Desformylflustrabromine Modulates $\alpha 4\beta 2$ Neuronal Nicotinic Acetylcholine Receptor High- and Low-Sensitivity Isoforms at Allosteric Clefts Containing the $\beta 2$ Subunit

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ABSTRACT

Alterations in expression patterns of $\alpha 4\beta 2$ nicotinic acetylcholine receptors have been demonstrated to alter cholinergic neurotransmission and are implicated in neurologic disorders, including autism, nicotine addiction, Alzheimer's disease, and Parkinson's disease. Positive allosteric modulators (PAMs) represent promising new leads in the development of therapeutic agents for the treatment of these disorders. This study investigates the involvement of the $\beta 2$ -containing subunit interfaces of $\alpha 4\beta 2$ receptors in the modulation of acetylcholine (ACh)-induced responses by the PAM desformylflustrabromine (dFBr). Eight amino acids on the principal face of the $\beta 2$ subunit were mutated to alanine to explore the involvement of this region in the potentiation of ACh-induced currents by dFBr. ACh-induced responses obtained from wild-type and mutant $\alpha 4\beta 2$

receptors expressed in *Xenopus laevis* oocytes were recorded in the presence and absence of dFBr using two-electrode voltage clamp electrophysiology. Wild-type and mutant receptors were expressed in both high and low ACh sensitivity isoforms by using biased injection ratios of 1:5 or 5:1 $\alpha 4$ to $\beta 2$ complementary RNA. Mutations were made in the B, C, and A loops of the principal face of the $\beta 2$ subunit, which are regions not involved in the binding of ACh. Mutant $\beta 2(Y120A)$ significantly eliminated dFBr potency in both isoform preparations. Several other mutations altered dFBr potentiation levels in both preparations. Our findings support the involvement of the principal face of the $\beta 2$ subunit in dFBr modulation of ACh-induced responses. Findings from this study will aid in the improved design of dFBr-like PAMs for potential therapeutic use.

Introduction

Neuronal nicotinic acetylcholine receptors (nAChRs) are integral membrane proteins involved in cholinergic transmission in the peripheral and central nervous systems (Taly et al., 2009). Dysregulation of nAChR has been postulated to be involved in neurological disorders, including Alzheimer's disease (Nordberg, 2001), schizophrenia (Adams and Stevens, 2007), Parkinson's disease (Aubert et al., 1992), autism (Martin-Ruiz et al., 2004), and nicotine addiction (Picciotto et al., 2001).

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Weltzin MM (2011) *Investigation of the Allosteric Modulators Desformylflustrabromine and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) Interactions on Nicotinic Acetylcholine Receptors*. Doctoral dissertation, University of Alaska Fairbanks, Fairbanks, AK.

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The predominant nAChR subtypes found in the brain are the homomeric $\alpha 7$ and the heteromeric $\alpha 4\beta 2$ receptors (Mudo et al., 2007). Studies using loose and concatenated subunits have demonstrated that the $\alpha 4\beta 2$ -nAChR forms at least two isoforms that have high (HS) and low (LS) sensitivity to acetylcholine (ACh) (Nelson et al., 2003; Zhou et al., 2003; Briggs et al., 2006a; Moroni et al., 2006; Zwart et al., 2006; Tapia et al., 2007; Mazzaferro et al., 2011; Eaton et al., 2014). It has been hypothesized that the HS isoform contains two $\alpha 4$ subunits and three $\beta 2$ subunits [$(\alpha 4)_2(\beta 2)_3$], whereas the LS isoform is composed of three $\alpha 4$ and two $\beta 2$ subunits [$(\alpha 4)_3(\beta 2)_2$] (Nelson et al., 2003; Briggs et al., 2006b; Moroni and Bermudez, 2006; Tapia et al., 2007; Mazzaferro et al., 2011; Eaton et al., 2014). The differences in isoform stoichiometries create distinctive subunit interfaces (Fig. 1A). The HS and LS isoforms have two ACh-binding sites located at the $\alpha 4$ +/ $\beta 2$ - interfaces and two non-ACh-binding interfaces at the $\beta 2$ +/ $\alpha 4$ - clefts. Additionally, the HS isoform contains a unique $\beta 2$ +/ $\beta 2$ - interface, and the $\alpha 4$ +/ $\alpha 4$ - interface on the LS stoichiometry binds ACh with low affinity (Mazzaferro et al., 2011; Eaton et al., 2014). Studies have provided evidence that similar HS and LS $\alpha 4\beta 2$ -nAChR isomers are expressed in the mammalian brain (Butt et al., 2002; Gotti et al., 2008) and can be altered by chronic exposure to nicotine (Moretti et al., 2010).

ABBREVIATIONS: ACh, acetylcholine; BZD, benzodiazepine; CaCC, calcium-activated chloride channel; cRNA, complementary RNA; dFBr, desformylflustrabromine; GABA_AR, GABA_A receptor; HS, high sensitivity; LS, low sensitivity; nAChR, neuronal nicotinic acetylcholine receptor; PAM, positive allosteric modulator; TEVC, two-electrode voltage clamp.

Fig. 1. Diagrams of the $\alpha 4\beta 2$ -nAChR stoichiometries, $\beta 2+$ / $\alpha 4-$ allosteric cleft and sequence alignments of the mutated residues investigated in this study. (A) Illustration of the low (left) and high (right) ACh sensitivity isoforms of the $\alpha 4\beta 2$ -nAChR. To encourage the expression of either isoform, biased cRNA ratios were used and are shown below each isoform diagram. Arrows indicate the ACh-binding sites at the $\alpha 4+$ / $\beta 2-$ and $\alpha 4+$ / $\alpha 4-$ clefts. Red (+) indicates the location of the mutants at the allosteric clefts. (B) A ribbon structure of the $\beta 2+$ / $\alpha 4-$ allosteric-binding cleft. The B loop is colored fuchsia, the C loop is colored green, and the A loop is colored blue (model based on the *Torpedo* AChR). (C) The structure of the positive allosteric modulator dFBr. (D) Alanine mutations were made on amino acids on the loops A [$\beta 2$ (D116), $\beta 2$ (Y120)], B [$\beta 2$ (W176), $\beta 2$ (T177), and $\beta 2$ (D179)], and C [$\beta 2$ (D217) and $\beta 2$ (D218)] located on the $\beta 2+$ face (colored boxes corresponding to the loop location). The amino acid sequences are shown for human nAChR and rat GABA subunit sequences. Highlighted residues are those found within the GABA $_A$ benzodiazepine site and the corresponding residues in $\alpha 4-$ and $\beta 2$ -nAChR subunits. Several $\beta 2$ -nAChR subunits are also highlighted because of sequence homology found in other nAChR subunits.

Injection of $\alpha 4$ and $\beta 2$ subunit complementary RNA (cRNA) in different proportions into *Xenopus* oocytes has been previously demonstrated to produce a predominance of either isoform whose function and pharmacology match concatenated receptors (Zwart and Vijverberg, 1998; Moroni et al., 2006; Mazzaferro et al., 2011; Eaton et al., 2014; Weltzin et al., 2014). We chose to use the loose subunit approach because it allows rapid and relatively inexpensive production of receptors, permitting multiple mutations to be easily investigated with high levels of expression.

Subtype selective ligands, such as positive allosteric modulators (PAMs), are potentially important therapeutic agents that could be useful in the treatment of pathologies involving alterations in nAChR expression. PAMs are ligands that bind at allosteric sites and alter responses to agonists, although they have no agonist properties of their own. Desformylflustrabromine (dFBr) (Fig. 1C) is a PAM that selectively enhances the efficacy of ACh stimulation (potentiation) of $\alpha 4\beta 2$ -nAChR by $> 265\%$ without altering ACh potency. ACh-induced currents were found to be inhibited, but not potentiated, on other common nAChR subtypes (Sala et al., 2005; Kim et al., 2007; Weltzin and Schulte, 2010). At high concentrations of dFBr ($> 10 \mu\text{M}$), $\alpha 4\beta 2$ receptors are inhibited by a mechanism that appears to involve dFBr open-channel block (Weltzin and Schulte, 2010). The mechanism of dFBr potentiation has been proposed to involve alteration of the equilibrium between open and desensitized receptor conformations, although a single channel analysis has not been reported (Sala et al., 2005; Weltzin and Schulte, 2010).

The current study investigates the mechanistic role of the complementary $\beta 2$ principal (+) subunit interface in the potentiation of ACh-induced responses by dFBr. The $\beta 2+$ face does not bind ACh but can be located next to ACh-binding interfaces (Fig. 1A). We hypothesize that the putative dFBr potentiation site is located on the $\beta 2+$ subunit interface in $\alpha 4\beta 2$ -nAChR and is similar to the benzodiazepine (BZD) binding site on GABA $_A$ receptors (GABA $_A$ Rs). We used

homology between the $\beta 2+$ / $\alpha 4$ complementary (–) subunit interface and the GABA $_A$ BZD binding site as a guide for mutagenesis (Fig. 1, B and D) (Wieland et al., 1992; Amin et al., 1997; Buhr et al., 1997a,b; Buhr and Sigel, 1997; Wagner and Czajkowski, 2001). Using site-directed mutagenesis, amino acids within the $\beta 2+$ -containing subunit interfaces were mutated to alanine and expressed in the HS and LS isoforms in *Xenopus laevis* oocytes, as described previously by Nelson et al. (2003). The effects of each mutation were evaluated using two-electrode voltage-clamp (TEVC) electrophysiology techniques. Data show that the $\beta 2+$ subunit interface is involved in the receptor's response to ACh. Furthermore, data obtained in the presence of dFBr support the conclusion that the dFBr potentiation mechanism involves the $\beta 2+$ face.

Materials and Methods

Receptors and cRNA. The cDNA sequences for the human $\alpha 4$ and $\beta 2$ (National Center for Biotechnology Information reference sequences NM_000744.5 and NM_000748.2, respectively) nAChR subunits were used to synthesize a full-length cDNA for each subunit. cDNA synthesis was conducted by GeneArt Inc. (Burlingame, CA). The $\beta 2$ subunit cDNA was ligated using T7 ligase (New England Biolabs, Ipswich, MA) into the pcDNA3.1/Zeo(+) mammalian expression vector using restriction enzyme cut sites NotI and XhoI (New England Biolabs). Similarly, the $\alpha 4$ cDNA was inserted into the pcDNA3.1/hygro myocin mammalian expression vector using restriction enzyme cut sites HindIII and BamHI (vectors procured from Invitrogen, Carlsbad, CA). $\beta 2$ mutant subunit cDNA was created using commercial mutagenesis services (DNA 2.0, Menlo Park, CA). The resulting DNA was inserted into the pcDNA3.1/Zeo(+) vector (Invitrogen) following the procedure as described for the wild-type $\beta 2$ subunit construct. All wild-type and mutant DNA constructs were fully sequenced and confirmed to be identical to the published sequences for each subunit (with the exception of the desired mutated amino acid).

Synthetic DNA was used to transform AG1 super-competent cells (Stratagene, La Jolla, CA) for production of cDNA. cDNA was isolated using Qiagen plasmid maxi kits (Valencia, CA) followed by an Fsp I

Fig. 2. Wild-type $\alpha 4 \beta 2$ -nAChR responses to ACh and dFBr. *Xenopus* oocytes were prepared using the high- and low-sensitivity receptor preparations (HS cRNA injected at a ratio of 1:5 $\alpha 4$: $\beta 2$; LS cRNA injection at a ratio of 5:1 $\alpha 4$: $\beta 2$). (A) Maximum ACh-induced currents measured in wild-type HS and LS isoform preparations (* $P < 0.05$). (B) ACh concentration-response profiles on HS and LS isoform preparations. The LS isoform preparation produced receptors that had a lower potency for ACh compared with the HS isoform preparations, thus verifying biased expression of the appropriate isoforms. (C) Increasing concentrations of dFBr coapplied with ACh (EC_{75}). Directly below (C) are current response profiles for wild-type receptors expressed in the HS (left) and LS (right) isoform preparations showing inward currents with application of ACh and ACh coapplied with dFBr. Responses obtained for the HS isoform preparation, with increasing concentrations of dFBr coapplied with 10 μ M ACh [dFBr concentrations: 0 μ M (a); 1 μ M (b); 10 μ M (c); 30 μ M (d); and 100 μ M (e)] (left). At concentrations greater than 10 μ M, dFBr produced a decrease in currents induced by ACh. Responses obtained for the LS isoform preparation, with increasing concentrations of dFBr coapplied with 100 μ M ACh [dFBr concentrations: 0 μ M (a); 0.3 μ M (b); 1.0 μ M (c); 3.0 μ M (d); 10 μ M (e); 30 μ M (f); and 100 μ M (g)] (right). Concentrations

linearization for production of cRNA. Synthetic cRNA transcripts for wild-type and mutant subunits were prepared using the T7 mMESSAGE mMACHINE high yield capped RNA transcription kit (Ambion, Austin, TX). The resulting cRNA was aliquoted and stored at -80°C until needed for oocyte injections.

Experimental Chemicals and Test Compounds. ACh, other salts, and buffering agents were obtained from Sigma-Aldrich (St. Louis, MO). dFBr was synthesized by Dr. Richard Glennon (Virginia Commonwealth University, Richmond, VA) according to a previously published procedure (Kim et al., 2007).

***X. laevis* Oocytes and Receptor Expression.** *X. laevis* frogs and frog food were purchased from Nacso (Fort Atkinson, WI). *X. laevis* frogs were housed in an isolated, quiet, temperature- and light-controlled environment consistent with their environment in the wild (12-hour light/dark cycle, 20 – 22°C). Frogs were housed and maintained for up to 1 year of age in a filtered water tank system with UV ray sterilization of the circulating water. No more than four surgeries were conducted on each frog. A recovery period greater than 6 weeks was allowed in between surgeries (*Xenopus* protocols were conducted at the University of Alaska and approved by the University of Alaska Fairbanks Institutional Animal Care and Use Committee; approval number 08-71).

Ovarian lobes were surgically removed from anesthetized *X. laevis* frogs (Finquel MS-222, *Tricaine methanesulfonate*), washed twice in Ca^{2+} free Barth's buffer (82.5 mM NaCl, 2.5 mM KCl, 1 mM MgCl_2 , and 5 mM HEPES, pH 7.4), and then gently shaken with 1.5 mg/ml collagenase (Sigma type II; Sigma-Aldrich) for 20 minutes at 20 – 25°C . Stage V oocytes were selected for microinjection.

Xenopus oocytes were injected with 50 nl of cRNA containing mixed ratios of $\alpha 4$ and $\beta 2$ receptor subunit cRNA. Expression of the HS isoform preparations was accomplished by injecting oocytes with a 1:5 $\alpha 4$ -to- $\beta 2$ cRNA subunit ratio (50 ng/ μ l of $\alpha 4$ cRNA to 250 ng/ μ l of $\beta 2$ cRNA). The LS isoform preparation was expressed in oocytes using a 5:1 $\alpha 4$ -to- $\beta 2$ cRNA injection ratio (250 ng/ μ l $\alpha 4$ cRNA to 50 ng/ μ l $\beta 2$ cRNA). Please note that throughout the text, injection ratios are presented with the quantity of the $\alpha 4$ subunit first followed by the $\beta 2$ subunit (i.e., 1:5 $\alpha 4$: $\beta 2$). Injected oocytes were incubated at 19°C for 36–72 hours prior to their use in TEVCs. The HS (1:5) and LS (5:1) isoform preparation pEC_{50} values obtained for ACh-induced currents from these injection ratios were verified by electrophysiology assays, as described below, and found to compare well with published values for the HS and LS $\alpha 4 \beta 2$ -nAChR isoforms (Zwart and Vijverberg, 1998; Moroni et al., 2006; Eaton et al., 2014).

Two-Electrode Voltage-Clamp Recordings. Current recordings were performed using an in-house-developed automated TEVC recording system incorporating an autoinjection system (Gilson, Middleton, WI), OC-725C oocyte clamp amplifier (Warner Instruments, Hamden, CT) coupled to a computerized data acquisition (Datapac 2000; RUN Technologies, Mission Viejo, CA). Recording and current electrodes with a resistance of 1–4 M Ω were filled with 3 M KCl. Details of the chambers and methodology employed for electrophysiological recordings have been described previously (Joshi et al., 2004). Oocytes were held in a vertical flow chamber of 200- μ l volume, clamped at a holding potential of -60 mV, and perfused with a modified ND-96 recording buffer. A phosphate ND-96 recording buffer was used in these experiments due to the findings that HEPES modulates the HS isoform (Weltzin et al., 2014). The phosphate ND-96 recording solution is similar to HEPES ND-96 buffer in all regards except with the omission of HEPES and the addition of phosphate (96 mM NaCl, 2 mM KCl, 1.8 mM CaCl_2 , 1 mM MgCl_2 , and 2 mM phosphate). Test compounds were dissolved in buffer and injected into the chamber at a rate of 20 ml/min using a Gilson autosampler injection system (Joshi et al., 2004).

greater than 10 μ M dFBr produced a decrease in currents induced by ACh. Data points represent averages ($\pm$ S.E.M.).

TABLE 1

Summary of ACh concentration-response data for wild-type and mutant $\alpha 4\beta 2$ -nAChR in HS (1:5) and LS (5:1) isoform preparations

The loop location for each mutation is shown, and the cRNA injection ratio indicates the biased receptor population (HS 1:5 and LS 5:1 $\alpha 4\beta 2$, respectively). In the LS isoform preparation (5:1 $\alpha 4\beta 2$), the ACh concentration-response data were best fit using either monophasic or biphasic fits. Specifically, mutants $\beta 2$ (W176A), $\beta 2$ (D179A), $\beta 2$ (D217A), and $\beta 2$ (Y127A) were best fit using a biphasic logistic equation (5:1 comparison of fits test results, where $P < 0.05$ resulted in rejection of the data being best fit using a monophasic logistic equation: $\alpha 4\beta 2$ $F_{3,37} = 0.5804$, $P = 0.6316$; $\alpha 4\beta 2$ (W176A) $F_{3,104} = 2.863$, $P = 0.0404$; $\alpha 4\beta 2$ (T177A) $F_{3,38} = 0.03193$, $P = 0.9922$; $\alpha 4\beta 2$ (D179A) $F_{3,32} = 5.328$, $P = 0.0043$; $\alpha 4\beta 2$ (D217A) $F_{3,42} = 2.937$, $P = 0.0442$; $\alpha 4\beta 2$ (D218A) $F_{3,47} = 0.5280$, $P = 0.6652$; $\alpha 4\beta 2$ (D116A) $F_{3,28} = 2.797$, $P = 0.0585$; $\alpha 4\beta 2$ (Y120A) $F_{3,38} = 2.542$, $P = 0.0707$; $\alpha 4\beta 2$ (Y127A) $F_{3,33} = 4.517$, $P = 0.0092$). $I_{\max}$ is the maximal ACh-induced current amplitude for each of the receptors. pEC_{50_HS} and pEC_{50_LS} is the apparent ACh potency for each receptor. The n_H values are the calculated Hill coefficients. Data are the mean $\pm$ S.E.M. Differences between groups were analyzed using one-way analysis of variance with Tukey's post hoc comparison (see *Materials and Methods*).

Subunits	Mutation Loop Location	Ratio ($\alpha 4\beta 2$)	ACh					HS-Phase Function
			$I_{\max} \pm$ S.E.M.	pEC _{50_HS} $\pm$ S.E.M.	$n_{H_HS} \pm$ S.E.M.	pEC _{50_LS} $\pm$ S.E.M.	$n_{H_LS} \pm$ S.E.M.	
			nA					%
High sensitivity								
$\alpha 4\beta 2$		1:5	310 $\pm$ 60	5.2 $\pm$ 0.1	1.9 $\pm$ 0.3	N/A	N/A	N/A
$\alpha 4\beta 2$ (W176A)	B	1:5	240 $\pm$ 30	6.0 $\pm$ 0.1**	0.8 $\pm$ 0.1	N/A	N/A	N/A
$\alpha 4\beta 2$ (T177A)	B	1:5	3290 $\pm$ 1050**	5.7 $\pm$ 0.2	5.7 $\pm$ 0.2	N/A	N/A	N/A
$\alpha 4\beta 2$ (D179A)	B	1:5	630 $\pm$ 190	6.0 $\pm$ 0.1**	0.7 $\pm$ 0.1	N/A	N/A	N/A
$\alpha 4\beta 2$ (D217A)	C	1:5	120 $\pm$ 8	5.8 $\pm$ 0.1*	1.1 $\pm$ 0.3	N/A	N/A	N/A
$\alpha 4\beta 2$ (D218A)	C	1:5	67 $\pm$ 8	5.5 $\pm$ 0.1	1.2 $\pm$ 0.1	N/A	N/A	N/A
$\alpha 4\beta 2$ (D116A)	A	1:5	460 $\pm$ 160	5.2 $\pm$ 0.1	0.9 $\pm$ 0.2	N/A	N/A	N/A
$\alpha 4\beta 2$ (Y120A)	A	1:5	370 $\pm$ 84	5.7 $\pm$ 0.2**	0.4 $\pm$ 0.1	N/A	N/A	N/A
$\alpha 4\beta 2$ (Y127A)	A	1:5	32 $\pm$ 1	6.4 $\pm$ 0.1***	0.5 $\pm$ 0.1	N/A	N/A	N/A
Low sensitivity								
$\alpha 4\beta 2$		5:1	4900 $\pm$ 1400	N/A	N/A	4.5 $\pm$ 0.1	1.4 $\pm$ 0.3	N/A
$\alpha 4\beta 2$ (W176A)	B	5:1	30 $\pm$ 3*	5.9 $\pm$ 0.2	1.1 $\pm$ 0.5	3.0 $\pm$ 1.0	~1	0.6 $\pm$ 0.4
$\alpha 4\beta 2$ (T177A)	B	5:1	24,200 $\pm$ 860***	N/A	N/A	3.6 $\pm$ 0.7	0.5 $\pm$ 0.2	N/A
$\alpha 4\beta 2$ (D179A)	B	5:1	7800 $\pm$ 1100	4.7 $\pm$ 0.8	1.2 $\pm$ 0.7	3.9 $\pm$ 0.1	2.0 $\pm$ 0.6	0.4 $\pm$ 0.5
$\alpha 4\beta 2$ (D217A)	C	5:1	3020 $\pm$ 990	5.6 $\pm$ 0.6	1.2 $\pm$ 1.4	4.5 $\pm$ 0.1	2.6 $\pm$ 1.3	0.3 $\pm$ 0.3
$\alpha 4\beta 2$ (D218A)	C	5:1	1820 $\pm$ 450	N/A	N/A	4.4 $\pm$ 0.1	0.8 $\pm$ 0.2	N/A
$\alpha 4\beta 2$ (D116A)	A	5:1	1200 $\pm$ 320	N/A	N/A	4.2 $\pm$ 0.1	1.1 $\pm$ 0.1	N/A
$\alpha 4\beta 2$ (Y120A)	A	5:1	1940 $\pm$ 370	N/A	N/A	4.0 $\pm$ 0.1	1.3 $\pm$ 0.2	N/A
$\alpha 4\beta 2$ (Y127A)	A	5:1	180 $\pm$ 26*	5.3 $\pm$ 0.3	~1	3.7 $\pm$ 0.1	3.4 $\pm$ 1.6	0.4 $\pm$ 0.2

* $P < 0.05$; ** $P < 0.01$; *** $P < 0.0001$.

Electrophysiology Concentration-Response Experiments.

ACh concentration-response data were collected for both wild-type and mutant receptors expressed in the HS (1:5 $\alpha 4\beta 2$) and LS (5:1 $\alpha 4\beta 2$) isoform preparations at ACh concentrations ranging from 0.1 μ M to 3 mM. The maximal induced current ($I_{\max}$) of wild-type and mutant receptors expressed in the HS (1:5 $\alpha 4\beta 2$) and LS (5:1 $\alpha 4\beta 2$) isoform expression preparations were examined at the maximally effective ACh concentration. All HS (1:5 $\alpha 4\beta 2$) isoform constructs were evaluated at 100 μ M ACh with the exception of $\beta 2$ (T177A), which reached maximal current at 30 μ M. Receptors expressed in the LS isoform preparation (5:1 $\alpha 4\beta 2$) responded maximally to 300 μ M ACh in $I_{\max}$ determinants.

Receptors expressed via the HS isoform preparation (1:5 $\alpha 4\beta 2$) were evaluated for dFBr modulation by coapplication of 10 μ M ACh (EC_{75}) with increasing concentrations of dFBr (0.001–100 μ M). Similarly, for receptors expressed via the LS isoform preparation (5:1 $\alpha 4\beta 2$), concentration-response curves for dFBr were determined by coapplication of 100 μ M ACh (EC_{75}) with increasing concentrations of dFBr (0.001–100 μ M). To compare responses from different oocytes, individual responses to drug application were normalized to the control responses elicited using either 10 or 100 μ M ACh for receptors expressed by the HS (1:5 $\alpha 4\beta 2$) or LS (5:1 $\alpha 4\beta 2$) isoform preparations, respectively, for both wild-type and mutated receptors. Data were collected from at least four replicate experiments using oocytes obtained from at least two different frogs.

Data Analysis and Statistics. pEC_{50} , pIC_{50} , Hill slopes (n_H), and $I_{\max}$ values were determined from individual oocytes. Concentration-response profiles were measured using nonlinear curve fitting and GraphPad Prism 4.0 (GraphPad Software, La Jolla, CA) with standard built-in algorithms. Unconstrained monophasic or constrained ($n_H > 1$) sigmoidal and biphasic logistic equations were used to fit all parameters, including pEC_{50}/IC_{50} values, n_H , and HS fractional contribution, to function in the 5:1 preparation, where applicable. Data were analyzed using Student's t test to compare pairs

of groups or by one-way analysis of variance and Tukey's multiple comparison tests to evaluate the means of three or more groups (Prism).

Results

Characterization of ACh and dFBr on Wild-Type HS and LS Isoform Preparations. To determine if our biased injection ratios expressed either predominantly the HS or LS $\alpha 4\beta 2$ -nAChR isoforms, responses to ACh were measured (peak currents and pEC_{50} values). Peak currents induced by ACh on oocytes expressing wild-type receptors were larger in the LS isoform preparation (5:1 $\alpha 4\beta 2$) (4900 $\pm$ 1400 nA) compared with the HS isoform preparation (1:5 $\alpha 4\beta 2$) (310 $\pm$ 60 nA) (Fig. 2A). These findings are consistent with previous studies using loose and concatenated receptor expression techniques (Moroni and Bermudez, 2006; Eaton et al., 2014). Eaton et al. (2014) showed, using concatenated receptors, that a pure population of the LS isoform (3100 $\pm$ 530 nA) produces currents four to five times larger than a pure population of the HS isoform (710 $\pm$ 60 nA).

ACh concentration-response curves were obtained for wild-type receptors using the HS (1:5 $\alpha 4\beta 2$) and LS (5:1 $\alpha 4\beta 2$) isoform preparations (Fig. 2B; Table 1). For both the HS and LS isoform preparations, EC_{50} values (6.3 and 32 μ M, respectively) were similar to previously published values in experiments using concatenated and loose subunit expression techniques (Zwart and Vijverberg, 1998; Moroni and Bermudez, 2006; Harpoe et al., 2011; Eaton et al., 2014).

To determine if dFBr modulated either of the $\alpha 4\beta 2$ isoforms differently, we performed concentration-response

experiments using dFBr with coapplication of ACh (see *Materials and Methods* for details). Application of dFBr without ACh produced no response in either the HS (1:5 $\alpha 4:\beta 2$) or LS (5:1 $\alpha 4:\beta 2$) isoform preparations. ACh-induced currents were potentiated and inhibited by dFBr in the HS (1:5 $\alpha 4:\beta 2$) and LS (5:1 $\alpha 4:\beta 2$) isoform preparations (Fig. 2C; Table 2), although dFBr displayed a higher potency on the LS isoform (5:1 $\alpha 4:\beta 2$) ($pEC_{50} = 6.4 \pm 0.2$) compared with the HS isoform (1:5 $\alpha 4:\beta 2$) ($pEC_{50} = 5.6 \pm 0.2$) (Fig. 2C; Table 2). dFBr potentiated ACh-induced responses of wild-type receptors expressed using the HS isoform preparation (1:5 $\alpha 4:\beta 2$) maximally by $350 \pm 20\%$, which is similar to receptors expressed via the LS isoform preparation (5:1 $\alpha 4:\beta 2$) ($350 \pm 30\%$) (Fig. 2C; Table 2). dFBr concentrations greater than $10 \mu M$ inhibited induced currents on both wild-type HS and LS isoform preparations. Similarities between dFBr actions on the two different isoforms suggest that they use similar dFBr-binding sites and mechanisms for the enhancement of ACh-induced currents.

Effects of the $\beta 2$ Subunit Mutations on ACh Maximal Induced Currents. To investigate the role of the $\beta 2$ subunit principal (+) face of $\alpha 4\beta 2$ -nAChR in mediating the effects of ACh-induced currents and modulation of these currents by dFBr, a series of amino acid residues located in the $\beta 2$ - $\alpha 4$ -interface were mutated to alanine. Alanine substitutions were chosen as a generic replacement residue because it is the most common amino acid found in proteins (Klapper, 1977) and it is frequently located in buried and exposed positions (Chothia, 1976; Rose et al., 1985). Additionally, alanine residues do not impose new hydrogen bonding, steric bulk, or unusual

hydrophobic side chains. Specific $\beta 2$ subunit residues were selected for mutagenesis based either on homology with other nAChR subunit residues or their location at equivalent positions to those previously identified in the binding pocket for BZDs on GABA_A receptors [GABA_A $\alpha 1$ (H101), $\alpha 1$ (Y159), $\alpha 1$ (S205), $\alpha 1$ (T206), $\gamma 1$ (M130), and γ (F77)] (Wieland et al., 1992; Amin et al., 1997; Buhr et al., 1997a,b; Buhr and Sigel, 1997; Wagner and Czajkowski, 2001) (Fig. 1D). Residues mutated on the $\beta 2$ + face include W176 [equivalent to GABA_A $\alpha 1$ (Y159)], T177, and D179 in the B loop; D217 and D218 [equivalent to GABA_A $\alpha 1$ (S205) and $\alpha 1$ (T206)] in the C loop; and D116, Y120 [equivalent to GABA_A $\alpha 1$ (H101)], and Y127 in loop A.

Mutant receptors expressed in the HS isoform (1:5 $\alpha 4:\beta 2$) preparation altered ACh maximal induced currents (I_{max}). Mutants $\beta 2$ (D218A) (C loop) and $\beta 2$ (Y127A) (A loop) reduced peak ACh-induced currents, whereas $\beta 2$ (T177A) (B loop) significantly enhanced ACh-induced currents (Fig. 3A; Table 1). In the LS isoform preparation (5:1 $\alpha 4:\beta 2$), the $\beta 2$ (W176A) (B loop) and $\beta 2$ (Y127A) (A loop) mutations significantly reduced ACh peak currents compared with wild-type $\alpha 4\beta 2$ receptors (Fig. 3B; Table 1), whereas mutant $\beta 2$ (T177A) (B loop) significantly enhanced ACh currents (Fig. 3B; Table 1). Since these mutations are not located in the ACh-binding sites, a change in responsiveness to ACh would indicate that subunit interfaces not binding ACh participate in the receptors' ability to respond to ACh. Similar to what has been observed for the BZD and oxantel/morantel binding sites in GABA_AR and $\alpha 3\beta 2$ -nAChR, respectively (Wagner and Czajkowski, 2001; Morlock and Czajkowski, 2011; Chrisman

TABLE 2

Summary of dFBr concentration-response data for wild-type and mutant $\alpha 4\beta 2$ -nAChR in HS (1:5) and LS (5:1) isoform preparations

The loop location for each mutation is shown, and the cRNA injection ratio indicates the biased receptor population (HS 1:5 and LS 5:1 $\alpha 4:\beta 2$, respectively). The amount of potentiation and the pEC_{50} for dFBr potentiation was determined from the curves shown in Fig. 6. dFBr does not elicit responses when applied alone, and thus potentiation is determined by coapplication of increasing concentrations of dFBr, with a fixed concentration of ACh (HS isoform preparation: $10 \mu M$ ACh; LS isoform preparation: $100 \mu M$ ACh). The percent potentiation by dFBr was calculated as the maximal response obtained by coapplication of dFBr and ACh compared with ACh alone. Thus, a 350% potentiation represents a 3.5-fold increase in efficacy by dFBr compared with that obtained for ACh alone. The pEC_{50} is the apparent dFBr potency for potentiation at each receptor. The n_H values are the calculated Hill coefficients for the dFBr potentiation concentration-response curve. Data represent the mean $\pm$ S.E.M. Differences between groups were analyzed using one-way analysis of variance with Tukey's post hoc comparison (see *Materials and Methods*).

Receptor Subunits	Mutation Loop Location	Ratio ($\alpha 4:\beta 2$)	dFBr Potentiation of ACh-Induced Currents		
			Percent Potentiation $\pm$ S.E.M.	pEC ₅₀ $\pm$ S.E.M.	$n_H \pm$ S.E.M.
High sensitivity					
$\alpha 4:\beta 2$		1:5	350 $\pm$ 20	5.6 $\pm$ 0.2	0.8 $\pm$ 0.2
$\alpha 4:\beta 2$ (W176A)	B	1:5	94 $\pm$ 2***	N/A	N/A
$\alpha 4:\beta 2$ (T177A)	B	1:5	210 $\pm$ 35**	6.5 $\pm$ 0.2	~ 2
$\alpha 4:\beta 2$ (D179A)	B	1:5	150 $\pm$ 22***	6.3 $\pm$ 0.3	1.0 $\pm$ 0.6
$\alpha 4:\beta 2$ (D217A)	C	1:5	280 $\pm$ 18	5.7 $\pm$ 0.1	1.6 $\pm$ 0.5
$\alpha 4:\beta 2$ (D218A)	C	1:5	350 $\pm$ 33	5.7 $\pm$ 0.1	1.3 $\pm$ 0.5
$\alpha 4:\beta 2$ (D116A)	A	1:5	230 $\pm$ 20*	6.4 $\pm$ 0.2	1.2 $\pm$ 0.4
$\alpha 4:\beta 2$ (Y120A)	A	1:5	130 $\pm$ 10***	N/A	N/A
$\alpha 4:\beta 2$ (Y127A)	A	1:5	230 $\pm$ 18*	5.8 $\pm$ 0.3	1.1 $\pm$ 0.5
Low sensitivity					
$\alpha 4:\beta 2$		5:1	350 $\pm$ 30	6.4 $\pm$ 0.2	1.1 $\pm$ 0.3
$\alpha 4:\beta 2$ (W176A)	B	5:1	230 $\pm$ 34	6.9 $\pm$ 0.2	~ 2
$\alpha 4:\beta 2$ (T177A)	B	5:1	210 $\pm$ 42	6.5 $\pm$ 0.3	1.7 $\pm$ 1.8
$\alpha 4:\beta 2$ (D179A)	B	5:1	350 $\pm$ 37	5.5 $\pm$ 0.2	1.1 $\pm$ 0.4
$\alpha 4:\beta 2$ (D217A)	C	5:1	190 $\pm$ 18*	7.0 $\pm$ 0.2	2.0 $\pm$ 1.5
$\alpha 4:\beta 2$ (D218A)	C	5:1	310 $\pm$ 53	6.4 $\pm$ 0.1	1.3 $\pm$ 0.4
$\alpha 4:\beta 2$ (D116A)	A	5:1	290 $\pm$ 33	6.5 $\pm$ 0.1	1.5 $\pm$ 0.6
$\alpha 4:\beta 2$ (Y120A)	A	5:1	93 $\pm$ 7**	N/A	N/A
$\alpha 4:\beta 2$ (Y127A)	A	5:1	1677 $\pm$ 380***	5.7 $\pm$ 0.7	1.7 $\pm$ 1.8

* $P < 0.05$; ** $P < 0.01$; *** $P < 0.0001$.

Fig. 3. Effects of the mutations of maximal ACh-induced function on $\alpha 4\beta 2$ -nAChR expressed in the HS and LS isoform preparations. Maximum ACh-induced currents were explored on $\alpha 4\beta 2$ -nAChR expressing *Xenopus* oocytes using injection ratios [HS, 1:5 $\alpha 4\beta 2$ cRNA; LS, 5:1 $\alpha 4\beta 2$ cRNA]. The effects of the mutations on ACh maximal induced currents for wild-type and mutant receptors expressed in the HS (A) and LS (B) isoform preparations are shown. Data points represent averages ($\pm$ S.E.M.). Differences in the ACh maximal induced current between the groups were analyzed using one-way analysis of variance with Tukey's post hoc comparison (see *Materials and Methods*). Values significantly different from wild-type receptors are indicated with * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.0001$, respectively.

et al., 2014), we observed a significant effect on ACh $I_{\max}$ for several of the mutants studied.

ACh Concentration-Response Profiles of Mutant $\alpha 4\beta 2$ -nAChR. Three out of the eight mutations did not affect apparent ACh potency in the HS isoform (1:5 $\alpha 4\beta 2$) preparation. Significant increases in ACh pEC_{50} values for $\beta 2(W176A)$ (B loop), $\beta 2(D179A)$ (B loop), $\beta 2(D217A)$ (C loop), $\beta 2(Y120A)$ (A loop), and $\beta 2(Y127A)$ (A loop) receptors were observed (Fig. 4, A–C; Table 1).

Expression of mutated $\beta 2$ subunits in the LS isoform preparation (5:1 $\alpha 4\beta 2$) produced concentration-response curves that were best fit using monophasic sigmoidal dose response curves, although four mutations produced biphasic curves (Fig. 4, D–F; Table 1). The B-loop $\beta 2(T177A)$, C-loop $\beta 2(D218A)$, A-loop $\beta 2(D116A)$, and $\beta 2(Y120A)$ receptors expressed in the LS isoform preparation (5:1 $\alpha 4\beta 2$) were best fit using a monophasic sigmoidal fit (Fig. 4, D–F). The B-loop $\beta 2(W176A)$ and $\beta 2(D179A)$, C-loop $\beta 2(D217A)$, and A-loop $\beta 2(Y27A)$ receptors expressed in the LS isoform preparation were best fit with a biphasic model and displayed HS- and LS-like ACh potency phases (Fig. 4, D–F). The HS-like phase pEC_{50} value (1.4–19 μM) was similar to the ACh

potency found in the HS isoform preparation (1:5 $\alpha 4\beta 2$) (5.8 μM) (Table 1). Wild-type LS-like (34 μM) potency was similar to what has been previously reported using loose subunit injection ratios and the concatenated approach (Zwart and Vijverberg, 1998; Moroni and Bermudez, 2006; Harpoe et al., 2011; Eaton et al., 2014). Mutants had LS-like potencies (34–971 μM) similar to those of wild-type nAChR.

These data indicate that positive face $\beta 2$ mutations were well tolerated in the LS isoform preparation, although some altered ACh sensitivity in the HS isoform preparation (1:5 $\alpha 4\beta 2$) was evident. The greater effects of $\beta 2$ mutations in the HS isoform preparation could be due to the larger number of $\beta 2$ subunits incorporated into the receptor in the HS isoform (two $\alpha 4$ and three $\beta 2$ subunits).

Effects of $\beta 2$ Subunit Mutations on dFBr Maximal Potentiation of ACh-Induced Currents. To determine if the $\beta 2$ mutations altered the ability of dFBr to potentiate ACh-induced currents, we evaluated the effect of each mutation on the apparent efficacy of dFBr for the potentiation of ACh-induced responses. As with wild-type receptors, application of dFBr alone did not induce responses in mutant receptors in either the HS or LS isoform preparations. Several $\beta 2$ subunit mutations significantly altered the ability of dFBr to potentiate ACh-induced currents (Fig. 5). In the HS isoform preparation (1:5 $\alpha 4\beta 2$), all B-loop [$\beta 2(W176A)$, $\beta 2(T177A)$, and $\beta 2(D179A)$] and A-loop [$\beta 2(D116A)$, $\beta 2(Y120A)$, and $\beta 2(Y127A)$] mutations significantly reduced or eliminated dFBr potentiation of ACh-induced currents (Fig. 5A; Table 2). Expression of the B-loop $\beta 2(W176A)$ mutation resulted in complete elimination of dFBr potentiation, and ACh-induced currents were inhibited by $\sim 37\%$ (Fig. 5A). Similarly, the $\beta 2(Y120A)$ mutant greatly reduced/eliminated dFBr potentiation ($\sim 20\%$ potentiation) (Fig. 5A; Table 2).

In the LS isoform preparation (5:1 $\alpha 4\beta 2$), the effects of mutations on dFBr potentiation were smaller than those observed in the HS isoform preparation (Fig. 5B; Table 2). We observed a trend in reduced dFBr potentiation of the B-loop $\beta 2(W176A)$ and $\beta 2(T177A)$ mutants (Fig. 5B; Table 2). The C-loop $\beta 2(D217A)$ and A-loop $\beta 2(Y120A)$ mutations significantly reduced dFBr-potentiated currents compared with wild-type receptors (Fig. 5B; Table 2). In contrast to other mutations, expression of $\beta 2(Y127A)$ in the LS isoform preparation produced receptors with increased potentiation by dFBr ($> 500\%$) compared with wild-type receptors (Fig. 5B; Table 2). A-loop mutants $\beta 2(Y120A)$ and $\beta 2(Y127A)$ significantly changed dFBr-potentiated currents in both of the HS and LS isoform preparations.

Effects of $\beta 2$ Subunit Mutations on dFBr-Positive Modulation. Several mutations expressed via the HS isoform preparation [1:5 ($\alpha 4\beta 2$)] produced significant effects on dFBr pEC_{50} values (Fig. 6, A–C; Table 2). The B-loop $\beta 2(W176A)$ mutation eliminated the ability of dFBr to potentiate ACh-induced responses and inhibited the receptor by $\sim 37\%$, with a pIC_{50} of 8.0 ± 0.4 (Fig. 6A). The A-loop $\beta 2(Y120A)$ produced only $\sim 20\%$ potentiation, which was too low to accurately determine an EC_{50} for dFBr potentiation (Fig. 6C; Table 2). The B-loop mutations $\beta 2(T177A)$ and $\beta 2(D179A)$ and the A-loop mutation $\beta 2(D116A)$ tended to enhance dFBr potency (Fig. 6, A–C; Table 2).

The effects of the $\beta 2$ mutations on dFBr modulation of mutant receptors expressed in the LS isoform preparation (5:1 $\alpha 4\beta 2$) were moderate but significant. The A-loop $\beta 2(Y120A)$ mutation significantly abolished dFBr potentiation and

Fig. 4. ACh concentration-response curves for HS and LS isoform preparations. *Xenopus* oocytes were prepared using the HS and LS isoform preparations (HS cRNA injected at a ratio of 1:5 $\alpha 4$: $\beta 2$; LS cRNA injection at a ratio of 5:1 $\alpha 4$: $\beta 2$). Expressing oocytes were exposed to increasing concentrations of ACh. ACh concentration-response curves for wild-type, B-loop (A), C-loop (B), and A-loop (C) mutant receptors expressed using the HS isoform preparation. ACh concentration-response curves for wild-type, B-loop (D), C-loop (E), and A-loop (F) mutant receptors expressed using the LS isoform preparation. Individual peak amplitudes were normalized to the $I_{\max}$ on the same oocyte. The ACh concentration-response values were determined using nonlinear curve fitting, as described in *Materials and Methods*, and are reported in Table 1. Data were collected from a minimum of four oocytes harvested from at least two batches of frogs. Data points represent averages ($\pm$ S.E.M.). Differences in ACh potency between groups were analyzed using one-way analysis of variance with Tukey's post hoc comparison (see *Materials and Methods*) and shown in Table 1.

inhibition of ACh-modulated currents (Fig. 6F; Table 2). The B-loop $\beta 2$ (D179A) mutation decreased the dFBr pEC_{50} by approximately one log unit ($pEC_{50} = 5.5 \pm 0.2$), but this change was not statistically different from that of wild-type receptors ($pEC_{50} = 6.4 \pm 0.2$) (Fig. 5D; Table 2). The B-loop $\beta 2$ (W176A) and C-loop $\beta 2$ (D217A) mutations tended to enhance dFBr potency (Fig. 6, D and E; Table 2).

Overall, the data indicate that mutation of residues on the + face of $\beta 2$ subunit alters the amount of potentiation produced by dFBr as well as the potency of dFBr for potentiation in $\alpha 4\beta 2$ -nAChR expressed in biased HS (1:5 $\alpha 4$: $\beta 2$) and LS (5:1 $\alpha 4$: $\beta 2$) isoform preparations. The effects observed on dFBr potency are localized primarily to the B and A loops in both preparations.

Discussion

We investigated the role of the principal face of the $\beta 2$ subunit in dFBr modulation of ACh currents on the $\alpha 4\beta 2$ -nAChR HS and LS isoforms. Our data indicate that mutation of residues in loops A and B alters both the magnitude of dFBr potentiation and pEC_{50} values. The $\beta 2$ residues W176 (B loop) and Y120 (A loop) were found to significantly alter dFBr apparent potency and eliminate dFBr potentiation, suggesting that these residues may be a part of the dFBr-binding site. These data provide clues to the mechanism of dFBr potentiation. Several mutations [$\beta 2$ (W176A), $\beta 2$ (D179A), $\beta 2$ (T177A), $\beta 2$ (D217A), $\beta 2$ (Y120A), and $\beta 2$ (Y127A)] also altered

ACh $I_{\max}$ and potency, even though they are not located in the ACh-binding cleft. Our findings provide evidence that suggests structural perturbations occurring within noncanonical binding interfaces alter the functional response to ACh on both $\alpha 4\beta 2$ -nAChR isoforms.

Previous mutations, specifically $\alpha 1$ (S205) and $\alpha 1$ (S206) [equivalent to nAChR $\beta 2$ (D217) and $\beta 2$ (D218)], $\gamma 2$ (Y72), $\gamma 2$ (D75), and $\gamma 2$ (F78) [equivalent to nAChR $\alpha 4$ (M83), $\alpha 4$ (N860), and $\alpha 4$ (V89)] located in the GABA_A BZD-binding site have been shown to increase or decrease GABA potency (Teissere and Czajkowski, 2001; Morlock and Czajkowski, 2011). Similarly, several recent studies have shown that mutation of residues $\alpha 3$ (E173), $\alpha 3$ (L158), $\alpha 3$ (A179), $\alpha 3$ (K183), $\beta 2$ (R46), and $\beta 2$ (A127) located in nonorthosteric interfaces of the rat $\alpha 3$ - and $\beta 2$ -nAChR subunits also increase or decrease ACh potency from 2- to 60-fold compared with wild-type $\alpha 3\beta 2$ -nAChR (Chrisman et al., 2014; Short et al., 2015). These results suggest that nonorthosteric clefts are involved in the receptors' conformational change in response to binding of ligands at orthosteric interfaces.

In the present study, several of the mutations [$\beta 2$ (W176A), $\beta 2$ (D179A), $\beta 2$ (D217A), and $\beta 2$ (Y127A)] altered ACh potency in both the HS (1:5 $\alpha 4$: $\beta 2$) and LS (5:1 $\alpha 4$: $\beta 2$) isoform preparations (Fig. 4; Table 1). Alanine mutations produced larger effects on ACh peak currents when expressed in the HS isoform preparation compared with the LS isoform preparation (Fig. 3; Table 1). Mutations that altered peak currents in the HS isoform preparation tended to also show similar

Fig. 5. Effects of $\beta 2$ + face mutations on dFBr potentiation of ACh-induced currents in HS and LS isoform preparations. *Xenopus* oocytes were prepared using the HS and LS isoform preparations (HS cRNA injected at a ratio of 1:5 $\alpha 4\beta 2$; LS cRNA injection at a ratio of 5:1 $\alpha 4\beta 2$). The max amount of dFBr potentiation is shown for mutants expressed in HS (A) and LS (B) isoform preparations. Unpotentiated wild-type response (ACh maximal response of 100%) is shown via the gray dotted line. Data points represent averages ($\pm$ S.E.M.). Differences in dFBr potentiation between groups were analyzed using one-way analysis of variance with Tukey's post hoc comparison (see *Materials and Methods*) and also shown in Table 2. Values significantly different from wild-type receptors are indicated with * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.0001$, respectively.

changes in mutant receptors expressed in the LS isoform preparation. These changes in receptor function could have been caused by the incorporation of an additional mutated $\beta 2$ subunit in the HS isoform [$(\alpha 4)_2(\beta 2)_3$]. Additionally, the investigated residues could have altered receptor expression levels. Given that the targeted residues are located in the extracellular domain and not in the intracellular domain, a region known to alter subunit expression (Jeanclos et al., 2001; Pollock et al., 2009), and that we observed alterations in ACh potency and $I_{\max}$ values, we speculate that these changes result from alterations in the signal transduction pathway rather than receptor expression. In agreement with our postulation, Dr. Mark M. Levandoski's group has recently shown evidence that the allosteric modulation is mediated by movement between subunits using mutational analysis and disulfide trapping of cysteine-substituted residues located in the allosteric clefts in the extracellular domain of $\alpha 3\beta 2$ -nAChR (Chrisman et al., 2014; Short et al., 2015).

nAChRs are capable of conducting calcium, which could potentially activate endogenous calcium-activated chloride channels (CaCCs) in *Xenopus* oocytes (Miledi, 1982; Barish and Thompson, 1983; Miledi and Parker, 1984) and alter our

recorded $I_{\max}$ values. It is possible that expression of the more functional mutant subunits could have caused a large enough calcium influx into the oocyte to activate CaCC. CaCC currents were initially measured in *Xenopus* oocytes to be less than +100 nA at 1.8 mM Ca^{2+} using a -60 mV holding potential, recording conditions comparable to the present study (Miledi, 1982). As CaCC produce *outward* currents at -60 mV, it is possible that their activation might slightly *reduce* measured $I_{\max}$ values. However, potential CaCC effects in the presented study would be nominal, resulting in a maximum of 0.5% reduction in our current measurements.

Contradictory reports in the literature have shown that the LS isoform, expressed via loose subunits, produces ACh concentration-response curves that are either monophasic or biphasic (Moroni et al., 2006, 2008; Harpsoe et al., 2011). In contrast, LS concatenated receptors display biphasic concentration-response profiles (Eaton et al., 2014). Presently, data from the wild-type LS (5:1 $\alpha 4\beta 2$) preparation were best fit using a monophasic concentration-response equation (Fig. 4; Table 1). Mutants $\beta 2(T177A)$ and $\beta 2(D218A)$ displayed a substantial decrease in n_H , suggesting that the mutant-containing receptors may have high and low affinities to ACh. Several of the investigated mutants [$\beta 2(W176A)$, $\beta 2(D179A)$, $\beta 2(D217A)$, and $\beta 2(Y127A)$] expressed in the LS isoform preparation produced ACh concentration-response curves that were best fit using a biphasic equation, displaying an enhanced HS-like phase. The surprising increase in ACh potency for these mutants suggests that the allosteric interfaces containing the $\beta 2$ + interface may underlie a fundamental structure/function component of receptor response to ACh and other ligands (including dFBr).

Residues involved in the putative dFBr-binding pocket will likely play different roles in the receptor response to modulating ligand potency and efficacy, similar to residues in the BZD-binding site in GABA_AR (Morlock and Czajkowski, 2011). Mutation of key receptor residues involved in the binding of a ligand will generally decrease ligand potency. Other residues may stabilize the binding site structure or mediate local structural movements, linking the modulation site to the agonist binding site. Previously, it was shown that BZDs allosterically modulate GABA currents by shifting the GABA_AR closed-state equilibrium to an open-state equilibrium (Downing et al., 2005; Rusch and Forman, 2005; Campo-Soria et al., 2006) or altered the receptors' binding affinity for GABA (Lavoie and Twyman, 1996; Mellor and Randall, 1997; Thompson et al., 1999; Hanson and Czajkowski, 2008; Hanson et al., 2008; Goldschen-Ohm et al., 2010). Morlock and Czajkowski (2011) investigated the role of 22 residues in GABA_AR found within the BZD binding pocket via TEVC and radioligand-binding studies. They found that six residues [$\alpha(A160)$ B loop, $\alpha(T206)$ and $\alpha(V211)$ C loop, $\gamma(R144)$ E loop, $\gamma(R197)$, and $\gamma(E189)$ F loop] altered BZD maximum potentiation of GABA-induced currents, three residues [$\alpha(G200)$ C loop, $\gamma(M130)$, and $\gamma(M132)$ E loop] altered BZD binding but not efficacy, and four residues [$\alpha(D97)$ and $\alpha(F99)$ A loop, $\alpha(G157)$ B loop, and $\alpha(Y209)$ C loop] altered both BZD binding and efficacy.

In the present study, many of the investigated residues did not alter dFBr potency or eliminate dFBr positive modulation of ACh-induced currents, excluding $\beta 2(W176A)$ (B loop) and $\beta 2(Y120A)$ (A loop) (Fig. 6; Table 2). Residue $\beta 2(Y120)$, when mutated, eliminated dFBr's ability to alter ACh currents in

Fig. 6. dFBr modulation concentration-response profiles for $\alpha 4\beta 2$ -nAChR mutant and wild-type receptors expressed using the HS and LS isoform preparations. dFBr's effects on wild-type and mutant HS (A–C) and LS (D–F) isoforms were investigated using oocytes injected with cRNA for $\alpha 4$ and $\beta 2$ subunits in a 1:5 ($\alpha 4$: $\beta 2$) (HS) or 5:1 ($\alpha 4$: $\beta 2$) (LS) ratio. Increasing concentrations of dFBr were coapplied with ACh EC₇₅, as described in *Materials and Methods*. The dFBr concentration-response values were determined using nonlinear curve fitting and are reported in Table 2. Data points represent averages ($\pm$ S.E.M.). Differences in dFBr modulation between groups were analyzed using one-way analysis of variance with Tukey's post hoc comparison and are shown in Table 2.

the HS isoform preparation and reduced dFBr potency in the LS isoform preparation. In the equivalent position within the BZD-binding site, $\alpha 1$ (H101) eliminated diazepam potentiation of GABA-induced currents (Benson et al., 1998). Mutation of residue $\beta 2$ (W176A) eliminated dFBr's ability to modulate ACh currents but only in the HS isoform preparation. Both of these residues appeared to transform dFBr from a positive to a weak negative modulator, or, alternatively, mutation of these residues eliminated dFBr's ability to potentiate ACh-induced currents. Our results suggest that these two residues may be in the dFBr-binding pocket.

Most other investigated residues decreased dFBr potentiation in both HS and LS isoform preparations, with the exception of the observed increase with $\beta 2$ (Y120A) (LS isoform preparation only) (Fig. 5; Table 2). The largest decreases in dFBr potentiation were seen using mutations within loops A and B. These findings suggest that the residues explored here [excluding $\beta 2$ (W176A) and $\beta 2$ (Y120A)] may not be a part of the dFBr-binding pocket but instead constitute part of the allosteric pathway coupling dFBr binding to modulation of receptor function.

Our results also suggest that dFBr may modulate $\alpha 4\beta 2$ receptors through other clefts. If only the $\beta 2$ +/ $\alpha 4$ - allosteric clefts were involved in the binding and potentiation mechanism of dFBr, then we would expect to see similar effects between the HS and LS isoforms. We observed greater effects

of the mutants when expressed in the HS isoform, suggesting that the $\beta 2$ +/ $\beta 2$ - interface, which is only found in the HS isoform, is involved as well. Involvement of the $\beta 2$ +/ $\beta 2$ - interface might explain the higher potency of dFBr on the HS isoform.

Allosteric clefts of $\alpha 4\beta 2$ -nAChR are presently understudied and could contain allosteric binding pockets for potential therapeutic ligands. Neuronal cholinergic transmission is required for central nervous system function and alterations in cholinergic function can result in cognitive impairment disorders, including Alzheimer's and Parkinson's disease. At a damaged cholinergic synapse, application of a PAM could be beneficial in that the PAM could enhance the ACh-induced responses of nAChR and restore function. To the best of our knowledge, this is the first study focused on the identification of the mechanism behind dFBr's modulatory actions on $\alpha 4\beta 2$ -nAChR isoforms and the role of the $\beta 2$ +/region in $\alpha 4\beta 2$ -nAChR modulation. Our data suggest that the modulation site of dFBr on the $\alpha 4\beta 2$ subtype may involve a region of the receptor homologous to the BZD-binding site in GABA_AR. Identifying residues that are involved in dFBr modulation are critical for elucidating the mechanisms that mediate the pharmacological effects of dFBr. This insight will aid the development of compounds similar to dFBr as well as identification of other classes of ligands for this domain. Understanding the basis of dFBr selectivity for the

$\beta 2$ -containing receptors may potentially also lead to the development of modulatory agents for other heteromeric subtypes of nAChRs.

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Authorship Contributions

Participated in research design: Weltzin, Schulte.

Conducted experiments: Weltzin.

Performed data analysis: Weltzin, Schulte.

Wrote or contributed to the writing of the manuscript: Weltzin, Schulte.

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