ 0.05	5	1.0, 0.004
$\beta$ - $\alpha$ & $\beta$ (D)	-207 $\pm$ 36	4	1.0	3.6 $\pm$ 0.6	4	1.0	0.66 $\pm$ 0.04	4	1.0, 0.003
$\beta$ - $\alpha$ & $\beta$ (E)	-8437 $\pm$ 1662	14	<0.001	3.0 $\pm$ 0.3	12	1.0	0.90 $\pm$ 0.04	4	0.4, 0.1
$\beta$ - $\alpha$ & $\beta$ (F)	-244 $\pm$ 77	4	1.0	3.2 $\pm$ 0.6	4	1.0	0.69 $\pm$ 0.01	3	1.0, 0.002
$\alpha$ - $\beta$ & $\beta$	-341 $\pm$ 98	32	–	2.9 $\pm$ 0.5	14	–	0.78 $\pm$ 0.07	10	–, 0.01
$\alpha$ - $\beta$ & $\beta$ (ABC)	-68 $\pm$ 27	4	0.6	5.7 $\pm$ 0.7	2	0.02	0.63 $\pm$ 0.00	2	0.7, <0.001
$\alpha$ - $\beta$ & $\beta$ (E)	-236 $\pm$ 51	4	1.0	7.6 $\pm$ 1.4	4	0.8	1.00 $\pm$ 0.07	4	0.2, 1.0
$\beta$ - $\alpha$ & $\alpha$	-14421 $\pm$ 1081	94	–	121 $\pm$ 10	59	–	1.38 $\pm$ 0.03	51	–, <0.001
$\beta$ - $\alpha$ & $\alpha$ (E)	-24237 $\pm$ 2810	24	0.007	3.7 $\pm$ 0.4	14	<0.001	0.53 $\pm$ 0.02	11	<0.001, <0.001
$\alpha$ - $\beta$ & $\alpha$	-7238 $\pm$ 1179	68	–	78 $\pm$ 5	47	–	1.51 $\pm$ 0.08	25	–, <0.001
$\alpha$ - $\beta$ & $\alpha$ (E)	-387 $\pm$ 105	6	0.09	3.1 $\pm$ 0.2	5	<0.001	0.59 $\pm$ 0.03	4	<0.001, 0.001
$\beta$ - $\alpha$ - $\beta$ - $\alpha$ - $\alpha$	-2020 $\pm$ 265	25	–	153 $\pm$ 12	9	–	1.43 $\pm$ 0.23	8	–, 0.1
$\beta$ - $\alpha$ - $\beta$ - $\alpha$ - $\alpha$ (E)	-1674 $\pm$ 507	9	0.5	4.6 $\pm$ 0.6	7	<0.001	1.08 $\pm$ 0.18	8	0.2, 0.7
$\beta$ - $\alpha$ - $\beta$ - $\alpha$ - $\beta$	-110 $\pm$ 23	20	–	3.2 $\pm$ 0.8	16	–	0.54 $\pm$ 0.04	6	–, <0.001
$\beta$ - $\alpha$ - $\beta$ - $\alpha$ - $\beta$ (E)	-265 $\pm$ 70	16	0.9	7.8 $\pm$ 1.3	11	0.002	1.20 $\pm$ 0.18	12	0.03, 0.3
$\beta$ - $\alpha$ - $\beta$ (E)- $\alpha$ - $\beta$	-2592 $\pm$ 677	12	<0.001	4.6 $\pm$ 0.4	7	0.6	1.01 $\pm$ 0.19	6	0.2, 1.0

Chimeric subunits are named as subunit contributing the amino terminus followed by the region swapped between subunits (in parentheses), so  $\beta$ (ABC) indicates a  $\beta$ 2 subunit containing  $\alpha$ 4 sequence for the A, B and C loops. The locations of the chimeras are shown in Figure 2. Means  $\pm$  S.E. and the number of cells are presented for the response to 1 mM ACh, for the concentration of ACh producing a half-maximal response (EC<sub>50</sub>), and for the ratio of the response to a low concentration of ACh in the presence of physostigmine to the response of the same cell to ACh in the absence of physostigmine. Dashes indicate that that particular subunit combination was the comparison value for the *P* computation.

<sup>a</sup>*P* values give the significance of the difference in value to that of the first entry in the set (one-way analysis of variance with Dunnett correction).

<sup>b</sup>The first *P* value is the significance of the difference to the first entry in the set, whereas the second value is the difference of the potentiation to a value of 1 (i.e., no effect; one-sample *t* test). Dashes indicate that that particular subunit combination was the comparison value for the *P* computation.

TABLE 4  
Effects of transferring the E loop on actions of other potentiators.

Subunits injected	Potentiator	Relative response		<i>P</i> Value <sup>a</sup>		
		Mean ± S.E.	No. of Cells	<i>P</i> to 1	<i>P</i> to pair	<i>P</i> to free
$\alpha 4\&\beta 2$ 8:1	GAL 0.5	0.94 ± 0.03	7	0.09	-	nd
$\beta$ - $\alpha$ & $\alpha$ (E)	GAL 0.5	0.95 ± 0.02	6	0.08	1.0	nd
$\alpha 4\&\beta 2$ 1:8	GAL 0.5	1.01 ± 0.04	9	0.85	-	nd
$\beta$ - $\alpha$ & $\beta$ (E)	GAL 0.5	0.91 ± 0.03	6	0.03	0.24	nd
$\alpha 4\&\beta 2$ 8:1	GAL 10	0.52 ± 0.02	6	<0.001	-	nd
$\beta$ - $\alpha$ & $\alpha$ (E)	GAL 10	0.88 ± 0.03	6	0.01	<0.001	nd
$\alpha 4\&\beta 2$ 1:8	GAL 10	0.90 ± 0.02	8	0.004	-	nd
$\beta$ - $\alpha$ & $\beta$ (E)	GAL 10	0.68 ± 0.06	5	0.005	0.001	nd
$\alpha 4\&\beta 2$ 8:1	ATR 100	1.51 ± 0.05	8	<0.001	-	nd
$\beta$ - $\alpha$ & $\alpha$ (E)	ATR 100	0.29 ± 0.03	7	<0.001	<0.001	nd
$\alpha 4\&\beta 2$ 1:8	ATR 100	0.35 ± 0.06	7	<0.001	-	nd
$\beta$ - $\alpha$ & $\beta$ (E)	ATR 100	0.55 ± 0.04	8	<0.001	0.002	nd
$\alpha 4\&\beta 2$ 8:1	ZN 100	1.97 ± 0.05	11	<0.001	-	-
$\alpha$ - $\beta$ & $\alpha$	ZN 100	2.98 ± 0.12	11	<0.001	-	<0.001
$\alpha$ - $\beta$ & $\alpha$ (E)	ZN 100	2.85 ± 0.17	6	<0.001	1.0	<0.001
$\beta$ - $\alpha$ & $\alpha$	ZN 100	1.38 ± 0.05	4	0.005	-	0.01
$\beta$ - $\alpha$ & $\alpha$ (E)	ZN 100	1.50 ± 0.07	7	<0.001	1.0	0.02
$\alpha 4\&\beta 2$ 1:8	ZN 100	0.21 ± 0.02	6	<0.001	-	-
$\alpha$ - $\beta$ & $\beta$ (E)	ZN 100	11.17 ± 0.84	6	<0.001	-	<0.001
$\beta$ - $\alpha$ & $\beta$	ZN 100	0.37 ± 0.03	7	<0.001	-	1.0
$\beta$ - $\alpha$ & $\beta$ (E)	ZN 100	3.59 ± 0.41	7	<0.001	<0.001	<0.001

Three additional potentiating drugs were tested: galantamine at 0.5  $\mu$ M (GAL 0.5) and 10  $\mu$ M (GAL 10), atropine at 100  $\mu$ M (ATR 100) and Zn<sup>2+</sup> at 100  $\mu$ M (ZN 100). Means ± S.E. and the number of cells are presented for the ratio of the response to a low concentration of ACh in the presence of physostigmine to the response of the same cell to ACh in the absence of physostigmine.

<sup>a</sup>Three *P* values are given: “to 1” indicates the *P* value for the difference to 1 (no effect) (one-sample *t* test), “to pair” indicates that the relative response differs from the value immediately above [e.g.,  $\alpha 4\&\beta 2$  1:8 to  $\beta$ - $\alpha$ & $\beta$ (E)] (one-way analysis of variance on all subunit combinations in the group with Bonferroni post hoc correction), and “to free” gives the *P* value for the difference from the value at the top of the group (one-way analysis of variance on all conditions in the group with Bonferroni post hoc correction). The 5 groups consisted of the 4 subunit combinations tested with 0.5  $\mu$ M galantamine, with 10  $\mu$ M galantamine, and with 100  $\mu$ M atropine, then the 5 subunit combinations starting with  $\alpha 4\&\beta 2$  8:1 tested with 100  $\mu$ M zinc and finally the 4 subunit combinations tested with 100  $\mu$ M zinc starting with  $\alpha 4\&\beta 2$  1:8.

Dashes indicate that that particular subunit combination was the comparison value for the *P* computation and “nd” indicates that the comparison was not made.

did not confer potentiation. These results are quite similar to those obtained with physostigmine.

Zinc ion (100  $\mu$ M) was chosen as a negative control potentiator, because it is a stoichiometry-dependent modulator while the residues identified as critical for its actions do not lie in the E loop (Moroni et al., 2008). We confirmed the stoichiometry dependence of the actions of Zn<sup>2+</sup> for receptors assembled from free subunits (Table 4). Expression of the  $\alpha 4$ (E) chimera with the  $\beta 2$ - $\alpha 4$  chimera somewhat reduces potentiation compared with free subunits (*P* = 0.02) but not compared with the level when wild-type  $\alpha 4$  was expressed with  $\beta 2$ - $\alpha 4$  (*P* = 1.0). However, transfer of the  $\alpha 4$  E loop to  $\beta 2$  conferred potentiation (Table 4). This is surprising, because the transferred E loop should contribute to the (−) side of a  $\beta 2/\beta 2$  interface (Fig. 2). Potentiation by Zn<sup>2+</sup> is the result of an interaction of zinc at the  $\alpha 4/\alpha 4$  interface [specifically between  $\alpha 4$ (H165) in the F loop on the (−) side and  $\alpha 4$ (E194) on the (+) side in the C loop] (Moroni et al., 2008). Accordingly, it would be expected that potentiation would not be affected, as we observed. However, inhibition reflects interaction of zinc at the  $\beta 2/\alpha 4$  interface [between  $\alpha 4$ (H165) on the (−) side and  $\beta 2$ (D193) on the (+) side], which should not have been altered when the  $\beta 2$ (E) chimera is expressed.

To examine the actions of Zn<sup>2+</sup> further, we expressed the chimeric subunits with the  $\alpha 4$ - $\beta 2$  dimer, to place the transferred E loop at an  $\alpha 4/\beta 2$  interface. Responses of receptors containing either the  $\alpha 4$ (E) or  $\beta 2$ (E) chimera were potentiated by 100  $\mu$ M Zn<sup>2+</sup> (Table 4). Indeed, when the  $\beta 2$ (E) chimera was expressed with this dimer, the level of potentiation was the largest we observed (Table 4), and the amplitude of the potentiated response actually exceeded the maximal response of the oocyte to 1 mM

ACh alone (the average relative potentiated response was 1.24 times the maximal response to ACh alone, *P* < 0.001).

Overall, the actions of transferring the E loop on potentiation by atropine were very similar to effects on physostigmine. Galantamine, in contrast, did not potentiate either form of the receptor when expressed in oocytes. However, the inhibition produced by galantamine at 10  $\mu$ M differed from the inhibition by physostigmine: galantamine inhibition was strongest on receptors containing three copies of  $\alpha 4$ . The effects of transferring the E loop on inhibition by galantamine are consistent with the idea that galantamine acts as an inverse agonist at the same site at which physostigmine acts as an agonist and results in potentiation. In contrast, the unexpected effects on modulation by Zn<sup>2+</sup> ions followed a different pattern, in that the transfer into the  $\beta 2$  subunit changed inhibition to potentiation while the reciprocal transfer had no effect. The consequences of the transfer were similar for Zn<sup>2+</sup> ions and physostigmine, in that it did not matter whether the affected interface formed a canonical binding site or occurred elsewhere in the pentamer.

## Discussion

Our initial observation was that the effect of physostigmine on currents elicited by ACh differed between the two stoichiometric forms of the nicotinic  $\alpha 4\beta 2$  receptor: physostigmine potentiated responses from receptors containing three copies of  $\alpha 4$  and inhibited receptors containing three copies of  $\beta 2$ .

The inhibition by physostigmine that we studied is unlikely to reflect open-channel block, based on three lines of evidence: low dependence on the concentration of physostigmine, weak

voltage dependence, and lack of effect when the membrane-spanning regions were swapped between the  $\alpha 4$  and  $\beta 2$  subunits. However, it is likely that this inhibition reflects an additional interaction between physostigmine and the  $\alpha 4\beta 2$  receptor, and physostigmine can also act as an open-channel blocker.

Our results indicate that the E loop of the  $\alpha 4$  and  $\beta 2$  subunits is a key determinant of the actions of physostigmine. Although transferring the E loop from  $\alpha 4$  to  $\beta 2$  did not confer potentiation, inhibition was reduced. In contrast, transferring the E loop from  $\beta 2$  to  $\alpha 4$  resulted in strong block of the response to ACh. Our data do not distinguish whether the potentiation and inhibition we have studied result from interaction of physostigmine with a single site (i.e., as agonist and inverse agonist) or at separate sites. Because potentiation, inhibition, or both could have been affected it is not clear whether the E loop has predominant effects on one or the other process.

A role for loops in the ACh binding site suggests that physostigmine might bind to the canonical ACh binding site, as proposed earlier (Zwart et al., 2000; Smulders et al., 2005). This is unlikely, however, since the E loop of the  $\alpha 4$  subunit does not contribute to the canonical site. Alternatively, recent studies have indicated that the pharmacology of nicotinic receptors is significantly influenced by sites located at inter-subunit interfaces in addition to the canonical ACh binding site found at the  $\alpha/\beta$  interface. These include a site located at an  $\alpha/\alpha$  interface (Harpsoe et al., 2011; Mazzaferro et al., 2011; Lucero et al., 2016) and sites at  $\beta/\alpha$  interfaces (Moroni et al., 2008; Seo et al., 2009). We examined the role that interface location plays by expressing chimeric subunits with concatemers of subunits and by incorporating chimeric subunits into pentameric concatemers. We found no indication that the position of the transferred E loop is critical in determining the actions of physostigmine, suggesting that interactions of physostigmine are not restricted to either canonical or noncanonical binding sites.

At this point we need to consider whether the overall data support the idea that physostigmine binds to the receptor in a site including the E loop. The results of photolabeling studies of the *Torpedo* nicotinic receptor strongly support the idea that binding does occur there. Other results indicate that the E loop plays a more general role in determining the properties of the receptor.

Turning first to the photolabeling studies, Hamouda et al. (2013) used the intrinsic photosensitivity of physostigmine to covalently label aromatic residues in the *Torpedo* electric organ nicotinic receptor (related to the muscle nicotinic receptor). They found that several residues in the canonical ACh binding site were labeled in the absence of a nicotinic agonist (carbamylcholine; 2-[(aminocarbonyl)oxy]-*N,N,N*-trimethylethanaminium chloride, CAS 51-83-2) or a competitive snake neurotoxin, whereas this labeling was blocked in the presence of carbamylcholine. However, they found other residues that were labeled even in the presence of agonist. None of this second group were located in the  $\alpha 1$  subunit, but several were found in or near the E loop of the  $\gamma$  subunit [in this receptor the  $\gamma$  subunit contributes the (–) side to a canonical ACh binding interface]. These residues are shown in Fig. 4. Galantamine also photolabeled the same residues in the presence of agonist. These results strongly support the idea that physostigmine and galantamine directly interact with

nicotinic receptors, in part with residues in the E loop. However, a significant caveat is that physostigmine is only a weak agonist on muscle nicotinic receptors (Shaw et al., 1985; Cooper et al., 1996; Militante et al., 2008), although one study reports that it can potentiate the response of skeletal muscle receptors to low concentrations of ACh (Svobodová et al., 2006).

On the other hand, the E loop plays a major role in determining the properties of the  $\alpha 4\beta 2$  receptor. We found that incorporating the  $\alpha 4(E)$  subunit had a significant effect on activation by ACh irrespective of the predicted interface at which it was placed. A previous study had mutated three residues in the  $\alpha 4$  E loop and found a similar shift in the  $EC_{50}$  for ACh (Harpsoe et al., 2011). In that study, receptors were expressed from free subunits at various ratios, and it was concluded that the mutations affected binding of ACh to the noncanonical binding site at the  $\alpha 4/\alpha 4$  interface. Recently, Lucero et al. (2016) extended these results using pentameric concatemers of subunits containing  $\alpha 4$  or  $\beta 2$  subunits with the same three mutations in the E loop of either the  $\alpha 4$  or  $\beta 2$  subunit. Lucero et al. (2016) found that incorporating a single copy of the mutated  $\alpha 4$  subunit greatly decreased the  $EC_{50}$  for ACh irrespective of whether the altered E loop contributed to a  $\beta/\alpha$  or an  $\alpha/\alpha$  interface. Our results agree well with both of these studies and confirm that a shift in the  $EC_{50}$  for ACh can occur even when the altered E loop is present at a single  $\beta 2/\alpha 4$  interface.

In terms of unexpected effects of swapping regions, the most surprising of our findings is that transferring the  $\alpha 4$  E loop to  $\beta 2$  conferred potentiation by zinc ions on the stoichiometric form of the receptor containing 3 copies of the  $\beta$  subunit. Based on the location of the residues implicated in potentiation and inhibition (see Fig. 4), this would definitely not have been expected. It is also surprising because inhibition is proposed to occur at the  $\beta 2/\alpha 4$  interface and potentiation at the  $\alpha 4/\alpha 4$  interface (Moroni et al., 2008), neither of which would be predicted to be altered by a change in the (–) side of the  $\beta 2$  subunit. In an earlier study, we found that transfer of a larger region, including the E loop, between the  $\alpha 2$  and  $\alpha 4$  subunits altered the efficacy of  $17\beta$  estradiol at potentiation (Jin and Steinbach, 2015). In that work, we proposed that the basis for the alteration was a change in the transduction process for potentiation by estradiol. These findings suggest that the E loop is a critical determinant of potentiation by drugs believed to bind elsewhere in the receptor, in addition to having significant actions on activation of the receptor by agonists. Accordingly, these considerations support the idea that the effects of swapping the E loop on the actions of physostigmine might well result from changes in either activation by ACh or transduction of the potentiating effect after physostigmine binding to a different region of the receptor.

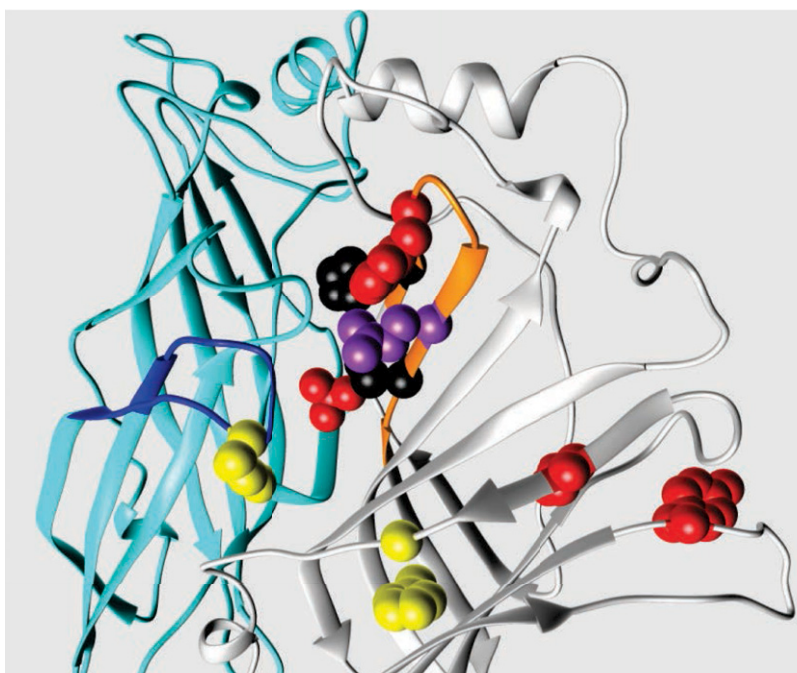
One significant issue in interpreting our results is that the effects of transferring E loops are independent of the interface affected. A possible interpretation is that the structure of binding sites may be influenced by regions at other interfaces. It has been proposed that interaction of a conotoxin with the canonical site on nicotinic  $\alpha 3\beta 2$  receptors depends on whether there is an  $\alpha 3/\alpha 3$  or  $\beta 2/\beta 2$  interface present in the receptor (Grishin et al., 2010). Furthermore, the apparent affinity for competitive antagonists at the canonical sites of  $\alpha 4\beta 2$  receptors is influenced by the presence of an  $\alpha 4/\alpha 4$  or a  $\beta 2/\beta 2$  interface in the receptor (Moroni et al., 2006). These studies of the actions of antagonists avoid the significant problem of



A

	A A A A A A A A A A A A	E E E E E E E E E E E E E E
$\alpha 4$	L I W R P D I V L Y N N A D G D F A V T H L T K A H L F H D G R V Q W T P P A I Y K S S C S I D V	
$\beta 2$	H I W L P D V V L Y N N A D G M Y E V S F Y S N A V V S Y D G S I F W L P P A I Y K S A C K I E V	
$\alpha 1$	D V W L P D L V L Y N N A D G D F A I V H M T K L L L D Y T G K I M W T P P A I F K S Y C E I I V	
$\delta$	L V W I P D I V L Q N N N D G Q Y H V A Y F C N V L V R P N G Y V T W L P P A I F R S S C P I N V	
$\gamma$	L L W L P D V V L E N N V D G Q F E V A Y Y A N V L V Y N D G S M Y W L P P A I Y R S T C P I A V	
	F F F F F F F F F F F F F F F F	C C C C C C C C C C C C C C C C
$\alpha 4$	L V N M H S R V D Q L D F W E S G E W V I V D A V G T Y N T R K Y E C C A E I Y P D I T Y A F V	
$\beta 2$	L K S E V A S L D D F T P S G E W D I V A L P G R R N E N P D D S T Y V D I T Y D F I	
$\alpha 1$	I S P E S D R P D L S T F M E S G E W V M K D Y R G W K H W V Y Y T C C P D T P Y L D I T Y H F I	
$\delta$	Y P I E W I I I D P E A F T E N G E W E I I H K P A K K N I Y P D K F P N G T N Y Q D V T F Y L I	
$\gamma$	E A V E W I H I D P E D F T E N G E W T I R H R P A K K N Y N W Q L T K D D T D F Q E I I F F L I	

B



**Fig. 4.** Location of residues photolabeled by physostigmine or implicated in the actions of  $Zn^{2+}$ . (A) Sections of aligned sequence for segments of the extracellular domains of the human  $\alpha 4$  and  $\beta 2$  subunits and the *Torpedo*  $\alpha 1$ ,  $\gamma$ , and  $\delta$  subunits. The letters on a blue background above the sequences indicate the locations of chimera segments at the (+) side of an interface (loops A and C), whereas those on the orange background indicate those on the (-) side (E and F). Residues are color coded to indicate that they were labeled by physostigmine only in the absence of carbamylcholine (green, in the  $\alpha 1$  subunit) or were labeled by physostigmine in the presence of carbamylcholine (red, in  $\gamma$  and  $\delta$  subunits). Note that no label was reported from the  $\beta 1$  subunit, and it was not possible to sequence the E loop of the  $\delta$  subunit. Yellow backgrounds indicate residues implicated in the actions of  $Zn^{2+}$ :  $\alpha 4H(165)$  participates in potentiation at the  $\alpha 4/\alpha 4$  interface and inhibition at the  $\beta 2/\alpha 4$  interface, whereas  $\alpha 4(E194)$  participates in potentiation and  $\beta 2(D193)$  in inhibition. The three residues in the E loops of  $\alpha 4$  and  $\beta 2$  highlighted in gray are the residues switched in the studies by Harpsøe et al. (2011) referred to in the Discussion. (B) A homology model of the extracellular domains of two  $\alpha 4$  subunits, based on the GluCl structure (3RIF; Hibbs and Gouaux, 2011) to provide an idea of the positions of the residues highlighted in (A). The view is from the outside of the receptor looking into the binding site for ACh, and the extracellular portion is at the top. The subunit contributing the (+) side is shown as a cyan ribbon. The C loop on the (+) side is colored blue, and the residue in  $\alpha 4$  contributing to the  $Zn^{2+}$  potentiating site is shown as yellow spheres. The residues in  $\alpha 1$  that were photolabeled by physostigmine are not shown. The subunit contributing the (-) side is shown as a light gray ribbon and the E loop is colored orange. The histidine contributing to both of the  $Zn^{2+}$  sites is shown as yellow spheres. Red spheres indicate the residues homologous to the residues photolabeled by physostigmine and for which carbamylcholine did not protect against labeling. Finally, the residues in the E loop interchanged by Harpsøe et al. (2011) are shown as black spheres. One residue was both interchanged and photolabeled; it is colored purple. Note that the residues contributing to the  $Zn^{2+}$  sites are relatively far from the E loop, whereas several residues photolabeled by physostigmine cluster near the E loop and to residues shown to be important in determining the properties of the receptor.

interpreting effects on activation by agonists, which necessarily are confounded by the problem of separating effects on affinity (potency) and transduction (efficacy) (Colquhoun, 1998). Most recently, Lucero et al. (2016) proposed that there is a strong interaction between adjacent subunits so that the structure at one interface (influenced by the structure of the E loop) can alter activation mediated by binding of ACh to neighboring subunits. The authors did not specify whether

the interaction alters binding or the coupling of binding to channel gating.

Perhaps the simplest summary of our results is that they confirm the physiologic relevance of the photolabeling studies, and they support the idea that physostigmine associates with the receptor at a site or sites including the E loop. This may also be true for galantamine and atropine. However, the lack of dependence on a specific interface and the presence of

multiple effects as a result of swapping the E loop both indicate the existence of greater complexity. The fact that there are significant changes in activation by ACh and in the pharmacology of unrelated modulatory drugs clearly indicates that many properties of the receptor have been altered, and it remains possible that physostigmine may associate with other (or additional) regions of the receptor to have its effects. The precise mechanisms mediating the actions of physostigmine require further study. There are similarities to the role that the E loop plays in activation of the receptor by ACh, suggesting that the structure-function relationship for the nicotinic  $\alpha 4\beta 2$  receptor involves extensive interactions of regions involving the entire pentamer.

#### Acknowledgments

The authors thank John Bracamontes for helpful comments and criticism and Daniel Shin for performing some of the oocyte recordings.

#### Authorship Contributions

Participated in research design: Jin, Akk, Steinbach.

Conducted experiments: Jin, McCollum, Germann.

Contributed new reagents or analytic tools: Jin.

Performed data analysis: Jin, Akk, Steinbach.

Wrote or contributed to the writing of the manuscript: Jin, Akk, Steinbach.

#### References

- Carbone AL, Moroni M, Groot-Kormelink PJ, and Bermudez I (2009) Pentameric concatenated (alpha4)(2)(beta2)(3) and (alpha4)(3)(beta2)(2) nicotinic acetylcholine receptors: subunit arrangement determines functional expression. *Br J Pharmacol* **156**:970–981.
- Colquhoun D (1998) Binding, gating, affinity and efficacy: the interpretation of structure-activity relationships for agonists and of the effects of mutating receptors. *Br J Pharmacol* **125**:924–947.
- Cooper JC, Gutbrod O, Witzemann V, and Methfessel C (1996) Pharmacology of the nicotinic acetylcholine receptor from fetal rat muscle expressed in *Xenopus* oocytes. *Eur J Pharmacol* **309**:287–298.
- Dani JA and Bertrand D (2007) Nicotinic acetylcholine receptors and nicotinic cholinergic mechanisms of the central nervous system. *Annu Rev Pharmacol Toxicol* **47**:699–729.
- Gotti C, Moretti M, Gaimarri A, Zanardi A, Clementi F, and Zoli M (2007) Heterogeneity and complexity of native brain nicotinic receptors. *Biochem Pharmacol* **74**:1102–1111.
- Grishin AA, Wang CI, Muttenthaler M, Alewood PF, Lewis RJ, and Adams DJ (2010) Alpha-conotoxin AuIB isomers exhibit distinct inhibitory mechanisms and differential sensitivity to stoichiometry of alpha3beta4 nicotinic acetylcholine receptors. *J Biol Chem* **285**:22254–22263.
- Hamouda AK, Kimm T, and Cohen JB (2013) Physostigmine and galanthamine bind in the presence of agonist at the canonical and noncanonical subunit interfaces of a nicotinic acetylcholine receptor. *J Neurosci* **33**:485–494.
- Harpsoe K, Ahring PK, Christensen JK, Jensen ML, Peters D, and Balle T (2011) Unraveling the high- and low-sensitivity agonist responses of nicotinic acetylcholine receptors. *J Neurosci* **31**:10759–10766.
- Hibbs RE and Gouaux E (2011) Principles of activation and permeation in an anion-selective Cys-loop receptor. *Nature* **474**:54–60.
- Hsiao B, Mihalak KB, Repicky SE, Everhart D, Mederos AH, Malhotra A, and Luetjke CW (2006) Determinants of zinc potentiation on the alpha4 subunit of neuronal nicotinic receptors. *Mol Pharmacol* **69**:27–36.
- Jin X, Bermudez I, and Steinbach JH (2014) The nicotinic  $\alpha 5$  subunit can replace either an acetylcholine-binding or nonbinding subunit in the  $\alpha 4\beta 2^*$  neuronal nicotinic receptor. *Mol Pharmacol* **85**:11–17.
- Jin X and Steinbach JH (2011) A portable site: a binding element for 17 $\beta$ -estradiol can be placed on any subunit of a nicotinic  $\alpha 4\beta 2$  receptor. *J Neurosci* **31**:5045–5054.
- Jin X and Steinbach JH (2015) Potentiation of neuronal nicotinic receptors by 17 $\beta$ -estradiol: roles of the carboxy-terminal and the amino-terminal extracellular domains. *PLoS One* **10**:e0144631.
- Lucero LM, Weltzin MM, Eaton JB, Cooper JF, Lindstrom JM, Lukas RJ, and Whiteaker P (2016) Differential  $\alpha 4(+)/(-)\beta 2$  agonist-binding site contributions to  $\alpha 4\beta 2$  nicotinic acetylcholine receptor function within and between isoforms. *J Biol Chem* **291**:2444–2459.
- Maelicke A and Albuquerque EX (2000) Allosteric modulation of nicotinic acetylcholine receptors as a treatment strategy for Alzheimer's disease. *Eur J Pharmacol* **393**:165–170.
- Mazzaferro S, Benallegue N, Carbone A, Gasparri F, Vijayan R, Biggin PC, Moroni M, and Bermudez I (2011) Additional acetylcholine (ACh) binding site at alpha4/alpha4 interface of (alpha4beta2)2alpha4 nicotinic receptor influences agonist sensitivity. *J Biol Chem* **286**:31043–31054.
- McGehee DS and Role LW (1995) Physiological diversity of nicotinic acetylcholine receptors expressed by vertebrate neurons. *Annu Rev Physiol* **57**:521–546.
- Militante J, Ma BW, Akk G, and Steinbach JH (2008) Activation and block of the adult muscle-type nicotinic receptor by physostigmine: single-channel studies. *Mol Pharmacol* **74**:764–776.
- Moroni M, Zwart R, Sher E, Cassels BK, and Bermudez I (2006) alpha4beta2 nicotinic receptors with high and low acetylcholine sensitivity: pharmacology, stoichiometry, and sensitivity to long-term exposure to nicotine. *Mol Pharmacol* **70**:755–768.
- Moroni M, Vijayan R, Carbone A, Zwart R, Biggin PC, and Bermudez I (2008) Non-agonist-binding subunit interfaces confer distinct functional signatures to the alternate stoichiometries of the alpha4beta2 nicotinic receptor: an alpha4-alpha4 interface is required for Zn<sup>2+</sup> potentiation. *J Neurosci* **28**:6884–6894.
- Samochocki M, Höfle A, Fehrenbacher A, Jostock R, Ludwig J, Christner C, Radina M, Zerlin M, Ullmer C, Pereira EF, et al. (2003) Galantamine is an allosterically potentiating ligand of neuronal nicotinic but not of muscarinic acetylcholine receptors. *J Pharmacol Exp Ther* **305**:1024–1036.
- Schrattenholz A, Godovac-Zimmermann J, Schäfer HJ, Albuquerque EX, and Maelicke A (1993) Photoaffinity labeling of Torpedo acetylcholine receptor by physostigmine. *Eur J Biochem* **216**:671–677.
- Seo S, Henry JT, Lewis AH, Wang N, and Levandoski MM (2009) The positive allosteric modulator morantel binds at noncanonical subunit interfaces of neuronal nicotinic acetylcholine receptors. *J Neurosci* **29**:8734–8742.
- Shaw KP, Aracava Y, Akaïke A, Daly JW, Rickett DL, and Albuquerque EX (1985) The reversible cholinesterase inhibitor physostigmine has channel-blocking and agonist effects on the acetylcholine receptor-ion channel complex. *Mol Pharmacol* **28**:527–538.
- Smulders CJ, Zwart R, Bermudez I, van Kleef RG, Groot-Kormelink PJ, and Vijverberg HP (2005) Cholinergic drugs potentiate human nicotinic alpha4beta2 acetylcholine receptors by a competitive mechanism. *Eur J Pharmacol* **509**:97–108.
- Storch A, Schrattenholz A, Cooper JC, Abdel Ghani EM, Gutbrod O, Weber KH, Reinhardt S, Lobron C, Hermsen B, Soskiç V, et al. (1995) Physostigmine, galanthamine and cocaine act as 'noncompetitive nicotinic receptor agonists' on clonal rat pheochromocytoma cells. *Eur J Pharmacol* **290**:207–219.
- Svobodová L, Krüsek J, Hendrych T, and Vyskocil F (2006) Physostigmine modulation of acetylcholine currents in COS cells transfected with mouse muscle nicotinic receptor. *Neurosci Lett* **401**:20–24.
- Triggle DJ, Mitchell JM, and Filler R (1998) The pharmacology of physostigmine. *CNS Drug Rev* **4**:87–136.
- Wachtel RE (1993) Physostigmine block of ion channels activated by acetylcholine in BC3H1 cells. *Mol Pharmacol* **44**:1051–1055.
- Zhou Y, Nelson ME, Kuryatov A, Choi C, Cooper J, and Lindstrom J (2003) Human alpha4beta2 acetylcholine receptors formed from linked subunits. *J Neurosci* **23**:9004–9015.
- Zwart R, van Kleef RG, Gotti C, Smulders CJ, and Vijverberg HP (2000) Competitive potentiation of acetylcholine effects on neuronal nicotinic receptors by acetylcholinesterase-inhibiting drugs. *J Neurochem* **75**:2492–2500.
- Zwart R and Vijverberg HP (1997) Potentiation and inhibition of neuronal nicotinic receptors by atropine: competitive and noncompetitive effects. *Mol Pharmacol* **52**:886–895.
- Zwart R and Vijverberg HPM (1998) Four pharmacologically distinct subtypes of alpha4beta2 nicotinic acetylcholine receptor expressed in *Xenopus laevis* oocytes. *Mol Pharmacol* **54**:1124–1131.

**Address correspondence to:** Joe Henry Steinbach, Department of Anesthesiology, Washington University School of Medicine, 660 South Euclid Avenue, St. Louis, MO 63110. E-mail: jhs@morpheus.wustl.edu