β subunits of GABA_\text{A} receptors form proton-gated chloride channels: Insights into the molecular basis

Aleksandra Garifulina_1, Theres Friesacher_1, Marco Stadler_1, Eva-Maria Zangerl-Plessl_1, Margot Ernst_2, Anna Stary-Weinzinger_1, Anita Willam_1,3 & Steffen Hering_1,3

Gamma-aminobutyric acid type A receptors (GABA_\text{A}Rs) are ligand gated channels mediating inhibition in the central nervous system. Here, we identify a so far undescribed function of β-subunit homomers as proton-gated anion channels. Mutation of a single H267A in β3 subunits completely abolishes channel activation by protons. In molecular dynamic simulations of the β3 crystal structure protonation of H267 increased the formation of hydrogen bonds between H267 and E270 of the adjacent subunit leading to a pore stabilising ring formation and accumulation of Cl\textsuperscript{-} within the transmembrane pore. Conversion of these residues in proton insensitive ρ1 subunits transfers proton-dependent gating, thus highlighting the role of this interaction in proton sensitivity. Activation of chloride and bicarbonate currents at physiological pH changes (pH\textsubscript{50} is in the range 6-6.3) and kinetic studies suggest a physiological role in neuronal and non-neuronal tissues that express beta subunits, and thus as potential novel drug target.
γ-Aminobutyric acid (GABA) type A receptors (GABA\(_{\text{A}R}\)) are ligand gated chloride channels mediating fast synaptic inhibition in the central nervous system (CNS)\(^1\) and are expressed in most tissues\(^2\). GABA\(_{\text{A}R}\) are pentamers that potentially can be assembled of 19 possible subunits (α(1–6), β(1–3), γ(1–3), δ, ε, θ, π and ρ(1–3))\(^3\) in mammals. The most abundant native receptors are heteropentamers consisting of two α1, two β2 and one γ2 subunit\(^5\). A large number of receptor subtypes comprising different subunit combinations is, however, likely to exist\(^5\). In heterologous expression systems β3 and ρ1 subunits form functional homomers\(^6\)–\(^8\).

The crystal structure of a homopentameric β3 receptor provided insights into the molecular architecture of pentameric ligand gated ion channels\(^9\). This study, together with the crystal structure of a GLIC-GABA\(_{\text{A}R}\) α1 subunit chimera\(^10\), and cryo-electron microscopy structures revealed detailed pictures of the extracellular domain and associated GABA binding loops at the β + /α− subunit interface, the central anion-conducting pore region in the TMD, potential locations of the channels activation and desensitisation gates and locations of drug binding sites\(^9,11\)–\(^14\).

Homomeric β3 receptors are lacking the β + /α− subunit interface and are therefore not sensitive to GABA. However, these receptors share properties with heteropentamers such as picrotoxin-sensitivity and contain binding determinants for histamine, propofol, pento barbital and other ligands, which makes them interesting research subjects\(^7,15\)–\(^19\).

The receptors subunit composition affects the kinetics of chloride currents (I\(_{\text{GABA}}\)), the potency of their agonists and the pharmacological properties\(^20\). GABA\(_{\text{A}R}\)s are targets for many drugs (such as benzodiazepines, barbiturates, propofol and volatile anaesthetics) that change I\(_{\text{GABA}}\) in a subunit-specific manner\(^21,22\).

Subunit-specificity is also a hallmark of GABA\(_{\text{A}R}\) modulation by protons. Acidification of the external solution differentially potentiates I\(_{\text{GABA}}\) through αβ1 or αβ2-receptors\(^26\). Hence, I\(_{\text{GABA}}\) potentiation is abolished if this residue on the β2 subunit in the TM is substituted (H267A)\(^20\). Site-specific mutagenesis later revealed that a lysine residue, K279 in the β subunit TM2–TM3 linker, is important for I\(_{\text{GABA}}\) modulation by alkaline pH\(^23\).

Fig. 1 Proton-induced currents (I\(_{\text{H}1}\)) through homomeric GABA\(_{\text{A}R}\) β1(S265N), β2 and β3 receptors induced at a holding potential of -70 mV from pH of 9. a, c Representative I\(_{\text{H}1}\) through β3 (human, rat), β1 and β2 subunit homomers elicited by rapidly switching the pH from 9 to the indicated values. b, d Normalized pH-response curve of I\(_{\text{H}1}\) through β3 homomers (pH\(_{50}\) = 6.09 ± 0.2, n\(_{H1}\) = 1.25 ± 0.37), β2 homomers (pH\(_{50}\) = 6.42 ± 0.01, n\(_{H1}\) = 1.25 ± 0.08), β1(S265N) homomers (pH\(_{50}\) = 6.03 ± 0.1, n\(_{H1}\) = 1.45 ± 0.21) receptors. The data are presented as mean values ±SEM, n = 5. The insets in (b) illustrate block of baseline current (left) and inhibition of I\(_{\text{H}1}\) (right).
inward currents (I_{H(β)}) (Fig. 1a) whose amplitudes increased with increasing proton concentration.

Expression of GABAA_β3 subunits in Xenopus oocytes resulted at pH = 7.4 in significant base line currents that were blocked by picrotoxin (Fig. 1b, inset). Raising the pH of the external solution to pH 9 reduced these currents significantly and pH 9 was thus used for most recordings of base line currents.

I_{H(β3)} activated with half-maximal activation (pH_{50}) of 6.09 ± 0.2 (β3 human) and 6 ± 0.01 of (β3 rat) and saturation at pH 5 (see Fig. 1a for typical current recordings and Fig. 1b for pH-response curves). Like baseline currents, proton-activated currents were inhibited by picrotoxin (Fig. 1b, inset). The current reversal potential indicates that chloride ions are the main charge carrier (Supplementary Fig. 1).

Proton-gated ion channel were also formed in mammalian cells. Making use of planar whole-cell patch clamp of CHO cells expressing human β3 GABAAR we observed pH-induced currents activating with a pH_{50} of 6.03 ± 0.19 (Supplementary Fig. 2).

Subsequent experiments revealed that other β subunits also form proton-gated channels. Figure 1c (left) illustrates similar activation of I_{H(β2)} at pH < 7 with pH_{50} 6.23 ± 0.13 (β2). However, large picrotoxin-sensitive baseline currents of up to several µA (at pH 7.4) in oocytes expressing wild type β1 subunits first prevented activation of I_{H(β1)}. These currents were reduced by shifting the pH to 9 but even under these conditions were too large for inducing I_{H(β1)}. We therefore made use of mutation S265N that is known to reduce constitutive activity of β1 homomers27. I_{H(β1(S265N))} activated at pH < 7.5 with pH_{50} of 6.03 ± 0.1 and served further as tool mutation to study I_{H(β1)} (Fig. 1c, d).

Functional properties of proton-activated chloride channels. Proton induced chloride currents through β3 homomers activate and desensitise with a slower time course than I_{GABA}, through heteropentameric α1β3 receptors. This is exemplified in Fig. 2a, b comparing current kinetics of I_{H(β3)} and I_{GABA} (α1β3 subunit heteropentamers) at saturating agonist concentrations (pH 5 and 100 µM GABA (pH_{GABA} 7.2)). Mean time to peak were 1.52 ± 0.06 s (I_{H(β3)}) and 0.63 ± 0.08 s (I_{GABA}) (p < 0.05, n = 5) and correspond mean desensitisation half-times amounted 48.65 ± 5.92 s and 32.16 ± 2.77 (p < 0.05, n = 5), respectively.

The steady-state desensitization of β homomers occurs with comparable mid points (pH of half-maximal desensitisation for β1(S265N) at 6.4 ± 0.02, β2 at 6.97 ± 0.06 and β3 at 6.6 ± 0.15, Fig. 2d). Superimposed activation and desensitisation curves illustrate that at a physiological pH a significant fraction of these channels is expected to sojourn in an activated non-desensitised state. This finding is in line with the observed picrotoxin sensitive “baseline” or “leak” currents at pH 7.4 in oocytes expressing β3 homomers (Fig. 1b, inset). A decrease in extracellular pH from 7.2 to 6.5 induces significant I_{H(β)} (exemplified for β3 homomers Fig. 2c).

Proton-activated currents were, however, also observed in oocytes expressing heteropentameric receptors comprising either α1β1, α1β2 or α1β3 subunits (Supplementary Fig. 3). pH-dependent activation of these currents occurred with similar kinetics and pH dependence as I_{H(β3)} (Supplementary Fig. 3) suggesting current flow through β homomers that are formed in addition to the heteropentamers. I_{H(α1β3)} activated with pH_{50} values of pH_{50} = 5.76 ± 0.04, n_{1} = 1.36 ± 0.11 (α1β1), pH_{50} = 6.14 ± 0.09, n_{1} = 1.09 ± 0.14 (α1β2) and pH_{50} = 6.06 ± 0.03, n_{1} = 1.73 ± 0.14 (α1β3).

Subunit concatenation (α1-β2/β2-α1-β2, α1-β3/β3-α1-β3 and α1-β3-α1-γ2-β3) prevented both the formation of β homomers and at the same time activation of I_{H(β)} (Supplementary Fig. 4).

Pivotal role of H267 in proton activation of β3 homomers. In heterologous GABAA receptors allosteric modulation of I_{GABA} by protons is largely attributed to protonation of a single histidine (H267 Fig. 3a26). In view of the strong effect of mutation H267A that completely prevents allosteric pH modulation of I_{GABA}26 it was tempting to analyse the impact of this residue on proton activation of β homomers.

As illustrated in Fig. 3b mutation H267A completely abolished activation of I_{H(β)} in β3 human homomers. Instead, switching the pH from 9.0 to 5 induced a reversible and pH-dependent inhibition of the baseline current. These data indicate that protonation of histidine in position 267 is not only essential for allosteric pH modulation of heteromeric GABAAR receptors but simultaneously has a key role in direct activation of β homomers by protons.

Structural analysis of the human homomeric GABAAR β3 receptor26 identified the interaction of H267 with a glutamate in position 270 at the neighbouring subunit. The motif HxxE is specific to β subunits and absent in other GABAAR receptor subunits (Fig. 3c). The authors conclude that a ring of salt bridges involving H267 and E270 from adjacent M2 helices contributes to stabilization of the extracellular portion of the pore in GABAAR-Rβ3cryst (see9 for details). To further investigate this interaction, we introduced several point mutations into position E270 of β3 subunits. However, mutation of E270 to alanine and glutamine resulted in non-functional channels. Furthermore, exchanging H267 and E270 (resulting in double mutant H267E-E270H) or other substitutions of H267 (H267K, H267R) did not lead to formation of functional homomers.

To further investigate the role of H267 (Fig. 3a) in proton activation, we performed 2 µs long MD simulations of the GABAAR receptor, carrying a protonated (GABAAR-prot) and deprotonated (GABAAR-deprot) form of H267, respectively. Protonation caused H267 to contact the residue E270 of the neighbouring subunit via hydrogen bonds (Fig. 3d). For GABAAR-prot, a substantial fraction of the simulation frames displayed 5 and more hydrogen bonds between these residues (Fig. 3d), which induced the formation of a ring-like structure at the apex of the transmembrane domain (TMD) of the pore (Fig. 3e) (see also9). This interaction was drastically reduced for GABAAR-deprot, which features 0–2 hydrogen bonds in most of the simulation frames and hence lacks the ring pattern (Fig. 3d, e).

Transfer of proton sensitivity to a ρ1 subunit. Further evidence for a crucial role of H267 - E270 interaction in proton activation was obtained when the corresponding residues of β3 subunits were inserted into a ρ1 subunit. Homomeric receptors formed of ρ1 subunits are not activated by protons (Fig. 4a). Conversion of both residues (G331H-A334E) resulted, however, in transfer of proton sensitivity with a comparable pH_{50} of μmol ± 0.1 (ρ1(G331H-A334E)) (Fig. 4c, d) while the sole transfer of the histidine (G331H) was insuficient (Fig. 4b).

Conformational changes induced by protonation of H267. In order to investigate further effects of H267 protonation on channel behaviour, we analysed the Cl- density, the pore radius and the hydrophobicity of the pore-facing residues in the two simulations (Fig. 5). The most striking difference between the two systems can be seen for the Cl- density. While GABAAR-prot is well-populated with Cl- nearly throughout the entire pore, we see an almost complete depletion of the Cl- density in the TMD of GABAAR-deprot. Another difference can be seen in the hydrophobicity of the pore-facing residues. Especially the region around and below the desensitisation gate is more hydrophobic.
in case H267 is deprotonated. Furthermore, the protonation state of H267 affects the pore diameter of the TMD. For GABAA-prot we see a narrowing of the channel, stretching from the apex of the TMD, where H267 is located, to the desensitization gate at the lower end of the pore. The difference in pore radius between the two systems reaches up to ~2 Å, observed below H267 and at the desensitization gate.

Picrotoxin sensitive, proton-induced currents in Jurkat cells. Two cell systems were studied to gain first insights into a potential physiological role of IH(βX). In Jurkat cells (an immortalised line of human T lymphocyte cells) that are known to express predominantly β-subunits of GABAA receptors28,29, we observed a fast activating (rapidly desensitising) inward current when pH was shifted from 7.2 to 5.0 (Supplementary Fig. 5a). This current was partially blocked by amiloride (200 µM) suggesting current through ASIC30. A remaining current in the presence of amiloride activated with slower kinetics and was completely blocked by 200 µM picrotoxin suggesting IH(βX) through proton-sensitive β-homopentamers in these cells. GABA (100 µM) did not activate chloride currents (Supplementary Fig. 5a, right panel) which is in line with the higher RNA levels of β subunits compared to those of α subunits28,29.

In iPS cell-derived GABANeurons shifting the pH to 5.0 also induced a rapidly activating ASIC-like current that was, however, completely inhibited by amiloride (200 µM, Supplementary Fig. 5b). Activation of IGABA in these cells indicated expression of heteromeric GABAA receptors which is known to reduce the formation of homooligomeric receptors31,32 and would explain the absence of residual picrotoxin-sensitive currents.

Discussion
In this study we identified a previously unknown function of GABAA receptor beta subunits – the formation of proton-activated homomeric anion channels (Fig. 1, Supplementary Fig. 2). Considering the many roles of pH in physiological processes, proton-gated anion channels can readily be envisioned to play many roles in rapid responses to changes in pH, or even to contribute to pH stabilization by way of their bicarbonate conductance.
\( I_{H(\beta x)} \) of all three receptor subtypes activate in *Xenopus* oocytes at \( pH < 7 \) with \( \text{pH}_{50s} \) ranging from 6.03 ± 0.1 (\( \beta 1(S265N) \)) to 6.23 ± 0.02 (\( \beta 2 \)) (Fig. 1a–d). Proton-gated anion channels with similar \( pH \) sensitivity were also formed in mammalian cells expressing \( \beta 3 \) subunits (Supplementary Fig. 2). It is currently not clear if the observed left shift of \( I_{H(\beta 1(S265N))} \) activation curve and its steeper slope result from mutation \( S265N \) or represent higher proton sensitivity of \( \beta 1 \) homomers (Fig. 1d). Acidiﬁcation at physiological \( pH \) (e.g. \( pH \) changes from 7.2 to 6.5) activate \( I_{H(\beta 3)} \) which highlights the high proton sensitivity of these channels (illustrated for \( I_{H(\beta 3)} \) in Fig. 2c, see also Supplementary Fig. 2).

| Gene      | Accession | Sequence                        | Length |
|-----------|-----------|---------------------------------|--------|
| GBRA1_HUMAN | 251       | SVPARTVFGVTTVLMTTTLISARNSLPKVE  | 280    |
| GBRA1_HUMAN | 246       | ASAARVALGTTVLMTTSTHLRETPKI     | 275    |
| GBRA1_HUMAN | 245       | ASAARVALGTTVLMTTINTHLRETPKI    | 274    |
| GBRA1_HUMAN | 246       | ASAARVALGTTVLMTTINTHLRETPKI    | 275    |
| GBRA2_HUMAN | 300       | AVPARVSLGTTVLMTTLSTIAKSLPKVE   | 329    |
| GBRA1_HUMAN | 310       | AVPARVPLGITTVLMSITGTVNASMPRV   | 339    |

![Diagram](image1)

**Figure 1:** (a) Schematic representation of the \( \beta 3 \)-H267A mutant. (b) Current-voltage relationships for different \( pH \) conditions. (c) Sequence alignment of \( \beta 1, \beta 2 \), and \( \beta 3 \) subtypes. (d) Frequency distribution of the number of hydrogen bonds between H267 and E270. (e) Structural representation of the \( \beta 3 \)-H267A mutant.
Fig. 3 Loss of function (β3(H267A)) mutation in GABA_A subunit homomers and a hydrogen bond formation between H267 and E270. a Location of H267 in the pore region of the crystal structure of β3 homomers (the position of H267 is indicated with violet spheres). b Mutation H267A in the human subunits β3 subunit prevented activation of I_{H(β3H267A)} but, instead, induced pH-dependent inhibition of large baseline currents (upper traces). Dotted line illustrates zero current level. Lower traces illustrate retained sensitivity for other agonists (100 μM pentobarbital, 1 mM histamine and 100 μM bicuculline). c Sequence alignment of the transmembrane M2 α-helix of α1, β1-3 (human), γ2 (human) and μ1 (rat) subunits (segment does not differ between rat and human). The location of H267 and E270 in β subunits and the corresponding homologous positions in the β1 subunit (double mutant p(G331H-A334E)) are illustrated. d Histogram of the number of hydrogen bonds between protonated and deprotonated H267 and E270. e Snapshots of the ring structure observed in the MD runs.

\[ a \]
\[ b \]
\[ c \]
\[ d \]
\[ e \]

Fig. 4 Gain of function mutations in GABA_A ρ1(G331H-A334E) subunit homomers. a Homomers of wild type ρ1 subunits are insensitive to protons but activated by GABA (10 μM, pH 7.2). b, c A single point mutation ρ1(G331H) was ineffective while double mutation ρ1(G331H-A334E) transferred proton sensitivity. d Normalized peak current values of \( I_{H(ρ1G331H-A334E)} \) at indicated pH fitted to the Hill equation (\( \rho_{H2} = 5.66 \pm 0.1, n_H = 0.7 \pm 0.12 \)). The data are presented as mean values ±SEM, n = 9. pH changes in all experiments from 9 to indicated values.

Intriguing by the key role of H267 (Fig. 3a) in allosteric potentiation of GABA_A receptors and structural data suggesting the formation of salt bridges between H267 and E270 at β3 subunit interfaces we substituted H267 in β homomers by alanine and investigated proton activation. Interestingly, H267A not only eliminated proton-induced receptor activation but additionally induced large picrotoxin-sensitive baseline currents that were blocked by protons, indicating the existence of molecular mechanisms for proton activation and inhibition in β homomers (Fig. 3b).

Substitutions of E270 by alanine or glutamine, exchanging H267 and E270 (mutant H267E-E270H) or other substitutions of H267 (H267K, H267R) did not lead to formation of functional channels, which prevented more detailed analyses of this interaction.

However, transfer of both residues to a proton insensitive ρ1 subunit resulted in gain of function, indicating a potential key role of the interaction of these residues in proton-induced gating (Fig. 4a, b).

In order to elucidate the effect of H267 protonation in proton-dependent gating, we conducted MD simulations of the human GABA_A β3 homopentamer (PDB: 4COF) exhibiting different protonation states at position 267 (Fig. 3a). Our simulations with GABA_A-prot unravel a ring-like structure at the apex of the transmembrane domain (TMD), which is stabilized by a formation of hydrogen bonds between protonated H267 and E270 of adjacent subunits (Fig. 3d, e). This interaction is accompanied by a narrow transmembrane pore with especially small radii at the potential desensitization gate.9,36 The conformations adopted by GABA_A β3_prot in our simulations are therefore reminiscent of the initial crystal structure 4COF9, which represents the agonist-bound, desensitized state of the receptor. The dual-gate model by Gielen and Corringer36 proposes that desensitization of GLICs is facilitated by a closing of the cytoplasmic end of the TMD. According to this model, our simulations with GABA_A β3_prot are likely to represent a post-activated, desensitized state. Importantly, comparison of inhibitory Cys-loop receptors37 in putative shut and open conformations identified a dilation at the cytoplasmic end of the pore to be associated with a closed channel conformation. Similarly, deprotonation of H267 causes a widening of the TMD and a relief of the constriction at the desensitization gate in our simulations.

Our computational investigations suggest an involvement of H267 protonation in switching between different gating conformations, and thus corroborate our experimental findings of an activation of GABA_A receptors by protonation.

Our simulations with GABA_A β3_deprot displayed an exclusion of Cl− from the TMD, while GABA_A β3_prot showed sizeable Cl− densities in both the extracellular and transmembrane domain. This finding further supports our experimental results of a receptor activation by protons and is reminiscent of a computational study on the glycine receptor (GlyR)38, which reported Cl− permeation though the transmembrane pore in the open structure, while the same region was cleared from Cl− in the closed conformation. Small differences in the hydrophobicity of the pore lining residues might be partially accountable for the different ion occupancies. However, more sampling, which go beyond the scope of this study, might be needed to make a hydrophobic gate visible.

1_{H(β3)} activate slower than 1_{GABA} with kinetics and proton sensitivities resembling the properties of bacterial GLIC and other member of pentameric bacterial ligand gated chloride channels (Fig. 2a–c, also see13).

Proton-induced activation and steady-state desensitisation revealed a window 1_{H(β3)} in the pH range between 7 and 6 (Fig. 2d). This current corresponds to a picrotoxin-sensitive leak chloride conductance that was first observed in oocytes expressing β1 homomers and later described for other β subunits. Proton-induced currents in oocytes expressing αβ heteropentamers display similar activation curves and pH_{50} as observed for 1_{H(β3)}, and are completely prevented by receptor concatenation (Supplementary Fig. 3, Supplementary Fig. 4). Both observations support the hypothesis that proton-activated currents in oocytes expressing heteropentamers result from the formation of a population of homopentamers35.

Intriguing by the key role of H267 (Fig. 3a) in allosteric potentiation of GABA_A receptors and structural data suggesting the formation of salt bridges between H267 and E270 at β3 subunit interfaces we substituted H267 in β homomers by alanine and investigated proton activation. Interestingly, H267A not only eliminated proton-induced receptor activation but additionally induced large picrotoxin-sensitive baseline currents that were blocked by protons, indicating the existence of molecular mechanisms for proton activation and inhibition in β homomers (Fig. 3b).

Substitutions of E270 by alanine or glutamine, exchanging H267 and E270 (mutant H267E-E270H) or other substitutions of H267 (H267K, H267R) did not lead to formation of functional channels, which prevented more detailed analyses of this interaction.

However, transfer of both residues to a proton insensitive ρ1 subunit resulted in gain of function, indicating a potential key role of the interaction of these residues in proton-induced gating (Fig. 4a, b).

In order to elucidate the effect of H267 protonation in proton-dependent gating, we conducted MD simulations of the human GABA_A β3 homopentamer (PDB: 4COF) exhibiting different protonation states at position 267 (Fig. 3a). Our simulations with GABA_A-prot unravel a ring-like structure at the apex of the transmembrane domain (TMD), which is stabilized by a formation of hydrogen bonds between protonated H267 and E270 of adjacent subunits (Fig. 3d, e). This interaction is accompanied by a narrow transmembrane pore with especially small radii at the potential desensitization gate.9,36 The conformations adopted by GABA_A β3_prot in our simulations are therefore reminiscent of the initial crystal structure 4COF9, which represents the agonist-bound, desensitized state of the receptor. The dual-gate model by Gielen and Corringer36 proposes that desensitization of GLICs is facilitated by a closing of the cytoplasmic end of the TMD. According to this model, our simulations with GABA_A β3_prot are likely to represent a post-activated, desensitized state. Importantly, comparison of inhibitory Cys-loop receptors37 in putative shut and open conformations identified a dilation at the cytoplasmic end of the pore to be associated with a closed channel conformation. Similarly, deprotonation of H267 causes a widening of the TMD and a relief of the constriction at the desensitization gate in our simulations.

Our computational investigations suggest an involvement of H267 protonation in switching between different gating conformations, and thus corroborate our experimental findings of an activation of GABA_A receptors by protonation.

Our simulations with GABA_A β3_deprot displayed an exclusion of Cl− from the TMD, while GABA_A β3_prot showed sizeable Cl− densities in both the extracellular and transmembrane domain. This finding further supports our experimental results of a receptor activation by protons and is reminiscent of a computational study on the glycine receptor (GlyR)38, which reported Cl− permeation though the transmembrane pore in the open structure, while the same region was cleared from Cl− in the closed conformation. Small differences in the hydrophobicity of the pore lining residues might be partially accountable for the different ion occupancies. However, more sampling, which go beyond the scope of this study, might be needed to make a hydrophobic gate visible.
In order to compare the similarity of the three β subunits in the H267 - E270 region, we also examined structures with β1 and β2 subunits. Of these, only heteromeric structures are available39,40. Thus, the individual β subunits were superposed for the best fit of the M2 segment. The amino acids of interest are structurally equivalent, and only slightly differ in sidechain rotamers. Thus, we expect the residues H267 and E270 play a structurally equivalent role in β1 and β2 homopentamers (Supplementary Fig. 6).

We propose that interaction between H267 and E270 might play a crucial role in the direct activation of homomeric GABA$_A$ β3 receptors by protons. Mutational studies on the GLIC channel suggests that proton activation occurs also in GLIC allosterically to the orthosteric site, but at the level of multiple loci11 and we thus cannot exclude that other residues than H267 contribute to proton-induced activation of β3 receptors. Furthermore, additional evidence suggests that other amino acid residues may be involved in proton-induced gating of GLIC where more detailed mechanisms of receptor activation are known42,43. There may be a similar picture to that of the GLIC, which has been described to have two electrostatic triads of amino acid residues in the extracellular domain (structurally highly conserved between pLGIC), which are part of a continuous network governing the gating transitions of the receptor. However, due to the absence of open/closed β3 homomeric receptor structures, it is difficult to speculate about the exact determinants and this question remains open for further investigation. The HxxE motif we describe here is unique to the β subunits (Fig. 3c) and absent in GLIC, and is necessary and sufficient for proton sensitivity as demonstrated by the conversion mutants, irrespective of the details of transduction. While MD simulations of GLIC have been conducted42, a side-by-side comparison with the same method would be needed to get more insight into similarities and differences in the proton interaction sites and transduction mechanisms.

Taken together, our study describes for the first time, that homomeric GABA$_A$ β1, β2 or β3 receptors form proton-gated chloride channels and that appropriate conversion mutants can induce proton gating to ρ1 homopentamers.

β-homomers form readily in recombinant expression systems, their existence in mammalian cells is currently unknown. First evidence for picrotoxin-sensitive I$_{H(\beta)}$ was obtained in Jurkat T cells (Supplementary Fig. 5a). These cells express β-subunits at much higher abundance than a GABA$_B$ subunits28 which would be expected to facilitate formation of β homomers. It is tempting to speculate that hyperpolarizing chloride currents upon acidification counterbalances depolarization via ASIC receptors and thus may have a role in immunomodulation29. However, this warrants further studies. Different activation states of T-cells might lead to variable expression of proton/ histamine sensitive and GABA-sensitive receptors, respectively29,31,32.

In studies on GABA-sensitive iPS cell-derived GABANeurons expressing heteropentamers we did not find evidence for I$_{H(\beta)}$ after 4–6 days in differentiating media (Supplementary Fig. 5b). These data warrant further research as the presence of heteromeric GABA$_A$ receptors in these cells (confirmed by I$_{GABA}$ -Supplementary Fig. 5b) may reduce formation of homooligomeric receptors31,32. It seems plausible that high expression levels of β-subunits are supportive for formation of β-homomers not only in heterologous expression systems. Given the large diversity of neuronal cell types, broad screens would be needed to potentially detect pH sensitive neuron types.

In fact, evidence for the existence of GABA insensitive receptors formed by β-subunits in neuronal preparations was obtained from studies with alternative agonists such as histamine36,18,44. Our results imply that GABA$_A$ β receptors are activated at physiological and pathological pH changes. It is therefore tempting to speculate that such pH sensitive channels formed by ubiquitously expressed subunits in the brain may exist and serve crucial functions under acidic conditions such as stroke and ischaemia where they can enhance neuroprotective hyperpolarization. Intriguingly, in an experimental model of stroke, upregulation of GABRB3 was connected with a putative

---

**Fig. 5 Protonation of H267 effects pore features as well as Cl$^-$ occupancy.** Cl$^-$ density, hydrophobicity of the pore-facing residues and radius of the pore calculated by the channel annotation programme (CHAP67). Right: Pore of β3 GABA$_A$R as detected by CHAP. Black dotted lines indicate the projection of the property curves on the pore. Pore properties on the left are the averaged over 2 µs MD simulation of β3 GABA$_A$R with H267 protonated and deprotonated β3 GABA$_A$R.
neuroprotective role\(^45\). Although macroscopic changes in extracellular pH in the brain are tightly controlled, pH fluctuations in specific micro-domains such as the synapse might be relevant\(^46\). For instance, synaptic vesicles have high proton concentrations (pH 5.7) so vesicle release during high neuronal activity could lower the pH in the synaptic cleft\(^47\). For instance, synaptic vesicles have high proton concentrations (pH 5.7) so vesicle release during high neuronal activity could lower the pH in the synaptic cleft\(^47\).

### Methods

**Animals and animal welfare.** All experiments involving animals were approved by the Austrian Animal Experimentation Ethics Board in compliance with the European convention for the protection of vertebrate animals used for experimental and other scientific purposes ETS no.: 123, which is in line with the EU Directive 2010/63/EU (GZ BMWFU-66.006/0021-V3b/2019). Every possible effort was taken to minimize the number of animals used.

Female *Xenopus laevis* frogs were purchased from NASCO (Fort Atkinson, USA) and kept in groups in temperature-controlled, continuous-flow water tanks (20 ± 1 °C). The temperature in the holding and testing facilities was fixed to 22 ± 2 °C; the humidity ranged between 40-60%; a 12 h light-dark cycle was in place. All compounds were dissolved in stock solutions to 100 mM (histamine in 0.1 N HCl, other compounds in DMSO). Oocytes with maximal current amplitudes >5 μA were discarded to exclude voltage-clamp errors.

Concentration–response curves were generated for different pH and the data were fitted by nonlinear regression analysis using Origin Software (OriginLab Corporation, USA).

Proton-induced currents \(I_{\text{Hh}}\) were normalized to the maximum current at pH range 5–4 (\(I_{\text{Hh,max}}\)) and the concentration–response relationship fitted with the Hill equation:

\[
\frac{I_{\text{Hh}}}{I_{\text{Hh,max}}} = \frac{1}{1 + \left(\frac{[\text{H}^+]}{K_{\text{H}^+}}\right)^n}
\]

where the \(pH_{50}\) represents the pH inducing 50% of the maximal current evoked by a saturating concentration and \(nH\) is the Hill coefficient. Each data point represents the mean ± SEM from at least 5 oocytes and 2–3 oocyte batches.

**Cell culture and transfection of cells.** Jurkat E 6.1 cells (Sigma Aldrich) were grown at a density of 5x10^5 cells/ml in RPMI 1640 medium supplemented with 10% foetal bovine serum (FBS) at 37 °C in a humidified atmosphere at 5% CO2. Every 2 days the cells were split when 80% confluence was reached. CHO cells (Sigma Aldrich) heat-inactivated in DMEM containing 10% horse serum (HS), 100 units/mL of penicillin and 100 μg/mL of streptomycin. Cells were grown in culture flasks at 37 °C in a 5% CO2-humidified incubator. Cells were then grown to allow to 70-80% confluence before transfection. Plasmid coding β3 (human) GABA\(_\text{A}\),β2 were transfected into CHO cells with TurboFect (ThermoFisher) transfection reagent according to the manufacturer’s protocol. Electrophysiological recordings were performed 24–48 h after transfection at room temperature.

**iPS cell-derived iCell GABAergic neurons culture and harvesting.** Frozen stocks of iCell GABA Neurons were obtained from CTD containing at least 4 million cells per well (catalogue number: R1013). Cells were thawed as per the manufacturer’s instructions at a density of at least 67,500 cells/cm\(^2\) in a 6-well plate. Before planting the cells, the plates were coated with poly-L-ornithine/laminin coating. In brief 6-well plates were coated with 0.01% poly-L-ornithine (Sigma Aldrich) for 1 h at room temperature; this was then removed, and the dishes were washed twice with sterile distilled water and coated with a 3.3-μg/ml solution of laminin (Sigma Aldrich). The dishes were incubated in a 37 °C incubator for at least 1 h. The laminin was aspirated shortly before addition of the cell suspension. A vial containing at least 4 million iCell GABA Neurons was placed in a 37 °C water bath for exactly 3 min. The cells were transferred to a 50-ml Falcon tube, and 1 ml Complete Maintenance Media (containing iCell Neurons medium supplement; CDI) was added slowly (dropwise) to the cells. A further 8 ml Complete Maintenance Media (CDI) was added slowly (dropwise) to the cells in the centrifuge tube. The cells were then centrifuged and pelleted once at a density of 70,000–100,000 cells/cm\(^2\). The cells were kept in a 37 °C incubator (5% CO2). After 24 h, media were exchanged for fresh Complete Maintenance Media (100% exchange). Cells could then be kept in a 37 °C incubator (5% CO2) for up to 4 weeks. A 50% media exchange was made every 2–3 days. Cells were cultured for at least 4 days and were typically used on the automated patch-clamp platform 7–10 days after plating. To harvest the cells, media was removed and the iCell GABA Neurons were washed with 2 ml phosphate-buffered saline (PBS) containing Ca\(^{2+}\)/Mg\(^{2+}\). After this, prewarmed (37 °C) Accutase (ThermoFisher) was added. The cells were incubated in Accutase for 5 min at 37 °C. The majority of the Accutase was then removed, leaving a thin film over the bottom of the well. Then, 0.5 ml Complete Maintenance Media were added in each well and pipetted up and down to ensure most of the cells were removed from the bottom of the well. An external recording solution (see below) was then added to the cell suspension in a 1:1 ratio before recording.

**Planar patch clamp of mammalian cells.** An automated patch clamp system (SynchroPatch 384, NIANON Technologies) for ionic current measurements on suspensions of CHO cells, Jurkat cells or iCell GABA Neurons was kindly provided by ChanPharm GmbH (Vienna, Austria). The recording was carried out at room temperature (22–24 °C) using PatchControl 384 and DataControl 384 (NION Technologies) software with an external solution containing 140 mM NaCl, 4 mM KCl, 2 mM CaCl\(_2\), 1 mM MgCl\(_2\), 5 mM glucose, and 10 mM HEPES (pH 7.4 with NaOH). Proton-induced currents were recorded in MES buffer (140 mM NaCl, 4 mM KCl, 2 mM CaCl\(_2\), 1 mM MgCl\(_2\), 5 mM glucose and 10 mM MES pH 7.4) equilibrated at the appropriate pHs with 1 M HCl. Microelectrodes were filled with 3 M KCl and had resistances between 1 and 3 MΩ.

---

**Fast perfusion during voltage clamp experiments.** External solution and drugs were applied by means of a fast perfusion system; drug or control solutions were applied as means of a TECAN Miniprep 60 enabling automation of the experiments (ScreeningTool, npi electronic, Tamm, Germany). To elicit chloride currents, the chamber was perfused with GABA- or solutions of different pH respectively, at a volume rate of 200 μL/s\(^50\).

All compounds were dissolved in stock solutions to 100 mM (histamine in 0.1 N HCl, other compounds in DMSO). Oocytes with maximal current amplitudes >5 μA were discarded to exclude voltage-clamp errors.

Concentration–response curves were generated for different pH and the data were fitted by nonlinear regression analysis using Origin Software (OriginLab Corporation, USA).
2. Akinci, M. K. & Schofield, P. R. Widespread expression of GABA receptor subunits in peripheral tissues. *Neurosci. Res.* 35, 145–153 (1999).
3. Macdonald, R. L. & Olsen, R. W. GABA receptor channels. *Annu. Rev. Neurosci.* 17, 569–602 (1994).
4. Sigel, E. & Steinmann, M. E. Structure, function, and modulation of GABA receptors. *J. Biol. Chem.* 287, 40224–40231 (2012).
5. Olsen, R. W. & Sieghart, W. International union of pharmacology. LXX. Subtypes of gamma-aminobutyric acid(A) receptors: classification on the basis of subunit composition, pharmacology, and function. *Updat. Pharm. Rev. 60*, 243–260 (2008).
6. Amin, I. & Weiss, D. S. Homeric rho 1 GABA channels: activation properties and domains. *Recept Channels 2*, 227–236 (1994).
7. Wooltorton, J. R., Moss, S. J. & Smart, T. G. Pharmacological and physiological characterization of murine homomeric beta3 GABA(A) receptors. *Eur. J. Neurosci.* 9, 2225–2235 (1997).
8. Davies, P. A., Kirkness, E. F. & Hales, T. G. Modulation by general anesthetics of rat GABAA receptors comprised of alpha 1 beta 3 and beta 3 subunits expressed in human embryonic kidney 293 cells. *Br. J. Pharm.* 120, 899–909 (1997).
9. Miller, P. S. & Aricescu, A. R. Crystal structure of a human GABAA receptor. *Nature 512*, 270–273 (2014).
10. Laverty, D. et al. Crystal structures of a GABA-receptor chimera reveal new endogenous neurosteroid-binding sites. *Nat. Struct. Mol. Biol. 24*, 977–985 (2017).
11. Corringer, P.-J. et al. Structure and pharmacology of pentameric receptor channels: from bacteria to brain. *Structure 20*, 941–956 (2012).
12. Laverty, D. et al. Cryo-EM structure of the human alpha1beta2 GABA receptor in a lipid bilayer. *Nature 565*, 516–520 (2019).
13. Malisius, S. et al. GABAA receptor signaling mechanisms revealed by structural pharmacology. *Nature 565*, 454–459 (2019).
14. Zhu, S. et al. Structure of a human synaptic GABAA receptor. *Nature 559*, 67–72 (2018).
15. Cestari, I. N., Uchida, I., Li, L., Burt, D. & Yang, J. The agonistic action of pentobarbital on GABAA beta-subunit homomeric receptors. *Neuroreport 7*, 943–947 (1996).
16. Yip, G. M. S. et al. A propofol binding site on mammalian GABAA receptors identified by photo-labelling. *Nat. Chem. Biol. 9*, 715–720 (2013).
17. Saras, A. et al. Histamine action on vertebrate GABAA receptors directs channel gating and potentiation of GABA responses. *J. Biol. Chem.* 283, 12833–12841 (2008).
18. Hoppel, P. et al. Mutagenesis and computational docking studies support the existence of a histamine binding site at the extracellular beta3+beta2-interface of homologomic beta3 GABAA receptors. *Neuropharmacology 108*, 252–263 (2016).
19. Nakane, T. et al. Single-particle cryo-EM at atomic resolution. *Nature 587*, 152–156 (2020).
20. Korpi, E. R., Grüneder, G. & Lüddens, H. Drug interactions at GABAA receptors. *Prog. Neurobiol. 6*, 113–159 (2002).
21. Sigel, E. Mapping of the benzodiazepine recognition site on GABA(A) receptors. *Curr. Top. Med Chem.* 2, 833–839 (2002).
22. Jayakar, S. S. et al. Positive and negative allosteric modulation of an alpha1beta3gamma2 GABAA receptor by binding to a site in the transmembrane domain at the γ–β2–interface. *J. Biol. Chem.* 290, 23432–23446 (2015).
23. Puthenkalam, R. et al. Structural studies of GABAA receptor binding sites: which experimental structure tells us what? *Front. Mol. Neurosci.* 9, 44 (2016).
24. Krishke, B. J., Moss, S. J. & Smart, T. G. Homomeric beta1 gamma2 GABA-A receptor: evolution of pharmacological and physiological properties. *Mol. Pharmacol. 49*, 494–504 (1996).
25. Wilkins, M. E., Hosie, A. M. & Smart, T. G. Proton modulation of recombinant GABAA receptors: influence of GABA concentration and the β subunit TM2–TM3 domain. *J. Physiol.*, 567, 365–377 (2005).
26. Wilkins, M. E., Hosie, A. M. & Smart, T. G. Identification of a beta subunit TM2 residue mediating proton modulation of GABA type A receptors, *J. Neurousci. 22*, 5328–5333 (2002).
27. Miko, A., Werby, E., Sun, H., Healey, I. & Zhang. L. A TM2 residue in the betal subunit determines spontaneous opening of homomeric and heteromeric gamma-aminobutyric acid-gated ion channels. *J. Biol. Chem.* 279, 22833–22840 (2004).
28. Dionisio, L., Arias, V., Bouzat, C. & Eand, M. del C. GABAA receptor plasticity in Jurkat T cells. *Biochimie* 95, 2376–2384 (2013).
29. Mendu, S. K., Bhandage, A., Jin, Z. & Birnin, B. Different subtypes of GABA-A receptors are expressed in human, mouse and rat T lymphocytes. *PLoS One 7*, e42959 (2012).
30. Waldmann, R., Champignon, G., Bassilana, F., Heutreax, C. & Ladzunski, M. A proton-gated cation channel involved in acid-sensing. *Nature 386*, 173–177 (1997).
31. Sieghart, W. & Sperk, G. Subunit composition, distribution and function of GABA(A) receptor subtypes. *Carr. Top. Med. Chem. 2*, 795–816 (2002).
32. Angelotti, T. P. & Macdonald, R. L. Assembly of GABAA receptor subunits: alpha 1 beta 1 and alpha 1 beta 1 gamma 25 subunits produce unique ion channels with diisummar single- channel properties. *J. Neurosci.* 13, 1429–1440 (1993).
33. Lahe, K. T., Ghosh, B. & Craikowski, C. Macroscopic kinetics of pentameric ligand gated ion channels: comparisons between two prokaryotic channels and one eukaryotic channel. *PLoS One* 8, e803322 (2013).
34. Davies, P. A., Kirkness, E. F. & Hele, T. G. The rat β1-subunit of the GABAA receptor forms a picrotoxin-sensitive anion channel open in the absence of GABA. *FEBS Lett.* 257, 377–379 (1989).
35. Boileau, A. J., Baur, R., Sharkey, L. M., Sigel, E. & Czajkowski, C. The relative amount of crRNA coding for gamma2 subunits affects stimulation by benzodiazepines in GABA(A) receptors expressed in Xenopus oocytes. *Neuropharmacology* **43**, 695–700 (2002).

36. Gielen, M. & Corringer, P.-J. The dual-gate model for pentameric ligand-gated ion channels activation and desensitization. *J. Physiol.* **596**, 1873–1902 (2018).

37. Gielen, M., Thomas, P. & Smart, T. G. The desensitization gate of inhibitory Cys-loop receptors. *Nat. Commun.* **6**, 6829 (2015).

38. Rao, S., Klesse, G., Lynch, C. I., Tucker, S. J. & Sansom, M. S. P. Molecular simulations of hydrophobic gating of pentameric ligand-gated ion channels: insights into water and ions. *J. Phys. Chem. B* **125**, 981–994 (2011).

39. Phulera, S. et al. Cryo-EM structure of the benzodiazepine-sensitive α1β2γ2 trimeric GABAAR receptor in complex with GABA. *Elife* **7**, e39835 (2018).

40. Kim, J. I. et al. Shared structural mechanisms of general anaesthetics and benzodiazepines. *Nature* **585**, 303–308 (2020).

41. Nemecz, Á. et al. Full mutational mapping of titratable residues helps to identify proton-sensors involved in the control of channel gating in the Glueoebacter violaceus pentameric ligand-gated ion channel. *PLoS Biol.* **15**, e2000470 (2017).

42. Rovšek, A. et al. Dynamic closed states of a ligand-gated ion channel captured by cryo-EM and simulations. *Life Sci. Alliance* **e20210110** (2021).

43. Hu, H. et al. Electrostatics, proton sensor, and networks governing the gating transition in GLIC, a proton-gated pentameric ion channel. *Proc. Natl Acad. Sci. U.S.A.* **115**, E11721–E11731 (2018).

44. Hatton, G. I. & Yang, Q. Z. Ionotropic histamine receptors and H2 receptors modulate supraoptic anterior pituitary excitability and dye coupling. *J. Neurosci.* **21**, 2974–2982 (2001).

45. Kaneko, Y., Pappas, C., Tajiri, N. & Borlongan, C. V. Oxytocin modulates GABAAR subunits to confer neuroprotection in stroke in vitro. *Sci. Rep.* **6**, 35659 (2016).

46. Miesenböck, G., De Angelis, D. A. & Rothman, J. E. Visualizing secretion and fluorescing fluorescent proteins. *Nature* **394**, 192–195 (1998).

47. Xu, E. et al. Intra-islet insulin suppresses glucagon release via GABA-GABAAR receptor system. *Cell Metab.* **3**, 47–58 (2006).

48. Ma, X. et al. Activation of GABAAR receptors in colon epithelium exacerbates acute colitis. *Front. Immunol.* **9**, 987 (2018).

49. Takano, K. et al. Characteristic expressions of GABAAR receptors and GABA producing/transporting molecules in rat kidney. *PLoS One* **9**, e105835 (2014).

50. Yocum, G. T. et al. Targeting the γ-Aminobutyric acid A receptor α4 subunit in airway smooth muscle to alleviate bronchoconstriction. *Am. J. Respir. Cell Mol. Biol.* **54**, 546–553 (2016).

51. Jin, Z., Mendu, S. K. & Birnir, B. GABA is an effective immunomodulatory molecule. *Amino Acids* **45**, 87–94 (2013).

52. Grinstein, S., Swallow, C. J. I. & Rotstein, O. D. Regulation of cytoplasmic pH in phagocytic cell function and dysfunction. *Clin. Biochem.* **24**, 241–247 (1991).

53. Khor, S. et al. Pharmacological properties of GABAAR receptors containing gamma1 subunits. *Mol. Pharmacol.* **69**, 640–649 (2006).

54. Baumann, S. W., Baur, R. & Sigel, E. Forced subunit assembly in alpha1beta2gamma2 GABAAR receptors. Insight into the absolute arrangement. *J. Biol. Chem.* **277**, 46200–46205 (2002).

55. Baburin, I., Beil, S. & Hering, S. Automated fast xenopsius on xenopus oocytes for drug screening. *Phylog. Arch.* **453**, 117–123 (2006).

56. Abraham, M. J. et al. GROMACS: High performance molecular simulations through multi-level parallelism from laptops to supercomputers. *SoftwareX* **1–2**, 19–25 (2015).

57. Lee, J. et al. CHARM-GUI membrane builder for complex biological membrane simulations with glycolipids and lipopolysaccharids. *J. Chem. Theory Comput.* **15**, 775–786 (2019).

58. Jo, S., Kim, T., Iyer, V. G. & Im, W. CHARM-GUI: a web-based graphical user interface for CHARM. *J. Comput. Chem.* **29**, 1859–1865 (2008).

59. Berger, O., Edholm, O. & Fahim, F. Molecular dynamics simulations of a fluid bilayer of dipoles from phosphatidylcholine at full hydration, constant pressure, and constant temperature. *Biophysical J.* **72**, 2002–2013 (1997).

60. Cordomi, A., Caltabiano, G. & Pardo, L. Membrane protein simulations using AMBER force field and berger lipid parameters. *J. Chem. Theory Comput.* **8**, 948–958 (2012).

61. Hornak, V. et al. Comparison of multiple Amber force fields and development of improved protein backbone parameters. *Proteins* **65**, 712–725 (2006).

62. Berendsen, H. J. C., Grigera, J. R. & Straatsma, T. P. The missing term in effective pair potentials. *J. Phys. Chem.* **91**, 6269–6271 (1987).