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# Differential Activation and Desensitization States Promoted by Noncanonical $\alpha 7$ Nicotinic Acetylcholine Receptor Agonists<sup>§</sup>

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## ABSTRACT

A series of dipicolyl amine pyrimidines (DPPs) were previously identified as potential  $\alpha 7$  agonists by means of a calcium influx assay in the presence of the positive allosteric modulator (PAM) 1-(5-chloro-2,4-dimethoxy-phenyl)-3-(5-methyl-isoxazol-3-yl)-urea (PNU-120596). The compounds lack the quaternary or strongly basic nitrogens of typical nicotinic agonists. Although differing in structure from typical nicotinic agonists, based on crystallographic data with the acetylcholine binding protein, they appeared to engage the site shared by such typical orthosteric agonists. Using oocytes expressing human  $\alpha 7$  receptors, we found that the DPPs were efficacious activators of the receptor, with currents showing rapid desensitization characteristic of  $\alpha 7$  receptors. However, we note that the rate of recovery from this desensitization depends strongly on structural features within the DPP family. Although the activation of receptors by DPP was blocked by the competitive antagonist methyllycaconitine (MLA), MLA had no effect on the DPP-induced desensitization, suggesting multiple modes of DPP binding. As expected, the desensitized conformational states could be reactivated by PAMs. Mutants made insensitive to acetylcholine by the C190A mutation in the agonist binding site

were weakly activated by DPPs. The observation that activation of C190A mutants by the DPP compounds was resistant to the allosteric antagonist (-)-*cis-trans*-4-(2,3,5,6-tetramethylphenyl)-3a,4,5,9b-tetrahydro-3H-cyclopenta[c]quinoline-8-sulfonamide supports the hypothesis that the activity of these noncanonical agonists in the orthosteric binding sites was not entirely dependent on the classic epitopes controlling activation by typical agonists and that perhaps they may access alternative modes for promoting the conformational changes associated with activation and desensitization.

## SIGNIFICANCE STATEMENT

This study reports a family of nicotinic acetylcholine receptor agonists that break the rules about what the structure of a nicotinic acetylcholine receptor agonist should be. It shows that the activity of these noncanonical agonists in the orthosteric binding sites is not dependent on the classical epitopes controlling activation by typical agonists and that through different binding poses, they promote unique conformational changes associated with receptor activation and desensitization.

## Introduction

The  $\alpha 7$  nicotinic acetylcholine receptor (nAChR) is one of the most well-studied subtypes of a pentameric family of allosteric transmembrane proteins (Bertrand et al., 2015; Bouzat et al.,

2018). It comprises five  $\alpha$  subunits forming a homopentamer around a central cation channel, which, when activated by acetylcholine (ACh) alone, has a relatively high permeability to calcium (Séguéla et al., 1993; Miller et al., 2020). Each subunit contributes to two possible agonist binding faces, one at each subunit interface. Although a three-dimensional cryogenic electron microscopy structure of the receptor has recently been published (Noviello et al., 2021), the degree to which the five putative orthosteric agonist (OA) binding sites contribute stoichiometrically and dynamically to activation is still unknown. Early structural data for  $\alpha 7$ , derived from mutant acetylcholine binding proteins (AChBP) (Hibbs et al., 2009), suggest that the binding sites

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**ABBREVIATIONS:** ACh, acetylcholine; AChBP, acetylcholine binding protein; aDPP, 2-amino dipicolyl amine pyrimidine; CAP, cholinergic anti-inflammatory pathway; DPP, dipicolyl amine pyrimidine; MLA, methyllycaconitine; nAChR, nicotinic acetylcholine receptor; NS6740, 1,4-diazabicyclo[3.2.2]nonan-4-yl(5-(3-(trifluoromethyl)-phenyl)-furan-2-yl)methanone; OA, orthosteric agonist; PAM, positive allosteric modulator; PNU-120596, 1-(5-chloro-2,4-dimethoxy-phenyl)-3-(5-methyl-isoxazol-3-yl)-urea; TMP-TQS, *cis-trans*-4-(2,3,5,6-tetramethylphenyl)-3a,4,5,9b-tetrahydro-3H-cyclopenta[c]quinoline-8-sulfonamide; WT, wild-type;  $\tau$ , time constant.

and subunit conformations become nonequivalent as the ligands bind sequentially.

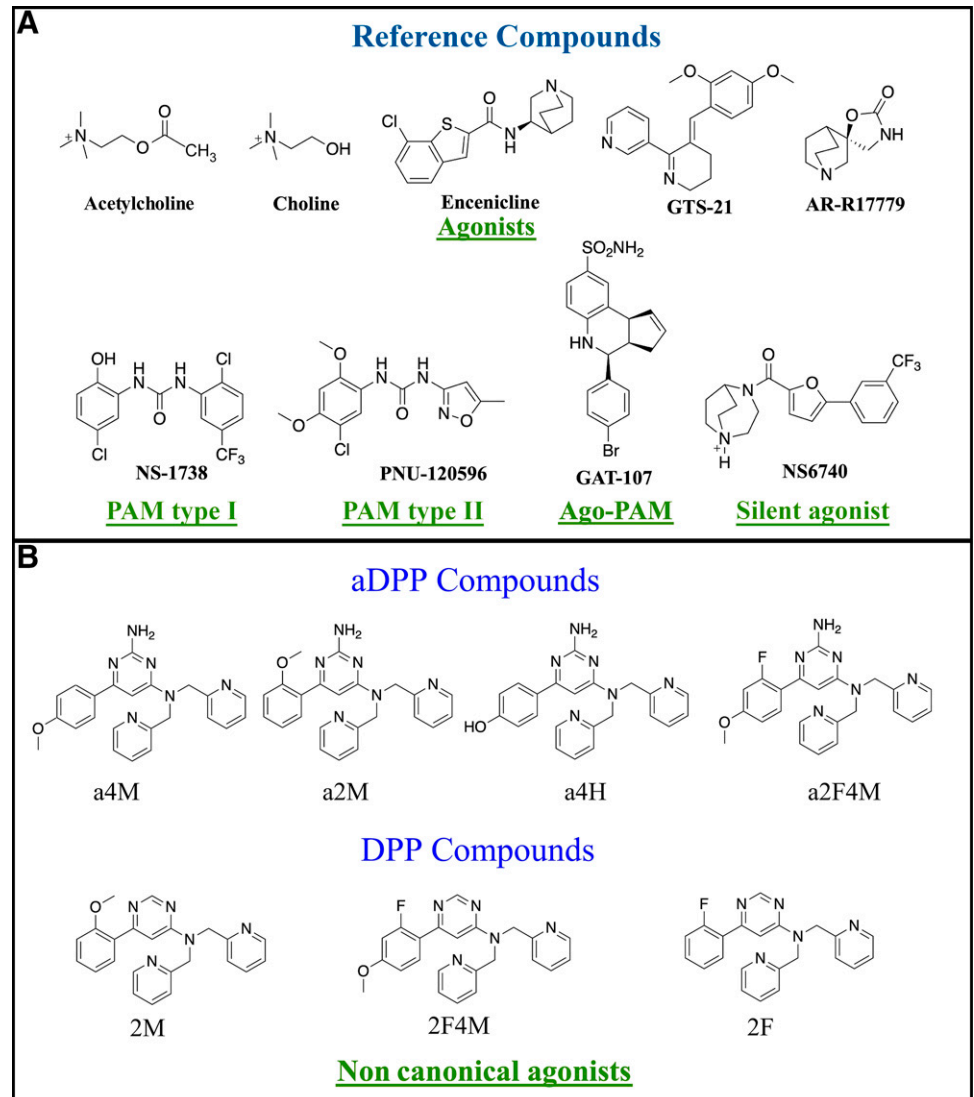
Compared with other nAChRs,  $\alpha 7$  receptors have a low probability of opening in response to ACh, as well as a rapid desensitizing profile that is unique and known to be concentration dependent (Papke and Porter Papke, 2002; Papke and Lindstrom, 2020). Specifically, the level of occupation of the binding sites by agonist appears to underlie the kinetics of this desensitization, such that high levels of ACh binding appear to explicitly promote closed (i.e., desensitized) states (Papke and Lindstrom, 2020). The  $\alpha 7$  receptors expressed in neurons have been implicated to be important for cognition, attention, regulation of aggression, and modulation of depression-like behaviors, whereas  $\alpha 7$  expression in nonneuronal cells has been shown to regulate inflammation (Jones et al., 1999; Wang et al., 2003; de Jonge and Ulloa, 2007; Bagdas et al., 2018; Lewis et al., 2018; Mineur et al., 2018).

Initially, most of the efforts targeting  $\alpha 7$  receptors were focused on orthosteric ligands and channel activation, resulting in the identification of several classes of  $\alpha 7$ -selective agonists, such as (-)-spiro[1-azabicyclo[2.2.2]octane-3,5-oxazolidin]-2-one

hydrochloride (AR-R17779) (Levin et al., 1999) and N-(3R)-1-azabicyclo[2.2.2]oct-3-yl-4-chlorobenzamide (PNU-282987) (Bodnar et al., 2005), and partial agonists, such as 3-(2,4-dimethoxybenzylidene)-anabaseine (GTS-21) (Sullivan et al., 1995) (Fig. 1A). Nonetheless, although diverse in structure, all  $\alpha 7$ -selective agonists previously identified share the structural features associated with the classic nicotinic pharmacophore (Beers and Reich, 1970), with a basic nitrogen in the core where a cationic species would prevail.

Presently, the exploration of ligands targeting  $\alpha 7$  extends beyond the OA binding sites to extracellular allosteric activation sites that are possibly accessed via the receptor vestibule, as well as putative allosteric sites in the transmembrane domains. Various types of allosterically acting ligands are known and include allosteric agonists like GAT107 (Fig. 1A) and positive allosteric modulators (PAMs) that bind in the transmembrane domains (Young et al., 2008). Type I PAMs like 1-(5-chloro-2-hydroxyphenyl)-3-(2-chloro-5-(trifluoromethyl)phenyl) urea (NS1738) (Fig. 1A) increase peak current responses to agonists, whereas more efficacious type II PAMs like 1-(5-chloro-2,4-dimethoxy-phenyl)-3-(5-methyl-isoxazol-3-yl)-urea (PNU-120596) (Fig. 1A) additionally convert agonist- and silent

**Fig. 1.** Compounds relevant to these studies. (A) Previously characterized compounds. (B) aDPP and DPP compounds used in these experiments.



agonist-induced nonconducting (desensitized) states of receptors to active states (Hurst et al., 2005).

Employing *Lymnaea stagnalis* AChBP (Brejc et al., 2001; Kaczanowska et al., 2017; Camacho-Hernandez et al., 2019; Camacho-Hernandez and Taylor, 2020) as a structural surrogate for  $\alpha 7$  nAChR, we identified a series of 2,4,6-substituted pyrimidine analogs (Fig. 1B) that have noncanonical agonist structures—that is, they are able to activate  $\alpha 7$  receptors selectively but lack characteristic chemical features associated with the nicotinic pharmacophore (Beers and Reich, 1970; Sheridan et al., 1986; Tønder et al., 2001). The core structure of these compounds with partial or full agonist activity is a dipicolyl amine pyrimidine (DPP). For reading convenience, here we abbreviate the nomenclature to the substitutions on the core structure. The prefix “a” indicates an amino group in the second position of the pyrimidine core, 2-amino dipicolyl amine pyrimidine (aDPP), and the rest of the abbreviated name is related to the substitutions on the phenyl ring in position 6 of the pyrimidine ring. Initial electrophysiological characterization of these ligands (Camacho-Hernandez et al., 2019) showed activation of  $\alpha 7$  and reduced postapplication ACh-evoked responses, indicating that, to varying degrees, these DPP compounds could induce a stabilization of nonconducting or desensitized states that could be converted by application of PNU-120596 to conducting states, somewhat similarly to the silent agonist 1,4-diazabicyclo[3.2.2]nonan-4-yl(5-(3-(trifluoromethyl)-phenyl)-furan-2-yl)methanone (NS6740) (Briggs et al., 2009). In this article, we investigate whether the activation and desensitizing properties of the DPP compounds rely on the OA binding sites in different binding modes or whether the compounds may also be binding to allosteric sites.

## Materials and Methods

**Chemicals and Reagents.** ACh and buffer chemicals were purchased from Sigma-Aldrich (St. Louis, MO). PNU-120596 was synthesized in the laboratory of Dr. Nicole Horenstein by Dr. Kinga Chojnacka following the published procedure (Hurst et al., 2005). The 2,4,6-substituted pyrimidine analogs (DPP series) were synthesized as previously documented (Kaczanowska et al., 2017; Camacho-Hernandez et al., 2019).

**Expression in *Xenopus* Oocytes.** The human  $\alpha 7$  nAChR clone was obtained from Dr. J. Lindstrom (University of Pennsylvania, Philadelphia, PA). The human resistance-to-cholinesterase 3 clone was obtained from Dr. M. Treinin (Hebrew University, Jerusalem, Israel) and coinjected with  $\alpha 7$  at a ratio of 2:1  $\alpha 7$ :resistance-to-cholinesterase 3 to enhance  $\alpha 7$  receptor expression without affecting the pharmacological

TABLE 1

Net-charge activity of test compounds on WT  $\alpha 7$  receptors.  $I_{\max}$  is normalized to ACh  $I_{\max}$ , and  $I_{\max}$  of postcontrol responses are normalized to 60  $\mu\text{M}$  ACh precontrol responses.

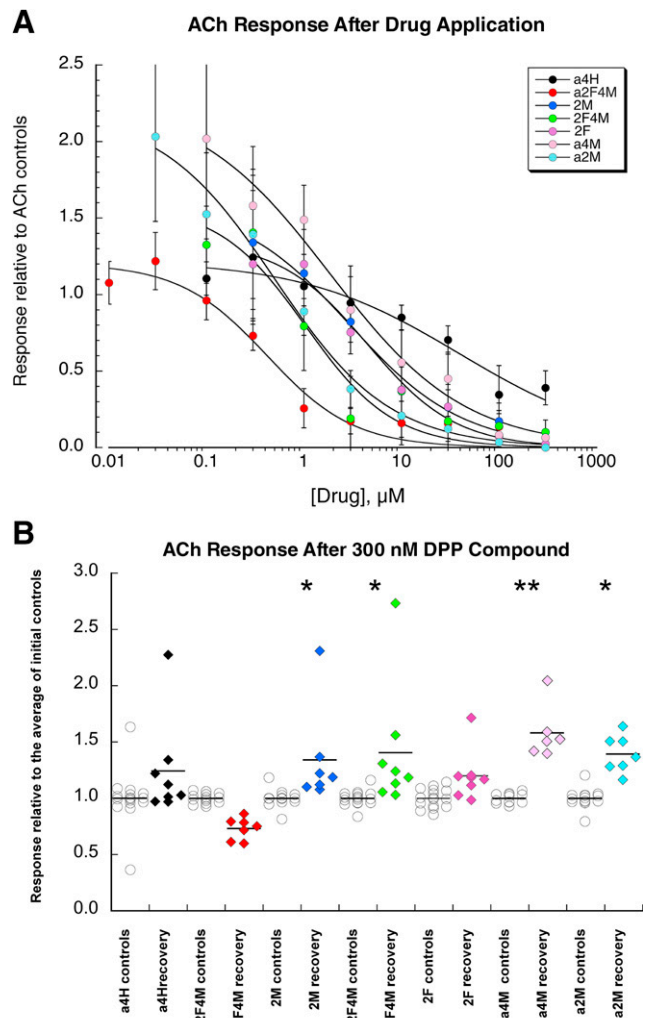
	Response $I_{\max}^a$	EC <sub>50</sub> , $\mu\text{M}^a$	Recovery $I_{\max}$	IC <sub>50</sub> , $\mu\text{M}$
a4H	0.64 ± 0.03	0.37 ± 0.08	1.22 ± 0.11	39.91 ± 20.05
a2F4M	0.78 ± 0.04	0.11 ± 0.03	1.20 ± 0.12	0.42 ± 0.17
2M	0.78 ± 0.02	2.98 ± 0.32	1.61 ± 0.21	2.95 ± 1.35
2F4M	0.72 ± 0.07	0.67 ± 0.27	1.59 ± 0.40	1.04 ± 0.82
2F	0.99 ± 0.03	2.55 ± 3.96 <sup>b</sup>	1.35 ± 0.14	4.47 ± 1.54
a4M	0.77 ± 0.03	0.92 ± 0.12	2.27 ± 0.31	1.85 ± 1.08
a2M	1.25 ± 0.36	1.35 ± 0.39	2.20 ± 0.21	0.50 ± 0.18

<sup>a</sup>See Supplemental Fig. 1.

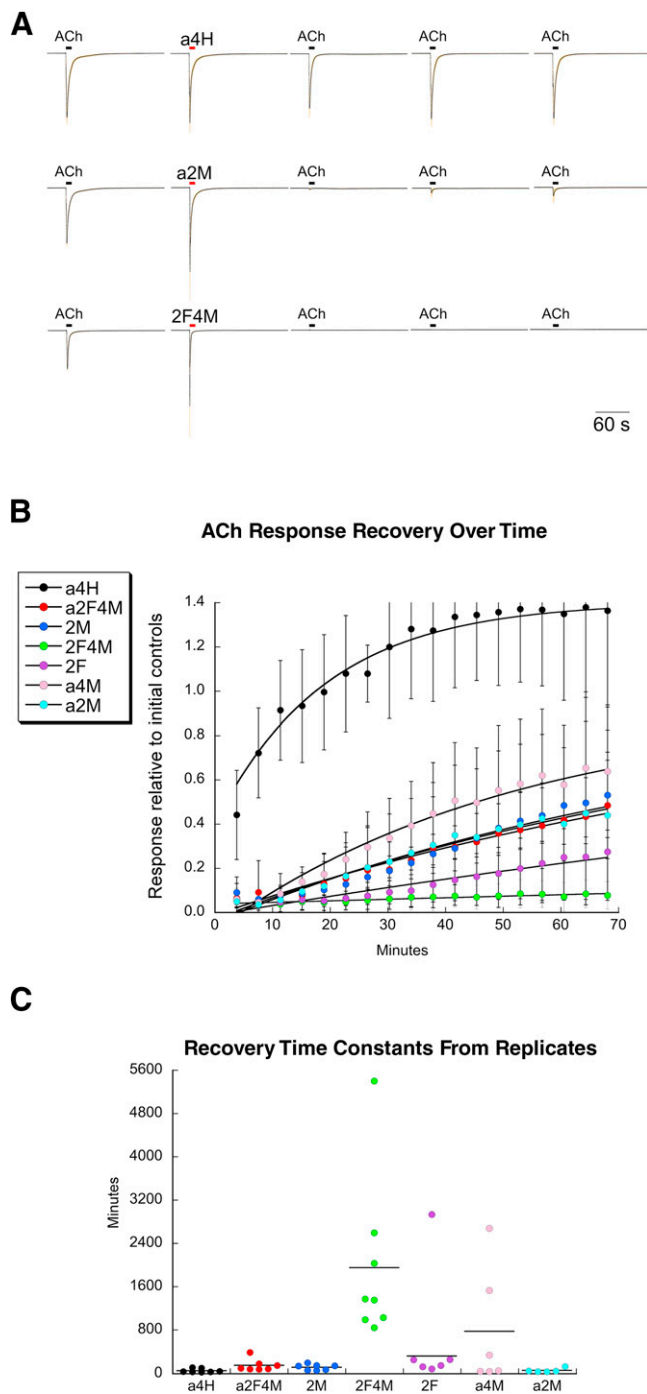
<sup>b</sup>These data were not well fit by the Hill equation, so that although the  $I_{\max}$  value is a reasonable indication of efficacy, the EC<sub>50</sub> value has an unsatisfactory error range.

properties of the receptors (Halevi et al., 2003). The  $\alpha 7\text{C190A}$  mutant was made as previously described with a C116S double mutation to prevent spurious disulfide bond formation with the free cysteine (Papke et al., 2011). Subsequent to linearization and purification of the plasmid DNAs, RNAs were prepared using the mMessage mMachine in vitro RNA transcription kit (Ambion, Austin, TX). A total of 6–9 ng RNA per oocyte was injected in 50 nl water. Oocytes were maintained in Barth's solution [88 mM NaCl, 1 mM KCl, 2.38 mM NaHCO<sub>3</sub>, 0.82 mM MgSO<sub>4</sub>, 15 mM HEPES, 0.41 mM CaCl<sub>2</sub>, 0.32 mM Ca(NO<sub>3</sub>)<sub>2</sub>, and 12 mg/l tetracycline, pH 7.6] at 16°C, and recordings were made 3–14 days after injection.

Oocytes were surgically removed from mature female *Xenopus laevis* frogs (Nasco, Ft. Atkinson, WI) as previously described (Pismataro et al., 2020). Frogs were maintained in the Animal Care Service facility of the University of Florida, and all procedures were approved by the University of Florida Institutional Animal Care and Use Committee (approval number 202002669).



**Fig. 2.** Enhancement and inhibition of postapplication ACh responses by DPP compounds. (A) Follow-up net-charge responses of  $\alpha 7$  receptors to 60  $\mu\text{M}$  ACh 4 minutes after single 12-second applications of the DPP compounds (*Materials and Methods*) at the indicated concentrations. Each point is the average ( $\pm$  S.D.) of 5–8 cells, normalized to the average of two initial ACh control responses obtained from the same cells. Data were fit to the Hill equation assuming a negative slope and allowing the initial  $I_{\max}$  to be fit as an experimental parameter (Table 1). (B) Statistical comparisons of the initial controls and the ACh responses following the application of the DPP compounds at a concentration of 300 nM. Significant increases in the ACh responses were noted for four of the compounds (\* $P$  < 0.05; \*\* $P$  < 0.0001, corrected for multiple comparisons). Full ANOVA provided in Supplemental Data.



**Fig. 3.** Varying recovery of ACh controls on oocytes expressing human  $\alpha 7$  nAChR following application of 100  $\mu\text{M}$  DPP and aDPP compounds. (A) Averaged raw data for ACh responses prior to and following 100  $\mu\text{M}$  application of representative DPP compounds. Data for individual cells ( $n = 8, 5,$  and 8 for a4H, a2M, and 2F4M, respectively) were normalized to the ACh peak current amplitudes from the same cells prior to the DPP applications. Data on averaged response (dark line) and S.E.M. (tan shaded areas) were calculated for each of 10,322 points over the 206.44-second acquisition periods. (B) Average net-charge values ( $\pm$  S.D.,  $n = 5$ –8 cells) of repeated ACh applications following 100  $\mu\text{M}$  DPP applications recorded at 4-minute intervals over the course of an hour. The averaged data were fit to an equation for exponential recovery of the response ( $R_t$ ) from a baseline (base), representing the extrapolated inhibition/desensitization immediately after the DPP application and  $\tau$  for recovery back to original control response amplitude:  $R_t = \text{base} + (1 - e^{-t/\tau})$ . Fit values and correlation coefficients are provided in Table 4. Also provided in Table 4 are the averaged fits ( $\pm$  S.D.) of the single-cell replicates (Supplemental Figs. 2–8). (C) Dot plots of fitted time constants for each replicate cell of each compound.

**Two-Electrode Voltage Clamp Electrophysiology.** Experiments were conducted using OpusXpress 6000A (Molecular Devices, Union City, CA) (Papke and Stokes, 2010). Both the voltage and current electrodes were filled with 3 M KCl. Oocytes were voltage-clamped at  $-60$  mV at room temperature ( $24^\circ\text{C}$ ). The oocytes were bath perfused with Ringer's solution (115 mM NaCl, 2.5 mM KCl, 1.8 mM  $\text{CaCl}_2$ , 10 mM HEPES, and 1  $\mu\text{M}$  atropine, pH 7.2) at 2 ml/min. Control and test solutions were applied from 96-well plates via disposable tips, 400  $\mu\text{l}$  at 2 ml/min. After a 30-second period of data recording to define baseline holding currents, drug applications were 12 seconds in duration followed by 181-second washout periods, during which time data were collected, followed by 17 seconds to acquire the next drug load, during which time data were not recorded. Data were collected at 50 Hz and filtered at 20 Hz. The responses were calculated as both peak current amplitudes and net charge relative to the baseline as previously described (Papke and Porter Papke, 2002).

**Data and Statistical Analysis.** Data were analyzed by Clampfit (Molecular Devices) and Excel (Microsoft, Redmond, WA). For wild-type (WT)  $\alpha 7$  receptors, to evaluate the experimental compounds, responses were normalized to control ACh-evoked responses, defined as the average of two initial applications of 60  $\mu\text{M}$  ACh made before test applications. The average of independent 1  $\mu\text{M}$  GAT107 responses was used as the control for the  $\alpha 7\text{C190A}$  mutant receptors each day. Especially for small responses, baselines may have been manually adjusted in Clampfit to correct for rising or falling baselines to record the actual response accurately.

Experiments each began with eight oocytes. For WT  $\alpha 7$  experiments, a preliminary application of control 60  $\mu\text{M}$  ACh was given to assess the health and expression of each cell. An oocyte would be changed for another if it was not good. This preliminary test was not available for the  $\alpha 7\text{C190A}$  cells, which do not respond to ACh, and exposure to GAT107 affects subsequent responses. An experimental cell would be excluded from results if the two precontrol responses were inconsistent by more than 50%, if an experimental solution accidentally missed delivery, or if voltage clamp was not maintained.

Data are expressed as means  $\pm$  S.D. from 5–8 oocytes for each experiment and plotted with Kaleidagraph 4.5.2 (Synergy Software, Reading, PA). To show actual data traces, means of the normalized data were calculated for each of the 10,322 points in each of the 206.44-second traces (acquired at 50 Hz), as well as the standard errors for those averages.

Net-charge responses are reported for  $\alpha 7$  nAChR due to their concentration-dependent rapid desensitization (Papke and Porter Papke, 2002).

Comparisons of results were made using one-way ANOVA or using  $t$  tests between the pairs of experimental measurements. In cases where multiple comparisons were made, a Bonferroni correction for multiple comparisons (Aickin and Gensler, 1996) was applied to correct for possible false positives. A value of  $P < 0.05$  was used to constitute a minimum level of significance. The statistics were calculated using an Excel template provided in Microsoft Office or ANOVA protocols in Kaleidagraph.

## Results

**Basic Characterization of the  $\alpha 7$  Activity of the DPP Compounds.** The structures of the series of 2,4,6-substituted DPPs previously identified as noncanonical  $\alpha 7$  nAChR-selective agonists are shown in Fig. 1B (Camacho-Hernandez et al., 2019). All of the compounds were efficacious full or partial agonists (Supplemental Fig. 1), with net-charge  $I_{\text{max}}$  values ranging from 0.64 to 1.25 compared with ACh  $I_{\text{max}}$  (Table 1) and potencies 10- to 100-fold greater than that of ACh, which has an  $\text{EC}_{50} \approx 30$   $\mu\text{M}$  (Papke and Porter Papke, 2002). All nicotinic agonists promote both activation and desensitization, and the apparent efficacy of a particular compound

measured with any specific experimental protocol will be affected by the balance of these processes throughout the duration of the measured response (Papke, 2010). In the case when the induced desensitization is particularly stable, the effects of the agonist may be longer than the duration of the currents stimulated. The data indicate that, to varying degrees, the DPP compounds were very effective at inducing relatively stable inhibition/desensitization of the receptors, such that after the compounds were applied to activate the receptors, the subsequent responses to ACh were markedly decreased in a concentration-dependent manner (Fig. 2A). This permitted the determination of  $IC_{50}$  values for the inhibition of the subsequent ACh controls (Camacho-Hernandez et al., 2019) (Table 1). Interestingly, however, although high concentrations inhibited subsequent responses to ACh, after application at low concentrations, some of the compounds potentiated the subsequent ACh responses (Fig. 2B).

The processes of activation and desensitization dynamically determine the magnitude and kinetics of these macroscopic responses, which are also strongly influenced by the drug application process itself (Papke and Porter Papke, 2002; Papke, 2010; Quadri et al., 2018). Activation of nAChRs is always a relatively transient process compared with desensitization, and this is especially true for  $\alpha 7$  receptors (Williams et al., 2011, 2012). Although the activation of  $\alpha 7$  receptors by the DPP compounds that we can measure will always be limited by rapid onset and concomitant integrated effects of desensitization, we have several approaches available to study the nature and duration of the desensitized states produced by these compounds.

**Effects of the DPP Compounds on  $\alpha 7$  ACh-Evoked Responses.** Although the induction of the  $\alpha 7$  desensitized states is rapid and influences the magnitude of the activation measured, the reversibility of apparent desensitization induced by the DPP compounds can be much slower. a4H was the least potent of the compounds for producing reduction of the postapplication ACh controls (Table 1), and following the application of 100  $\mu$ M a4H, ACh-evoked responses returned to their original amplitude within 8 minutes (Fig. 3A), by the time of the second postapplication ACh

delivery. In contrast, there was essentially no recovery of ACh control responses after the application of 100  $\mu$ M 2F4M over the course of an hour (Fig. 3B). The other compounds fell between these two extremes defined by a4H and 2F4M. The Bonferroni means comparison of the responses recorded 4 minutes and 64 minutes after the DPP applications are reported in Table 2 and 3, respectively. The data for these recoveries could be fit to an exponential function to define time constants ( $\tau$ ) for recovery, either based on the fit of the averaged data or of the individual replicates (Supplemental Figs. 2–8; Table 4). The ANOVA on the time constants of replicate data (Fig. 3C) is provided in the Supplemental Data ANOVA results.

**Differing Effects of Methyllycaconitine on 2F4M Activation and Desensitization.** We tested whether the  $\alpha 7$ -selective competitive antagonist methyllycaconitine (MLA) (Alkondon et al., 1992) would block activation by 2F4M and, by doing so, prevent the induction of long-lived inhibition/desensitization produced by this compound. Since the  $\alpha 7$  agonist-evoked responses occur during the leading edge of the drug applications (Papke and Porter Papke, 2002), we first preapplied 100  $\mu$ M MLA for 30 seconds before coapplying 100  $\mu$ M MLA and 10  $\mu$ M 2F4M. Although MLA effectively blocked the 2F4M activation, the inhibition/desensitization of subsequent ACh-evoked responses was indistinguishable from that observed when 10  $\mu$ M 2F4M was applied alone (Fig. 4). This suggests that the activation and desensitization produced by 2F4M may arise from different binding modes, one MLA sensitive and the other MLA insensitive, respectively.

**PAM-Sensitive Desensitization Induced by DPP Compounds.** Of all nAChR,  $\alpha 7$  receptors have the lowest probability of opening and normally open for extremely brief periods of time, if at all, before entering into desensitized states (Williams et al., 2011). The extent to which the responses of  $\alpha 7$  receptors are regulated by desensitization (Uteshev et al., 2002; Papke and Lindstrom, 2020) has been revealed by the effects of type II PAMs like PNU-120596, which can reactivate previously desensitized receptors and promote bursts of currents lasting many seconds (Williams et al., 2011; Andersen et al., 2016). The use of type II PAMs have also shown that specific ligands can induce extremely stable desensitized

TABLE 2  
Data for the Bonferroni means comparison of data in Fig. 3B  
Significant differences between responses to ACh at  $t = 4$  minutes<sup>a</sup>.

Comparison	Difference	Q	P	95% Confidence Interval
a4H versus 2F4M	0.779	16.89	<.0001	0.577–0.981
a4H versus a2M	0.774	15.229	<.0001	0.551–0.997
a4H versus 2M	0.771	17.309	<.0001	0.576–0.967
a4H versus 2F	0.769	16.679	<.0001	0.567–0.971
a4H versus a4M	0.766	15.899	<.0001	0.554–0.976
a4H versus a2F4M	0.764	16.567	<.0001	0.562–0.967

<sup>a</sup>All other pairwise comparisons were not significantly different.

TABLE 3  
Data for the Bonferroni means comparison of data in Fig. 3B  
Significant differences between responses to ACh at  $t = 64$  minutes<sup>a</sup>.

Comparison	Difference	Q	P	95% Confidence Interval
a4H versus 2F4M	1.180	9.933	<.0001	0.659–1.700
a4H versus 2F	1.033	8.695	<.0001	0.512–1.553
a4H versus a4M	0.859	6.928	<.001	0.316–1.402
a4H versus 2M	0.858	7.480	<.0001	0.355–1.361
a4H versus a2F4M	0.829	6.9799	<.0001	0.308–1.350

<sup>a</sup>All other pairwise comparisons were not significantly different.

TABLE 4  
Recovery from DPP inhibition/desensitization

Drug	Values from Replicates					Values from Fits in Fig. 3B				
	$\tau$ , s	S.D.	Base	S.D.	n	$\tau$ , s	Error	Base	Error	r
a4H	47.65	35.84	0.62	0.20	7	19.19	2.53	0.40	0.03	0.984
a2F4M	146.34	111.60	-0.03	0.04	7	110.15	6.59	-0.01	0.01	0.9862
2M	111.03	53.78	-0.05	0.07	7	91.54	9.54	-0.04	0.03	0.967
2F4M	1952.09	1512.73	-0.03	0.07	8	1410.50	150.01	0.04	0.03	0.902
2F	319.23	1320	-0.01	0.06	7	224.85	17.81	-0.01	0.01	0.967
a4M	776.93	1097.07	-0.07	0.05	6	51.78	4.47	-0.08	0.03	0.989
a2M	53.18	39.59	-0.06	0.07	5	98.59	5.25	-0.03	0.01	0.990

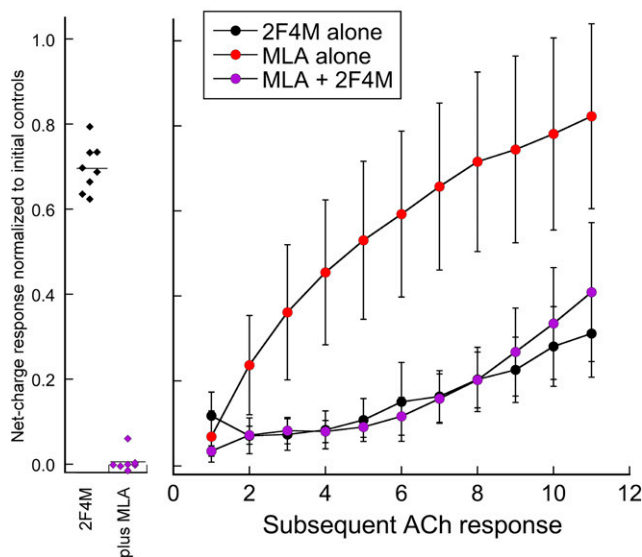
states, still detectable an hour or longer after drug application (Pismataro et al., 2021). To test the hypothesis that the DPP compounds that produced long-lasting inhibition of ACh responses did so by inducing stable desensitization that was in part PAM sensitive, we applied the compounds at 30  $\mu\text{M}$  and, without further applications of ACh, washed the cells for 30 minutes before applying 10  $\mu\text{M}$  PNU-120596 alone (Fig. 5A). The concentration of 30  $\mu\text{M}$  was chosen because it was approximately the  $\text{IC}_{50}$  for a4H, the least potent compound for residual

inhibition (Table 1), and 30 minutes showed a good separation of predicted recoveries, based on Fig. 3B. All compounds that effectively reduced ACh responses (i.e., all but a4H) primed the receptors to respond to the delayed application of the PAM (Fig. 5B; see Supplemental Data ANOVA results). The log magnitude of the delayed PNU-120596 responses was well correlated to the amount of residual inhibition (Fig. 5C,  $R = 0.985$ ).

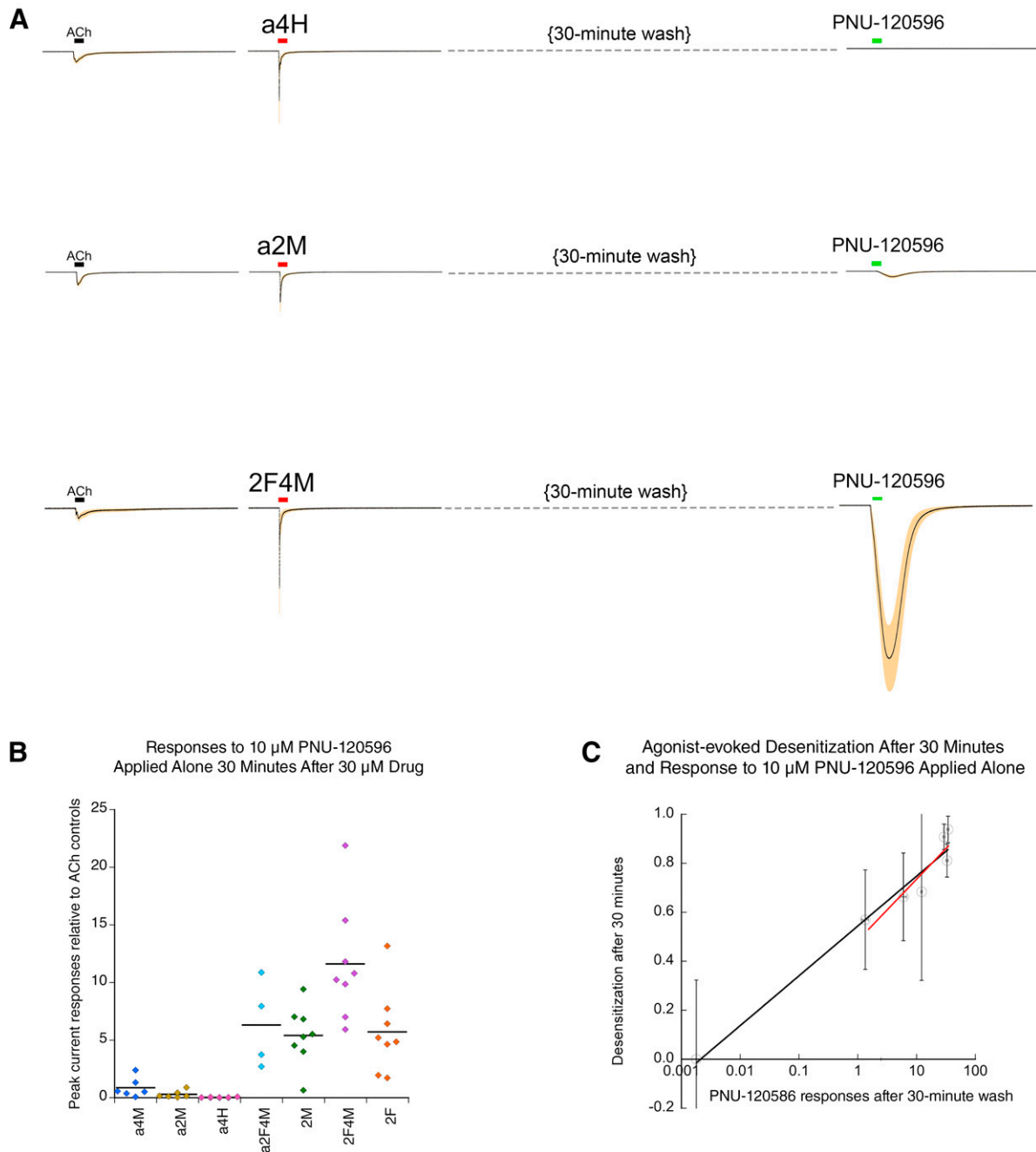
**Cis-trans-4-(2,3,5,6-tetramethylphenyl)-3a,4,5,9b-tetrahydro-3H-cyclopenta[c]quinoline-8-sulfonamide Sensitivity of 2F4M-Primed Activation by PNU-120596.** To test the hypothesis that 2F4M may bind to a previously identified allosteric activation site of  $\alpha 7$  receptors, we determined whether (-)-cis-trans-4-(2,3,5,6-tetramethylphenyl)-3a,4,5,9b-tetrahydro-3H-cyclopenta[c]quinoline-8-sulfonamide ((-)-TMP-TQS) (Gill et al., 2012; Papke et al., 2020), an antagonist of the allosteric agonist GAT107 [the active isomer of 4-(4-bromophenyl)-3a,4,5,9b-tetrahydro-3H-cyclopenta[c]quinoline-8-sulfonamide] (Gill et al., 2011; Thakur et al., 2013), would be able to block the 2F4M-primed responses to delayed applications of PNU-120596. As shown in Fig. 6, coapplication of 100  $\mu\text{M}$  of (-)-TMP-TQS totally blocked responses to an application of PNU-120596 primed by the earlier application of 10  $\mu\text{M}$  2F4M. However, this inhibition was transient and did not perturb the desensitization induced by the initial application of 2F4M since cells treated with (-)-TMP-TQS were as sensitive to a second PNU-120596 application as cells that received just two applications of the PAM (see Supplemental Data ANOVA results). This indicates that the prolonged effects of 2F4M were not perturbed by this antagonist binding to an allosteric site. These results are consistent with 2F4M binding either in a unique manner to the orthosteric sites or to other unidentified sites to produce long-term desensitization.

**Activity of DPP Compounds on C190A Mutants.** We have previously characterized the C190A  $\alpha 7$  mutant as being nonorthosterically activatable due to its insensitivity to ACh (Gulsevina et al., 2019). However, considering that the test compounds are so structurally divergent from ACh and other typical agonists, we examined the activity of specific DPP compounds on the C190A mutant, utilizing a range of concentrations higher than required for activation of WT  $\alpha 7$  receptors. When applied at sufficiently high concentrations, 2F4M could activate  $\alpha 7\text{C190A}$  receptors; although, without PAM coapplications, the magnitudes of the responses were relatively small compared with the 1  $\mu\text{M}$  GAT107 control responses, with peak currents typically less than 1  $\mu\text{A}$  (Fig. 7). To test whether 2F4M was working at the allosteric site to activate and desensitize C190A receptors, we coapplied 2F4M and varying

### Recovery of ACh Responses, Effects of 10 $\mu\text{M}$ 2F4M and MLA



**Fig. 4.** MLA effects on 2F4M activation and desensitization of  $\alpha 7$ . After recording two initial ACh control responses, cells were treated with 100  $\mu\text{M}$  MLA 30 seconds without washout from the chamber prior to standard application of 100  $\mu\text{M}$  MLA alone or coapplied with 10  $\mu\text{M}$  2F4M. Subsequently, 11 further applications of 60  $\mu\text{M}$  ACh were made to evaluate recovery from MLA inhibition and/or 2F4M-induced desensitization/inhibition. For comparison, other cells were treated with 10  $\mu\text{M}$  2F4M without MLA and evaluated for their subsequent ACh responses. As shown to the left, the MLA treatment (purple diamonds) eliminated 2F4M activation observed when the drug was applied alone (black diamonds). The inhibition of ACh responses following treatment with MLA alone fully reversed through the course of the experiment (red circles), whereas the desensitization/inhibition produced by 2F4M was essentially identical for cells with (purple circles) or without MLA coapplication (black circles). All points are the average net-charge responses ( $\pm$  S.D.) of eight cells. ANOVA (Supplemental Data) indicated that following MLA, the ACh responses were greater in cells receiving MLA alone than cells receiving 2F4M and MLA ( $P < 0.001$ ) and that there were no significant differences at any timepoint between the two 2F4M groups.

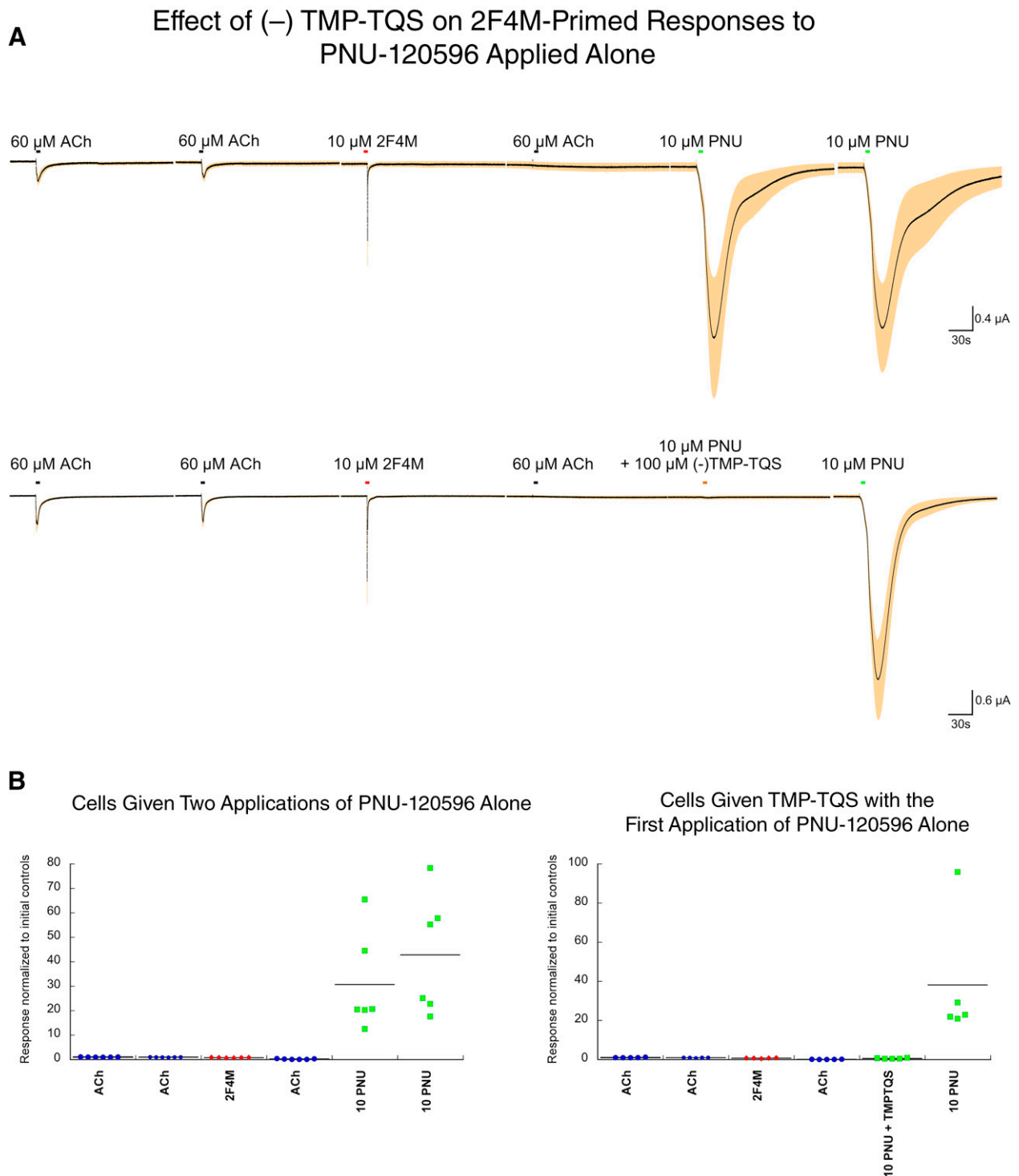
Responses of Wild-type  $\alpha 7$  to 10  $\mu\text{M}$  PNU-120596 30 Minutes After 30  $\mu\text{M}$  DPP

**Fig. 5.** Decreased controls correspond to increased sensitivity to PNU-120596 applied alone to  $\alpha 7$ -expressing cells. (A) Averaged raw data (*Materials and Methods*) for the three of the DPP compounds shown in Fig. 3 indicating initial ACh controls, the responses to 30  $\mu\text{M}$  DPP applications, and, after a 30-minute wash, the responses to 10  $\mu\text{M}$  PNU-120596 alone ( $n = 8, 6,$  and  $7$  for a4H, a2M, and 2F4M, respectively). (B) Dot plots showing each net-charge response of  $\alpha 7$ -expressing cells ( $n = 6-8, \pm$  S.E.M.) to 10  $\mu\text{M}$  PNU applications 30 minutes after 30  $\mu\text{M}$  DPP and aDPP compound applications, normalized to the initial ACh controls. Black bars indicate the means. (C) Scatter plot of the PNU-120596 responses shown in (B) to the residual inhibition after 30 minutes (data taken from Fig. 3B). Residual inhibition was calculated as 1 minus the response at the 30-minute timepoint in Fig. 3B. The fit function was inhibition =  $0.544 + 0.203 \log(\text{PNU-120596 response})$ ,  $R = 0.9825$ . Note that the left-most point is quite distant from the others, so we also evaluated the fit if that point was omitted. In that case, the fit function was inhibition =  $0.503 + 0.240 \log(\text{PNU-120596 response})$ ,  $R = 0.908$ . This alternative fit is represented by the red line in the plot.

concentrations of (-)TMP-TQS (Papke et al., 2020) to  $\alpha 7\text{C190A}$  (Fig. 7A). Either 100 or 300  $\mu\text{M}$  2F4M was applied alone or coapplied with 30, 100, or 300  $\mu\text{M}$  (-)TMP-TQS. The (-)TMP-TQS applications were entirely ineffective at reducing either

the direct activation of the C190A mutant (Fig. 7, A and C, left) or the priming of the receptors to subsequent application of 10  $\mu\text{M}$  PNU-120596 alone (Fig. 7, B and C, right). Note that this lack of (-)TMP-TQS effect during the 2F4M application is





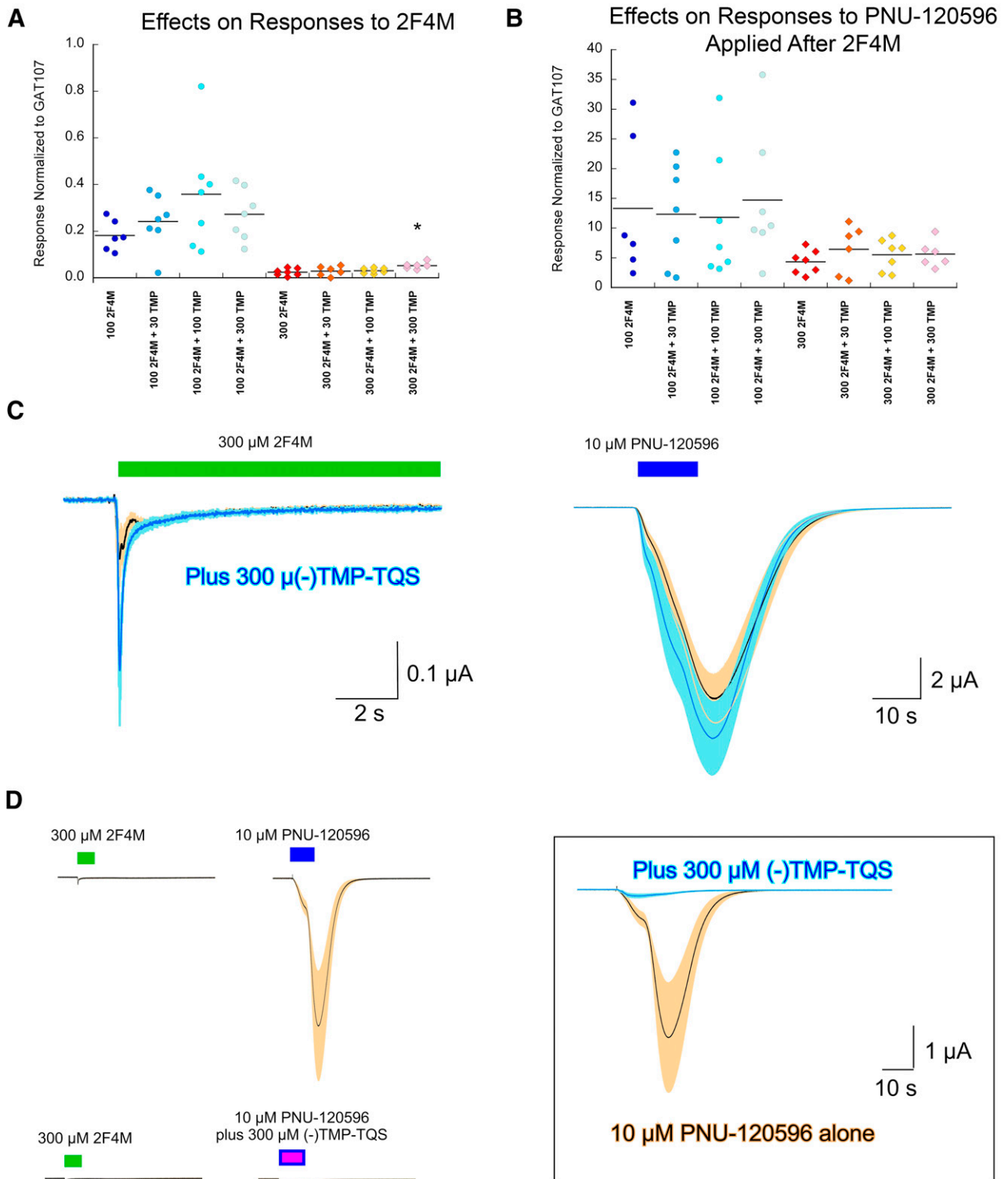
**Fig. 6.** TMP-TQS inhibition of 2F4M-primed responses of  $\alpha 7$  nAChR to PNU-120596. (A) Averaged net-charge responses (black lines) of six cells ( $\pm$  S.E.M., shaded areas) to control applications of 60  $\mu$ M ACh, followed by 10  $\mu$ M 2F4M, a subsequent application of 60  $\mu$ M ACh, and then two applications of 10  $\mu$ M PNU-120596, at 4-minute intervals. The upper traces ( $n = 5$ ) are from cells receiving PNU-120596 alone on the last two applications. The cells represented in the lower traces ( $n = 6$ ) received 100  $\mu$ M of the most active (-) isomer of TMP-TQS during the first application of PNU-120596. (B) Dot plots of the averaged net-charge data ( $\pm$  S.E.M.) from the cells in (A) (ANOVA information available in Supplemental Data).

in great contrast with the effects of (-)TMP-TQS when coapplied with the PNU-120596 following 2F4M priming (Fig. 7D).

The observation that the 2F4M-induced desensitization persisted when 2F4M was coapplied with the allosteric antagonist indicates that the desensitization induced by 2F4M, and detected with the PNU-120596 applications, was not likely

due to 2F4M binding to the allosteric site targeted by (-)TMP-TQS.

**Dynamics of Response Potentiation and Recovery from Desensitization.** We investigated whether there were dynamic interactions between PAM-mediated activation of 2F4M-desensitized receptors and the recovery of ACh sensitivity.

Effects of (-) TMP-TQS on  $\alpha 7$  C190A Responses to 2F4M

**Fig. 7.** Effects of coapplications of TMP-TQS on responses of C190A  $\alpha 7$  mutants. Data are normalized to the average responses to 1  $\mu$ M GAT107 on the same day from the same batch of injected oocytes. (A) Dot plots with means indicated for net-charge responses to 2F4M applications of 100–300  $\mu$ M alone or with increasing concentration of (-)TMP-TQS. The only significant effect of TMP-TQS coapplication ( $P < 0.05$ , see Supplemental Data ANOVA results) was a small increase in the 300  $\mu$ M response. (B) Responses to 10  $\mu$ M PNU-120596 applied alone 4 minutes after (A) responses. Data are the net-charge responses of 5–8 oocytes, normalized to the average responses of  $\alpha 7$ C190A-expressing cells to 1  $\mu$ M GAT107 obtained on the same day with cells from the same injection set. Note the differences in scale between (A) and (B), reflecting the small size and transient nature of the responses to 2F4M applied alone. (C) The averaged raw data traces ( $n = 6$ ) for the responses to 300  $\mu$ M 2F4M, applied alone or coapplied with 300  $\mu$ M (-)TMP-TQS. Traces on the left are the responses to the initial 2F4M application. Traces on the right are

We sought to determine if recovery was strictly time dependent or could be accelerated by episodes of PAM-potentiated activation. We induced a less-than-total level of desensitization with a single application of 30  $\mu\text{M}$  2F4M, which permitted partial recovery of ACh responses over the course of an hour (Fig. 8A). We made alternating applications of 60  $\mu\text{M}$  ACh and 10  $\mu\text{M}$  PNU-120596 at 4-minute intervals (Fig. 8, B and C). Although the single application of 30  $\mu\text{M}$  2F4M reduced ACh responses for over an hour (Fig. 8A), repeated applications of PNU-120596 promoted the recovery of ACh responses after the single 30  $\mu\text{M}$  application (Fig. 8B). Note that to keep the ACh responses in register between the two experiments, only every other ACh response is shown in Fig. 8A. When the ACh responses were alternated with PNU-120596 applications, the net charge of the ACh responses became nearly three times the original values (Fig. 8C). For the PAM to have these augmented effects, it is likely that it was retained at least to some degree at the transmembrane binding site or otherwise remained available to rebind. There is also evidence for persistent effects of 2F4M at its binding site since applications of PNU-120596 alone continued to produce activation that was not statistically different from the initial PNU responses (Supplemental Fig. 9). The switch from reduced to the enhanced ACh responses (Fig. 8C) suggests that the PNU-120596 applications either allowed orthosteric sites to become available for binding ACh or restored the ability of ACh binding to promote channel activation.

**Repeated PNU-120596 Effects on ACh Responses in the Absence of 2F4M Desensitization.** When applied alone, PNU-120596 fails to activate  $\alpha 7$  receptors, and in general, its effects as a potentiator are transient and only apparent when applied to previously desensitized receptors or when coapplied with an agonist. However, we observed in a control experiment (Fig. 9A) that when PNU-120596 was applied repeatedly in a protocol similar to that illustrated in Fig. 8B, but without the desensitizing effects of 2F4M, there was a progressive increase in ACh control responses (Fig. 9B). It may be the case that PNU-120596 accumulated at sites on the receptor or in the chamber itself or partitioned into the oocyte membrane as nicotine can (Jia et al., 2003). It is interesting, though, that effects on ACh responses by the repeated PNU-120596 applications paralleled the increased ACh responses in the experiment with 2F4M, although beginning from a different baseline (Fig. 9C). The potentiating effects of alternating applications of 10  $\mu\text{M}$  PNU-120596 were essentially the same when the PNU-120596 was coapplied with 30  $\mu\text{M}$  (-)TMP-TQS (Supplemental Fig. 10). It is interesting to note that there were proportionately greater increases in net charge compared with peak currents. Although this was more apparent after 2F4M (Fig. 9D), the differences were not significant.

## Discussion

State functions for the nAChR in muscle were initially defined by Katz and Thesleff in 1957, when they described recovery from desensitization as slow, whereas dissociation of

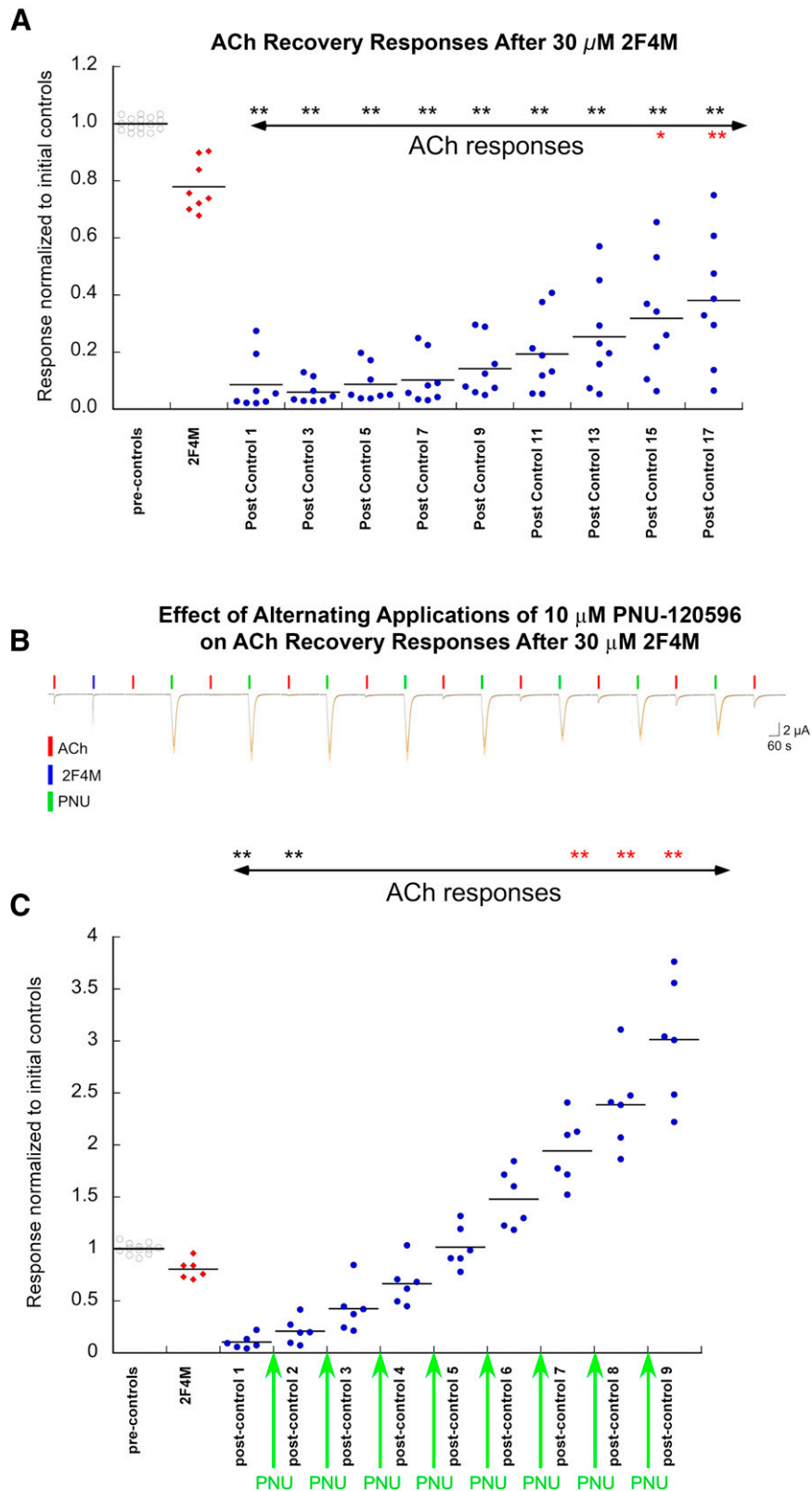
the ligand was more rapid (Katz and Thesleff, 1957). Studies of direct pharmacological relevance came to light in the muscle nicotinic receptor by Rang and Ritter (1970a, 1970b), who demonstrated that desensitization would be extended to other ligands by defining metaphilic antagonists that not only blocked the receptor but promoted desensitization. Subsequently, Sine and Taylor (1979) were able to show that local anesthetics enhanced the affinity of orthosteric agents and that in the muscle receptor there were two defined sites, presumably at the  $\alpha$  subunit interfaces, that promoted agonist responses, and occupation of only one of the two sites was necessary for competitive antagonism by snake toxins (Sine and Taylor, 1980, 1981). The extension of these concepts to the  $\alpha 7$  receptor might have been expected to yield structural simplification in employing a homomeric pentamer for structural studies; however, each monomer in the pentamer may experience local conformational freedom, resulting in a certain amount of structural stochasticity. That is, based on ligand binding, intersubunit interactions, or thermodynamic flexibility, each monomer within a pentamer may adopt more than one conformation, resulting in a certain amount of structure variability within the population of receptors. Indeed, we have shown through molecular dynamic studies that the pentamer does not maintain a fivefold axis of symmetry while interacting with ligands (Gulsevin et al., 2019, 2020). These considerations notwithstanding, more complexity enters the equation since addition of ligands in a polynomial fashion creates additional asymmetry at the binding site interfaces, heterogeneous stoichiometry of site occupation, and unresolved questions of possible subunit cooperativity.

Although lacking the typical features of nAChR agonists, the substituted pyrimidine compounds appear to function as  $\alpha 7$ -selective agents, hypothetically by binding in unique ways to the subunit interfaces associated with the binding sites for more typical orthosteric agonists. It was demonstrated that the 2-dipicolyl amine substitution at the 4-position in the pyrimidine core is crucial for agonism and that the substitution in the 6-position is important to modulate this agonism (Camacho-Hernandez et al., 2019; Camacho-Hernandez and Taylor, 2020). Hence, this family of congeneric compounds can serve as agonists and antagonists and promote transitions among at least three states: activatable closed channels, active open channels, and desensitized nonfunctional channels. The molecular and structural determinants of the varying desensitization produced by the DPP compounds remains to be resolved, although in general, the aDPP compounds were less desensitizing than the DPP compounds, suggesting that the 2-amino moiety plays a role either by direct interaction and/or by affecting the  $\text{pK}_a$  of the pyrimidine ring.

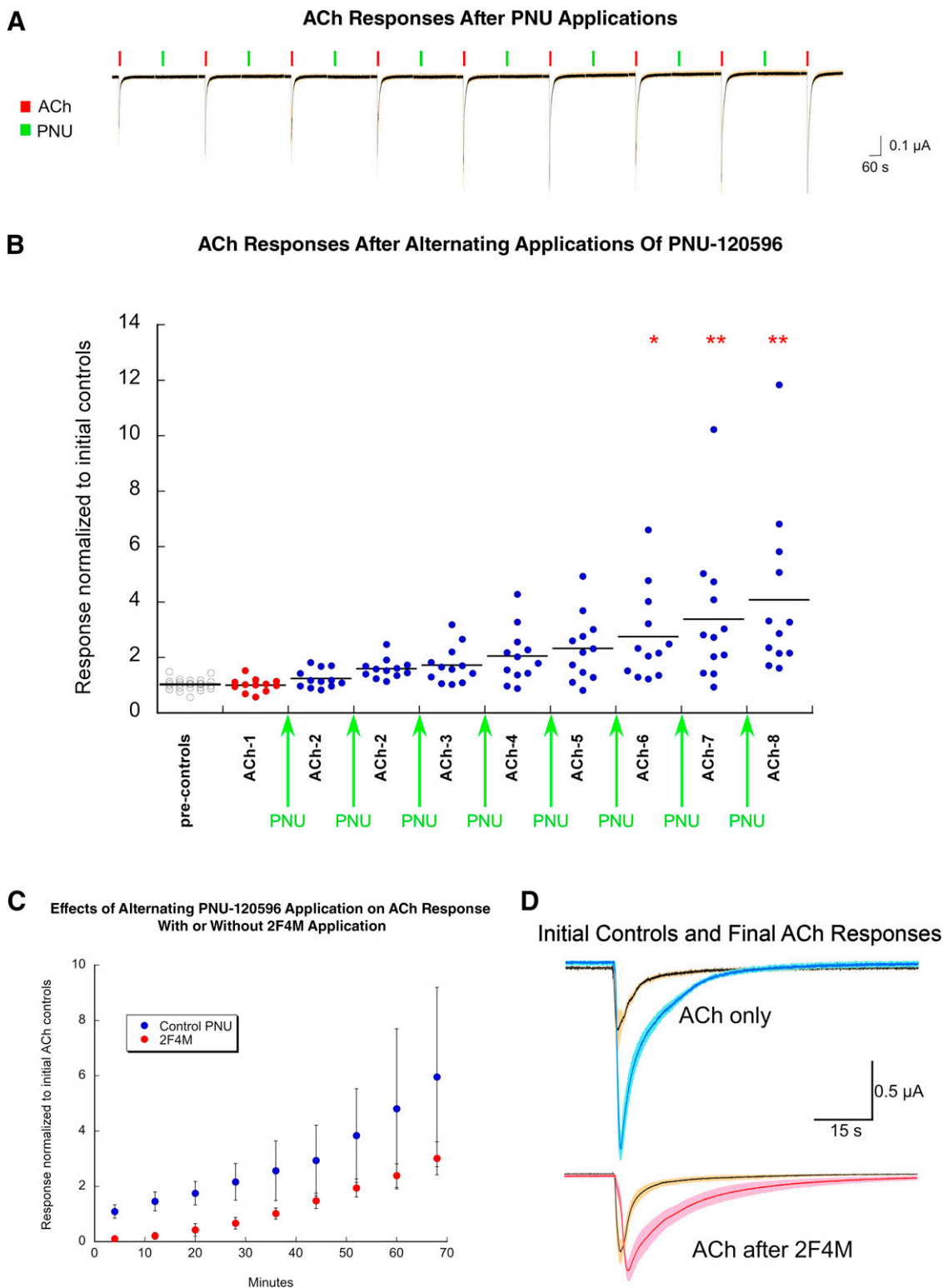
It is difficult to define an analog of the classic pharmacophore for a nicotinic agonist within the structures of the DPP compounds. This raises the questions of not only the stoichiometry but the pose of these compounds when bound to the nAChR. This is particularly so for the DPP series since a cationic center is integral to virtually all orthosteric agonists associating with the  $\alpha 7$  nAChR, but the  $\text{pK}_a$ s of the pyrimidine

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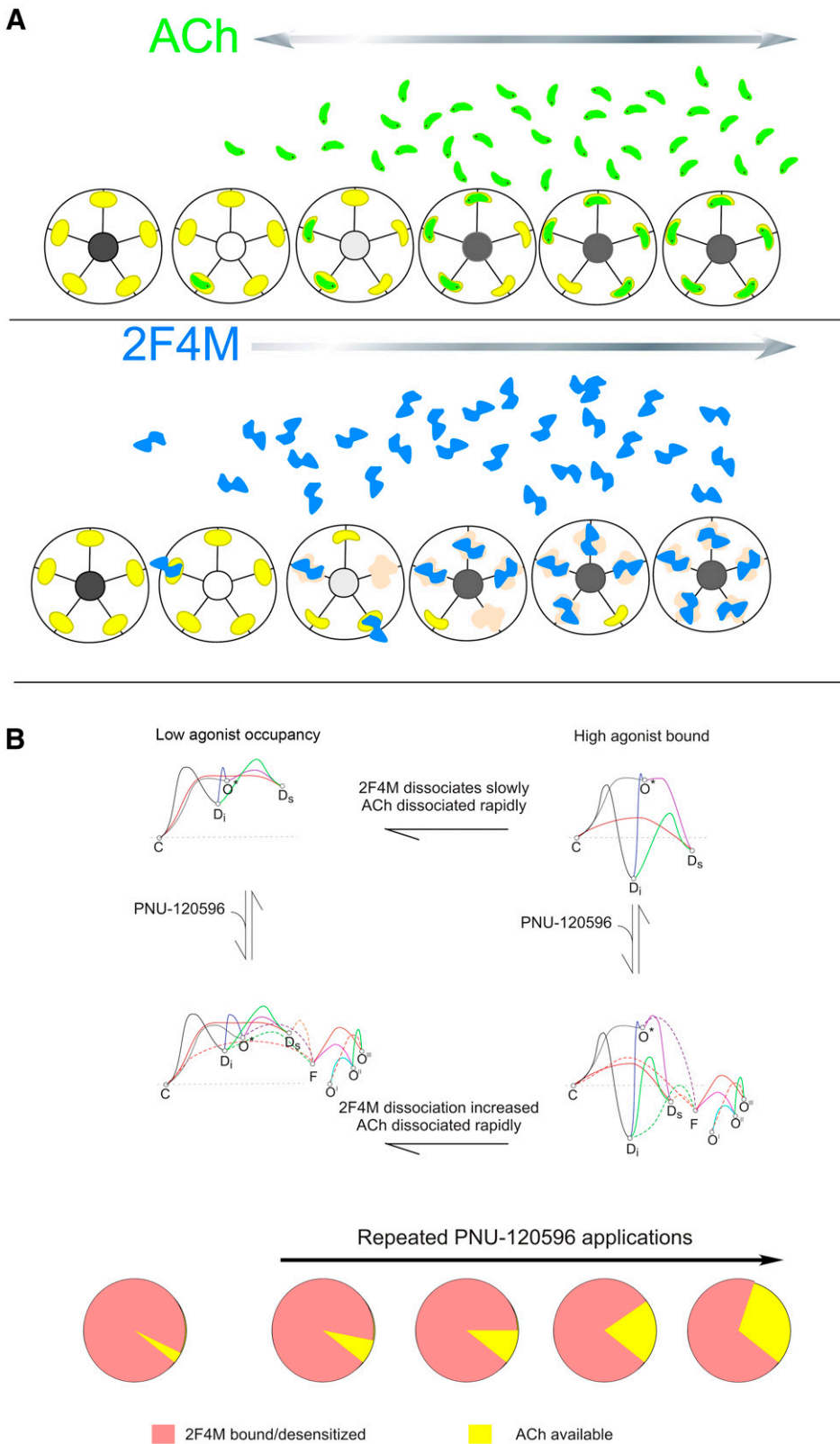
the responses to 10  $\mu\text{M}$  PNU-120596 applied 4 minutes after the initial 2F4M applications. Black lines indicate the mean response to 2F4M alone, with tan S.E.M., and blue lines show the mean response to 2F4M coapplied with (-)TMP-TQS, with aqua S.E.M. (D) The effects of (-)TMP-TQS coapplication with PNU-120596 on  $\alpha 7\text{C}190\text{A}$  responses primed by a prior application of 300  $\mu\text{M}$  2F4M. The group of traces on the left shown the averaged raw data ( $n = 5$ ,  $\pm$  S.E.M.) for applications of 300  $\mu\text{M}$  2F4M applied alone followed up with applications of 10  $\mu\text{M}$  PNU-120596, alone or coapplied with 300 (-)TMP-TQS. On the right are shown a superimposition of the PNU-120596 responses, illustrating the significant effect of (-)TMP-TQS when coapplied ( $P < 0.01$ ).



**Fig. 8.** Effect of repeated PNU-120596 applications on ACh recoveries of  $\alpha 7$ -expressing cells. (A) The inhibition and slow recovery of ACh responses after 30  $\mu$ M 2F4M. All responses were reduced compared with the initial ACh responses (black asterisks, ANOVA with Dunnett's multiple comparisons), and the last two ACh responses are increased compared with the first postapplication response (red asterisks). Note that only every other ACh response in the complete sequence is shown, to be in register with the data in (C). (B) The averaged raw data from cells treated alternately with ACh and 10  $\mu$ M PNU-120596 after a single application of 30  $\mu$ M 2F4M ( $n = 6$ ). Prior to averaging, the raw data were normalized based on the peak of the ACh control obtained from the same cells. (C) Net-charge responses to the ACh and 2F4M applications in the sequence of response illustrated in (B). Compared with the initial ACh controls (ANOVA with Dunnett's multiple comparisons), the first two ACh applications were significantly reduced (black asterisks). However, with repeated applications of PNU-120596, responses increased, and the last three applications were significantly increased (red asterisks). Note that the size of the PNU-120596 responses in this experiment did not change significantly in this experiment (Supplemental Fig. 9).



**Fig. 9.** Effects of control experiment PNU-120596 applications on 60  $\mu$ M ACh responses of  $\alpha 7$ -expressing cells. (A) The averaged raw data from a control experiment with alternating applications of 60  $\mu$ M ACh and 10  $\mu$ M PNU-120596. (B) The sequence of ACh net-charge responses compared with the initial ACh controls. Significant increases, compared with the ACh prior to the first PNU-120596 application (red circles), are indicated by red asterisks (ANOVA with Dunnett's multiple comparisons, Supplemental Data). (C) Comparison of ACh increases due to alternating PNU-120596 applications under control conditions in (B) (blue circles) and following initial inhibition by 30  $\mu$ M 2F4M (red circles, data taken from Fig. 8C). Plotted are the averages  $\pm$  S.D from the data in the dot plots. (D) Averaged raw data comparisons of initial controls (black lines with tan representing the S.E.M.) and ACh responses after augmentation by alternating applications of 10  $\mu$ M PNU-120596. The upper traces are from the experiment shown in (A) and (B), with the average final ACh response in red and the S.E.M. in pink. The lower traces are the from the experiment illustrated in Fig. 8, with the average final ACh response in red and the S.E.M. in pink.



**Fig. 10.** Models. (A) Hypothetical effects of increasing effects of receptor binding to ACh (upper scheme) or 2F4M (lower scheme), representing changes in binding sites and channel gating. ACh binding is assumed to be more rapidly reversible than 2F4M binding. (B) Hypothetical energy landscapes of state transitions as functions of agonist occupancy and PNU-120596 binding [adapted from Papke and Horenstein (2021)]. If 2F4M can dissociate more rapidly from the PNU-120596-activated channels than from receptors not bound by PNU-120596, then repeated bouts of PNU-120596 activation might free up sites for ACh binding and activation.

and pyridine nitrogens are low in the DPP series, making it likely that the nitrogens are unprotonated to varying degree at physiologic pH (Camacho-Hernandez et al., 2019). These

considerations led us to speculate that the DPP compounds might be influenced by, or working through, allosteric sites of  $\alpha 7$  to produce activation and/or desensitization rather than

strictly through the OA sites common to nAChR and the AChBP.

A hypothetical model for  $\alpha 7$  state transitions with progressive levels of ACh binding is shown in Fig. 10A. As ligands bind, it is likely that the symmetry and equivalence of the ligand binding sites change, with some sites adopting higher affinity for agonists, associated with desensitized forms of the receptor. Additional factors may influence the state transitions regulated by the binding of DPP compounds like 2F4M, consistent with the differential effects of MLA on activation and desensitization. The binding mode associated with activation may be only a low-affinity interaction within the traditional binding pocket since activation is seen as a transient process for all of the DPP compounds. However, the desensitizing effects may come from associations with an as yet undefined binding pocket, and for some of the DPP compounds, this may represent a higher affinity binding, consistent with the stability of the desensitization produced by agents like 2F4M. In the absence of PAM-dependent activation, these binding actions may remain stable and prevent ACh from binding or at least from producing activation. The condition at the end of the sequence of alternating applications of 2F4M and PNU-120596 (Fig. 8C) seems paradoxical since the recovery of sensitivity to ACh might suggest a relief of desensitization, whereas the persistent activity of PNU-120596 applied alone suggests that the receptors remain desensitized. A likely resolution of this apparent paradox is suggested by the character of the ACh responses obtained at the end of the sequence of applications (Fig. 9D). The ACh responses do not resemble those of receptors in the normal resting state but rather those of receptors that have been allosterically potentiated by residual/accumulating PAM, so, in effect, they are consistent with PAM-dependent activation of desensitized receptors.

Shown in Fig. 10B is a proposed model for PNU-120596 activation of  $\alpha 7$  at different levels of agonist occupancy [adopted from Papke and Horenstein (2021)]. Our data suggest that the high-affinity binding of 2F4M, which is stabilized in a desensitized conformation, may be modified as a consequence of the conformational changes associated with PAM-dependent destabilization of the desensitized state so that the repeated presentations of PNU-120596 alter the nature of the 2F4M-induced desensitization. It may be that the ligand slips out of the high-affinity binding site that is created during the desensitization process and that as the receptor “breathes” (fluctuates among activated and desensitized states), the ligand can dissociate, or the receptor otherwise relax, making some of the sites available to bind ACh or alternatively allowing ACh binding to be more effectively coupled to channel activation via the effects of the accumulating PAM. Under these conditions, the ACh activity is not typical since it clearly benefits from, and likely depends on, the residual PNU-120596 activity, as was seen in the control experiment (Fig. 9B). The results of PNU-120596 selectively promoting greater net-charge responses suggests that there are fundamental effects on the  $\alpha 7$  activation processes yet without relieving the desensitization required for PNU-120596 to activate the receptors when applied alone.

Initially, we considered the hypothesis that the prolonged desensitization profile of the DPP compounds might be due to interactions at allosteric sites, which was encouraged by the unique structural features departing from traditional orthosteric agonists. However, the observation that TMP-TQS produced

only transient block of PAM priming by 2F4M with the WT receptors suggested that the prolonged 2F4M-induced desensitization was not a result of a direct interaction with the allosteric site targeted by TMP-TQS but rather due to an alternative mode of binding at sites related to orthosteric activation. Binding of DPP compounds to the  $\alpha 7$  receptor apparently breaks some of the rules associated with the canonical nAChR agonist pharmacophore, consistent with their ability to activate ACh-insensitive C190A receptors.

The identification of the role played by  $\alpha 7$  in the cholinergic anti-inflammatory pathway (CAP) (Tracey, 2007; Bagdas et al., 2018) has indicated that  $\alpha 7$  has both ionotropic and metabotropic properties and suggested that channel activation may not be critical for the anti-inflammatory profile of ligands targeting  $\alpha 7$  (Thomsen and Mikkelsen, 2012). This, in turn, led to the discovery of silent agonists such as NS6740 (Fig. 1A) as potential therapeutics for inflammation.

In some ways, the activity profile of 2F4M resembles that of the strongly desensitizing partial agonist NS6740, which is an effective activator of CAP (Papke et al., 2015) but is actually an antagonist of the procognitive effects of other, more efficacious  $\alpha 7$  agonists such as A-582941 (Briggs et al., 2009) and BMS-902483 (Pieschl et al., 2017). The basis for the effectiveness of NS6740, and possibly DPP compounds like 2F4M, in CAP is that the nonconducting states associated with ion-channel desensitization may, in fact, be signal-transducing states that mediate G-protein function (King et al., 2017) or other intracellular second-messenger pathways (Kabbani and Nichols, 2018; King et al., 2018). Therefore, it will be of immediate interest to determine the pharmacokinetic profiles of the DPP compounds, such as metabolic stability and blood-brain barrier penetration, and whether they are effective as  $\alpha 7$  drugs in vivo.

In conclusion, the family of DPP compounds are a major structural departure and an opportunity to investigate a potentially important paradigm shift in the development of nAChR pharmacology. Our nascent understanding of their function as  $\alpha 7$  activators and desensitizers challenges many of our current models, and reconsideration of those models is likely to lead to new insights and further exploration of alternative models.

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Oocyte recordings were conducted by Lu Wenchi Corrie.

#### Authorship Contributions

*Participated in research design:* Stokes, Camacho-Hernandez, Taylor, Papke.

*Conducted experiments:* Stokes.

*Contributed new reagents or analytic tools:* Camacho-Hernandez, Thakur, Wu, Taylor.

*Performed data analysis:* Stokes, Papke.

*Wrote or contributed to the writing of the manuscript:* Stokes, Camacho-Hernandez, Taylor, Papke.

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