Neurosteroids are endogenous brain sterols, and allosteric modulators of pentameric ligand gated ion channels (pLGICs), including GABA<sub>A</sub> receptors (GABA<sub>A</sub>R) (1), nicotinic acetylcholine receptors (2,3), and glycine receptors (4,5). Modulation of these channels is thought to underlie the effect of neurosteroids on neuronal excitability (6,7), and their pharmacologic potential as anesthetics (8) and treatments for epilepsy (9) and psychiatric disorders (10,11). Neurosteroids have variable effects on different pLGICs. 3α-Hydroxypregnane neurosteroids potentiate and directly activate the GABA<sub>A</sub>R (12), while 3β-hydroxysteroids and 3-sulfated neurosteroids inhibit the GABA<sub>A</sub>R (13,14). Certain synthetic neurosteroid analogues both potentiate and inhibit the GABA<sub>A</sub>R (15). In addition, neurosteroids can modulate nicotinic acetylcholine receptors (16) and glycine receptors, which may contribute to their potential for the treatment of chronic pain (17). Understanding the structural basis of neurosteroid actions in pLGICs is essential for exploiting their pharmacologic benefits.

Potentiation of the GABA<sub>A</sub>R by neurosteroids is thought to occur by binding to the transmembrane domains (TMDs) (18-20). A photo-affinity labeling study identified F301 in the TM3 membrane-spanning domain of the β3 homopentameric GABA<sub>A</sub>R as a potential neurosteroid binding site (21). Recently, crystal
structures of a GLIC-GABA\textsubscript{R}\textalpha{1} chimera and GABA\textsubscript{R}\textbeta{3}-\textalpha{5} chimera identified an equivalent site between TM3 and TM1 of adjacent subunits as a neurosteroid potentiating site (22,23). However, multiple lines of evidence suggest that potentiation of the GABA\textsubscript{R} by 3\textalpha{-hydroxy}pregnane neurosteroids is mediated by more than one site. These include the complex effects of neurosteroids on GABA\textsubscript{R} gating (24), mutations that alter neurosteroid action (25), and radioligand binding (26). Notably, point mutations in the GABA\textsubscript{R} that reduce potentiation or direct activation by 3\textalpha{-hydroxy}pregnane neurosteroids localize to disparate regions that have been modeled as two binding sites (25). However, a mutagenesis strategy cannot differentiate between direct effects of mutations on ligand binding and indirect effects on binding or transduction of the ligand signal.

Photo-affinity labeling (PAL) is a more direct approach for identifying ligand binding sites, where ligands modified to be photo-reactive covalently label binding sites. Labeled sites are then identified using mass spectrometry (MS). While PAL has been an effective approach to identify binding sites of various ligands including anesthetics (27,28), identification of neurosteroid labeled sites has been impeded by the difficulty of recovering and analyzing steroid-modified TMD peptides by MS. These difficulties result from the hydrophobicity of these photolabeled peptides and their tendency to undergo neutral loss by tandem MS (29,30). To date, only one neurosteroid photolabeled residue in a pLGIC has been reported (21) despite the availability of multiple neurosteroid photolabeling reagents (21,31-33).

Here, we employ an innovative approach that combines a series of 3\textalpha{-hydroxy}pregnane neurosteroid photolabeling reagents with top-down MS to determine the stoichiometry, sites and orientation of neurosteroid binding in a pLGIC. We previously demonstrated the utility of top-down MS to determine the stoichiometry of steroid photolabeling in a membrane protein (29,30). We now introduce a sensitive and effective middle-down MS strategy, which involves analysis of large TMD peptides for the identification of neurosteroid photolabeled sites.

In this study, we analyze neurosteroid photolabeling of the bacterial \textit{Gloeobacter} ligand gated ion channel (GLIC). GLIC is an important structural model of pLGICs (34,35), and has provided valuable insights on the interaction of allosteric modulators with pLGICs using X-ray crystallography (36-38), molecular modeling (39-42), and PAL (43). GLIC was used in this initial study of pLGICs because: 1) it can be readily expressed and purified in milligram quantities, and 2) the absence of glycosylation modifications makes it readily applicable to top-down MS. We demonstrate that GLIC is efficiently labeled by neurosteroid photolabeling reagents with a stoichiometry of two per subunit, and delineate distinct intersubunit and intrasubunit binding pockets. By using complementary photo-reactive moieties positioned throughout the neurosteroid backbone, we map the preferred orientation of neurosteroids within these sites. Mutational analysis demonstrates that both photolabeled sites mediate neurosteroid inhibition of GLIC channel activity. The findings of this study provide a structural model for multi-site neurosteroid modulation of the prototypic pLGIC, GLIC, which may be applicable to mammalian pLGICs.

**RESULTS**

Neurosteroids photolabel the TMD of GLIC with a stoichiometry of two. To determine the stoichiometry of neurosteroid binding in a pLGIC, we photolabeled purified, n-dodecyl-\textbeta{-D}-maltoside (DDM)-solubilized GLIC with (3\textalpha,5\textalpha)-6-azi-pregnanolone (5\textalpha,6-AziP) (Fig. 1A), a photo-reactive analogue of the endogenous neurosteroid allopregnanolone, and analyzed the labeled protein using top-down MS. MS analysis of intact photolabeled GLIC demonstrates efficient labeling by 5\textalpha,6-AziP at 100 \mu M (Fig. 1C; Table S1), and at 100 \mu M labeled three times, with a labeling stoichiometry of two (i.e. two neurosteroids per GLIC subunit) (Fig. 1B and 1D). Top-down fragmentation by higher-energy collisional dissociation (HCD) of the singly labeled GLIC species yields a series of 5\textalpha,6-AziP-containing y-ions that localize at least one of the labeled sites to the C-terminal end of TM3 or TM4 (Fig. 1E). While analysis of intact GLIC clearly demonstrates the stoichiometry of labeling, top-down fragmentation yields poor sequence
coverage of the GLIC protein, and thus inadequate localization of the labeled sites.

To further localize 5α-6-AziP labeling, we applied a middle-down MS strategy by digesting GLIC with specific endoproteinases. Digestion of GLIC by AspN, an endoproteinase that cleaves at the N-terminal end of aspartate residues (44), predominantly cleaves at an aspartate between the extracellular domain (ECD) and TMD leaving each domain intact (Table S1). MS analysis of the ECD and TMD of GLIC labeled with 5α-6-AziP reveals no detectable labeling in the ECD, and efficient labeling of the TMD with a stoichiometry of two (Fig. 2A and 2B). HCD fragmentation of the singly labeled TMD species yields extensive sequence coverage with 5α-6-AziP-containing b- and y-ions that identify two photolabeled sites located in the N-terminal end of TM1 and the C-terminal end of TM3, respectively (Fig. 2C). Thus, systematic top-down and middle-down MS analyses of GLIC reveal a labeling stoichiometry of two within the TMD.

Identification of two neurosteroid photolabeled sites in the GLIC TMD. To identify the residues labeled by 5α-6-AziP in the GLIC TMD, we applied a middle-down MS analysis using trypsin. Trypsin digestion of DDM-solubilized GLIC yields three large peptides composed of transmembrane helix 1 and 2 (TM1+2), TM3, and TM4. We find that keeping these TMD peptides in DDM for LC-MS analysis is an effective method for maintaining the solubility and stability of neurosteroid-labeled peptides. In addition, due to the large size of these peptides, neurosteroid-labeled peptides show minimal neutral loss by collision-induced dissociation (CID) or HCD fragmentation, facilitating localization of the labeled residues. Tryptic digests of GLIC photolabeled with 100 μM 5α-6-AziP show labeling of all three TMD peptides with efficiencies of 5% for TM1+2, 32% for TM3, and 1.0% for TM4 (Fig. 3A). As expected, the more hydrophobic 5α-6-AziP-labeled peptides are shifted to higher retention times with reverse phase LC-MS.

CID or HCD fragmentation spectra for these three 5α-6-AziP-labeled TMD peptides show that 5α-6-AziP labels E272 in TM3 (Fig. 3B); labeling was localized to one of three adjacent residues (Q193-F195) in TM1 (Fig. 3C), and one of three adjacent residues (F315-F317) at the C-terminus of TM4 (Fig. 3D). Examination of these labeled residues in the GLIC crystal structure (45) reveals two distinct foci of labeling: one formed by the C-terminal end of TM3 (E272) and another between the N-terminal end of TM1 (Y194) and the C-terminal end of TM4 (F315; G316 and F317 are not resolved in the GLIC crystal structure) (Fig. 3E). E272 in TM3 is near an intersubunit neurosteroid binding site previously defined by labeling in the β3 homopentameric GABAR (21) and GABAR chimera crystal structures with bound 3α,5α-tetrahydro-deoxycorticosterone (THDOC) and pregnanolone neurosteroids (22,23). Since 5α-6-AziP is an aliphatic diazirine that may preferentially label nucleophilic side chains such as glutamate (28,29), we tested labeling of the E272A GLIC mutant. The DDM-solubilized E272A mutant was purified as a pentamer, and is also labeled by 5α-6-AziP with a stoichiometry of two (Fig. 4A; Table S1). As in WT, E272A is labeled by 5α-6-AziP at the C-terminal end of TM1 and TM4 (Fig. 4C and 4D), but, in contrast to WT, E272A is labeled at Y278 in TM3 (Fig. 4B), which supports neurosteroid binding to an intersubunit site between TM3 and TM1 of adjacent subunits (Fig. 3E). Although the exact labeled residue was not identified in TM1, we favor labeling of Y194 since the side chain points towards an intrasubunit site encompassed by the C-terminus of TM4, and Y194 is the only nucleophile in the Q193-Y194-F195 segment where 5α-6-AziP labels. Docking simulations were performed for allopregnanolone in the TMD region of a GLIC pentamer, revealing a dominant cluster of poses located in an intersubunit site adjacent to E272 and Y278 (Fig. 3E). To search for additional sites, a second allopregnanolone molecule was docked to the GLIC TMD with one allopregnanolone molecule placed within the intersubunit site. This yielded additional binding clusters including one located in an intrasubunit site encompassed by Y194 and F315 (Fig. 3E). Thus, our photolabeling results reveal two putative neurosteroid binding sites: an intersubunit site between TM3 and TM1 of adjacent subunits, and an intrasubunit site between TM1 and TM4.
Mapping the orientation of neurosteroid binding within both sites. The structure of neurosteroids, particularly at the 3 and 5 positions of the steroid backbone, are critical determinants of modulation of pLGICs (4,46,47), suggesting that neurosteroids bind to sites in these channels with a particular orientation. To map the orientation of neurosteroid binding in GLIC, we synthesized 5α-12-AziP and 5α-15-AziP where the photo-reactive diazirine is placed in the 12 and 15 positions of the steroid backbone, respectively (Fig. 5A). Analysis of GLIC labeled with 100 μM 5α-6-AziP, 5α-12-AziP, or 5α-15-AziP shows significantly higher photolabeling efficiency for 5α-6-AziP compared to 5α-12-AziP or 5α-15-AziP for TM3 and TM1+2 (Fig. 5B). In contrast, non-specific labeling of the peptide YGGFLRF by these reagents shows similar labeling efficiencies, indicating that they are equally photo-reactive (Fig. 5B). 5α-12-AziP and 5α-15-AziP both label TM3 at E272 (Fig. 5C and 5D), and no detectable labeling of TM1+2 was observed for these reagents. To explore potential binding poses, allopregnanolone was docked to the intersubunit and intrasubunit sites, which revealed poses where the neurosteroid has opposite orientations with respect to the 3 and 17 positions (Fig. 6). For the intersubunit site, approximately equal numbers of poses were obtained with allopregnanolone in either orientation (Fig. 6A), whereas the intrasubunit site showed a predominant docking orientation with the 3-hydroxy pointing extracellular (Fig. 6B). The higher labeling efficiency for 5α-6-AziP compared to 5α-12-AziP or 5α-15-AziP indicates that the 6-position of the neurosteroid is most proximal to E272 in TM3 and Y194 in TM1. Thus, the photolabeling data is consistent with a neurosteroid binding orientation where the 3-hydroxy is pointing intracellular for the intersubunit site and extracellular for the intrasubunit site (Fig. 7A and 7B). Although the equal number of allopregnanolone docking poses obtained for each orientation at the intersubunit site does not suggest a preferred orientation, we believe that photolabeling data provides better discrimination of orientation than docking to a static crystal structure. Careful inspection of allopregnanolone docking poses in both sites shows the nature of the interactions for these preferred binding modes (Fig. 6). For the intersubunit site, allopregnanolone forms closer van der Waals interactions with W317 when the 3-hydroxy points intracellular; the 3-hydroxy may also form a hydrogen bond interaction with the Y278 aromatic ring. For the intrasubunit site, the preferred orientation results in hydrogen bonds between the 3-hydroxy of allopregnanolone and backbone carbonyl of F121, and between the 20-carbonyl of allopregnanolone and Y254 hydroxyl. In contrast, one weak hydrogen bond is present between the 20-carbonyl of allopregnanolone and Y194 hydroxyl when allopregnanolone is in the opposite orientation. 5α-6-AziP, 5α-12-AziP, and 5α-15-AziP were also docked to GLIC at both sites. In each case, among the most populated binding clusters are poses with the 6-diazirine closest to E272 for the intersubunit site and Y194 for the intrasubunit site (Fig. 7). Given the inherent preference of 5α-6-AziP, 5α-12-AziP, and 5α-15-AziP for nucleophilic residues, we synthesized two additional neurosteroid photolabeling reagents, KK200 and CW12 (48) (Fig. 8A and 8B, Supporting Material), which contain a trifluoromethylphenyl-diazirine (TPD) group in the 17 and 11 position, respectively, and are expected to not show the same preference for nucleophiles as aliphatic diazirines (28,49). Similar to 5α-6-AziP, 100 μM KK200 efficiently labels GLIC (22% efficiency) and shows a labeling stoichiometry of two (Fig. 8A). In contrast, CW12 labels GLIC with low efficiency (<1%) (Fig. 8B). Tryptic analysis of GLIC labeled with KK200 shows labeling of TM3 and TM4 peptides (efficiencies of 7% and 29%, respectively), and fragmentation spectra localize KK200 labeling to F267 in TM3 (Fig. 8C) and N307 in TM4 (Fig. 8D). F267 is located near the intersubunit binding site and labeling of this residue by KK200 is consistent with the orientation proposed above for allopregnanolone, where the neurosteroid lies in the interface between TM3 of one subunit and TM1 of the adjacent subunit with the 17-position pointing toward the extracellular surface (Fig. 6A). N307 is located near the intrasubunit site and labeling of this residue by KK200 is consistent with an orientation where the 17-position points toward the intracellular surface (Fig. 6B). No KK200 photolabeling was identified in the ECD using a
Neurosteroid Binding Sites in GLIC

PEAKS search (97% sequence coverage), confirming that the TPD group does not point extracellular and label the ECD from within the intrasubunit site. Docking of KK200 to both sites reveals binding poses in agreement with these findings where the diazirine lies adjacent to F267 and N307 (Fig. 7). Docking poses of CW12 to both sites suggest that the 11-TPD group is likely pointing away from GLIC towards the lipid bilayer, consistent with the low labeling efficiency of CW12 in GLIC (Fig. 7).

Using a series of neurosteroid photolabeling reagents, we have precisely mapped two neurosteroid photolabeled sites in GLIC. To confirm that neurosteroids bind to these photolabeled sites, we tested the ability of neurosteroids to competitively prevent labeling. We examined the labeling efficiency of TM3 by 10 µM 5α-6-AziP (intersubunit site) and TM4 by 10 µM KK200 (intrasubunit site) in the absence and presence of 100 µM allopregnanolone and CW12. 5α-6-AziP labeling of TM3 and KK200 labeling of TM4 were examined because these gave the highest labeling efficiencies at 10 µM. For 5α-6-AziP labeling of TM3, allopregnanolone and CW12 significantly reduce the labeling efficiency (Fig. 9A). KK200 labeling of TM4 is significantly reduced by CW12 while allopregnanolone causes a reduction of labeling that is not statistically significant (Fig. 9B). Thus, a neurosteroid analogue, CW12, prevents labeling at both sites. Prevention of KK200 labeling at the intrasubunit site by allopregnanolone is not statistically significant possibly because it has a lower affinity for this site compared to KK200. Indeed, we have found that competition of photolabeling by neurosteroid-based reagents is often difficult to demonstrate, and suspect that this is due to the fact that labeling is an irreversible process and neurosteroids bind with relatively low affinity (21,27,29). CW12 prevention of labeling at both sites supports the interpretation that the low labeling efficiency of this reagent is due to the position of the TPD photolabeling moiety in the steroid backbone and not an inability of this reagent to bind to these sites.

Both neurosteroid binding sites mediate modulation of GLIC activity. We next examined the functional effect of allopregnanolone on GLIC channel activity using two-electrode voltage clamp recordings of Xenopus oocytes. At pH7.5 for activation, 30 µM of allopregnanolone as well as the photolabeling reagents, 5α-6-AziP, 5α-12-AziP, and 5α-15-AziP, KK200, and CW12 significantly inhibit GLIC activity ranging from 8-86% inhibition (Fig. 10; Tables 1 and 2). To determine the functional involvement of the intersubunit and intrasubunit photolabeled sites in mediating the inhibitory effect of neurosteroids, we mutated residues within both binding pockets as predicted by the allopregnanolone docking model (Fig. 11A). In the intersubunit site, we generated I271W, W213A and W217A; however, only I271W produced functional receptors. I271W is predicted to introduce significant steric hindrance to the intersubunit site. In the intrasubunit site, we generated F121A and Y254A. F121A is predicted to remove key van der Waals interactions with the neurosteroid ring structure, and Y254A is predicted to remove a hydrogen bond interaction between Y254 and the neurosteroid 20-carbonyl. Inhibition by 5α-6-AziP was abolished in F121A (resulting in potentiation) and Y254A, but not I271W (Fig. 11B and 11C; Table 2). In contrast, inhibition by KK200, which was the strongest inhibitor of all neurosteroid analogues tested, was abolished in F121A and significantly reduced in I271W and Y254A (Fig. 11B and 11C; Table 2). Inhibition by allopregnanolone was abolished in F121A and Y254A (resulting in potentiation), but unaffected by I271W (Fig. 11C; Table 2). Thus, the results obtained with KK200 support the hypothesis that binding of certain neurosteroid analogues to either the intersubunit or intrasubunit site results in allosteric inhibition of GLIC activity. There is a loss of inhibition in F121A and Y254A but no significant effect in I271W for 5α-6-AziP or allopregnanolone suggesting that binding of these compounds to the intrasubunit site predominantly mediates their inhibitory effect. To distinguish whether the increased inward current evoked by 5α-6-AziP (and allopregnanolone) in F121A channels represents potentiation of low pH activation or direct activation, we examined the effect of 30 µM 5α-6-AziP on the holding current at pH 7.6 (n=5). The absence of any effect on the holding current suggests that 5α-6-AziP potentiates the effect of low pH rather than...
directly activating the channel. To test whether potentiating of F121A by allopregnanolone and 5α-6-AziP is due to unmasking of a potentiating intersubunit site, we generated the F121A/I271W double mutant, but found that this construct does not express currents in oocytes.

**DISCUSSION**

Neurosteroids are potent endogenous molecules that modulate the activity of neuronal pLGICs, and are being developed as antiepileptics, anti-depressants, general anesthetics, analgesics for chronic pain, and neuroprotective agents (7). This study demonstrates precise mapping of two neurosteroid binding sites in a prototypic pLGIC, GLIC, using a series of novel neurosteroid photolabeling reagents. Confident identification of the stoichiometry and sites of labeling was enabled by analysis with top-down and middle-down MS approaches. Two recent studies report crystal structures of a GLIC-GABAAR α1 chimera and GABAAR β3–α5 chimera with a single bound THDOC and pregnanolone, respectively, at the same intersubunit site identified by our photolabeling reagents (22,23). In contrast, we report a second functionally relevant 3α-hydroxypregnane neurosteroid binding site in the pLGIC GLIC. We think that this discrepancy is due the challenge of obtaining crystallized structures of membrane proteins complexed with steroids, and anticipate that the photolabeling approach introduced in this study will enable sensitive identification of neurosteroid binding sites in other integral membrane proteins. Of note, the intrasubunit site identified in this study for allopregnanolone is distinct from an intrasubunit site identified for the inhibitory neurosteroid, pregnenolone sulfate, in a GLIC-GABAAR α1 chimera crystal structure between the intracellular end of TM3 and TM4 (22).

Identification of two neurosteroid binding sites that modulate GLIC activity is consistent with the complex effects of neurosteroids in the GABAAR and glycine receptor, which suggest that multiple binding sites also exist in these receptors (4,24,50). The intersubunit and intrasubunit sites identified in this study may be neurosteroid binding pockets shared among pLGICs, and thus provide a structural framework for understanding neurosteroid interactions with pLGICs in general. The presence of the intersubunit neurosteroid site in GLIC is consistent with the finding that the Q241L mutation in the GABAAR α1 subunit abolishes neurosteroid potentiation (25). This site was previously identified by the neurosteroid photolabeling reagent 5β-6-azi-pregnanolone in the β3 homopentameric GABAAR (21), and recently, by neurosteroid-bound GABAAR chimera crystal structures (22,23). In the GABAAR, the neurosteroid 3-hydroxy forms a hydrogen bond with Q241 in TM1, which is the opposite orientation than we determined for neurosteroid binding in this site in GLIC. We propose that the preferred orientation of neurosteroids within these sites will vary for different pLGICs depending on the exact molecular composition of the sites, and that key interactions with the protein will determine transduction of neurosteroid binding to effect potentiation or inhibition. For example, while allopregnanolone, 5α-6-AziP, and KK200 all bind to the intersubunit site in GLIC, only KK200 inhibition is reduced by the I271W mutation suggesting that interaction of the KK200 TPD group within this site results in an inhibitory effect.

The presence of an intrasubunit neurosteroid site at the extracellular end of TM1 and TM4 in GLIC is also consistent with prior mutagenesis studies in other pLGICs. Mutations of N407 and Y410 in TM4 of the GABAAR α1 subunit reduce neurosteroid potentiation (25), and mutation of the C-terminus of the GABAAR γ2 subunit (24) or nicotinic acetylcholine receptor α4 subunit (51) reduce potentiation of these pLGICs by neurosteroids or an estrogenic steroid, respectively. The intrasubunit neurosteroid site is located at the extracellular end of TM1 and TM4 and, in GLIC, is also comprised of F121 from the β6-β7 loop in the ECD. The F121A mutant reduces inhibition by all tested neurosteroid analogues- allopregnanolone, 5α-6-AziP and KK200, which is consistent with our model that shows this residue forming close van der Waals interactions with the neurosteroid ring structure. Contribution of an ECD loop to a neurosteroid binding site is novel to our current understanding of neurosteroid interactions with pLGICs, and has implications for understanding neurosteroid interactions with pLGICs generally.
modulation of prokaryotic-ECD/eukaryotic-TMD pLGIC chimeras. For example, a recently characterized GLIC-GABAr1 chimera recapitulates neurosteroid stereoselectivity at the 5-position characteristic of r1 receptors. However, while r1 receptors are potentiated by 5α-THDOC, the chimera and GLIC are both inhibited by 5α-THDOC (52). This was attributed to the impact of ECD-TMD coupling interactions that affect gating, but may also result from an ECD contribution to a neurosteroid binding site. Indeed, we find that the directionality of neurosteroid effect is determined, in part, by the structure of the intrasubunit binding site, since the F121A mutant reverses the effect of 5α-6-AziP and allopregnanolone from inhibition to potentiation. An alternative explanation to this finding is that the F121A mutation unmasks the effect of a neurosteroid potentiating site for 5α-6-AziP and allopregnanolone, which may be the intersubunit site. It is also intriguing that mutations at either the intersubunit or intrasubunit site nearly abolish KK200 inhibition of GLIC. This indicates that, in the case of KK200, both sites are necessary for inhibition, and suggests that there is either a cooperative interaction between these sites or that mutations at one site affect the other.

Two neurosteroid binding sites in GLIC raises the question as to whether these sites, particularly the intrasubunit site, mediate potentiating or inhibitory effects in mammalian pLGICs. While the intersubunit site is an established potentiating site of certain endogenous neurosteroids in the GABA AR, we hypothesize that both sites mediate either potentiation or inhibition for different neurosteroids in pLGICs. Understanding the pharmacology of each respective site will be important for structure-based design of neurosteroid therapeutics. For example, certain neurosteroids may cause potentiation at one site and inhibition at the other at varying concentrations: such opposing effects may be useful to widen the therapeutic window of novel neurosteroids.

It is interesting to speculate as to the functional significance of these sterol binding sites in GLIC since bacterial membranes do not contain neurosteroids or other sterols. The equivalent of eukaryotic sterols in bacteria are triterpenoid lipids known as hopanoids, which may have a role in determining the function of prokaryotic pLGICs such as GLIC (53). Furthermore, examination of the GLIC crystal structure shows that both neurosteroid binding sites identified in this study are occupied by phospholipid densities (34). We hypothesize that these sites are conserved hydrophobic pockets that are occupied by hopanoids in bacterial membranes. These pockets were then assumed by neurosteroids and possibly other sterols in eukaryotic membranes to affect the structural stability and function of mammalian pLGICs. Consistent with this hypothesis, the intrasubunit site identified here was previously noted to satisfy the criteria for a CARC or cholesterol recognition motif, and molecular docking showed that both the hopanoid, diploptene, and cholesterol bind favorably to this site (53).

**EXPERIMENTAL PROCEDURES**

**Synthesis of neurosteroid photolabeling reagents.** The syntheses 5α-6-AziP, 5α-12-AziP, 5α-15-AziP, and KK200, are detailed in the Supplemental Note.

**Expression and purification of GLIC.** pET26-MBP-GLIC was a gift from Raimund Dutzler (Addgene plasmid # 20887) and was used for WT GLIC expression. Mutagenesis was performed by oligo-directed mutagenesis using Phu polymerase (Thermo Scientific) verified by sequencing. GLIC was expressed and purified as previously described (54) in OverExpress™ C43(D3) E. coli (Lucigen), derived from BL21(DE3) cells. Briefly, GLIC cultures were grown using Terrific Broth (Sigma), and induced with 0.2 mM IPTG. Cell pellets were reconstituted in Buffer A (50 mM Tris pH8, 150 mM NaCl), cOmplete EDTA-free protease inhibitor cocktail (Roche), and DNAase, lysed, and solubilized with 1% DDM. Solubilized protein was purified using Ni-NTA and eluted using Buffer A, 500 mM Imidazole and 0.02% DDM. His-MBP-GLIC was digested overnight with HRV 3c protease (Invitrogen), cleaned up using a reverse Ni-NTA purification, and injected onto a Sephadex 200 Increase 10/300 column, which yielded pentameric GLIC protein in Buffer A + 0.02% DDM.

**Photolabeling of GLIC and top-down MS analysis.** Purified GLIC was photolabeled and analyzed by
top-down MS as previously described (29). For top-down MS analysis, 50 µg GLIC was mixed with neurosteroid photolabeling reagent at 100 µM, 300 µM, or 100 µM added three times with UV irradiation after each addition. 100 µl samples were irradiated in a quartz cuvette with >320 nm UV light, treated with 250 mM DTT, then precipitated with chloroform/methanol/water. The precipitated protein was washed three times with equal volumes of water and methanol, centrifuged and the protein pellet reconstituted in 3 µl of 90% formic acid followed by 50-100 µl of 4:4:1 chloroform/methanol/water. AspN digests were performed by digesting 50 µg of photolabeled GLIC with 2 µg of AspN at RT for 15 or 30 h. These digests were then precipitated and reconstituted as described above. Reconstituted samples were then analyzed in an Orbitrap Elite mass spectrometer (Thermo Scientific) by direct injection at 3 µl/min using a Max Ion API source with a HESI-II probe. Full spectra of photolabeled GLIC were acquired on the LTQ using spray voltage of 4 kV, capillary temp of 320 °C, and SID of 30 V. HCD fragmentation spectra were acquired on the Orbitrap at 60,000 resolution, with an AGC target of 5 x10^5, normalized energy of 10, and 3 m/z isolation window. Deconvolution of intact GLIC spectra was performed using MagTran(55). Ion assignments of HCD fragmentation spectra were performed by MASH searching with a mass accuracy of 1.1 Da to account for the “delta 1Da” error(56); each fragment ion was manually verified and accepted within 10 ppm.

Tryptic middle-down MS analysis. 15 µg of photolabeled GLIC was reduced with 5 mM tris(carboxymethyl)phosphine (TCEP) for 30 min, alkylated with 5 mM iodoaceticamide for 30 min, and quenched with 5 mM DTT. Samples were then digested with 2-6 µg of trypsin for 7 days at 4°C- extended digestion at low temperature was necessary to obtain maximal recovery of TMD peptides. Next, formic acid was added to 1%, followed directly by LC-MS analysis on an Orbitrap Elite mass spectrometer. 10 µl samples were injected into a home-packed PLRP-S (Agilent) column (10 cm x 75 µm, 300 Å), separated with an 85 min gradient from 10% to 95% ACN, and introduced to the mass spectrometer at 800 nl/min with a nanospray source. MS acquisition was set as an MS1 Orbitrap scan (res 60,000) followed by top 20 MS2 Orbitrap scans (res 15,000) using data dependent acquisition, 15 s dynamic exclusion, and exclusion of 1+ and 2+ precursors. Fragmentation was performed using CID and HCD with normalized collision energies of 35 and 30, respectively. Analysis of data sets was performed using Xcalibur (Thermo Scientific) to manually search for TM1+2, TM3 or TM4 tryptic peptides with or without neurosteroid photolabel modifications. Photolabeling efficiency was estimated by generating extracted chromatograms of unlabeled and labeled peptides, determining the area under the curve, and calculating the abundance of labeled peptide/(unlabeled+labeled peptide). Competitive inhibition of labeling was performed by labeling GLIC with 10 µM 5α-6-AziP or KK200 in the presence of 100 µM allopregnanolone, CW12 or 17-PA. Relative photolabeling efficiency in the absence or presence of competitor was determined for TM3 for 5α-6-AziP and for TM4 for KK200. Analysis of statistical significance comparing the photolabeling efficiency of 5α-6-AziP, 5α-12-AziP and 5α-15-AziP for GLIC, TM3 and TM1+2 or for the competitive inhibition assay was determined using a one-way ANOVA with post-hoc corrections using Dunnett’s test.

MS2 spectra of photolabeled TMD peptides were analyzed by manual assignment of fragment ions with and without photolabel modification. Fragment ions were accepted based on the presence of a monoisotopic mass within 10 ppm mass accuracy. In addition to manual analysis, PEAKS database searches were performed for data sets of GLIC photolabeled with 5α-6-AziP and KK200 primarily to search for photolabeled ECD tryptic peptides. Search parameters were set for a precursor mass accuracy of 10 ppm, fragment ion accuracy of 0.1 Da, up to 3 missed cleavages on either end of the peptide, and variable modifications of methionine oxidation, cysteine carbamidomethylation, and 5α-6-AziP or KK200 on any amino acid.

Electrophysiology Experiments. The cDNAs for oocyte expression were subcloned into the pcDNA3 vector with the modification that the
MBP was replaced with the signal peptide of the human beta 3 GABA-A subunit. The T7 promoter was used for RNA synthesis and linearized by XbaI (New England Biolabs). The cRNAs were produced using mMessage mMachine (Ambion). Oocytes were injected with 5 ng cRNA and incubated for 1-3 days prior to recording. The electrophysiological experiments were conducted using standard two-electrode voltage clamp. Voltage and current electrodes were borosilicate patch-clamp electrodes (G120F-4, OD=1.20 mm, ID=0.69 mm, Warner Instruments, Hamden, CT) that were filled with 3 M KCl and had resistance of less than 1 MΩ. The oocytes were clamped at -60 mV. Solutions were gravity-applied from 30-ml glass syringes via Teflon tubing to reduce adsorption. Oocytes were perfused continuously with ND96 (96 mM NaCl, 2 mM KCl, 1 mM MgCl₂, 1.8 mM CaCl₂, 5 mM HEPES) solution at pH 7.6 at approximately 5 ml/min. Proton-elicited currents were measured by exposing the cells to ND96 buffered at pH 7.0 to 3.5. At lower pH values, HEPES was replaced with 5 mM MES (pH 6-5.45) or 5 mM Na citrate (pH 5.0-3.5). Solutions were switched manually.

The pH-response relationships were determined by exposing an oocyte to 4-7 different pH solutions. The concentration-response data were fit to the following equation:

\[ I = \frac{I_{MAX}}{1 + 10^{\left(\frac{pH - pH_{50}}{nH}\right)}} \]

where I is the peak response to a given pH, Iₘₐₓ is the maximal fitted amplitude, pHₕₒ is the pH eliciting half-maximal response, and nₜ is the Hill coefficient.

The effects of steroid on the function of GLIC were studied at pH 50. Each oocyte was additionally exposed to picrotoxinin (in pH 50), a known blocker of GLIC (57), to exclude any drastic effects of mutations on receptor function that may have masked the effects on modulation by steroids. Exposure to 50 μM picrotoxinin reduced the pHₕₒ current levels to 19 ± 1% of control in WT, and 30 ± 3% (P<0.05 vs. wild-type; ANOVA with Dunnett's post hoc correction), 13 ± 1% (P>0.55), or 26 ± 5% (P>0.23) of control in I271W, F121A, and Y254A, respectively.

A typical experiment (Fig. 4b) consisted of recording of a 10-20 s baseline at pH 7.6, followed by exposure to pHₕₒ until a steady response was obtained. The receptor was then exposed to 30 μM steroid in pHₕₒ (maximal application duration 65 s), followed by recovery in the pHₕₒ solution and an application of 50 μM picrotoxinin. WT GLIC exposed to KK200 exhibited strong block and slow washout; accordingly, the effect of picrotoxinin was tested before steroid application (Fig. 4b). The F121A mutant exhibited unstable transient responses upon switch from pHₕₒ to pHₕₒ + 5α-6-AziP. For this combination, the effect of steroid was determined by comparing peak responses to applications of pHₕₒ or pHₕₒ + 5α-6-AziP, separated by a brief wash in pH 7.6 solution (Fig. 4b).

The concentration of DMSO in final steroid solutions was 0.3% (v/v). In control experiments we tested the effect of 0.3% DMSO on GLIC function. Oocytes expressing wild-type or mutant receptors activated by pHₕₒ showed inhibition in the presence of DMSO. The average inhibitory effect was 7 ± 1%, 10 ± 1%, 23 ± 3%, or 25 ± 2% (n = 5 cells for each) for wild-type, I271W, F121A, and Y254A, respectively. The data for steroid effects are reported after subtraction of the DMSO-only effect.

Current responses were amplified with an OC-725C amplifier (Warner Instruments), filtered at 40 Hz, digitized with a Digidata 1200 series digitizer (Molecular Devices) at a 100 Hz sampling rate, and stored using pClamp (Molecular Devices). The traces were subsequently analyzed with Clampfit (Molecular Devices) to determine the maximal amplitude of current response.

GLIC was expressed in oocytes from the African clawed frog (Xenopus laevis). Frogs were purchased from Xenopus 1 (Dexter, MI), and housed and cared for in a Washington University Animal Care Facility under the supervision of the Washington University Division of Comparative Medicine. Harvesting of oocytes was conducted in accordance with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the National Institutes of Health. The protocol is approved by the Animal Studies Committee of Washington University in St. Louis (Approval No. 20170071).
Docking Simulations. Docking was performed using AutoDock 4.2 (58). The GLIC template was prepared using pdb 4FHI in AutoDock Tools by deleting waters, adding hydrogens, computing Gasteiger charges, and merging non-polar hydrogens. Allopregnanolone was prepared by converting the sdf file from pubchem into a pdb file using Open Babel (59), and Gasteiger charges and free torsion angles were determined by AutoDock Tools. Structures for $5\alpha$-12-AziP, $5\alpha$-15-AziP, CW12 and KK200 were obtained by modifying the allopregnanolone in Maestro (Academic version, Schrodinger) using the 2D draw and 3D conversion function, after which Gasteiger charges and free torsion angles were determined by AutoDock Tools. Four docking simulations were performed for allopregnanolone using templates of: 1) the entire TMD of two adjacent GLIC subunits (50 Å x 42 Å x 50 Å), 2) the entire TMD of two adjacent GLIC subunits (50 Å x 42 Å x 50 Å) with one allopregnanolone molecule within the intersubunit site (Note: this was necessary to adequately sample binding modes outside of the intersubunit site), 3) the intersubunit site (36 Å x 32 Å x 34 Å) and 3) the intrasubunit site (32 Å x 30 Å x 32 Å). The photolabeling reagents were docked to grid boxes encompassing only the intersubunit and intrasubunit sites. All simulations were performed with 1 Å grid spacing, using a genetic algorithm with 250 runs, and otherwise default parameters. Results were clustered using a 2 Å RMSD. For each neurosteroid photolabeling ligand, the three most populated clusters were examined and shown are poses where the photolabeling moiety is in closest proximity to the labeled residue.

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CONFLICTS OF INTEREST
The authors declare that they have no conflicts of interest with the contents of the article.

AUTHOR CONTRIBUTIONS
W.W.C. and Z.W.C. contributed to the study conception and design, acquisition and analysis of data, and writing of the manuscript. A.S.E., D.F.C., and G.A. contributed to the study conception and design, and writing of the manuscript. J.R.B. contributed to the study design and molecular biology. M.M.B contributed to the study design and interpretation of data. D.J.S. contributed to the acquisition and analysis of electrophysiology data. D.F.C, K.K., X.J. and C.W. contributed to the synthesis of neurosteroid photolabeling reagents.

REFERENCES
1. Lambert, J. J., Belelli, D., Hill-Venning, C., and Peters, J. A. (1995) Neurosteroids and GABAA receptor function. Trends Pharmacol Sci 16, 295-303
2. Bertrand, D., Valera, S., Bertrand, S., Ballivet, M., and Rungger, D. (1991) Steroids inhibit nicotinic acetylcholine receptors. Neuroreport 2, 277-280
3. Gillo, B., and Lass, Y. (1984) The mechanism of steroid anaesthetic (alphaxalone) block of acetylcholine-induced ionic currents. Br J Pharmacol 82, 783-789
4. Maksay, G., Laube, B., and Betz, H. (2001) Subunit-specific modulation of glycine receptors by neurosteroids. Neuropharmacology 41, 369-376
5. Jiang, P., Yang, C. X., Wang, Y. T., and Xu, T. L. (2006) Mechanisms of modulation of pregnanolone on glycineric response in cultured spinal dorsal horn neurons of rat. Neuroscience 141, 2041-2050
6. Belelli, D., and Lambert, J. J. (2005) Neurosteroids: endogenous regulators of the GABA(A) receptor. Nat Rev Neurosci 6, 565-575
7. Reddy, D. S., and Estes, W. A. (2016) Clinical Potential of Neurosteroids for CNS Disorders. Trends Pharmacol Sci 37, 543-561
8. Harrison, N. L., and Simmonds, M. A. (1984) Modulation of the GABA receptor complex by a steroid anaesthetic. Brain Res 323, 287-292
9. Reddy, D. S., and Rogawski, M. A. (2012) Neurosteroids - Endogenous Regulators of Seizure Susceptibility and Role in the Treatment of Epilepsy. in Jasper's Basic Mechanisms of the Epilepsies (Noebels, J. L., Avoli, M., Rogawski, M. A., Olsen, R. W., and Delgado-Escueta, A. V. eds.), 4th Ed., Bethesda (MD). pp 9-11.

10. Zorumski, C. F., Paul, S. M., Izumi, Y., Covey, D. F., and Mennerick, S. (2013) Neurosteroids, stress and depression: potential therapeutic opportunities. Neurosci Biobehav Rev 37, 109-122.

11. Vallee, M. (2016) Neurosteroids and potential therapeutics: Focus on pregnenolone. J Steroid Biochem Mol Biol 160, 78-87.

12. Shu, H. J., Eisenman, L. N., Jinadasa, D., Covey, D. F., Zorumski, C. F., and Mennerick, S. (2004) Slow actions of neuroactive steroids at GABAA receptors. J Neurosci 24, 6667-6675.

13. Park-Chung, M., Malayev, A., Purdy, R. H., Gibbs, T. T., and Farb, D. H. (1999) Sulfated and unsulfated steroids modulate gamma-aminobutyric acidA receptor function through distinct sites. Brain Res 830, 72-87.

14. Wang, M., He, Y., Eisenman, L. N., Fields, C., Zeng, C. M., Mathews, J., Benz, A., Fu, T., Zorumski, E., Steinbach, J. H., Covey, D. F., Zorumski, C. F., and Mennerick, S. (2002) 3beta-hydroxypregnane steroids are pregnenolone sulfate-like GABA(A) receptor antagonists. J Neurosci 22, 3366-3375.

15. Li, P., Covey, D. F., Steinbach, J. H., and Akk, G. (2006) Dual potentiating and inhibitory actions of a benz[e]indene neurosteroid analog on recombinant alpha1beta2gamma2 GABA(A) receptors. Mol Pharmacol 69, 2015-2026.

16. Paradiso, K., Sabey, K., Evers, A. S., Zorumski, C. F., Covey, D. F., and Steinbach, J. H. (2000) Steroid inhibition of rat neuronal nicotinic alpha4beta2 receptors expressed in HEK 293 cells. Mol Pharmacol 58, 341-351.

17. Mensah-Nyagan, A. G., Meyer, L., Schaeffer, V., Kibaly, C., and Patte-
25. Hosie, A. M., Wilkins, M. E., da Silva, H. M., and Smart, T. G. (2006) Endogenous neurosteroids regulate GABAA receptors through two discrete transmembrane sites. *Nature* **444**, 486-489

26. Evers, A. S., Chen, Z. W., Manion, B. D., Han, M., Jiang, X., Darbandi-Tonkabon, R., Kable, T., Bracamontes, J., Zorumski, C. F., Mennerick, S., Steinbach, J. H., and Covey, D. F. (2010) A synthetic 18-norsteroid distinguishes between two neuroactive steroid binding sites on GABAA receptors. *J Pharmacol Exp Ther* **333**, 404-413

27. Woll, K. A., Dailey, W. P., Brannigan, G., and Eckenhoff, R. G. (2016) Shedding Light on Anesthetic Mechanisms: Application of Photoaffinity Ligands. *Anesth Analg* **123**, 1253-1262

28. Das, J. (2011) Aliphatic diazirines as photoaffinity probes for proteins: recent developments. *Chem Rev* **111**, 4405-4417

29. Budelier, M. M., Cheng, W. W., Bergdoll, L., Chen, Z. W., Janetka, J. W., Abramson, J., Krishnan, K., Mydock-McGrane, L., Covey, D. F., Whitelegge, J. P., and Evers, A. S. (2017) Photoaffinity labeling with cholesterol analogues precisely maps a cholesterol-binding site in voltage-dependent anion channel-1. *J Biol Chem*

30. Budelier, M. M., Cheng, W. W., Bergdoll, L., Chen, Z. W., Abramson, J., Krishnan, K., Qian, M., Covey, D. F., Janetka, J. W., and Evers, A. S. (2017) Click Chemistry Reagent for Identification of Sites of Covalent Ligand Incorporation in Integral Membrane Proteins. *Anal Chem*

31. Savechenkov, P. Y., Chiara, D. C., Desai, R., Stern, A. T., Zhou, X., Ziembia, A. M., Szabo, A. L., Zhang, Y., Cohen, J. B., Forman, S. A., Miller, K. W., and Bruzik, K. S. (2017) Synthesis and pharmacological evaluation of neurosteroid photoaffinity ligands. *Eur J Med Chem* **136**, 334-347

32. Darbandi-Tonkabon, R., Hastings, W. R., Zeng, C. M., Akk, G., Manion, B. D., Bracamontes, J. R., Steinbach, J. H., Mennerick, S. J., Covey, D. F., and Evers, A. S. (2003) Photoaffinity labeling with a neuroactive steroid analogue. 6-azi-pregnanolone labels voltage-dependent anion channel-1 in rat brain. *J Biol Chem* **278**, 13196-13206

33. Chen, Z. W., Wang, C., Krishnan, K., Manion, B. D., Hastings, R., Bracamontes, J., Taylor, A., Eaton, M. M., Zorumski, C. F., Steinbach, J. H., Akk, G., Mennerick, S., Covey, D. F., and Evers, A. S. (2014) 11-trifluoromethyl-phenyldiazirinyl neurosteroid analogues: potent general anesthetics and photolabeling reagents for GABAA receptors. *Psychopharmacology (Berl)* **231**, 3479-3491

34. Bocquet, N., Nury, H., Baaden, M., Le Poupon, C., Changeux, J. P., Delarue, M., and Corringer, P. J. (2009) X-ray structure of a pentameric ligand-gated ion channel in an apparently open conformation. *Nature* **457**, 111-114

35. daCosta, C. J., and Baenziger, J. E. (2013) Gating of pentameric ligand-gated ion channels: structural insights and ambiguities. *Structure* **21**, 1271-1283

36. Pan, J., Chen, Q., Willenbring, D., Mowrey, D., Kong, X. P., Cohen, A., Divito, C. B., Xu, Y., and Tang, P. (2012) Structure of the pentameric ligand-gated ion channel GLIC bound with anesthetic ketamine. *Structure* **20**, 1463-1469

37. Nury, H., Van Renterghem, C., Weng, Y., Tran, A., Baaden, M., Dufresne, V., Changeux, J. P., Sonner, J. M., Delarue, M., and Corringer, P. J. (2011) X-ray structures of general anaesthetics bound to a pentameric ligand-gated ion channel. *Nature* **469**, 428-431

38. Fourati, Z., Ruza, R. R., Laverty, D., Drege, E., Delarue-Cochin, S., Joseph, D., Koehl, P., Smart, T., and Delarue, M. (2017) Barbiturates Bind in the GLIC Ion Channel Pore and Cause Inhibition by Stabilizing a Closed State. *J Biol Chem* **292**, 1550-1558

39. Murail, S., Howard, R. J., Broemstrup, T., Bertaccini, E. J., Harris, R. A., Trudell, J. R., and Lindahl, E. (2012) Molecular mechanism for the dual alcohol modulation of Cys-loop receptors. *PLoS Comput Biol* **8**, e1002710
40. Howard, R. J., Murail, S., Ondricek, K. E., Corringer, P. J., Lindahl, E., Trudell, J. R., and Harris, R. A. (2011) Structural basis for alcohol modulation of a pentameric ligand-gated ion channel. *Proc Natl Acad Sci U S A* **108**, 12149-12154

41. Chen, Q., Cheng, M. H., Xu, Y., and Tang, P. (2010) Anesthetic binding in a pentameric ligand-gated ion channel: GLIC. *Biophys J* **99**, 1801-1809

42. Laurent, B., Murail, S., Shahsavar, A., Sauguet, L., Delarue, M., and Baaden, M. (2016) Sites of Anesthetic Inhibitory Action on a Cationic Ligand-Gated Ion Channel. *Structure* **24**, 595-605

43. Chiara, D. C., Gill, J. F., Chen, Q., Tillman, T., Dailey, W. P., Eckenhoff, R. G., Xu, Y., Tang, P., and Cohen, J. B. (2014) Photoaffinity labeling the propofol binding site in GLIC. *Biochemistry* **53**, 135-142

44. Giansanti, P., Tsiatsiani, L., Low, T. Y., and Heck, A. J. (2016) Six alternative proteases for mass spectrometry-based proteomics beyond trypsin. *Nat Protoc* **11**, 993-1006

45. Sauguet, L., Poitevin, F., Murail, S., Van Renterghem, C., Moraga-Cid, G., Malherbe, L., Thompson, A. W., Koehl, P., Corringer, P. J., Baaden, M., and Delarue, M. (2013) Structural basis for ion permeation mechanism in pentameric ligand-gated ion channels. *EMBO J* **32**, 728-741

46. Harrison, N. L., Majewska, M. D., Harrington, J. W., and Barker, J. L. (1987) Structure-activity relationships for steroid interaction with the gamma-aminobutyric acidA receptor complex. *J Pharmacol Exp Ther* **241**, 346-353

47. Li, W., Jin, X., Covey, D. F., and Steinbach, J. H. (2007) Neuroactive steroids and human recombinant rho1 GABAC receptors. *J Pharmacol Exp Ther* **323**, 236-247

48. Chen, Z.-W., Wang, C., Krishnan, K., Manion, B. D., Hastings, R., Bracamontes, J., Taylor, A., Eaton, M. M., Zorumski, C. F., and Steinbach, J. H. (2014) 11-trifluoromethyl-phenyl diazirinyl neurosteroid analogues: potent general anesthetics and photolabeling reagents for GABAA receptors. *Psychopharmacology* **231**, 3479-3491

49. Brunner, J. (1993) New photolabeling and crosslinking methods. *Annu Rev Biochem* **62**, 483-514

50. Akk, G., Covey, D. F., Evers, A. S., Mennerick, S., Zorumski, C. F., and Steinbach, J. H. (2010) Kinetic and structural determinants for GABA-A receptor potentiation by neuroactive steroids. *Curr Neuropharmacol* **8**, 18-25

51. Paradiso, K., Zhang, T. W., and Czajkowski, C. (2017) A chimeric proaryotic-eukaryotic pentameric ligand gated ion channel reveals interactions between the extracellular and transmembrane domains shape neurosteroid modulation. *Neuropharmacology* **125**, 343-352

52. Barrantes, F. J., and Fantini, J. (2016) From hopanoids to cholesterol: Molecular clocks of pentameric ligand-gated ion channels. *Prog Lipid Res* **63**, 1-13

53. Hilf, R. J., and Dutzler, R. (2009) Structure of a potentially open state of a proton-activated pentameric ligand-gated ion channel. *Nature* **457**, 115-118

54. Zhang, Z., and Marshall, A. G. (1998) A universal algorithm for fast and automated charge state deconvolution of electrospray mass-to-charge ratio spectra. *J Am Soc Mass Spectrom* **9**, 225-233

55. Guner, H., Close, P. L., Cai, W., Zhang, H., Peng, Y., Gregorich, Z. R., and Ge, Y. (2014) MASH Suite: a user-friendly and versatile software interface for high-resolution mass spectrometry data interpretation and visualization. *J Am Soc Mass Spectrom* **25**, 464-470

56. Alqazzaz, M., Thompson, A. J., Price, K. L., Breitinger, H. G., and Lummis, S. C. (2011) Cys-loop receptor channel blockers also block GLIC. *Biophys J* **101**, 2912-2918
58. Morris, G. M., Huey, R., Lindstrom, W., Sanner, M. F., Belew, R. K., Goodsell, D. S., and Olson, A. J. (2009) AutoDock4 and AutoDockTools4: Automated docking with selective receptor flexibility. *J Comput Chem* **30**, 2785-2791

59. O'Boyle, N. M., Banck, M., James, C. A., Morley, C., Vandermeersch, T., and Hutchison, G. R. (2011) Open Babel: An open chemical toolbox. *J Cheminform* **3**, 33
TABLE 1: pH dependence of GLIC WT and mutants.

|      | $pH_{50}$ | SD  | Hill coefficient | SD  | n  |
|------|-----------|-----|------------------|-----|----|
| WT   | 4.97      | 0.12| 2.55             | 0.18| 6  |
| L271W| 5.48      | 0.11| 1.97             | 0.29| 5  |
| F121A| 4.31      | 0.11| 3.45             | 0.70| 6  |
| Y254A| 4.19      | 0.32| 1.80             | 0.63| 5  |
TABLE 2: Inhibition of GLIC WT and mutants by neurosteroids.

|        | Average % Effect | SEM | n  | Average DMSO-corrected % Effect | SEM |
|--------|------------------|-----|----|----------------------------------|-----|
| WT     |                  |     |    |                                  |     |
| Allopregnanolone | -17 | 2   | 9  | -10                             | 2   |
| 5α-6-AziP | -25 | 2   | 5  | -18                             | 2   |
| 5α-12-AziP | -27 | 2   | 6  | -20                             | 2   |
| 5α-15-AziP | -18 | 2   | 5  | -11                             | 2   |
| KK200  | -93              | 1   | 5  | -86                             | 2   |
| CW12   | -15              | 1   | 5  | -8                              | 1   |
| I271W  |                  |     |    |                                  |     |
| Allopregnanolone | -18 | 1   | 6  | -8                              | 1   |
| 5α-6-AziP | -30 | 6   | 5  | -20                             | 6   |
| KK200  | -17              | 2   | 5  | -7                              | 2   |
| F121A  |                  |     |    |                                  |     |
| Allopregnanolone | -10 | 1   | 8  | 13                              | 3   |
| 5α-6-AziP | 14  | 4   | 6  | 37                              | 5   |
| KK200  | 23               | 4   | 5  | 0                               | 5   |
| Y254A  |                  |     |    |                                  |     |
| Allopregnanolone | -13 | 1   | 9  | 12                              | 2   |
| 5α-6-AziP | -27 | 2   | 5  | -2                              | 3   |
| KK200  | -34              | 2   | 4  | -9                              | 3   |
FIGURE 2

A) GLIC

B) Neurosteroid Binding Sites in GLIC

C) Neurosteroid Binding Sites in ECD and TMD
FIGURE 3

A

B

C

D

E

Neurosteroid Binding Sites in GLIC
FIGURE 5

A

5α-12-AzIP

5α-15-AzIP

B

Photolabeling Efficiency

0.6

0.4

0.2

0

6AzIP

12AzIP

15AzIP

6AzIP

12AzIP

15AzIP

TM3 (E272)

TM1+2 (Y194)

YGGFLRF Peptide

C

D

TPYMTYGAFFLYFLuya

LVLE (12-AzIP)

LVLE (15-AzIP)

y17y21y23y24y25

y18y20y22

y16

y26y27y28y29

y23

y22

y24

y25

y26

y27

y28

y29

TM1

TM2

TM3

TM4

m/z

Intensity (x10^5)

200 600 1,000 1,400 1,800
FIGURE 6

A

Intersubunit Site

Cluster 1 - 52% of poses
F267  F210
I271  W213
E272  W217
Y278

Cluster 2 - 24% of poses
F267  F210
I271  W213
E272  W217
Y278

Cluster 3 - 24% of poses
F267  F210
I271  W213
E272  W217
Y278

B

Intrasubunit Site

Cluster 1 - 93% of poses
Y194  F315
F121  Y254
I202  N307

Cluster 2 - 7% of poses
Y194  F315
F121  Y254
I202  N307
FIGURE 7

A

Intersubunit Site

5α-6-Azip

5α-12-Azip

5α-15-Azip

E272

F267

E272

TPD

E272

TPD

Allopregnanolone

B

Intrasubunit Site

5α-6-Azip

5α-12-Azip

5α-15-Azip

Y194

Y194

Y194

TPD

TPD

Allopregnanolone

KK200

KK200

CW12

CW12

N307
FIGURE 9

A

Intersubunit Site

Relative TM3 Labeling Efficiency

100 μM Cntrl AlloP CW12
10 μM 5α-6-AziP

B

Intrasubunit Site

Relative TM4 Labeling Efficiency

100 μM Cntrl AlloP CW12
10 μM KK200
FIGURE LEGENDS

FIGURE 1: Top-down analysis of GLIC photolabeled with 5α-6-AziP. (A) Structure of allopregnanolone and 5α-6-AziP. (B) Full spectrum of GLIC labeled with 100 µM 5α-6-AziP three times, acquired on an Elite LTQ. (C) Deconvoluted spectrum of GLIC photolabeled with 100 µM 5α-6-AziP. Asterisks denote the number of 5α-6-AziP labels per GLIC subunit. (D) Deconvoluted spectrum of GLIC photolabeled with 100 µM of 5α-6-AziP three times. (E) Fragment ions from top-down HCD of the 32+ charge state of singly labeled GLIC from the spectrum in (B). The grey b- and y-ions are fragment ions that contain no 5α-6-AziP, and the black y-ions contain 5α-6-AziP. The TMDs are color coded.

FIGURE 2: AspN middle-down analysis of GLIC photolabeled with 5α-6-AziP. (A) Deconvoluted spectra of GLIC photolabeled with 300 µM 5α-6-AziP showing intact GLIC, and the ECD and TMD after AspN digestion. (B) HCD fragment ion assignments of the unlabeled ECD peptide shown in (A). The grey lines represent b- and y-ions that do not contain 5α-6-AziP. (C) HCD fragment ion assignments of the singly labeled TMD species in (A). The red and black lines represent b-ions and y-ions, respectively, that contain 5α-6-AziP.

FIGURE 3: Trypsin middle-down analysis of GLIC photolabeled with 5α-6-AziP. (A) Extracted chromatograms of unlabeled and 5α-6-AziP-labeled (denoted by arrows) TM1+2, TM3 and TM4 peptides, normalized to the intensity of each unlabeled peptide. Inset graph shows the labeling efficiency of each peptide. Schematic highlights in red the TMD being analyzed and the asterisk denotes the approximate location of 5α-6-AziP. (C) HCD MS2 spectrum of TM3 labeled with 5α-6-AziP from GLIC. (D) CID MS2 of TM4 labeled with 5α-6-AziP from GLIC. The b22+ and b23+ represent neutral loss of adduct. (E) GLIC structure (pdb 4HFI) highlighting the photolabeled residues in the intersubunit (purple spheres) and intrasubunit sites (green spheres) and docking poses for allopregnanolone (purple sticks) at both sites.

FIGURE 4: MS analysis of E272A GLIC photolabeled with 5α-6-AziP. (A) Deconvoluted spectra of GLIC E272A labeled with 100 µM 5α-6-AziP (left) or 100 µM three times (right). The asterisks denote GLIC E272A labeled with one or two 5α-6-AziP. (B) CID MS2 spectrum of TM3 labeled with 5α-6-AziP from E272A GLIC. Red and black b- and y- ions do and do not contain 5α-6-AziP, respectively. Schematic highlights in red the TMD being analyzed and the asterisk denotes the approximate location of 5α-6-AziP. (C) HCD MS2 spectrum of TM1+2 peptide from GLIC E272A labeled with 5α-6-AziP. (D) CID MS2 spectrum of TM4 peptide labeled with 5α-6-AziP from GLIC E272A.

FIGURE 5: MS Analysis of GLIC photolabeled with 5α-12-AziP and 5α-15-AziP. (A) Structure of the photolabeling reagents 5α-12-AziP and 5α-15-AziP. (B) Photolabeling efficiency of 5α-6-AziP, 5α-12-AziP, and 5α-15-AziP for TM3 and TM1+2 of WT GLIC, and the peptide YGGFLRF (n=7 for 5α-6-AziP, n=3 for 5α-12-AziP and 5α-15-AziP). *SD, ** indicates p<0.01. (C) HCD MS2 spectrum of TM3 peptide labeled with 5α-12-AziP from GLIC WT. Red and black fragment ions do and do not contain 5α-12-AziP, respectively. (D) Same as (C) for 5α-15-AziP.

FIGURE 6: 5α-6-AziP photolabeled residues are consistent with docking poses within an intersubunit and intrasubunit binding site. (A) Clusters of poses for allopregnanolone docked within the intersubunit site indicating the percent of total poses represented by each cluster. Cluster 1 is most consistent with the preferred binding orientation from the photolabeling data. (B) Same as (A) for the intrasubunit site. Cluster 1 is most consistent with the preferred binding orientation from the
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photolabeling data. Cluster 1 shows hydrogen bond interactions between the neurosteroid 3-hydroxy and backbone carbonyl of F121 (distance of 2.3 Å), and the neurosteroid 20-carbonyl and Y254 hydroxyl (distance of 2.0 Å). Cluster 2 shows a weak hydrogen bond between the neurosteroid 20-carbonyl and the Y194 hydroxyl (distance of 4.0 Å). (B) Same as (A) for the intersubunit site.

FIGURE 7: Docking poses of allopregnanolone and photolabeling reagents in the intersubunit and intrasubunit sites. (A) Binding poses selected from among the three most populated clusters for allopregnanolone, 5α-6-AziP, 5α-12-AziP, 5α-15-AziP, KK200 and CW12 in the intersubunit site. The photolabeled residues, E272 (5α-6-AziP, 5α-12-AziP, 5α-15-AziP) and F267 (KK200), are shown as purple spheres. The positions of the allopregnanolone steroid backbone or the photolabeling groups are labeled. (B) Same as (A) for the intrasubunit site where the photolabeled residue, Y194 (5α-6-AziP) and N307 (KK200), are shown as purple spheres.

FIGURE 8: MS Analysis of GLIC photolabeled with KK200 and CW12. (A) Structures and deconvoluted spectra of intact GLIC photolabeled with 100 µM KK200. Asterisk denotes number of photolabels. (B) Same as (A) for CW12. (C) CID MS2 spectrum of TM3 labeled with KK200 from GLIC. The unlabeled y21+, y22+, and y23+ ions represent neutral loss of adduct. Red and black fragment ions do and do not contain KK200, respectively. (D) HCD MS2 spectrum of TM4 labeled with KK200 from GLIC.

FIGURE 9: Competitive prevention of neurosteroid labeling at both sites (A) Photolabeling efficiency of TM3 in WT GLIC by 10 µM 5α-6-AziP in the presence of no competitor (Cntrl), 100 µM allopregnanolone (n=7) or CW12 (n=4). Efficiencies are normalized to that of control. ±SD, * indicates p<0.05, ** p<0.01. (B) Photolabeling efficiency of TM4 in WT GLIC by 10 µM KK200 in the presence of no competitor (Cntrl), 100 µM allopregnanolone (n=4) or CW12 (n=4).

FIGURE 10: Neurosteroid inhibition of GLIC WT (A) Sample current traces from GLIC WT activated by pH 5.00 (pH50) showing the effects of 30 µM allopregnanolone (AlloP), 5α-12-AziP (12AziP), 5α-15-AziP (15AziP), or CW12, and 50 µM picrotoxinin (PTX). Traces for 5α-6-AziP (6AziP), and KK200 in GLIC WT are shown in Fig. 11. (B) Summary of the functional effects of different neurosteroid analogues on GLIC WT. The effects are shown as DMSO-effects subtracted from the effects of steroids in the presence of DMSO. * indicates p<0.05 and ** p<0.001 for the difference in effect between each neurosteroid analogue and DMSO (±SD, n at least 5).

FIGURE 11: Both neurosteroid binding sites mediate modulation of GLIC channel activity. (A) Structure of intersubunit and intrasubunit sites highlighting residues that contribute to the allopregnanolone binding pocket. The preferred allopregnanolone docking pose is shown in purple sticks. Residues that were mutated are shown in yellow sticks. (B) Sample currents from GLIC WT, I271W and F121A in the absence and presence of 5α-6-AziP, KK200, and picrotoxinin (PTX). (C) Summary of the functional effects of 5α-6-AziP, KK200, and allopregnanolone in WT, I271W, F121A and Y254A. The effects are shown as DMSO-effects subtracted from the effects of steroids in the presence of DMSO (±SD, n at least 5). * indicates p<0.05, ** p<0.001.
Mapping Two Neurosteroid Modulatory Sites in the Prototypic Pentameric Ligand Gated Ion Channel GLIC

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