Determination of the Residues in the Extracellular Domain of the Nicotinic α Subunit Required for the Actions of Physostigmine on Neuronal Nicotinic Receptors

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ABSTRACT

Physostigmine can potentiate and inhibit neuronal nicotinic receptors, in addition to inhibiting the activity of acetylcholinesterase. We found that receptors containing three copies of the $\alpha 2$ subunit are inhibited by low concentrations of physostigmine in contrast to receptors containing three copies of the $\alpha 4$ subunit that are potentiated. We exploited this observation to determine the regions required for the actions of physostigmine. Chimeric constructs of the $\alpha 2$ and $\alpha 4$ subunits located two regions in the extracellular amino-terminal domain of the subunit: the E loop (a loop of the transmitter-binding domain) and a

region closer to the amino-terminus that collectively could completely determine the different effects of physostigmine. Point mutations then identified a single residue, $\alpha 2$ (I92) versus $\alpha 4$ (R92), that, when combined with transfer of the E loop, could convert the inhibition seen with $\alpha 2$ subunits to potentiation and the potentiation seen with $\alpha 4$ subunits to inhibition. In addition, other point mutations could affect the extent of potentiation or inhibition, indicating that a more extensive set of interactions in the amino-terminal domain plays some role in the actions of physostigmine.

Introduction

Physostigmine has been used in clinical practice to raise acetylcholine (ACh) levels in the brain because of its ability to inhibit the enzyme acetylcholinesterase but also 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). In addition to acting as an agonist and potentiator, physostigmine also can inhibit nicotinic receptors (Zwart et al., 2000; Smulders et al., 2005). There has been recent interest in the ability of physostigmine and other drugs to act as “positive allosteric modulators” for neuronal nicotinic receptors, with possible therapeutic uses in, for example, the treatment of dementias including Alzheimer’s disease (Triggle et al., 1998; Maelicke and Albuquerque, 2000).

The site at which physostigmine acts on neuronal nicotinic receptors is not known, although it has been proposed that it may interact with the ACh-binding site (Smulders et al., 2005; however, see Militante et al., 2008). The ACh-binding site is in the extracellular domain of the receptor, located at the interface between the α and β subunits (Fig. 1). The site comprises regions from the α subunit (the so-called A, B, and C loops) and the β subunit (D, E, and F loops). We recently found in studies involving

the $\alpha 4$ and $\beta 2$ subunits that the E loop of the ACh-binding site is a key determinant of the actions of physostigmine (Jin et al., 2017). However, finer mapping of the critical residues was difficult because of the sequence divergence between the $\alpha 4$ and $\beta 2$ subunits. In the course of other studies, we found that physostigmine did not potentiate receptors comprising $\alpha 2$ and $\beta 2$ receptors and pursued this preliminary observation in the present work. The $\alpha 4$ and $\alpha 2$ subunits are highly homologous in terms of amino acid sequence (Jin and Steinbach, 2015) and occupy the same position in the assembled pentameric receptor so that it was possible to examine the structural differences between the $\alpha 2$ and $\alpha 4$ subunits that underlay this pharmacological difference. We used chimeric constructs between the $\alpha 2$ and $\alpha 4$ subunits to first determine that the basis for the different responses to physostigmine lay in the extracellular domain of the subunit. A series of finer chimeras identified two regions of the extracellular domain that were required for both inhibition and potentiation by physostigmine. The first is the E loop that was necessary for both actions but, by itself, could not confer full potentiation or inhibition. The second is a region containing the D loop and stretching to the A loop (the D-A region). These two regions could quantitatively account for the differences in the actions of physostigmine on the $\alpha 2$ and $\alpha 4$ subunits. Within the D-A region a single residue, R92 in the $\alpha 4$ subunit and I92 in the $\alpha 2$ subunit, could largely duplicate the effects of exchanging the entire D-A region in association with the E loop. However, other mutations in the D-A region also affected physostigmine actions, indicating that other interactions were important.

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ABBREVIATIONS: ACh, acetylcholine; ANOVA, analysis of variance; TM, transmembrane domain.

Fig. 1. Summary of subunit structure and arrangement in the pentameric receptor. The figure shows cartoon representations of the structure of neuronal nicotinic receptors. (A) Main structural regions of the primary sequence for both α and β subunits. Each subunit has a similar overall structure (A, bottom) with a relatively large N-terminal extracellular domain that contains the loops of the ACh-binding site. This is followed by three TMs; the channel is lined by the TM2 domains from each subunit. There is then a variable-length intracellular domain followed by a fourth TM and a short C-terminal extracellular domain. (B) The arrangement of subunits in the pentameric receptor viewed from the extracellular side (left); a canonical ACh-binding site (indicated by a star) is formed at an interface between an α subunit (“+” surface) counterclockwise to a β subunit (“-” surface). The α subunit contributes the A, B, and C loops (right), whereas the β subunit contributes the D, E, and F loops. (C) The location of the $\alpha 2$ subunit when expressed with the $\alpha 4$ - $\beta 2$ and $\beta 2$ - $\alpha 4$ dimeric constructs. Note that the nature of the interfaces formed by the $\alpha 2$ subunit depends on the particular constructs used.

Materials and Methods

The methods used were generally as described previously (Jin and Steinbach, 2015; Jin et al., 2017).

Generation and Expression of Constructs. Constructs of the coding region were provided by Dr. Jon Lindstrom (University of Pennsylvania, Philadelphia, PA) for the human $\alpha 4$ and $\beta 2$ subunits and by Dr. Jim Boulter (University of California, Los Angeles) for the mouse $\alpha 2$ and $\beta 4$ subunits. The mouse subunits were supplied in pSGEM vector, and all other subunits were in the pcDNA3 vector (Invitrogen, Carlsbad CA). Chimeric subunits were constructed by overlap extension, smaller mutations were constructed using QuikChange (Stratagene, La Jolla CA), and the complete coding region was sequenced to determine that only the desired changes had been made. Concatemers of the human $\alpha 4$ and $\beta 2$ subunits were described previously (Jin and Steinbach, 2011).

Complementary RNA was synthesized using the mMessage mMachine T7 kit (Ambion, Austin TX), and the concentration of RNA was estimated from the optical density 260 value. Subunits were injected at a ratio of 20:1 (α/β) for combinations of an α subunit with $\beta 4$ to ensure a subunit ratio of 3 α :2 β , unless otherwise noted in *Results*. We tested responses of receptors containing either the human or mouse form of the $\beta 4$ subunit and found no differences (data not shown), and so the data are pooled as in the *Results* section. Oocytes were injected with 12 to 15 ng of complementary RNA in a volume of 18–23 nl. Oocytes were maintained at 18°C for 2–4 days before physiologic study.

Xenopus oocytes were prepared in Dr. C. Zorumski’s laboratory (Washington University in St. Louis, St. Louis, MO). The protocols for animal use have been approved by the Washington University Animal Studies Committee. Animals are purchased from approved suppliers and are cared for in a Washington University Animal Care Facility. This facility is operated under the supervision of the Washington University Division of Comparative Medicine. This is an approved animal care facility providing housing and veterinary care for animals. Frogs were anesthetized with 0.1% tricaine (3-aminobenzoic acid ethyl ester) during a partial ovariectomy. Eggs are harvested twice per frog. Frogs are killed under tricaine anesthesia by rapid decapitation. This is an approved method of euthanasia.

Recording and Data Analysis. Standard methods were used for two-electrode voltage clamp of *Xenopus* oocytes (Jin and Steinbach, 2015; Jin et al., 2017). Records were digitized at 50 Hz with a Digidata 1200 interface (Molecular Devices, Sunnyvale, CA), stored using pClamp 8.0 (Molecular Devices), and analyzed with Clampfit (Molecular Devices). Oocyte recordings were performed in an RC-1Z chamber (Warner Instruments, Hamden, CT) that was continuously perfused with saline. Drug applications were made using a manually controlled perfusion system. The bath solution contained the following (in mM): 96 NaCl, 2 KCl, 1.8 BaCl₂, 1 MgCl₂, and 10 HEPES, pH 7.3. External Ca²⁺ was replaced with Ba²⁺ to avoid activation of Ca²⁺-activated channels.

The Hill equation was used to characterize the concentration-response relationship for activation by ACh ($Y/[ACh] = Y_{max}([ACh]/([ACh] + (EC_{50})^{nHill})$), where Y is the response to a concentration of ACh, Y_{max} is the maximal response, and nHill is the Hill coefficient. Concentration-response data for each cell were normalized to the response to 1 mM ACh for that cell and fit with the Hill equation, then overall mean values were calculated for oocytes that were injected with that set of constructs.

To test the effect of physostigmine, a low concentration of ACh was chosen for each subunit combination, which was able to evoke less than 20% of the maximal current. A test consisted of first applying the low concentration of ACh alone. After a stable response was reached, the application was switched to ACh plus physostigmine (10 or 15 μ M physostigmine). The response was terminated by the application of bath solution. The relative response in the presence of physostigmine to that in the absence of physostigmine was then calculated. Applications were separated by 3–4 minutes to allow full washout.

Values are presented as the arithmetic mean $\pm$ S.E.M. (number of observations).

Multiple regression analysis of the chimeric constructs was conducted using the sources of the sequence in the swapped regions as the independent variables, assigning the number 0 to a region containing the sequence from the $\alpha 2$ subunit and 1 to a region containing sequence from the $\alpha 4$ sequence.

The published structure of the nicotinic receptor containing two copies of the $\alpha 4$ subunit and three copies of $\beta 2$ (5kxi.PDB; Morales-Perez et al., 2016) was viewed, and figures were made using Chimera version 1.11 (<http://www.rbvi.ucsf.edu/chimera>).

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°C . Physostigmine (physostigmine hemisulfate; CAS 64-47-1) was purchased from Tocris Bioscience (Ellisville, MO). Physostigmine was prepared as a 10 mM stock solution in deionized water and stored frozen at -20°C . Working solutions were prepared on the day of experiments.

Results

Neuronal Nicotinic Receptors Containing the $\alpha 2$ Subunit Are Inhibited by Physostigmine. Our initial observation was that receptors containing the $\alpha 2$ subunit were inhibited by physostigmine when either two or three copies of $\alpha 2$ were present (Fig. 2; Table 1). This contrasts to receptors

Fig. 2. Effect of transplanting the D-A and E loop regions between the $\alpha 4$ and $\alpha 2$ subunits. Current traces are shown for responses when ACh was initially applied alone, and then the perfusion was switched to ACh plus physostigmine (application of ACh alone is indicated by the bottom bar above the trace, and the time of the application of both ACh and physostigmine is shown by the top bar). The constructs injected are shown above the trace (20:1 ratio for α/β subunits); the horizontal bar shows 20 second for all traces, whereas 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. The physostigmine concentration was 10 μM . The ACh concentrations used were as follows: $\alpha 4\beta 4$, 10 μM ; $\alpha 2\beta 4$, 20 μM ; $\alpha 4(\text{D-A+E})\beta 4$, 3 μM ; and $\alpha 2(\text{D-A+E})\beta 4$, 10 μM .

containing the $\alpha 4$ subunit that were potentiated by physostigmine when three copies of $\alpha 4$ were present and inhibited when only two copies were present (Table 1). These results were obtained when α subunits were expressed with either the $\beta 2$ or $\beta 4$ subunit. The $\alpha 2$ subunit expressed poorly with the $\beta 2$ subunit (Table 1), so all subsequent work was performed using the $\beta 4$ subunit. In the rest of the experiments, α constructs were expressed with $\beta 4$ at a 20:1 α/β ratio, so that receptors containing three copies of $\alpha 4$ showed potentiation by physostigmine.

The Presence of a Single $\alpha 2$ Subunit in the Receptor Affects Potentiation. We also expressed $\alpha 2$ with two concatemeric constructs of the $\alpha 4$ and $\beta 2$ subunits (Jin and Steinbach, 2011) to assess whether the position of the $\alpha 2$

subunit in the pentameric receptor affected responses to physostigmine (Fig. 1). These receptors both had the stoichiometry $\alpha 4_2\beta 2_2\alpha 2$, but when expressed with the $\alpha 4$ - $\beta 2$ concatemer the $\alpha 2$ subunit occupied a position between $\alpha 4$ and $\beta 2$ (when viewed from the extracellular medium, the subunit order is counterclockwise $\alpha 4$ - $\alpha 2$ - $\beta 2$) whereas with the $\beta 2$ - $\alpha 4$ concatemer the order was $\beta 2$ - $\alpha 2$ - $\alpha 4$. Drug-binding sites are formed at subunit interfaces such that one subunit contributes the “+” side whereas the counterclockwise subunit contributes the “−” side. A canonical ACh-binding site is formed when the order is α - β , whereas additional drug binding sites have been identified at β - α and α - α interfaces (Seo et al., 2009; Harpsøe et al., 2011; Mazzaferro et al., 2011; Lucero et al., 2016). Accordingly, with the $\alpha 4$ - $\beta 2$ concatemer the $\alpha 2$ subunit participated in a canonical interface ($\alpha 2$ - $\beta 2$) and a noncanonical interface ($\alpha 4$ - $\alpha 2$), whereas with the $\beta 2$ - $\alpha 4$ concatemer it participated in two noncanonical interfaces ($\beta 2$ - $\alpha 2$ and $\alpha 2$ - $\alpha 4$).

The effect of physostigmine did not differ depending on which concatemer the $\alpha 2$ subunit was expressed with (Table 2) ($P = 0.16$, two-tailed t test with unequal variances), indicating that it did not matter which interfaces the $\alpha 2$ subunit participated in. In both cases, the receptors were potentiated by physostigmine, whereas the potentiation was less than when the $\alpha 4$ subunit was expressed with either concatemer and was greater than when $\beta 2$ was expressed with either concatemer (Table 2).

The Difference in Effect Does Not Depend on Sequence Differences in the Transmembrane Domains.

We first asked whether differences in the transmembrane domains (TMs) were important. The $\alpha 2$ and $\alpha 4$ subunits are identical over the first three TMs (TM1 to TM3), so we transferred the region containing TM4 and the short C-terminal tail to produce the two chimeric constructs $\alpha 4(\text{TM4-C})$ and $\alpha 2(\text{TM4-C})$, where the construct is identified as the parent subunit (region transferred into it). Swapping the TM4 regions had no effect on the actions of physostigmine. For the $\alpha 4$ subunit, physostigmine potentiated both $\alpha 4$ [1.51 ± 0.04 (29)] and $\alpha 4(\text{TM4-C})$ [1.50 ± 0.02 (6); $P = 0.85$] [data are given as the mean $\pm$ S.E. (number of oocytes tested); P value that the two values are the same were assessed using a two-tailed t test with unequal variance]. For the $\alpha 2$ subunit, physostigmine inhibited both $\alpha 2$ [0.81 ± 0.03 (14)] and $\alpha 2(\text{TM4-C})$ [0.83 ± 0.03 (6); $P = 0.58$].

TABLE 1

Physostigmine does not potentiate responses of receptors containing the $\alpha 2$ subunit

The first column shows the subunit combination injected and the ratio of α to β subunits. The column headed “Maximal Response” gives the mean $\pm$ S.E.M. (number of cells) for the peak amplitude in response to 1 mM ACh. The column headed “ACh EC_{50} ” gives for the concentration of ACh producing EC_{50} values. The column headed “Physostigmine Effect” gives 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. The column headed “ P in pair” gives the P value that the parameter (EC_{50} or effect ratio) is the same for the injections at different ratios (two-tailed t test with unequal variances).

Subunits	Maximal Response	ACh EC_{50}	P in pair	Physostigmine Effect	
	nA	μM		Ratio	P in pair
$\alpha 4$ and $\beta 2$ 8:1	$-13,042 \pm 1303$ (51)	132 ± 10 (33)	<0.001	1.42 ± 0.04 (38)	<0.001
$\alpha 4$ and $\beta 2$ 1:8	-1029 ± 193 (47)	5 ± 1 (20)		0.69 ± 0.03 (15)	
$\alpha 2$ and $\beta 2$ 8:1	-130 ± 21 (26)	217 ± 42 (15)	<0.001	0.68 ± 0.01 (6)	0.05
$\alpha 2$ and $\beta 2$ 1:8	-190 ± 35 (59)	4 ± 0 (36)		0.78 ± 0.04 (13)	
$\alpha 4$ and $\beta 4$ 10:1	$-10,607 \pm 473$ (118)	31 ± 2 (21)	<0.001	1.51 ± 0.03 (31)	<0.001
$\alpha 4$ and $\beta 4$ 1:10	$-14,898 \pm 1221$ (27)	14 ± 2 (21)		1.10 ± 0.05 (27)	
$\alpha 2$ and $\beta 4$ 20:1	-929 ± 102 (85)	91 ± 6 (28)	<0.001	0.78 ± 0.03 (14)	0.25
$\alpha 2$ and $\beta 4$ 1:8	$-11,509 \pm 1635$ (22)	22 ± 3 (16)		0.90 ± 0.07 (9)	

TABLE 2

The response to physostigmine is less when the $\alpha 2$ subunit replaces a single $\alpha 4$ subunit

The first column gives the subunit combination injected. The concatemer construct $\alpha 4$ - $\beta 2$ has the $\alpha 4$ subunit at the amino-terminal end joined to the $\beta 2$ subunit by a linker, whereas the $\beta 2$ - $\alpha 4$ concatemer has the $\beta 2$ subunit at the amino-terminal end. The concatemers assemble in a clockwise fashion (N-terminal to C-terminal) as viewed from the extracellular space (Zhou et al., 2003; Jin and Steinbach, 2011). The column headed "P to $\alpha 2$ " gives the P value that the parameter (EC_{50} or effect ratio) is the same for the concatemer expressed with the studied subunit as with the $\alpha 2$ subunit (two-tailed t test with unequal variances).

Subunits	ACh EC_{50}		Physostigmine Effect	
	μM	P to $\alpha 2$	Ratio	P to $\alpha 2$
$\alpha 4$ - $\beta 2$ and $\alpha 2$	294 ± 22 (5)		1.20 ± 0.05 (5)	
$\alpha 4$ - $\beta 2$ and $\alpha 4$	78 ± 5 (47)	<0.001	1.51 ± 0.08 (25)	0.003
$\alpha 4$ - $\beta 2$ and $\beta 2$	2.9 ± 0.5 (14)	<0.001	0.78 ± 0.07 (10)	0.001
$\beta 2$ - $\alpha 4$ and $\alpha 2$	96 ± 6 (8)		1.12 ± 0.01 (10)	
$\beta 2$ - $\alpha 4$ and $\alpha 4$	121 ± 10 (59)	0.04	1.38 ± 0.03 (51)	<0.001
$\beta 2$ - $\alpha 4$ and $\beta 2$	3.7 ± 0.5 (57)	<0.001	0.72 ± 0.05 (28)	<0.001

Data are the mean $\pm$ S.E.M. (number of cells).

Some of the constructs we made were also used in a previous study of potentiation by 17β -estradiol (Jin and Steinbach, 2015) and examined the effects of the presence of either a tryptophan ($\alpha 4$: PPWLAGMI) or phenylalanine ($\alpha 2$: PPFLAGMI) in the carboxyl-terminal tail immediately after TM4. The lack of effect of swapping the TM4 to the C region indicates that the W to F difference is not important for the actions of physostigmine. We also verified a lack of difference for subunits in which both the W and F forms were tested [data are given as the mean $\pm$ S.E. (number of oocytes tested)]: $\alpha 4$ [1.51 ± 0.04 (29)] versus $\alpha 4$ (F)

[1.51 ± 0.10 (2); ($P = 0.96$) and $\alpha 2$ [0.81 ± 0.03 (14)] versus $\alpha 2$ (W) [0.73 ± 0.06 (9); ($P = 0.22$)]. Accordingly we pooled results for N-terminal constructs made with either W or F present in the C-terminal tail.

These results indicated that the difference in the responses to physostigmine were determined by residues in the amino-terminal extracellular region, as we had previously found in the case of the $\alpha 4$ and $\beta 2$ subunits (Jin et al., 2017).

Substituting Regions of the $\alpha 2$ N-Terminal Domain with Homologous Regions from the $\alpha 4$ Subunit Can Remove Inhibition and Confer Potentiation.

The $\alpha 2$ and $\alpha 4$ subunits are quite similar in sequence in the amino-terminal extracellular region (Fig. 3). Therefore, it seemed likely that we could make relatively large exchanges of sequence and still obtain constructs that would produce functional receptors. We constructed a set of overlapping chimeras that covered the extracellular domain. The chimeras are identified in terms of the domains contributing to a canonical binding site for ACh: the A, B, and C loops contributed by an α subunit on the "+" side of the interface, and the D, E, and F loops contributed by a β subunit on the "-" side. For example, a chimera based on the $\alpha 2$ subunit but containing the sequence from the $\alpha 4$ subunit covering the region from the amino terminus to the A loop is named $\alpha 2$ (N-A). The chimeras made are diagrammed in Fig. 3.

We first examined the $\alpha 2$ subunit and sought to confer potentiation. The results are shown in Fig. 4 and presented in Table 3. When the extreme amino-terminal region from the $\alpha 4$ subunit (N-terminal to the A loop) was swapped into the $\alpha 2$ subunit [$\alpha 2$ (N-A)], inhibition was reduced but potentiation

Fig. 3. The extracellular domains of $\alpha 2$ and $\alpha 4$ subunits. Sequences of the human $\alpha 4$ (accession number NP_000735.1) and mouse $\alpha 2$ (accession number NP_659052.1) subunits are shown for the amino-terminal extracellular region. In the line below the sequences ("Differ") a "+" indicates that the residues differ at that position in the two subunits, whereas the approximate locations of the loops in the canonical ACh-binding site are shown above the sequences ("Loops"). Loops A, B, and C are contributed by the subunit at the "+" side of the interface, whereas loops D, E and F are contributed by the subunit on the "-" side. The regions transferred in the various chimeras are indicated by the lines above the loops, and identified at the left (e.g., "A-TM1" starts at the end of the A loop and extends to the start of the first TM). Finally, the residues mutated in the D-A region are indicated by solid arrows below the sequences in the line marked "Mutations," and the residues in the E loop are indicated by hollow arrows.

was not conferred. When the remaining portion of the extracellular domain was swapped [$\alpha 2$ (A-TM1)], inhibition was also reduced but potentiation was not conferred (Table 3). However, chimeras with the joining point further from the amino terminus did not affect inhibition [$\alpha 2$ (B-TM1) and $\alpha 2$ (C)]. These results suggested that the region conferring inhibition was relatively widely distributed but was located between the amino terminus and the B loop.

Our previous study of the $\alpha 4$ and $\beta 2$ subunits had indicated that the E loop is a key determinant of the actions of physostigmine (Jin et al., 2017), so we examined the consequences of swapping this domain. When the E loop from $\alpha 4$ was inserted into $\alpha 2$ block was fully removed, again potentiation was not conferred. This suggested that the E loop was the basis for the effects of the $\alpha 2$ (A-TM1) chimera. However, the results had indicated that a region further to the N-terminus was also important in determining the effects of physostigmine. When we combined transfer of the D-A region with the E loop, strong potentiation was conferred [$\alpha 2$ (D-A+E)] (Fig. 4; Table 3). We also made additional chimeras incorporating regions of the $\alpha 4$ subunit between the E loop

and the start of the TMs without significant differences to the chimera involving the E loop alone [$\alpha 2$ (E+B-TM1) and $\alpha 2$ (E+C)] or to the D-A+E chimera [$\alpha 2$ (D-A+E+C)] (Fig. 4; Table 3).

The E Loop and D-A Region Contain All the Determinants Necessary to Confer Full Potentiation. The physostigmine effect on receptors containing the $\alpha 2$ (D-A+E) chimera was comparable to that for $\alpha 4$ (1.42 ± 0.05 versus 1.51 ± 0.03 ; $P = 0.14$, two-tailed t test determining that the effects are the same), indicating that these regions contained all the determinants necessary to confer full potentiation.

Linear multiple regression of the physostigmine relative response on the origin of the regions transferred into the $\alpha 2$ subunit (see *Materials and Methods*) indicated that the largest effects were due to the presence of regions from $\alpha 4$ in the D-A region ($P = 2 \times 10^{-19}$ that the correlation arose by chance) and the E loop region ($P = 5 \times 10^{-19}$). In both cases, the presence of the $\alpha 4$ sequence increased potentiation. Other regions did not show strong effects ($P > 0.005$).

The Effects of Mutations on the Background of Chimeric $\alpha 2$ Subunits. We then refined the residues required by making point mutations in the D-A region on the background of $\alpha 2$ chimeric receptors. We performed the mutations symmetrically, seeking residues in the D-A region for which mutations increased the potentiation of the $\alpha 2$ (E) chimera to that of the $\alpha 2$ (D-A+E) chimera and, conversely, decreased the potentiation of the $\alpha 2$ (D-A+E) chimera to that of the $\alpha 2$ (E) chimera. We selected four pairs of mutations to make one in the D loop [$\alpha 4$ (V61) versus $\alpha 2$ (L61)], one immediately after the D loop [$\alpha 4$ (H66) versus $\alpha 2$ (N66)], and a group of three adjacent residues in the middle of the D-A region [$\alpha 4$ (D76,Y77,E78) versus $\alpha 2$ (E76,F77,G78)] and one near the A loop [$\alpha 4$ (R92) versus $\alpha 2$ (I92)]. These were chosen because the residues differ between $\alpha 2$ and $\alpha 4$ but are the same for $\alpha 2$ and $\beta 2$ (L61, E76, F77) or because there is a major difference in side-chain properties (H to N, R to I). On the background of $\alpha 2$ (E), the residue was changed from the $\alpha 2$ to the $\alpha 4$ residue (e.g., L61 to V61), whereas on the background of $\alpha 2$ (D-A+E), it was changed from $\alpha 4$ to $\alpha 2$ (V61 to L61).

Fig. 4. Effects of N-terminal region chimeras on the actions of physostigmine. The left-hand panel shows a cartoon representation of the structure of the N-terminal region of the various chimeras tested. Regions derived from the $\alpha 2$ subunit are shown in red, whereas regions from the $\alpha 4$ subunit are shown in blue. Regions that are identical between the two subunits are in gray. The chimeras are identified in the left-hand boxes; those based on the $\alpha 2$ subunit are shown in the bottom portion, whereas those based on the $\alpha 4$ subunit are shown in the top portion. The chimera joining points are shown in Fig. 3. The mean $\pm$ S.E. physostigmine effect is shown in the bar to the right, whereas the asterisks give the P value indicating that the effect is identical to the effect for the wild-type subunit (one-way ANOVA with Dunnett's correction; *** $P < 0.001$, ** $P < 0.01$). The solid vertical line shows an effect ratio of 1 (no effect), whereas the dashed lines show the mean effects for wild-type $\alpha 2$ and $\alpha 4$ subunits. Data values are given in Tables 3 and 5. All α subunits were expressed with $\beta 4$ at a 20:1 ratio.

TABLE 3

The D-A region and the E loop are important in determining the effect of physostigmine

Chimeric subunits are named as the subunit contributing to the amino terminus followed by the region swapped between subunits (in parentheses), so $\alpha 2$ (D-A) indicates an $\alpha 2$ subunit containing the $\alpha 4$ sequence for the D-A region. The joining points for the chimeras are shown in Fig. 3. The data are arranged in terms of increasing physostigmine effect. The column headed "P" gives the P value indicating that the parameter value is the same as that for the control for that group, indicated by X (one-way ANOVA with Dunnett's correction). All α subunits were injected with $\beta 4$ at a ratio of 20:1.

α Subunit	ACh EC ₅₀		Physostigmine Effect	
	μ M	P	Ratio	P
$\alpha 2$	93 ± 4 (38)	X	0.78 ± 0.03 (23)	X
$\alpha 2$ (D-A)	52 ± 6 (10)	0.3	0.79 ± 0.03 (10)	1.0
$\alpha 2$ (C)	37 ± 2 (9)	0.03	0.80 ± 0.02 (14)	1.0
$\alpha 2$ (B-TM1)	36 ± 2 (9)	0.02	0.86 ± 0.02 (20)	0.2
$\alpha 2$ (N-A)	96 ± 3 (7)	1	0.93 ± 0.01 (14)	0.002
$\alpha 2$ (A-TM1)	211 ± 32 (7)	<0.001	0.95 ± 0.02 (20)	<0.001
$\alpha 2$ (E)	209 ± 16 (3)	<0.001	0.98 ± 0.01 (12)	<0.001
$\alpha 2$ (E+C)	99 ± 5 (7)	0.9	1.01 ± 0.03 (30)	<0.001
$\alpha 2$ (E+B-TM1)	159 ± 6 (5)	0.006	1.05 ± 0.02 (31)	<0.001
$\alpha 2$ (D-A+E+C)	93 ± 14 (13)	0.9	1.31 ± 0.03 (20)	<0.001
$\alpha 2$ (D-A+E)	257 ± 12 (23)	<0.001	1.42 ± 0.05 (10)	<0.001

Data are the mean $\pm$ S.E.M. (number of cells).

TABLE 4

The effect of mutations in the D-A region differs depending on the background

The first two columns give the background chimera [$\alpha 2(E)$ or $\alpha 2(D-A+E)$] and the mutation made on that background. The data are arranged in order of the location of the mutation, with the site closest to the amino-terminus given first. The column headed " P " gives the P value indicating that the parameter value is the same as that for the control for that group, indicated by X (one-way ANOVA with Dunnett's correction). The locations of mutations and the joining points for the chimeras are shown in Fig. 3.

$\alpha 2$ Background	Mutation	ACh EC ₅₀		Physostigmine Effect	
		μM	P	Ratio	P
$\alpha 2(E)$		209 ± 16 (3)	X	0.98 ± 0.01 (12)	X
$\alpha 2(E)$	L61V	ND	ND	1.00 ± 0.01 (5)	0.9
$\alpha 2(E)$	N66H	124 ± 4 (2)	0.07	0.67 ± 0.04 (5)	<0.001
$\alpha 2(E)$	EFG $\rightarrow$ DYE	231 ± 4 (2)	0.7	0.68 ± 0.05 (5)	<0.001
$\alpha 2(E)$	I92R	235 ± 8 (5)	0.5	1.19 ± 0.05 (7)	<0.001
$\alpha 2(D-A+E)$		257 ± 12 (23)	X	1.42 ± 0.05 (10)	X
$\alpha 2(D-A+E)$	V61L	ND	ND	1.13 ± 0.03 (5)	0.005
$\alpha 2(D-A+E)$	H66N	121 ± 50 (3)	0.001	1.32 ± 0.11 (5)	0.6
$\alpha 2(D-A+E)$	DYE $\rightarrow$ EFG	92 ± 13 (7)	<0.001	1.38 ± 0.04 (5)	1.0
$\alpha 2(D-A+E)$	R92I	184 ± 10 (5)	0.02	0.99 ± 0.01 (6)	<0.001

Data are the mean $\pm$ S.E.M. (number of cells). ND, not determined.

The results are shown in Table 4 and Fig. 5. There was one pair of mutations that showed the predicted pattern: $\alpha 2(E+I92R)$ showed potentiation and $\alpha 2(D-A+E+R92I)$ showed a loss of potentiation. The physostigmine effect on $\alpha 2(E)$ was increased by the mutation I92R to 1.19 ± 0.05 compared with 0.98 ± 0.01 for $\alpha 2(E)$ ($P = 0.01$ that the physostigmine effects are equivalent based on a one-way analysis of variance (ANOVA) with Bonferroni correction for all the constructs shown in Table 4). However, the physostigmine effect was less than that for $\alpha 2(D-A+E)$ (1.42 ; $P = 0.01$). The converse mutation $\alpha 2(D-A+E+R92I)$ reduced the physostigmine effect to 0.99 ± 0.01 , which did not differ from that for $\alpha 2(E)$ ($P = 1$) and was significantly less than that for $\alpha 2(D-A+E)$ ($P < 0.001$).

However, the other pairs showed discordant results. On the background of $\alpha 2(E)$, both the N66H and EFG to DYE mutations significantly increased inhibition by physostigmine, whereas on the $\alpha 2(D-A+E)$ background neither the H66N nor the DYE to EFG mutations had significant effects on potentiation. In contrast, the L61V mutation had no effect on the $\alpha 2(E)$ background, whereas V61L significantly reduced potentiation on the $\alpha 2(D-A+E)$ background. This pattern of results is difficult to interpret. Overall, it appears that there are several regions in the D-A region that interact to influence the binding of physostigmine and/or the transduction of binding into functional effect.

The Effects of Mutations in the E Loop of the $\alpha 2$ Subunit. We also mutated two residues in the E loop of wild-type $\alpha 2$ subunits: $\alpha 2(H122Q)$ and $\alpha 2(V124T)$. The mutations change two of the residues in the E loop that previous work has shown are critical in determining the difference in EC₅₀ for ACh between receptors containing three copies of $\alpha 4$ and three copies of $\beta 2$ (Harpsøe et al., 2011; Lucero et al., 2016). The third residue studied by these authors [$\alpha 2(H114)$] is identical between $\alpha 2$ and $\alpha 4$. The double mutant $\alpha 2(Q122, T124)$ was inhibited by physostigmine (ratio 0.92 ± 0.01 , $N = 5$). Inhibition was reduced compared with $\alpha 2$ wild-type subunits (ratio 0.81 ± 0.03 ; $P = 0.001$ from a t test with unequal variance) but was larger than that for the $\alpha 2(E)$ chimera (ratio 0.98 ± 0.01 ; $P = 0.004$). The double mutant had an EC₅₀ for ACh of $242 \pm 9 \mu M$ (5 cells), compared with $91 \mu M$ for $\alpha 2$ wild-type ($P = 0.01$) and $209 \mu M$ for $\alpha 2(E)$ ($P = 0.2$), supporting the role of these residues in determining the EC₅₀.

When we made the $\alpha 2(I92R)$ mutation on the background of $\alpha 2(Q122, T124)$ the physostigmine response was increased to 1.04 ± 0.01 ($N = 5$), a level that was greater than that for the double mutant alone (0.92 ± 0.01 ; $P = 5 \times 10^{-5}$) but still less

Fig. 5. Effects of mutations in the D-A region. The figure is similar to Figure 4. In the left panel, a cartoon of the region from the D loop to the end of the E loop is shown, with regions derived from the $\alpha 2$ subunit in red and regions from the $\alpha 4$ subunit in blue. The A loop (gray) is identical in these subunits, whereas the region between the A and E loops (white) was not mutated. The mutations were made on the background of the $\alpha 2(E)$ chimera (top four lines, hatched bars) or the $\alpha 2(D-A+E)$ chimera (bottom 4 lines, solid black bars), whereas the middle four lines show results for relevant chimeras and wild-type $\alpha 2$ subunits. The approximate location of the residues mutated is shown by the colored bars shown in the cartoons (left). On the background of the $\alpha 2(E)$ chimera, the mutation changed a residue found in the $\alpha 2$ sequence to that found in the $\alpha 4$ subunit and so is shown as red and vice versa. The dashed lines show the mean effects for the background chimeras, and the asterisks indicate the P value that the effect is identical to that for the background chimera (one-way ANOVA with Dunnett's correction; *** $P < 0.001$, ** $P < 0.01$). For locations of chimera joining points and mutations, see Fig. 3. Data are shown as the mean $\pm$ S.E.; data values are given in Table 4.

than that for the $\alpha 2(\text{E} + \text{I92R})$ construct (1.19 ± 0.05 ; $P = 0.02$). These observations suggest that these residues in the E loop contribute to determining the effects of physostigmine but are not the sole determinants in the E loop.

Substituting Regions of the $\alpha 4$ N-Terminal Domain with Homologous Regions from the $\alpha 2$ Subunit Can Remove Potentiation and Confer Block. We also constructed four constructs based on the $\alpha 4$ subunit. Transfer of both the D-A and E regions fully removed potentiation and conferred block (Fig. 4; Table 5), whereas the separate transfer of the regions produced partial effects. The physostigmine effect on receptors containing the $\alpha 4(\text{D-A} + \text{E})$ chimera (0.73 ± 0.02) was comparable to that for receptors containing $\alpha 2$ (0.78 ± 0.03 ; $P = 0.02$, two-tailed t test determining that the effects are the same). These consequences are the inverse of the effect of the transfers into the $\alpha 2$ subunit and support the idea that these regions are the full basis for the differences in the actions of physostigmine.

Receptor Activation by ACh. Many of the constructs based on the $\alpha 2$ subunit showed EC_{50} values for ACh that differed significantly from wild type (Tables 3 and 4), with values spread over almost a 10-fold range for the 17 constructs tested. However, there was not a strong relationship between the change in activation by ACh and the effect of physostigmine. There was a relatively weak rank correlation between the physostigmine effect and EC_{50} ($P = 0.03$), indicating that a larger physostigmine ratio and higher EC_{50} values tend to be associated. It seems likely that the correlation between the magnitude of the physostigmine effect and the EC_{50} values reflects the strong tendency of the $\alpha 4$ sequence in the E loop to increase both parameters. There was no significant rank correlation between the maximal current and the physostigmine effect ratio for these constructs ($P = 0.4$; data not shown).

Multiple linear regression (as described for the physostigmine effect) for the 11 chimeras shown in Table 3 showed no significant effect of the origin of the D-A region on the EC_{50} value ($P = 0.3$), whereas both the E loop and C regions showed strong effects ($P = 8 \times 10^{-25}$ and 2×10^{-19} , respectively). When $\alpha 4$ sequence was in the E loop, the EC_{50} value was larger, whereas when it was in the C region it was lower. The effects of sequence on activation differed from those on physostigmine, in that no dependence on the D-A region was present. Furthermore, the C region strongly affected the ACh EC_{50} value but not the physostigmine effect.

These results indicate that there is no strong relationship between the effects of the various molecular manipulations on activation by ACh and modulation by physostigmine. Although regions of the α subunit that contribute to the ACh-binding sites do contain residues that affect modulation by physostigmine, the importance of the regions is not identical for effects on physostigmine and ACh.

Discussion

Our initial observation was that the effect of physostigmine differed between neuronal nicotinic receptors containing three copies of the $\alpha 2$ subunit versus three copies of the $\alpha 4$ subunit. We found that we could fully convert the responses by interchanging regions in the amino-terminal extracellular regions of the subunits.

The inhibition of responses seen with the $\alpha 2$ subunit was not influenced by the residues in the transmembrane region. The

TABLE 5

Reciprocal exchanges in the $\alpha 4$ subunit have inverse effects

The data are arranged in terms of increasing physostigmine effect. The column headed " P " gives the P value indicating that the parameter value is the same as that for the control for that group, indicated by X (one-way ANOVA with Dunnett's correction). All α subunits were injected with $\beta 4$ at a ratio of 20:1.

α Subunit	ACh EC_{50}		Physostigmine Effect	
	μM	P	Ratio	P
$\alpha 4(\text{D} - \text{A} + \text{E})$	16 ± 2 (6)	0.005	0.73 ± 0.02 (13)	<0.001
$\alpha 4(\text{E})$	7 ± 0 (4)	<0.001	0.96 ± 0.01 (12)	<0.001
$\alpha 4(\text{N} - \text{A})$	ND	ND	1.08 ± 0.01 (13)	<0.001
$\alpha 4(\text{D} - \text{A})$	74 ± 6 (8)	<0.001	1.27 ± 0.02 (24)	<0.001
$\alpha 4$	31 ± 2 (24)	X	1.51 ± 0.03 (31)	X

Data are the mean $\pm$ S.E.M. (number of cells). ND, not determined.

$\alpha 2$ and $\alpha 4$ subunits are identical in the TM1 to TM3 regions, and swapping the TM4 region did not affect the responses to physostigmine. The various chimeric constructs also indicate that inhibition is fully accounted for by the effects of residues in the amino-terminal extracellular domain. Our previous study (Jin et al., 2017) of the actions of physostigmine on receptors containing $\alpha 4$ and $\beta 2$ subunits obtained similar results. In that work, the inhibition seen with low concentrations of ACh and physostigmine for receptors containing two copies of $\alpha 4$ and three copies of $\beta 2$ was unlikely to result from open channel block. We did not examine the properties of inhibition in the present study, but it seems highly likely that the inhibition seen with receptors containing $\alpha 2$ subunits also does not result from open channel block.

Swapping the E loops between $\alpha 2$ and $\alpha 4$ subunits to produce the $\alpha 4(\text{E})$ and $\alpha 2(\text{E})$ chimeras resulted in receptors that showed weak inhibition in both cases. This result makes it more likely that interchange of the E loop affected both potentiation and inhibition, because an effect on only one action would not result in the ablation of both potentiation and inhibition. Further, it indicates that the E loop is necessary but not sufficient for either action of physostigmine. To fully interconvert the inhibition found with the $\alpha 2$ subunit and the potentiation found with the $\alpha 4$ subunit, we had to exchange both the D-A region and the E loop.

We identified one residue in the D-A region that interacted with the E loop in a fashion that we had expected based on the interactions between the full D-A region and the E loop. If a residue in the D-A region underlay the difference in the actions of physostigmine on the $\alpha 2(\text{E})$ and $\alpha 2(\text{D-A} + \text{E})$ constructs, then in the simplest case swapping the residue would swap the effects of physostigmine. The residue at position 92 [$\alpha 2(\text{I92})$ versus $\alpha 4(\text{R92})$] essentially meets this criterion. These results clearly implicate this residue as a critical element in determining the effects of physostigmine in concert with the E loop. However, other mutations in the D-A region resulted in significant changes in the physostigmine effect that were not reciprocal between the background chimeras. This implies that additional interactions are important, although the rules appear obscure. For example, on the background of $\alpha 2(\text{E})$ mutating the residues E76, F77, and G78 of the $\alpha 2$ sequence to DYE of the $\alpha 4$ sequence actually significantly increased inhibition by physostigmine. If a simple mapping of the $\alpha 4$ sequence to increased potentiation (or decreased inhibition) existed, this result would be completely unexpected. It could be that some residues in the D-A region more strongly influence processes resulting in inhibition and others resulting in potentiation.

Fig. 6. Location of mutated residues in the N-terminal domain of the $\alpha 4$ subunit. The figure shows views of the extracellular N-terminal domain of the nicotinic $\alpha 4\beta 2$ receptors (Morales-Perez et al., 2016) to indicate the position of the residues mutated. The color coding is kept constant for all panels. (A) and (B) provide overall orientation. (A) A view from the extracellular fluid looking down at the receptor. The $\alpha 4$ subunits are shown as blue ribbons, whereas the $\beta 2$ subunits are shown in green. The yellow arrows show the locations of canonical ACh-binding sites ($\alpha 4$ counterclockwise, contributing the “+” side). The red bracket indicates the $\beta 2$ - $\alpha 4$ interface shown in the remaining panels. (B) A side view of a $\beta 2$ - $\alpha 4$ pair from the outside of the receptor with the extracellular fluid at the top. The approximate position of the cell membrane is shown by the black bracket on the right. The E loop of the $\alpha 4$ subunit is shown in orange, and the D-A region is shown in cyan. (C) The region indicated by the red box in (B), enlarged and with the $\beta 2$ subunit removed to reveal the $\alpha 4$ subunit. The view is from the outside of the receptor, looking into the cavity that binds drugs. The face of the “-” side of the binding pocket is shown. The peptide chain of the $\alpha 4$ subunit is shown as a blue ribbon, with the E loop shown in orange and the D-A region shown in cyan. The mutated residues

Three residues in the E loop have been identified as critical in determining the pharmacological differences between stoichiometric forms of the $\alpha 4\beta 2$ receptor: $\alpha 4$ (H114), $\alpha 4$ (Q122), and $\alpha 4$ (T124) (Harpsøe et al., 2011; Lucero et al., 2016). H114 is present in both $\alpha 2$ and $\alpha 4$, whereas the other two residues differ [$\alpha 2$ (H122) and $\alpha 2$ (V124)]. We made the double mutation to the wild-type $\alpha 2$ subunit [$\alpha 2$ (Q122, T124)] and found that physostigmine still inhibited the response to ACh, although to a lesser extent than that for $\alpha 2$. The addition of the $\alpha 2$ (I92R) mutation on the background of $\alpha 2$ (Q122, T124) conferred a small level of potentiation, although less than that seen with the $\alpha 2$ (E+I92R) construct. These observations suggest that the E loop has residues affecting the actions of physostigmine in addition to those that affect activation by ACh.

In the structure of the $\alpha 4$ subunit (Morales-Perez et al., 2016), the D-A region curves around the E loop (Fig. 6, B and C), suggesting that extensive physical interactions could take place. The $\alpha 4$ (R92) residue lies behind the E loop and is oriented to approach the conserved tryptophan at position 123. The residues in or near the D loop [$\alpha 4$ (V61) and $\alpha 4$ (H66)] are near the carboxy-terminal end of the E loop, although not oriented directly toward the E loop. However, the sidechains of the triplet starting at D76 are oriented toward the initial α helix, far from the E loop itself. None of these residues is positioned in such a way as to indicate that they participate in forming a binding site also involving the E loop, suggesting that they either participate in a transduction process or alter the conformation of the receptor to facilitate the interaction of physostigmine with the receptor. The residues are also located in positions that are unlikely to associate with an adjacent subunit, suggesting that all the interactions take place within a single subunit.

There was no indication that the loops contributed by the α subunit to the “+” side of an interface played a role in determining the effects of physostigmine. The A and B loops are identical for the $\alpha 2$ and $\alpha 4$ subunits (Fig. 3), and swapping the region containing the C loop had no effect (Table 2).

The E loop of the α subunit does not participate in a canonical ACh-binding site as it lies on the “-” or complementary side of the interface. This side is contributed by a β or other non- α subunit in a canonical binding site. A surprising result from previous studies of $\alpha 4$ and $\beta 2$ subunits (Jin et al., 2017) was that swapping the E loop from $\beta 2$ to $\alpha 4$ resulted in physostigmine becoming an inhibitor regardless of whether the chimeric $\alpha 4$ subunit was placed counterclockwise from an $\alpha 4$ subunit so that the chimera provided the “-” side to an $\alpha 4$ - $\alpha 4$ interface or with a $\beta 2$ subunit to contribute to a $\beta 2$ - $\alpha 4$ interface. This appears to be the case for the $\alpha 2$ subunit as well. We expressed free $\alpha 2$ subunits with concatemers of the $\alpha 4$ and $\beta 2$ subunits

are shown in stick format: V61 and H66 residues are shown in green to the right of the E loop; D76, Y77, and E78 residues are shown in black at the top of the panel; and R92 residue is shown in red behind the E loop. (D–F) Enlarged views of mutated residues. (D) The V61 and H66 residues (green stick format) seen from a perspective similar to (A). Although these residues are close to the E loop, they do not appear to be in a position to directly interact or extend into the drug-binding pocket. (E) The D76, Y77, and E78 residues (black stick format) seen from a perspective further into the extracellular milieu. These residues appear to be close to the initial α helix of the subunit and far from the E loop. (F) The R92 residue is shown in red seen from a perspective approximately 180° from that in (A), where R92 is behind the E loop and appears to approach the W123 sidechain in the E loop (shown in orange stick format).

constructed so that the free $\alpha 2$ subunit would contribute the “–” side to either an $\alpha 4$ - $\alpha 2$ interface or a $\beta 2$ - $\alpha 2$ interface (Fig. 1) and obtained similar low levels of potentiation by physostigmine.

Although the nature of the amino acids in the E loop affects both the actions of physostigmine and activation by ACh, there does not seem to be a mechanistic connection between the two effects. There was no connection or a weak relationship between them in terms of the effects of the various constructs on physostigmine effects and activation parameters, and the regions of the α subunit implicated in mediating effects were not identical.

Our experiments focused on the properties of the α subunit, but inspection of Table 1 suggests that the nature of the β subunit has some effect on the response to physostigmine: in every expression context, the $\beta 4$ subunit resulted in larger potentiation or less inhibition than the $\beta 2$ subunit.

The present results agree with our previous studies of physostigmine effects on receptors containing $\alpha 4$ and $\beta 2$ subunits (Jin et al., 2017) and confirm the role of the E loop. In combination, the D-A region and the E loop are able to fully explain the differences in physostigmine effect between the $\alpha 2$ and $\alpha 4$ subunits. Studies of the related nicotinic receptor from *Torpedo* electroplax have shown that physostigmine photolabels residues near the E loop (Hamouda et al., 2013), which is consistent with the idea that physostigmine binds at or near the E loop. The additional residues we have identified in the D-A region appear likely to be involved in the transduction of binding into either potentiation or inhibition.

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Authorship Contributions

Participated in research design: Jin, Akk, and Steinbach.
Conducted experiments: Jin, Germann, and Shin.
Contributed new reagents or analytic tools: Jin and Germann.
Performed data analysis: Jin, Germann, Shin, Akk, and Steinbach.
Wrote or contributed to the writing of the manuscript: Jin, Akk, and Steinbach.

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