Nicotinic acetylcholine receptor (nAChR) ligands that lack agonist activity but enhance activation in the presence of an agonist are called positive allosteric modulators (PAMs). nAChR PAMs have therapeutic potential for the treatment of nicotine addiction and several neuropsychiatric disorders. PAMs need to be selectively targeted toward certain nAChR subtypes to tap this potential. We previously discovered a novel PAM, (R)-7-bromo-N-(piperidin-3-yl)benzothiophene-2-carboxamide (Br-PBTC), which selectively potentiates the opening and closing of nAChR subunits. The pharmacological effect of Br-PBTC depends on the interaction of a subunit. E282Q abolishes Br-PBTC potentiation. Using transmembrane helix were found to be crucial for Br-PBTC’s binding site for the positive allosteric modulator Br-PBTC.

Br-PBTC and its derivatives should prove useful as cognitive enhancement agents. nAChRs are pentameric ligand-gated ion channels widely expressed in muscle and neuronal systems (1). Muscle α1, β1, γ, δ, and ε and neuronal α2–α7, α9–α10, and β2–β4 nAChR subunits have been found in humans (2). Subunits assemble in combinations of five to form various nAChR subtypes. Upon binding an agonist, nAChRs assume a short-lived conductive state before transitioning into nonconductive desensitized states with agonist still bound. Multiple desensitized states have been reported, which differ in onset and stability (3, 4).

The distinct nAChR subunits exhibited different selectivity for Br-PBTC. The nAChR subunits that exhibited the highest selectivity for Br-PBTC were α4β2 and α4β4, with α2β2 exhibiting less selectivity (2). The selectivity for binding sites within nAChR subtypes was determined using crystallography and cryo-electron microscopy (cryo-EM), photoaffinity labeling, mutagenesis, and docking in a subtype-selective manner to treat major neurological disorders, including Alzheimer’s disease, Parkinson’s disease, autism, schizophrenia, and nicotine addiction (7–14). Enhancing activation of α5* nAChRs by subtype-selective PAMs could be more effective for nicotine addiction (7, 8, 10), whereas α7*- and α4β2*-selective PAMs may be effective for cognitive enhancement (11, 14).

Previously, we reported a novel subtype-selective type II PAM, Br-PBTC (Fig. 1), which enhanced the opening of α2- and α4-containing subtypes and reactivated desensitized α4-containing subtypes (15). Br-PBTC is selective and efficacious in potentiating channel opening for both α2* and α4* subtypes. There was no detectable PAM effect on α3*, α6*, and α7* nAChR subtypes that lack an α2 or α4 subunit. Reactivation of desensitized α4* nAChRs is most effective for a stoichiometry with three α4 subunits, (α4β2)2α4 (15).

Understanding the structural features of Br-PBTC’s interactions with nAChRs will be crucial for developing subtype-selective nAChR PAMs. There are several methods to study ligand–protein interactions, including co-crystalization, cryo-electron microscopy (cryo-EM), photoaffinity labeling, mutagenesis, and docking (16, 17). Co-crystallography and cryo-EM are the most direct methods for finding the Br-PBTC–binding site, but...
existing structures of α4β2 nAChRs may not represent functional nAChRs because they were solved without lipids and with truncated cytoplasmic domains (18–21). The lack of native lipid in structure determination complicates interaction of Br-PBTC with nAChRs. Lipid–nAChR interactions greatly influence conformational changes at the extracellular end of the transmembrane domain where Br-PBTC is likely to act (15). In addition, crystallization tends to resolve nAChRs in the most stable desensitized conformation (19, 22), which may not carry a high-affinity Br-PBTC–binding site. Although type II PAMs can reactivate desensitized nAChRs, their effect on long-term desensitized nAChRs is limited (3, 15, 23, 24). Therefore, they may not bind to the desensitized nAChRs obtained by crystallography. Photoaffinity labeling requires a photoreactive ligand. Modifying Br-PBTC to include photoreactive groups may reduce its binding affinity and produce false positives from nonspecific binding. To locate the Br-PBTC–binding site, we used mutagenesis of α4 subunits combined with a consensus docking approach. Consensus docking involves searching for binding sites identified by two different suites of docking software (25). Docking with one software can produce results that are biased by the idiosyncrasies of that docking software. Consensus docking can mitigate the biases of each individual software.

Our docking templates were homology models of α4 derived from corrected crystal structures of the Torpedo marmorata muscle-type nAChR structure in an open state (25). Torpedo remains the only Cys-loop receptor structure solved in its native membrane rather than after detergent solubilization (26, 27). The registry of transmembrane domains was corrected by remapping the electron potential map, producing the template structure (25) that we use to create the α4 homology model in this study.

We located a Br-PBTC–binding site in an intrasubunit cavity between the extracellular ends of the four α4 transmembrane domains using consensus docking, which successfully predicted α4 mutations that reduce Br-PBTC activity: E282Q and F286S. We also discovered derivatives of Br-PBTC that can act on α5 subunits. In (α4β2)2α4, agonist occupancy of the accessory site at the α4/α4 subunit interface increases Br-PBTC potency more than 9-fold. We find that Br-PBTC acts synergistically between the accessory and primary α4 subunits within (α4β2)2α4 to reactivate desensitized nAChRs.

Results

Pharmacological comparison of Br-PBTC and dFBr

Recent studies of various nAChR PAMs suggest that Br-PBTC has a similar potentiation profile to those of 17β-estradiol and dFBr (15, 28). Bermudez and co-workers (28) have shown that 17β-estradiol and dFBr communicate with the Cys-loop through Ile-601 in α4. When the last four α4 C-tail residues were mutated from AGMI to AAC, potentiation of α4β2 activity by Br-PBTC decreased by 80% (15). These data suggest that Br-PBTC, dFBr, and 17β-estradiol are all sensitive to the α4 C-tail (28).

We first compared the effects of Br-PBTC and dFBr on activation and desensitization of (α4β2)2α4 and (α4β2)2β2 nAChRs (Fig. 2 and Table 1). We used two cell lines expressing nAChRs in defined stoichiometries by using concatemeric subunit pairs (Fig. 2A) (15). Agonist sites form at the α4β2 subunit interfaces within the concatemers. These α4 subunits are primary α4 subunits. The fifth free subunit is termed the accessory subunit. An accessory α4 subunit can form a third α4/α4 agonist-binding site (29–31). Br-PBTC and dFBr (structures shown in Fig. 1) increased activation of both α4β2 stoichiometries in a dose-dependent manner (Fig. 2B). Br-PBTC is 7.24-fold more potent and 3.97-fold more efficacious than dFBr at potentiating (α4β2)2α4 nAChRs. We showed that Br-PBTC reactivated long-term desensitized (α4β2)2α4 but had little effect on (α4β2)2β2 nAChRs (15). Interestingly, dFBr reactivates both stoichiometries, with slightly better potency on (α4β2)2α4 nAChRs (Fig. 2C). dFBr is 3.31-fold less potent than Br-PBTC at reactivating desensitized (α4β2)2α4 nAChRs.

We next investigated the effects of two mutants, I601F and I601W, on Br-PBTC potentiation (Table 2). We chose to mutate isoleucine to aromatic amino acids because these mutants greatly reduced PAM activity of dFBr (28). Because PAM activity is evaluated in the presence of agonists, we studied whether these mutants affected activation by ACh. We chose the (α4β2)2β2 stoichiometry for initial screening of the mutants’ effects to simplify the study because this stoichiometry only has one kind of agonist site, i.e., the high-sensitivity site at the α4β2 subunit interface (29–31). Potencies of ACh were mutated whether these mutants affected activation by ACh. We showed that Br-PBTC, dFBr, and 17β-estradiol are all sensitive to the α4 C-tail (28).

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Table 1

| Potency and efficacies of Br-PBTC and dFBr on activation and desensitization of (α4β2)α4 and (α4β2)β2 nACHRs |
|--------------------------------------------------|
| (α4β2)α4 | (α4β2)β2 |
| Br-PBTC | dFBr | Br-PBTC | dFBr |
| EC50 (μM) | Imax | Hill | EC50 (μM) | Imax | Hill | EC50 (μM) | Imax | Hill |
| 0.196 | 0.552 | 0.374–1.11 | 0.965 | 1.26 | 0.875–1.83 | 1.42 | 0.939 | 0.686–1.258 | 84.4 | 61.0 | NA |
| 335 | 113 | 96.3–147 | 1.26 | 1.22–3.66 |
| 0.633–1.14 | 0.79–10 | 0.8 | 100 | 100 | 100 |

Table 2

| Potencies and efficacies of Br-PBTC and dFBr on activation and desensitization of (α4β2)α4 and (α4β2)β2 on activation and desensitization of (α4β2)α4 and (α4β2)β2 nACHRs. |
|--------------------------------------------------|
| (α4β2)α4 | (α4β2)β2 |
| Br-PBTC | dFBr | Br-PBTC | dFBr |
| EC50 (μM) | Imax | Hill | EC50 (μM) | Imax | Hill | EC50 (μM) | Imax | Hill |
| 0.514 | 2.05 | 1.36–3.16 | 92.5 | 25.6 | NA | 1.53 | 1.71 | 1.02 |
| 0.194 | 0.658–1.69 | 0.8 | 100 | 100 | 100 |
| 85.9–101 | 7.81 | 6.49–9.34 | 48.3 | 48.5 | NA | 1.77 | 2.00 |
| 0.611–1.22 | 2.30 | 2.30–3.99 |

These data suggest that the binding site or mode of allosteric potentiation of Br-PBTC is slightly different from that of dFBr and 17β-estradiol. Experimental conditions in studies from Bermudez and co-workers are different from ours (28). They used a mixture of two stoichiometries at EC10 concentrations of ACh to evaluate mutant effects on dFBr. They biased expression to the (α4β2)β2 stoichiometry and used an EC90 concentration of ACh. Our WT experiments using both PAMs on the two α4β2 stoichiometries indicate that the two PAMs act similarly, but not identically.

Identifying the α4 Br-PBTC–binding site using mutagenesis

Previously, we found that exchanging the M4 transmembrane domain and C-tail of α3 with α4 allowed Br-PBTC to potentiate the chimeric α3 subunit (15). M1–M3 transmembrane domains are relatively conserved between α3 and α4. Because the M1–M3 domains are less conserved between α4 and α7, we tested Br-PBTC on an α71–463α4951–601 chimera. Responses to 3 μM Br-PBTC co-applied with 1,000 μM ACh were within 100 ± 5% of responses evoked by ACh alone. The absence of Br-PBTC’s PAM activity in α71–463α4951–601 chimera suggests that the M1–M3 domains of α4 are also important for potentiation by Br-PBTC in addition to the α4 M4 and C-tail. We hypothesized that nonconserved residues in α7 M1–M3 domains were responsible for preventing Br-PBTC potentiation in the α71–463α4951–601 chimera.

Because mutating the last four amino acids at the C-tail of the α4 subunit greatly reduced Br-PBTC potentiation (15), we hypothesized that the extracellular ends of M1–M3 in α4 were responsible for Br-PBTC’s PAM effect. The extracellular ends of M1–M3 in α4 are close to the C-tail in α4. We mutated four α4 amino acids that are conserved in both α3 and α4 but are not conserved between α4 and α7: E282Q, F286S, T140R, and L275D (Fig. 3). None of these mutations greatly influenced the activation of (α4β2)β2 nACHRs by ACh (Table 1). These mutations showed variable effects on Br-PBTC. Mutations T140R and L275D did not alter the potency or efficacy of Br-PBTC on potentiation activity. Mutations E282Q and F286S greatly decreased the magnitude of Br-PBTC potentiation. (α44591–601)β2 showed 9-fold lower maximum potentiation by Br-PBTC compared with WT (α4β2)β2. Br-PBTC had no detectable potentiation on (α4E282Qβ2)β2 nACHRs. Because Br-PBTC is more efficacious when there are more α4 subunits (15), we next investigated the (α4E282Qβ2)α4E282Q mutant to test whether there is any potentiation of the α4 mutant E282Q by Br-PBTC that is undetectable in (α4E282Qβ2)β2. To ensure expression of a defined stoichiometry, we used a β2(QAP)7, α4E282Q concatamer with α4E282Q to express (α4E282Qβ2)β2 nACHRs in Xenopus oocytes. There was no potentiation (<1% increase in responses from 500 μM ACh) by Br-PBTC tested at 0.006–25 μM.

We next made an α71–463α4951–601 chimera with Q282E/S286F double mutations to investigate whether these two critical residues were sufficient to rescue the PAM effect of Br-PBTC. Br-PBTC did not potentiate activation of α71–463α4951–601 with Q282E/S286F double mutations. Responses of 3 μM Br-PBTC co-applied with 500 μM ACh were within 100 ± 10% of responses evoked by ACh alone. Therefore, residues...
Besides Glu-282, Phe-286, and the C-tail in α4 are also important for potentiation. These results suggest that Br-PBTC acts at a cavity in the transmembrane domain of α4 bordering the extracellular domain.

**Consensus docking between SwissDock and Autodock Vina**

We used a consensus docking approach (25, 32, 33) to locate the Br-PBTC–binding site within the α4 subunit. In consensus docking, we searched for the lowest root mean square difference (RMSD) between pairs of docked conformations from two different docking programs: SwissDock and Autodock Vina. Each pair contains one pose from the lowest energy cluster revealed by SwissDock and one pose from the top 20 poses reported by Autodock Vina. Both programs are free and widely used docking programs (34). Autodock Vina uses iterated local search and allows side chains to be flexible to predict binding poses of ligands within a customer-defined region of the protein. SwissDock uses exhaustive ranking and clustering of tentative binding modes in the vicinity of all target cavities. Low-energy docked conformations and binding pockets identified by two docking programs are likely more accurate than the results of a single docking program (25, 32, 33). In addition, we hypothesize that dFBr is likely to bind in the same basic site as Br-PBTC because dFBr is sensitive to a Y283F mutation in α4 that is adjacent to E282Q and near a F286S mutation that inhibits Br-PBTC (28).

We first attempted to dock dFBr and Br-PBTC to the α4 subunit of one crystal and two cryo-EM structures of desensitized (α4β2)2β2 and (α4β3)2α4 (19, 22). We did not find a consensus pose using SwissDock and Autodock Vina within 10 Å of Glu-282 and Phe-286. Estimated binding energy was generally lower, and consensus RMSD was generally higher for those in desensitized α4β2 structures compared with the α4 homology model based on the open-state Torpedo α1 structure (Table 3). Although the RMSD for Br-PBTC poses in the cryo-EM structure of (α4β2)2β2 is only 1.897 Å, Br-PBTC is too far from Glu-282 and Phe-286 in these poses to form any meaningful interactions. The charged nitrogen of Br-PBTC is 18.53 Å away from the side-chain oxygen in Glu-282 in Autodock Vina and 17.44 Å in SwissDock. The benzothiophene ring of Br-PBTC is more than 14.45 Å away from the phenyl ring of Phe-286 in Autodock Vina and 14.98 Å in SwissDock. The transmembrane domains in the desensitized structure were too closely packed near Glu-282 and Phe-286, preventing Br-PBTC from accessing these critical residues. This suggests the desensitized structure likely does not possess a Br-PBTC–binding site (Fig. 4).

![Figure 3. Location of α4 point mutations on the amino acid sequence and an α4 homology model. Mutated residues in the α4 subunit are shown as spheres with appropriate van der Waal radii. The α4 model shown here is derived from a revised Torpedo nAChR structure (25). Pink spheres represent carbon of side chains. The AGMI C-tail sequence is highlighted in red.](image-url)
is also known that Br-PBTC does not reactivate long-term desensitized (α4β2)2β2 (15), which suggests Br-PBTC may not bind to desensitized (α4β2)2β2. In addition, these crystal and cryo-EM structures could represent an unnatural “uncoupled” state due to detergent solubilization used to prepare the purified nAChR (18, 22, 25). Br-PBTC does reactivate desensitized (α4β2)α4, but the effect is transient (15). There are multiple desensitized states (3, 4), and Br-PBTC may not act on the conformation of desensitized (α4β2)α4 resolved by cryo-EM.

We next built an α4 homology model using the corrected cryo-EM structure of T. marmorata α subunit in the open conformation, the only known nAChR structure solved in its native membrane (25). There is a registration mistake in the transmembrane domains in Unwin’s structure, which is revised and remodeled in the corrected α1 structure (25). When we docked Br-PBTC and dFBr to our open-state α4 model, we saw an excellent consensus between SwissDock and Autodock Vina (Table 3). Both SwissDock and Autodock Vina placed dFBr and Br-PBTC derivatives within the same binding pocket that is formed by a triad of transmembrane domains: M1, M3, and M4 (Figs. 5A and 6A).

Both Autodock Vina and SwissDock position Br-PBTC and dFBr close to Glu-282 and Phe-286. Br-PBTC and dFBr bound with their aryl planes (benzothiophene or indole) oriented roughly parallel to the transmembrane helices. The positively charged piperidine moiety of Br-PBTC was closer to the extracellular domain, whereas the aromatic ends of Br-PBTC dock deeper into the transmembrane domain. dFBr locates its secondary amine at a similar level as its aromatic end. The docking site of dFBr is consistent with previous studies, which also observe dFBr docking in between the helix of Glu-282 and Phe-286 (28). The carboxylate group of Glu-282 is 7.20 Å away from Br-PBTC and 3.03 Å away from dFBr in Autodock Vina. In SwissDock, this distance is 4.21 Å for Br-PBTC and 2.83Å for dFBr. These suggest a possible coulombic interaction for binding of Br-PBTC and dFBr. Despite the relatively long distance, this salt bridge is plausible because it is shielded from solvent at the center of the binding pocket (35). The flanking hydrophobic residues from M1 and M4 could block solvent from screening and disrupting the electrostatic interaction between the charged nitrogen and Glu-282. This salt bridge could help explain the total loss of Br-PBTC PAM activity with the mutation E282Q. In the consensus poses for Br-PBTC and dFBr, the ligands’ aromatic ring systems were nearly parallel with the aromatic face of Phe-286 (Figs. 5 and 6). This is consistent with a π-stacking interaction (36) and explains the deleterious effect of F286S on Br-PBTC and dFBr potentiation (28). Consensus poses for both ligands are too far from Leu-275 and Thr-140 to form any significant interaction. This is consistent with mutagenesis data showing no effect of mutations L275D and T140R on Br-PBTC potentiation (Table 1).

Our consensus poses place Br-PBTC and dFBr too far from the AGMI C-tail sequence to form direct contact. Docked Br-PBTC and dFBr instead form direct contacts with M4 right before the C-tail begins. Our published results show that the C-tail sequence is critical for Br-PBTC function (15). Mutating the C-tail α4 from AGMI to AAC reduced potentiation by 80% (15). Instead of forming direct contacts with Br-PBTC, we believe C-tail mutations affect Br-PBTC potentiation by disturbing contacts between the C-tail and Cys-loop of α4, a possibility suggested in another study of dFBr (28).

Docking has limitations in finding precise bound conformations. Docking is heavily dependent on choice of receptor, treatment of ligand and side-chain rotations, and receptor backbone movements. Our homology modeling approach cannot accurately account for subtle differences in peptide backbone and tertiary structure between the template α1 subunit and α4 model. Docking is not a substitute for structural determination experiments, but by relying on consensus between multiple programs, we account for some idiosyncratic inaccuracies of each docking program and provide a reasonable hypothesis for Br-PBTC binding in the α4 subunit consistent with our mutagenesis data on Br-PBTC activity.

Br-PBTC derivatives acting on the α5 subunit

Promoting activation of α5 nAChR subtypes holds promise for the treatment of nicotine dependence (8, 10). Currently, there are no known α5-selective nAChR agonists or PAMs.

**Table 3**

| Ligand                  | RMSD between consensus poses | Estimated binding energy |
|-------------------------|-----------------------------|--------------------------|
|                         | SwissDock                  | Autodock Vina            |
| α4 homology model       |                            |                          |
| from open-state of      |                            |                          |
| Torpedo nAChRs          |                            |                          |
| Br-PBTC                 | 2.276                      | -18.363                  |
| dFBr                    | 2.515                      | -10.715                  |
| Desensitized α4β2 crystal structure (PDB code 5KXI) | | |
| Br-PBTC                 | 5.277                      | -6.343                   |
| dFBr                    | 3.201                      | -6.919                   |
| Desensitized (α4β2)β2 cryo-EM structure (PDB code 6CN) | | |
| Br-PBTC                 | 1.897                      | -1.699                   |
| dFBr                    | > 10.0                     | -0.590                   |
| Desensitized (α4β2)α4 accessory α4 cryo-EM structure (PDB code 6CNK) | | |
| Br-PBTC                 | 6.532                      | -4.570                   |
| FBr                     | 3.112                      | -0.113                   |

**Table 3**

RMSD between consensus poses of SwissDock and Autodock Vina in different α4 structures/models

Listed are the RMSD and calculated binding free energy for consensus poses of Br-PBTC and dFBr from SwissDock cluster and Autodock Vina. The absolute difference in energy between SwissDock and Autodock Vina is high due to different approaches calculating binding energy in each program.
Because Br-PBTC can act from a single α subunit, and each nAChR naturally contains only one α5, Br-PBTC could be a good lead molecule for developing α5-selective PAMs. Therefore, we investigated whether Br-PBTC or its derivatives can act from the α5 subunit.

To avoid effects on α4 subunits, we used the mutant form (α4E282Qβ2)2α5, where both α4 subunits are Br-PBTC–inactive (Fig. 7A). SR14271 and SR14273 showed a PAM effect, whereas Br-PBTC, SR13521, SR14270, and SR19678 did not (Fig. 7D). Structures of these compounds are shown in Fig. 1. All six ligands potentiated activation of α4β2 subtypes (37). The potentiation of activation of α5 by Br-PBTC analogs, SR14271 and SR14273, cannot be explained by PAM activity on α4E282Q or β2 subunit because neither Br-PBTC nor SR14273 can potentiate (α4E282Qβ2)2β2 (Fig. 7B).

Among the Br-PBTC derivatives that potentiated activation of (α4E282Qβ2)2α5 nAChRs, a common feature was 4-trifluoromethylation of the benzothiophene ring. The α5–selective PAMs SR14271 and SR14273 are trifluoromethylated derivatives of two non-α5–selective PAMs, SR14270 and SR19678 (Fig. 1), respectively. This suggests that trifluoromethylation is critical for α5–selective PAM activity.

Based on our α4 model and sequence homology between α4 and α5, we hypothesized that a nonconserved tyrosine, Tyr-430, in the α5 M4/C-tail region (Fig. 3) is important for α5–selectivity for Br-PBTC derivatives. The α5Y430A mutant was potentiated more by SR14270, SR14271, SR19678, and SR14273 than in WT α5, but not by SR13521 (Fig. 7E). Because not all ligands were affected by the Y430A mutant, the mutation’s effects are likely specific for Br-PBTC derivatives potentiating α5 subunits rather than a nonspecific global effect on all PAMs. These data support the existence of a PAM site for Br-PBTC derivatives in the α5 subunit.

The position of Tyr-430 in the C-tail of α5 suggests that it is too far from Glu-282 and Phe-286 to directly contact the PAM. Because Tyr-430 is at the base of the α5 C-tail, Tyr-430 could be involved in transmitting the PAM effect to the rest of the subunit. This is similar to the role of the α4 C-tail that did not contact Br-PBTC in docking experiments. Despite the long distances from Br-PBTC in α4, mutations at Ile-601 and the AGMI C-tail sequence still affected Br-PBTC’s PAM function. Tyr-430 in α5 may function similarly, ensuring the PAM effect is only transmitted for certain α5–selective PAMs. Y430A, like other M4/C-tail mutations, probably loosens selectivity between α5 and α4 subunits by subtly modifying the structural linkage that communicates PAM binding to the ion channel gating.
It is known that accessory α subunits in heteromeric nAChRs confer unique pharmacological properties to the pentamer (29). In α4β2 nAChRs, there are two primary α4 subunits, each of which forms a high-affinity agonist-binding site with an adjacent β2 subunit. The fifth accessory subunit position forms a low-affinity agonist-binding site when it is occupied by an α4 or
Figure 7. Br-PBTC derivatives can potentiate through α5 accessory subunits. A, (α4β2)2, (α4E282Qβ2)2, (α4β2)2α5, (α4E282Qβ2)2α5, and (α4E282Qβ2)2α5Y430A were expressed in Xenopus oocytes. In (α4E282Qβ2)2α5, any observed potentiation can only come from PAM activity on α5 subunits because PAM sites on all α4 subunits are disabled. Varying concentrations of Br-PBTC and SR14273 were co-applied with a saturating 500 μM ACh. To test for potentiation, 1.56, 6.25, and 25 μM of SR13521, SR14270, SR14271, SR14273, and SR19678 were co-applied with 500 μM ACh, a saturating agonist concentration. All five compounds are known to potentiate (α4β2)2 and (α4β2)2α5 subtypes. Only SR14271 demonstrated agonist activity against (α4β2)2α5 at 25 μM. Potentiation was observed in (α4β2)2α5 at lower concentrations of SR14271. Results are presented as mean ± S.E. Sample size: B, n = 3; C, n = 3–4; D, n = 5; E, n = 5.

Figure 8. Br-PBTC acts with higher potency on (α4β2)2α4 but not (α4β2)2β2 nAChRs as agonist concentration increases. Thus, the accessory ACh-binding site is critical for agonist-induced increase in sensitivity to potentiation by Br-PBTC. HEK cells expressing (α4β2)2 or (α4β2)2β2 were co-applied with varying concentrations of Br-PBTC in the presence of nicotine, ACh, or A85380. Responses were measured as fluorescence from a membrane potential-sensitive dye using a FlexStation benchtop fluorimeter. Results are presented as mean ± S.E. Sample size is n = 4.
Figure 9. Br-PBTC acts more than 10-fold more potently against (α4β2)4 and (α4β2)4E282Q when co-applied with ACh rather than sazetidine-A (Saz-A). Activation by ACh increased sensitivity to potentiation by Br-PBTC, but sazetidine did not. ACh binds to primary and accessory ACh sites, but sazetidine binds only to primary ACh sites. Thus, activation of the accessory site is critical for agonist-induced sensitivity to Br-PBTC. Activation of the accessory ACh site increases sensitivity to potentiation by Br-PBTC even when the PAM-binding site on the accessory α4 subunit is blocked by the E282Q mutation. Xenopus oocytes were injected with 1:2 mass ratio of ACh to Br-PBTC. Br-PBTC was co-applied with either saturating ACh (500 μM) or saturating sazetidine-A (0.01 μM). The percent increase in peak current was used to assess Br-PBTC potentiation. Pentamer diagrams are presented on the right of the Br-PBTC concentration response curve for each nAChR construct. ACh-, sazetidine-A-, and Br-PBTC–binding sites are noted in the graph. Results are presented as mean ± S.E. Sample size is n = 4.

no agonist site when it is occupied by a β2 subunit (19, 29–31). Previously, we found that Br-PBTC potentiates activation of both stoichiometries of α4β2. Now we find that activation of the (α4β2)4 stoichiometry selectively increases the potency of Br-PBTC.

Using nAChR-expressing human embryonic kidney 293 tsA 201 (HEK) cell lines, we evaluated the EC50 of Br-PBTC on (α4β2)2α4 and (α4β2)2β2 nAChRs when co-applied with three agonists: ACh, nicotine, and A85380 (Fig. 8). The agonist concentrations tested span the EC10–100 for each agonist against (α4β2)2α4 to achieve various occupancies of primary and accessory agonist sites. In (α4β2)2α4, Br-PBTC potency significantly increased as agonist concentration escalated. The EC50 of Br-PBTC is 9-fold lower from 0.348 to 0.037 μM as nicotine concentration increased from 0.2 to 100 μM. EC50 of Br-PBTC is 3.5-fold lower from 0.147 to 0.039 μM as ACh concentration increased from 1 to 300 μM. EC50 of Br-PBTC declined by 4-fold from 0.207 to 0.049 μM as A85380 concentration increased from 100 μM to 1 μM (Fig. 8). The EC50 of Br-PBTC in (α4β2)2β2 did not change with increasing agonist concentrations. This suggests that occupying the accessory ACh-binding site increases sensitivity of (α4β2)2β2 to Br-PBTC.

To confirm involvement of the accessory ACh-binding site in increasing Br-PBTC’s potency, we characterized potency of Br-PBTC in the presence of EC50 ACh (500 μM) or sazetidine-A (10 nM) using (α4β2)2α4 nAChRs expressed in oocytes. Sazetidine-A only binds primary agonist-binding sites formed by α4β2 pairs, whereas ACh acts from both primary and accessory agonist sites (14). We found that the Br-PBTC EC50 was 9-fold lower with ACh than with sazetidine-A (Fig. 9A). Even when the accessory PAM site was abolished by mutant E282Q, the EC50 of Br-PBTC was still 17-fold lower in (α4β2)2α4E282Q with ACh compared with sazetidine-A (Fig. 9B). This suggests that agonist binding to the accessory ACh-binding site causes the entire pentamer to enter a higher-affinity state for Br-PBTC. Single-channel studies support the existence of intermediate states between resting and open-state (α4β2)2α4 attributed to agonist binding at the accessory site (38, 39). These intermediate states could explain how agonist binding at the accessory site leads to a high-affinity conformation of (α4β2)2α4 for Br-PBTC.

Another important effect of the accessory α4 is that it allows desensitized (α4β2)2α4 to be efficiently reactivated by Br-PBTC (15). The presence of an accessory α4 is critical for reactivation because very small reactivation by Br-PBTC was observed in (α4β2)2β2, which lacks an accessory α4 (15). We selectively deleted Br-PBTC sites on several α4 subunits in (α4β2)2α4 by inserting the E282Q mutation. This mutation did not greatly change the potency of agonist to activate nAChRs.
The EC50 values of nicotine for WT and (α4β2)4α4 mutants are as follows in μM (95% CI in parentheses): WT, 17 (12–25); (α4E282Qβ2)2α4, 12 (9.3–14); (α4β2)2α4E282Q, 13 (10–18); (α4E282Qβ2)2α4E282Q, 8.2 (5.9–11); and (α4E282Qβ2)(α4β2)α4, 10 (6.9–14). However, Br-PBTC reactivation efficiency decreased 90% from that in WT (α4β2)2α4 when either all primary α4 subunits or only the accessory α4 carried an E282Q mutation. Reactivation was unaffected if only one PAM site in primary α4 was removed, leaving PAM sites in one other primary α4 and the accessory α4 intact (Fig. 10). This shows that the primary and accessory α4 PAM sites exhibit synergistic binding to reopen desensitized (α4β2)2α4. What matters is not merely the number of PAM sites but also the intrinsic pharmacological characteristics of each PAM site with regard to Br-PBTC.

Discussion

Using mutagenesis and consensus docking, we located Br-PBTC’s binding site to a pocket between M1, M3, and M4 transmembrane domains in α4. We found that Glu-282, Phe-286, and Ile-601 were important for Br-PBTC potentiation in α4, whereas mutations in Thr-140 and Leu-275 did not affect Br-PBTC efficacy or EC50. The docked conformations of Br-PBTC, two Br-PBTC derivatives, and dFBr exhibited excellent overlap in both Autodock Vina and SwissDock. Br-PBTC could reactivate desensitized (α4β2)2α4 (15) and was found to have improved potency in (α4β2)2α4 with increased agonist occupancy of the accessory α4 agonist site. However, these features were not observed in (α4β2)2β2 that lacks an accessory α4 subunit. Probing Br-PBTC function in the accessory α4 by using Br-PBTC disabling α4 mutations, we found that the accessory α4 was functionally distinct from primary α4 subunits with regard to the reactivation of desensitized (α4β2)2α4.

The Br-PBTC–binding site we located overlapped well with that of other known nAChR PAMs that have been docked within the transmembrane domains (31, 40). Some PAMs bind at similar intrasubunit cavities as Br-PBTC and dFBr (40), and some bind at intersubunit cavities in the transmembrane domains (25, 40, 41). A common transmembrane–binding region suggests that perturbation of the transmembrane domains is a critical structural feature needed to potentiate nAChR opening. It is known that nAChR function is very sensitive to conformational changes in the transmembrane domain M4, which directly connects to the C-tail (42, 43). Subtle alterations in the tertiary structure of M4 could easily alter the conformation of the C-tail, thereby affecting nAChR function. It was shown that a single C418W mutation in M4 of α1 increased sensitivity to ACh by 16-fold (42). M4 is also believed to act as a “lipid sensor” in α1 (43). LY2087101, an α4- and α7-selective PAM, is also sensitive to mutations at Glu-282 and Phe-286 in α4 like Br-PBTC (40). dFBr, an α4-selective PAM, is affected by mutations at Phe-286 and Ile-601 (28). 17β-Estradiol, an α4*-selective PAM, has also been shown to depend on the α4 C-tail for its function (28). Swapping the C-tail of β2 with that of α4 allowed 17β-estradiol to potentiate the chimeric β2 (44). This demonstrates the importance of conformational changes in α4 transmembrane domains for potentiating nAChR responses.

These mutagenesis results also led us to identify and confirm novel PAMs that act on the α5 subunit to potentiate activation of nAChRs. Probing for α5-selective compounds is technically challenging because the α5 subunit can only function in the presence of both α4 and β2 subunits. Unfortunately, α4 and β2 also self-assemble into functional nAChRs devoid of α5. (α4β2)2α5 nAChRs expressed from free subunits may form a mixed population of (α4β2)2α4, (α4β2)β2, and (α4β2)2α5. This led us to mis-categorize NS9283 as an α5 PAM (24) initially, which was later disproved by a detailed concatamer study (45). Using the Br-PBTC–inactive α4 mutant E282Q, we identified two new α5-selective PAMs, SR14271 and SR14273.
Although there are α5-selective antagonists (46), SR14271 and SR14273 are the first PAMs that promote activation of α5-containing nAChRs. They need to be further optimized for α5-selectivity by eliminating their potentiation on α4-containing nAChRs.

We propose that Br-PBTC and similar nAChR PAMs that bind in the transmembrane domains potentiate nAChR opening by slowing down the closing of a desensitization gate in M2 and reactivate desensitized nAChRs by reopening this desensitization gate. In a published structure of desensitized (α4β2)_2β2 (22), the five-leucine gate associated with activation near the middle of M2 creates an opening wide enough for a hydrated sodium cation to fit through, but another ring of five glutamates near the cytoplasmic end of M2 forms a ring too small to pass a hydrated cation. The presence of a desensitization gate was also observed in nAChRs and other Cys-loop receptors such as glycine and GABA_A receptors (47, 48). In (α4β2)_2β2, the activation gate is shut in the resting state, and both the activation and desensitization gates are open in the conductive state; but in the desensitized state, the desensitization gate remains shut while the activation gate is held open. Because Br-PBTC can reactivate desensitized (α4β2)_2α4, it must be opening the desensitization gate while keeping the activation gate open. Reactivation of long-term desensitized (α4β2)_2α4 by Br-PBTC is transient (15). After reactivation, nAChRs again assume a desensitized state. The desensitization gate theory is consistent with the crystal structures of various Cys-loop receptors solved in open and desensitized states (47, 48).

Although the (α4β2)_2β2 and (α4β2)_2α4 structures provided valuable structural information about ion channel gating (19, 22), they were not optimal for docking our ligands. Br-PBTC does not reactivate long-term desensitized (α4β2)_2β2, nor does it have a long-lasting effect on desensitized (α4β2)_2α4 (15), and the structures are assumed to be in a desensitized state (19, 22). Therefore, we are not surprised that Br-PBTC did not dock well to these structures. Newcombe et al. (25) found that an α7 model derived from the same crystal structure of (α4β2)_2β2 bound to nicotine did not allow them to produce consensus docking results for their α7 PAMs. Similarly, we found that Br-PBTC could not dock to α4 subunits from the (α4β2)_2β2 structure. They attributed this docking failure to conformational disruptions in (α4β2)_2β2 crystals resulting from detergent solubilization. Detergent solubilization of muscle-type nAChRs is believed to produce an “uncoupled” state (18). The extracellular domain and ion channel of the (α4β2)_2β2 and (α4β2)_2α4 structures may remain intact in detergent due to their water solubility, but the transmembrane domains are in intimate contact with lipids or detergent. This makes transmembrane domains especially sensitive to the chemical identity of membrane constituents. This suggests the (α4β2)_2β2 and (α4β2)_2α4 structure transmembrane domains may carry significant structural distortions. The transmembrane domains of an α4 homology model derived from a native-membrane muscle-type nAChR structure carry a sizable binding pocket, whereas the desensitized (α4β2)_2β2 M1, M2, and M3 transmembrane domains are tightly compacted with the M4 domain extending away the nAChR body. The significant structural differences between the structures solved in native membrane and detergent suggest that detergent solubilization has significant effects on the transmembrane domain structure.

We used a single α4 subunit for docking because the position of Glu-282 in our α4 model and its homolog in the corrected Torpedo structure point toward an intrasubunit cavity and could not have reached into the intersubunit junction between α4 and β2 subunits. Br-PBTC’s strong dependence on Glu-282 bounded our search area so that Br-PBTC could form a direct contact with Glu-282.

Based on our experiments with reactivation from desensitized states and lowering Br-PBTC’s EC50 in (α4β2)_2α4, we hypothesize that Br-PBTC potentiates nAChR opening by preferentially stabilizing the open state and slowing the transition to the desensitized state, similar to previously proposed models of PAM activity in nAChRs (49). nAChRs bound with Br-PBTC eventually desensitize. This suggests that the open state is not made more stable than the desensitized state by PAM binding, but the equilibrium between open and desensitized states is shifted by Br-PBTC temporarily (5). For reactivation from desensitized states, Br-PBTC clearly induces a transient transition to the open state. The ensuing desensitization is most likely a transition to a new Br-PBTC–bound desensitized state. This suggests Br-PBTC binding to α4β2 opens a new thermodynamic path to more stable Br-PBTC–bound open and desensitized states, with the Br-PBTC-bound desensitized state being the most stable.

In summary, we combined functional studies and mutagenesis with computational techniques to find a novel PAM-binding site in the transmembrane domains. A common dependence on Glu-282, Phe-286, and Ile-601 for Br-PBTC, dFBr, and LY2087101 potentiation combined with good overlap in docked Br-PBTC derivatives and dFBr suggest the binding site is likely to be accurate. Further refinement of our knowledge of the Br-PBTC–binding site will be important for designing new subtype-selective nAChR PAMs. Such PAMs may be useful for treating several neurological diseases.

Experimental procedures

Reagents

Synthesis of Br-PBTC and its derivatives were described previously (15, 37). A85380 was purchased from Tocris Bioscience. Other reagents were purchased from Sigma or Thermo Fisher Scientific unless stated otherwise.

cDNA and RNA

Human α4, α5, and β2 subunit cDNAs were cloned in this laboratory (29, 49–51). nAChR subunit concatamers were generated by connecting the C-tail of one subunit with the N terminus of the second subunit using a (QAP)_n linker (15, 29). The β2(QAP)₅α4 dimer is abbreviated as β2-α4. The β2(QAP)₅α4(QAP)₅β2 trimer is abbreviated as β2-α4-β2. The α4(QAP)₅α4 dimer is abbreviated as α4-α4. After linearization and purification of cDNAs, mRNA was produced for WT and mutant α4, α5, β2-α4, β2-α4-β2, and α4-α4 subunits using the SP6 mMessage mMACHINE kits (Ambion, Austin, TX).
Mutagenesis
All mutations were made using the QuikChange™ hot-start PCR technique (52) with PfuUltra high-fidelity polymerase. An E282Q mutation was introduced into the α4 subunits of the β2-α4 dimer and the β2-α4-β2 trimer with the same oligonucleotide used to generate E282Q in a single α4 subunit. All mutations were confirmed by sequencing.

Xenopus oocyte extraction
Oocytes were removed surgically from Xenopus laevis and defolliculated using published procedures (29).

RNA injection
Extracted and defolliculated oocytes were injected with mRNAs less than 36 h after surgery. To express (α4β2)β2, a 20-ng mixture of WT α4 and β2 mRNA was injected at a 1:5 α4 and β2 mass ratio to force expression of the (α4β2)β2 stoichiometry. WT or α4 mutant (α4β2)α5 was expressed by injecting a 30-ng mixture of α4, β2, and α5 subunit mRNA in a 1:2:5 ratio. (α4β2)α4 was expressed by injecting a 30-ng mixture of β2-α4 and α4 mRNA at a 1:2 ratio. The (β2α4E282Q)β2(α4α4) mutant nAChr was created by injecting 30 ng of a 1:1 ratio of β2-α4E282Q-β2 and α4-α4 mRNA. Function was tested 4–5 days after injection.

Two-electrode voltage clamp on Xenopus oocytes
Currents were measured using the OpusXpress 6000A (Molecular Devices, Union City, CA), an automated two-electrode voltage-clamp amplifier that can record up to eight oocytes simultaneously (29). All oocytes were clamped to a holding potential of −50 mV. Fresh dilutions of drugs were made daily in recording buffer (ND-96: 96 mM NaCl, 2 mM KCl, 1.8 mM CaCl₂, 1.0 mM MgCl₂, 5 mM HEPES, pH 7.6) with 0.5 μM atropine. Agonists and PAMs were applied over 4 s at 3 ml/min to oocytes via the sidewall of each recording chamber. Between drug applications, oocytes received 253 s of buffer washing at a rate of 3 ml/min.

To compensate for variable nAChr expression in oocytes, peak current amplitudes of experimental responses were normalized to peak current amplitudes from ACh or nicotine. The extent of potentiation or Br-PBTC enhancement of nAChr opening was defined as the percent increase in peak current from co-application of Br-PBTC and agonist compared with application of the same concentration of agonist alone as shown in Equation 1.

\[
\% \text{potentiation} = \left( \frac{\text{peak current with Br-PBTC}}{\text{peak current agonist alone}} - 1 \right) \times 100 \% \quad \text{(Eq. 1)}
\]

Dilutions of Br-PBTC and its derivatives in ND-96 were freshly prepared daily from 10 mM stock in DMSO. Mean and standard error were calculated for all concentrations of agonist and Br-PBTC. At least three oocytes were used per experimental group. When testing for Br-PBTC enhancement of nAChr opening, Br-PBTC was co-applied with an agonist, ACh, or nicotine. When testing for Br-PBTC’s efficiency in reactivating desensitized nAChr, oocytes were perfused with 0.2 or 100 μM nicotine in ND-96 with 0.5 μM atropine at a rate of 2.0 ml/min. Concentration–response curves were fitted to the Hill equation to determine the EC₅₀ for any compound tested (15).

FLEXstation assay
Functional testing of nAChr was carried out in HEK cells permanently transfected with nAChr cDNA using a FlexStation (Molecular Devices, Sunnyvale, CA) bench-top plate reader as described (53). All cells were maintained as described previously (51). Creation of HEK cell lines expressing defined stoichiometries of α4β2 were described previously (15).

To enhance HEK cell expression of (α4β2)α4 or (α4β2)β2 nAChr, 96-well plates of cells were incubated at 29 °C for 16–24 h prior to testing to increase nAChr expression and thus enhance the fluorescent signal. Blue membrane potential sensitive fluorescent dye from Molecular Devices was used to measure membrane potential changes upon addition of agonists according to the manufacturer’s instructions.

Homology modeling
The α4 subunit homology model was made from a corrected α₂ structure of Torpedo nAChr at open state (27) using SWISS-MODEL (54–56). The sequence alignment for homology modeling was obtained from Clustal Omega (57). α₄ refers to the α subunit that forms an ACh-binding site in conjunction with the γ subunit in Torpedo nAChr.

Docking
The α4 subunit in the desensitized nAChr crystal structure (PDB code 5KXI) and cryo-EM structures (PDB codes 6CNJ and 6CNK) were used directly in docking (22). Before docking, atomic coordinates of ligands were prepared using the “build structure” function in UCSF Chimera (58) and subjected to 5,000 steepest descent and 5,000 conjugated gradient minimization steps with a steepest descent and conjugate gradient step size of 0.02 Å.

In SwissDock, the α4 homology model and a minimized mol2 file of the desired ligand were directly input into the SwissDock server (59). Over a hundred docked ligand poses were sorted and clustered by location and conformational similarity. Each cluster was ranked by its average energy.

We used flexible docking function in Autodock Vina (version 1.1.2) (16) via a graphic interface MGLTools (Scripps Molecular Graphics Laboratory, San Francisco) (60). A 21 × 24 × 21 Å box encompassing the C-tail, Glu-282, Phe-286, and parts of each α4 transmembrane domain was set as the search space. Ten amino acid side chains in the search space were allowed to rotate freely: Tyr-220, Leu-224, Ile-266, Leu-275, Leu-279, Glu-282, Tyr-283, Phe-286, Leu-593, and Leu-597. Rotatable residues were distributed throughout the search space rather than concentrated in one region of the search space. This protects from biased docking toward the region containing the most rotatable side chains. Top 20 lowest energy poses were outputted for each ligand. To find a pair of consensus poses for a given ligand, we searched for the pose from Autodock Vina that had the lowest RMSD from the lowest energy SwissDock cluster.
Binding site for the PAM Br-PBTC

Author contributions—J. N., J. W., A. K., A. L., T. M. K., and J. L. conceptualization; J. N. and J. W. data curation; J. N. and J. W. formal analysis; J. N., A. K., A. L., and J. L. investigation; J. N., J. W., A. K. A. L., and J. L. methodology; J. N., J. W., and J. L. writing—original draft; J. N., J. W., A. K., A. L., T. M. K., and J. L. writing—review and editing; J. W., A. K., A. L., and J. L supervision; A. K., C. D., and T. M. K. resources; T. M. K. and J. L. funding acquisition.

Acknowledgments—We thank Dr. Esmail Haddadian and Dr. Pei Tang for advice on modeling.

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