Dual activation of neuronal G protein-gated inwardly rectifying potassium (GIRK) channels by cholesterol and alcohol

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Activation of G protein-gated inwardly rectifying potassium (GIRK) channels leads to a hyperpolarization of the neuron’s membrane potential, providing an important component of inhibition in the brain. In addition to the canonical G protein-activation pathway, GIRK channels are activated by small molecules but less is known about the underlying gating mechanisms. One drawback to previous studies has been the inability to control intrinsic and extrinsic factors. Here we used a reconstitution strategy with highly purified mammalian GIRK2 channels incorporated into liposomes and demonstrate that cholesterol or intoxicating concentrations of ethanol, i.e., >20 mM, each activate GIRK2 channels directly, in the absence of G proteins. Notably, both activators require the membrane phospholipid PIP$_2$ but appear to interact independently with different regions of the channel. Elucidating the mechanisms underlying G protein-independent pathways of activating GIRK channels provides a unique strategy for developing new types of neuronal excitability modulators.

Many modulatory neurotransmitters in the brain, such as dopamine, acetylcholine, serotonin and GABA, inhibit neuronal activity by stimulating G protein-coupled receptors (GPCRs) that couple to G protein-gated inwardly rectifying (GIRK, also referred to as Kir3) channels. The activation of GIRK channels hyperpolarizes the neuron's membrane potential, and thereby reduces action potential firing. GIRK channels are widely expressed in the brain, existing as predominantly heterotetramers of three different GIRK channel subunits, GIRK1, GIRK2 and/or GIRK3, or as homotetramers of the GIRK2 subunit. GIRK channels have been implicated in the pathophysiology of several human neurological disorders. Genome-wide association studies (GWAS) of people with schizophrenia have identified single nucleotide polymorphisms (SNPs) in the $Kcnj3$ (GIRK1) gene. SNPs in $Kcnj6$ (GIRK2) have been linked to alcohol and nicotine dependence, reduced opioid withdrawal and increased opioid requirement for analgesia. Recently, a mutation in $Kcnj6$ has been proposed to contribute to Keppen-Lubinsky syndrome, a severe developmental disorder with cognitive deficits.

Prior work on GIRK channels has focused on delineating the G protein-dependent pathway for activation of GIRK channels. Following stimulation of GPCRs that couple to pertussis toxin-sensitive G$_i/o$ G proteins, the G protein G$\beta\gamma$ subunits bind directly to the channel, and induce a conformational change that opens the channel in a manner that depends on the membrane phospholipid PI(4,5)P$_2$ (referred to as PIP$_2$). Recently, this interaction was confirmed in an atomic resolution structure based on x-ray crystallography of GIRK2 in complex with G$\beta\gamma$. Activation of GIRK channels through so-called ‘G protein-independent’ pathways, however, are poorly understood. For example, GIRK channels are activated by alcohol and, similar to G$\beta\gamma$ activation, requires PIP$_2$. However, while previous studies with heterologous expression systems and neurons suggested that alcohol directly activates GIRK channels, the role of endogenous G$\beta\gamma$ subunits could not be unequivocally dismissed. Furthermore, alternative mechanisms through which alcohol could exert its effects were possible, such as increasing the fluidity of the plasma membrane. Another example of a putative G protein-independent modulator is cholesterol. Cholesterol, which accounts for up to 50% of the total lipid membrane content, has been shown to modulate the activity of potassium channels, including cardiac GIRK channels, i.e., GIRK1/GIRK4 subunits.
However, whether cholesterol affects brain GIRK channels, comprised mostly of GIRK2-containing tetramers, requires Gβγ subunits, and interacts with alcohol-dependent activation is unknown.

In previous studies using heterologous expression systems, e.g., Xenopus oocytes, HEK-293 cells, or neurons, the contribution of endogenous proteins could not be entirely eliminated or controlled. For example, cholesterol concentration in plasma membranes is affected by numerous factors and varies between expression systems. To elucidate more definitively the mechanisms of GIRK activation, a different approach is needed in which the lipid components, G proteins and activators can be all controlled experimentally. In the current study, we implemented a methodology in which we purified GIRK2 channels, reconstituted the channels into liposomes with different lipid compositions and then probed channel function with exogenous ligands. We demonstrate that alcohol and cholesterol directly activate neuronal GIRK2 channels, in the absence of G protein Gβγ subunits but requiring PIP2. Interestingly, although PIP2 has been considered a co-factor that is necessary, but not sufficient, for channel activation, here we demonstrate that PIP2 is sufficient to activate GIRK2 channels in the presence of Na+.

Determining the mechanism of activation of GIRK channels by modulators that bypass G protein-gating could provide a new therapeutic strategy for treating a variety of human diseases.
Results

**PIP₂ is necessary and sufficient to activate GIRK2 channels.** Mouse GIRK2 channels were expressed in *Pichia pastoris*, purified, and reconstituted into liposomes of defined compositions (Fig. 1a), following a protocol described previously. GIRK2 channels express efficiently in *Pichia pastoris* and retain their activation by G\_\beta\_\gamma subunits. To study K\textsuperscript{+} flux through GIRK2 channels, we used a fluorescence-based K\textsuperscript{+} flux assay with a plate-reader equipped with a fluidic system to add compounds acutely. In GIRK2-containing liposomes loaded with K\textsuperscript{+} and incubated with the membrane permeable pH-sensitive ACMA dye, the addition of the proton ionophore CCCP results in quenching of the ACMA dye if GIRK channels are open, i.e., H\textsuperscript{+} enters via CCCP if K\textsuperscript{+} can exit the proteoliposome. Thus the decrease in fluorescence, i.e., quenching, provides a measure of the relative K\textsuperscript{+} flux that correlates directly with GIRK2 channel activity.

Purified GIRK2 channels, containing amino acids 52 - 380, were reconstituted into liposomes containing 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine (POPE) and 1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-(1’-rac-glycerol) (POPG) at a 3:1 ratio. PE:PG lipids were found previously to support both G\_\beta\_\gamma and Na\textsuperscript{+}-dependent activation. We first examined the requirement of PIP₂ for channel activation. PIP₂ is found in the plasma membrane at concentrations up to 1% and has been shown previously to be required for GIRK activity. We measured the relative K\textsuperscript{+} flux through GIRK2 channels reconstituted in either the absence or presence of 1% brain PIP₂, under conditions that saturate the cytoplasmic Na\textsuperscript{+} binding site in GIRK2. GIRK2-containing liposomes containing brain PIP₂ exhibited a robust quenching of fluorescence upon addition of diC\textsubscript{8}-PIP₂ (30\(\mu\text{M}\)) revealed potent activation, i.e., increase in the rate of quenching (Fig. 1c). To quantify the change in K\textsuperscript{+} flux, we measured both the steady state...
fluorescence at the end of 15 minutes and the rate of quenching following the addition of diC8-PIP2 (see methods for details). Using both analyses, diC8-PIP2 clearly activates GIRK2 channels (Figs 1d and 2a,b).

To determine the PIP2 sensitivity, we examined the effect of applying different concentrations of diC8-PIP2 to GIRK2-containing liposomes (Fig. 2a). Note the progressively faster rate of quenching with higher diC8-PIP2 concentrations. Plotting the rate of K+ flux (1/τau) as a function of the diC8-PIP2 concentration reveals a concentration-dependent increase in the rate of quenching. The best-fit with the Hill equation indicates an apparent EC50 of 25.1±3.3 μM and a Hill coefficient of 1.7±0.1 (n=10) (Fig. 2b). The EC50 with diC8-PIP2 is higher than for that for GIRK2 reconstituted in bilayers in the presence of Gβγ (≈15 μM)34 and lower than that for GIRK1/GIRK4 channels expressed heterologously (≈ 45 μM)39.

To probe the sensitivity of GIRK2 channel activation by brain PIP2, we examined the effect of neomycin, which is a competitive inhibitor of PIP240, on the K+ flux with GIRK2 channels. Increasing concentrations of neomycin applied acutely to GIRK2-containing liposomes with brain PIP2 attenuated the rate of quenching (Fig. 2c). The IC50 for neomycin inhibition is 11.1±2.4 μM (n=4), with a Hill coefficient of 1.3±0.3 (Fig. 2d). Taken together, these experiments demonstrate that both brain PIP2 and soluble diC8-PIP2 activate GIRK2 channels in a dose-dependent manner, in the absence of G proteins or other ligands, indicating that GIRK2 channels are directly gated by PIP2 in the presence of Na+.

Figure 3. Alcohol directly activates GIRK2 channels in absence of G proteins. (a,b) Normalized fluorescent traces (mean ± SEM) show K+ flux for GIRK2-containing liposomes with 1% brain PIP2 and increasing concentrations of ethanol (EtOH) (a) (n = 12) (green traces) or propanol (PrOH) (b) (n = 4–6) (blue traces). (c) The normalized rate of K+ flux is plotted as a function of ethanol (green) and propanol (blue) concentration. Inset, zoom of the dose-response curves over the physiological alcohol concentrations. * p < 0.05 vs. 10 mM using one-way ANOVA and Dunnett’s post hoc test (n = 5–6 for PrOH and 11–12 for EtOH). (d) Normalized fluorescent traces (mean ± SEM) show K+ flux for GIRK2 in the presence of 100 mM PrOH in the absence (light blue, n = 3) or presence of 50 μM diC8-PIP2 (dark blue, n = 4). GIRK2 in the absence of both PrOH and diC8-PIP2 is shown for comparison (black, n = 4). (e) Normalized fluorescent traces (mean ± SEM) show K+ flux for GIRK2-containing liposomes with 1% brain PIP2 and 1 mM β-OG (left) (n = 4, magenta) or 30 μM capsaicin (right) (n = 4, blue). MTS-HE inhibition is shown for comparison (red). (f) The normalized rate of K+ flux is plotted as a function of different β-OG (pink) or capsaicin (blue) concentrations, and is compared to 100 mM EtOH (green). * not significant vs. 3 μM Capsaicin, † not significant vs. 0.3 mM β-OG, ** P < 0.05 vs. 3 μM Capsaicin or 0.3 mM β-OG using one-way ANOVA and Dunnett’s post hoc test.

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Thus, the apparent affinity for PIP₂ increases nearly 2-fold in the presence of cholesterol. In GIRK2 proteoliposomes that alcohol-dependent activation of GIRK channels requires only PIP₂, and no other proteins, e.g., G-βγ to increase membrane elasticity, e.g., β-OH-glucoside (β-OH) and capsaicin. Direct action of cholesterol on brain GIRK2 channels. Cholesterol does not affect the alcohol sensitivity of GIRK2 channels.

**Figure 4.** Cholesterol directly activates GIRK2 channels. (a) Normalized fluorescent traces (mean ± SEM) show K⁺ flux for GIRK2-containing liposomes with 1% brain PIP₂ in the absence (black) or presence of 5% cholesterol (orange) (n = 6). Inset, chemical structure of cholesterol is shown. (b) Bar graph shows the increase in the rate of K⁺ flux with different cholesterol concentrations (1%, 5%, and 10%). Statistical significance *p < 0.05, **p < 0.01 (n = 6).

Direct action of alcohol on brain GIRK2 channels. In addition to G proteins, GIRK channels are activated by alcohols. In order to address whether this property is retained in the purified protein, we examined the effect of directly applying ethanol to GIRK2-containing liposomes with brain PIP₂. Ethanol, added at increasing concentrations, produced a dose-dependent increase in the relative rate of K⁺ flux (Fig. 4a). Similarly, increasing concentrations of propanol also enhanced the relative rate of K⁺ flux (Fig. 3b). We calculated the change in the rate of K⁺ flux, relative to 0 mM alcohol, and plotted the normalized rate as a function of alcohol concentration (Fig. 3c). Note the increase in the normalized rate of K⁺ flux with concentrations greater than 10 mM, a rank order of activation with propanol greater than ethanol, and no apparent saturation at 200 mM. These properties of alcohol activation are remarkably similar to those with GIRK2 channels expressed heterologously in HEK293 cells. Importantly, alcohol-dependent activation required brain PIP₂, and no other proteins, e.g., G-βγ₁ subunits.

Using the lipid-reconstitution system, we could now investigate the possibility that changes in lipid membrane dynamics could affect GIRK2 function. We tested the effect of compounds demonstrated previously to increase membrane elasticity, e.g., β-octyl glucoside (β-OH) and capsaicin. Direct application of β-OH (1 mM) or capsaicin (30 μM) did not alter the rate of K⁺ flux for GIRK2 channels reconstituted into liposomes with brain PIP₂ (Fig. 3e). There was no significant change in the rate of K⁺ flux with increasing concentrations of β-OH or capsaicin, in contrast to the change in K⁺ flux with ethanol (Fig. 3f). Taken together, these experiments demonstrate that known lipid-membrane disruptors have little effect on GIRK2 channel activity, providing additional support for the direct activation of GIRK2 by ethanol.

Direct action of cholesterol on brain GIRK2 channels. Cholesterol inhibits constitutively open inwardly-rectifying potassium channels but appears to activate cardiac (GIRK1/GIRK4) and brain (GIRK2) channels. Whether cholesterol acts directly on brain GIRK2 channels in the absence of G-βγ is unknown. Using GIRK2-containing liposomes that have brain PIP₂ (1%), we examined the effect of cholesterol on the relative K⁺ flux. The basal rate of K⁺ flux through GIRK2 channels was significantly enhanced in membranes containing 5% cholesterol (Fig. 4a). Both 5% and 10% cholesterol significantly increased the relative rate of K⁺ flux, while 1% was ineffective (Fig. 4b). Interestingly, the relative rate of K⁺ flux was similar for 5% and 10% cholesterol, suggesting near saturation for this response.

GIRK2 channels reconstituted in cholesterol-containing liposomes (5%) lacking brain PIP₂ exhibited no basal K⁺ flux, indicating that PIP₂ is required for cholesterol activation, similar to alcohol activation. We hypothesized that cholesterol may enhance GIRK2 activity by altering the sensitivity to PIP₂. To test this, we measured the rate of K⁺ flux in GIRK2-containing liposomes containing 5% cholesterol that were directly exposed to different concentrations of diC₈-PIP₂ (Fig. 5a). The dose-response curve for diC₈-PIP₂ in the presence of cholesterol shifted to lower concentrations, compared to that without cholesterol (Fig. 5b). The EC₅₀ for PIP₂ activation of GIRK2 in the presence of cholesterol was 12.2 ± 2.5 μM (n = 6), compared to an EC₅₀ of 25.1 ± 3.3 μM without cholesterol. Thus, the apparent affinity for PIP₂ increases nearly 2-fold in the presence of cholesterol. In GIRK2 proteoliposomes containing both brain PIP₂ (1%) and 5% cholesterol, acute application of neomycin decreased the rate of K⁺ flux, with an IC₅₀ of 15.0 ± 4.6 μM (Fig. 5c,d). The IC₅₀ for neomycin inhibition is indistinguishable from that measured in the absence of cholesterol (Fig. 2d), suggesting little difference in the off-rate of PIP₂. Thus, in addition to alcohol, cholesterol appears to directly activate GIRK2 channels through enhancement of the interaction with PIP₂.

Cholesterol does not affect the alcohol sensitivity of GIRK2 channels. Both alcohol and cholesterol activate GIRK2 channels by increasing the apparent affinity for PIP₂. We next investigated the interaction between alcohol and cholesterol on GIRK2 activation. With GIRK2-containing liposomes in 5% cholesterol (a saturating concentration in our experiments), ethanol enhanced the relative K⁺ flux to a greater extent than for...
cholesterol alone or ethanol alone (Fig. 6a). The rate of $K^+$ flux with 5% cholesterol increased over a range of ethanol concentrations (Fig. 6b). However, the dose-response curves completely overlap over the physiological range of activation, e.g., 10–100 mM, after adjusting for the cholesterol-dependent increase in the basal $K^+$ flux (Fig. 4b), (Fig. 6b, inset). These results indicate that the effects of alcohol and cholesterol on GIRK channel are additive, suggesting these two modulators increase channel activity via separate structural sites in the channel.

**Discussion**

GIRK channels provide a major pathway for inhibition in the brain that is important in both normal and diseased states. The canonical pathway for GIRK channel activation is mediated by G protein $G^{i/o}$ subunits, which occurs following stimulation of a $G^{i/o}$-coupled GPCRs, such as GABA-B, dopamine D2 or mu opioid receptors. Recent crystal structures of GIRK2 in complex with $G^{i/o}$ and PIP$_2$ have provided structural and mechanistic details of how $G^{i/o}$ associates with GIRK channels. It has become increasingly evident that G protein-independent pathways also exist for modulating GIRK channels; however, much less is known about the mechanism of action. A majority of the identified compounds that modulate GIRK channels independently of G proteins appear to inhibit GIRK channel activity. Conversely, the number of drugs that directly activate GIRK channels is small. Here, we demonstrate for the first time that ethanol and cholesterol directly activate GIRK2 channels via changes in the interaction with PIP$_2$, and do not require the presence of G proteins or other endogenous proteins. In previous studies, extracellular application of alcohol enhanced GIRK channel activity, both with heterologously expressed channels, or with nativey expressed channels in cerebellar neurons. Alcohol activation was determined to be insensitive to pertussis toxin (PTX) or antibodies to the G protein $G^{i/o}$ subunit, suggesting that G proteins were not involved. Further, deletion studies, the identification of an alcohol pocket in x-ray crystallographic structures and alcohol-tagging studies all indicated that alcohol activation was likely mediated by the direct interaction of alcohol with the channel. However, the involvement of endogenous proteins, e.g. $G^{i/o}$ subunits, could not be ruled out with heterologous expression systems, such as oocytes or HEK293 cells, or native cell systems, i.e., neurons. In addition, a component of ethanol activation could include an alteration in the lipid bilayer fluidity, since this was not directly tested. By reconstituting purified GIRK2 channels
in liposomes, controlling the components of the bilayer, and adding alcohol in isolation of any other proteins, we could overcome these previous limitations and demonstrate that intoxicating concentrations of ethanol, i.e., >20 mM, directly activate GIRK2 channels in the presence of PIP2. For reference, a blood alcohol level of 0.08% is ~17 mM, binge-drinking levels can reach 50 mM, and the anesthetic concentration is around 190 mM52. On the other hand, we find that two types of lipid disruptive amphiphiles, e.g., β-octyl glucoside (β-OG) and capsaicin, do not activate GIRK2 channels. This, thus, mechanism of ethanol activation of GIRK2 channels is unlikely to involve an indirect component from an alcohol-dependent changes in the membrane lipids or from Gβγ subunits.

Using mammalian GIRK2 channels reconstituted in defined lipid membranes, we also determined that cholesterol directly activates GIRK2 channels. By contrast, cholesterol inhibits the activity of many different types of ion channels, including other members of the inwardly rectifying potassium channel family (Kir)24, 26, 53, 54. Like cholesterol directly activates GIRK2 channels. By contrast, cholesterol inhibits the activity of many different types of ion channels, including other members of the inwardly rectifying potassium channel family (Kir)24, 26, 53, 54. Like alcohol, cholesterol activation of GIRK2 depends on the presence of PIP2. Both activators appear to increase the apparent affinity of the channel for PIP2, similar to Gβγ activation46, suggesting that a tighter association of PIP2 with the channel may contribute to the increase in channel activity. In addition, we demonstrate that PIP2 is not only necessary, but is sufficient to activate GIRK2 channels with cytoplasmic Na+ in the absence of Gβγ subunits. As such, PIP2 could be considered an ‘agonist’ for GIRK channels. Moreover, molecules typically described as channel activators, i.e., Gβγ subunits and alcohol, could be mechanistically referred to as positive allosteric modulators (PAMs). PAMs are molecules that cannot activate the channel on their own but increase the efficacy or potency of an agonist, i.e., PIP2. For example, benzodiazepine is a PAM for GABA_A receptors; it does not activate GABA_A receptors but shifts the EC50 for agonist, i.e., GABA, activation46. However, the endogenous levels of PIP2 are low relative to the sensitivity of GIRK channels, resulting in a small basal channel activity. GIRK channels are considered to have a relatively low affinity for PIP2, compared to constitutively active Kir2.1 channels56. Consistent with this, the EC50 for activation of GIRK2 with diC8-PIP2 is ~25μM, which compares to a 10-fold lower EC50 of ~2μM for Kir2.157. Interestingly, the GIRK channel activators, i.e., Gβγ, alcohol and cholesterol, do not alter the levels of PIP2 but instead change the apparent affinity of the channel for PIP2. The shift in apparent PIP2 affinity with these activators likely occurs through allosteric changes in the channel that alter the rates of association and dissociation of PIP2. On the other hand, stimulation of GPCRs that couple to Gq G proteins can reversibly lower the levels of membrane PIP2, leading to a decrease in GIRK channel activity56.

Similar to its effects on brain GIRK2 channels, cholesterol enhances acetylcholine activated potassium (KAC) channels in the heart, which are composed of GIRK1 and GIRK4 subunits51, 52. Interestingly, this effect was proposed to be independent of Gβγ but with little change in the apparent PIP2 affinity51. In these experiments, the relative association of PIP2 with GIRK1/GIRK4 heteromeric or GIRK4 homomeric channels was inferred from measuring the rate of current inhibition following activation of a PIP2-depleting phosphatase65. The rate was similar in the absence and presence of cholesterol51. By contrast, we find that cholesterol activates the neuronal GIRK2 channel activity by increasing the apparent association with PIP2 in the absence of Gβγ subunits. Whether this difference in PIP2 dependence is unique to GIRK2 remains to be determined.

A high-resolution structural view of the binding site for cholesterol in GIRK2 is not available. By contrast, a structural pocket has been identified in GIRK2 channels for alcohol activation66, 51. The alcohol pocket is located at the interface of the cytoplasmic domain of two adjacent channel subunits, and is formed by hydrophobic amino acids in the N-terminal domain, the βL-βM strands, and the βD-βE strand18. This binding pocket overlaps with the region involved in Gβγ subunits activation15, 59. Molecular dynamics (MD) simulations combined with functional studies have suggested cholesterol may be coordinated by residues in the transmembrane spanning regions60, 61. Recently, Bukiya et al. (2017) conducted cholesterol-docking experiments and identified four amino acids that potentially interact directly with cholesterol, V99, V101, L174 and L183. Interestingly, these sites are physically close to the binding pocket for PIP2 in the GIRK2 closed structure52, raising the possibility that cholesterol may enhance the access of PIP2 to the channel. Thus, the site for cholesterol modulation is unlikely to be the same as that for alcohol (i.e. membrane vs. cytoplasmic). In addition, we noted functional differences in the concentration dependence of GIRK2 activity in response to alcohol and cholesterol.

**Figure 6.** Cholesterol does not affect the ethanol sensitivity of GIRK2 channels. (a) Normalized fluorescent traces (mean ± SEM) show the K+ flux for GIRK2-containing liposomes with 1% brain PIP2 (black trace), and either 100 mM EtOH (green), 5% cholesterol (orange), or both 100 mM EtOH and 5% cholesterol (blue trace) (n = 4–12) (b) The rate of K+ flux for GIRK2 with PIP2 and 5% cholesterol (blue) or PIP2 alone (green) is plotted as a function of different EtOH concentrations. Inset, the normalized rate of K+ flux is plotted as a function of EtOH concentration.

**a**

**b**

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EtOH

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[EtOH], mM

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between alcohol and cholesterol activation. First, there was no apparent shift in ethanol sensitivity in the presence of cholesterol. A direct or allosteric effect of alcohol on cholesterol activation might be reflected in differences in the dose–response curves. Second, the basal rate of K\(^+\) flux through GIRK2 channels in the presence of alcohol and a saturating level of cholesterol was higher than the rate for alcohol alone. Together, these results suggest that alcohol and cholesterol interact structurally and mechanistically with different regions of the channel.

Cholesterol is ubiquitous in plasma membranes, accounting for up to 50% of the lipid mass\(^{22}\). Changes in brain cholesterol are implicated in numerous neurodegenerative diseases, such as Alzheimer’s, Huntington’s, Parkinson’s, and Niemann-Pick disease\(^{62}\). Cholesterol metabolism and utilization in the brain is significantly different than the rest of the body. Although the brain only makes up 2-5% of the body mass, almost 25% of the total body cholesterol resides in the brain\(^{63}\). Moreover, cholesterol degradation in the brain is low. An increase of cholesterol in the brain has been reported to decrease the firing rate in hippocampal neurons, suggesting it may reduce excitability\(^{64}\). Notably, some types of GIRK channels are localized in lipid rafts, which are enriched in cholesterol\(^{65,66}\). Thus, changes in membrane cholesterol content may alter the basal activity of neuronal GIRK channels. Consistent with this, Bukiya et al. (2017) reported that cholesterol enrichment increased baclofen-induced, tertiapin-inhibited GIRK-like currents in hippocampal neurons. Similarly, cholesterol (33%) enhanced the basal activity of a constitutively-active mutant GIRK2 channel\(^{45}\). GIRK channels are emerging as a potential drug target for treating alcoholism and other neurological disorders\(^1\). Interestingly, chronic alcohol exposure leads to increased cholesterol levels and more widespread distribution of cholesterol in the membrane leaflets\(^{67,68}\). Moreover, increases in cholesterol have been also reported in cases of fetal alcohol exposure\(^{69}\). The potentiation of GIRK channel activity by cholesterol could amplify the effects of alcohol, perhaps contributing to a comorbidity of cholesterol and alcohol. With a better understanding of the molecular mechanism underlying the direct activation of GIRK channels by small compounds like ethanol and cholesterol, it should be possible to design new drugs that specifically modulate the activity of GIRK channels through these G protein-independent pathways.

**Methods**

**Molecular Biology.** We used a variant of GIRK2 shown previously to express efficiently in *Pichia pastoris*; mouse GIRK2 (containing amino acids 52–380) is linked in-frame with an HRV 3 C protease site, green fluorescent protein (GFP) and a decahistidine (HIS10) tag (a generous gift from R. MacKinnon, The Rockefeller University, New York, NY). GIRK2 clones in pPICZ were transformed into *Pichia pastoris* using the lithium chloride transformation method\(^{70}\) or electroporation (according to manufacturer protocols). Transformants were screened based upon Zeocin resistance (>500 μg/ml) and GFP emission. Highest expressing clones were selected for large-scale purification.

**Protein purification and reconstitution.** All GIRK channels were expressed and purified in *P. pastoris* as described previously\(^{15,32}\). Briefly, the highest-expressing clone was grown in BMGY medium and induced in BMM medium containing 1% methanol. Cells were harvested, resuspended in buffer (50 mM HEPES, pH 7.4; 150 mM KCl; 1 mM TCEP; 1 mM AEBSF and Complete EDTA-free protease inhibitor tablets (Roche)), dripped into liquid nitrogen, and placed at −80 °C. Frozen cells were lysed in a Mixer Mill (Retsch) 5-times for 3 minutes at 25 Hz and solubilized in 50 mM HEPES, pH 7.35; 150 mM KCl; 1 mM TCEP; 1 mM AEBSF; 3% (w/v) n-Dodecyl-β-D-maltoside (DDM; Anatrace) and Complete ULTRA EDTA-free protease inhibitor tablets (Roche) with gentle stirring at 4 °C. Unsolubilized material was separated by centrifugation at 40,000 × g for 40 min at 4 °C. The supernatant was injected onto a HISPan HP column (GE Healthcare) equilibrated in wash buffer (50 mM HEPES, pH 7.0; 150 mM KCl; 0.4% DDM; 20 mM imidazole) connected to an AKTA pure (GE Healthcare) chromatography system and eluted in buffer containing 300 mM imidazole. The HISPan column eluate was pooled, exchanged into imidazole-free buffer and digested overnight at 4 °C with HRV 3 C protease, purified as described below.\(^2\) (a generous gift of Dan Axelrod, UCSF, San Francisco, CA). Digested protein was concentrated and run on a Superdex-200 gel filtration column in 20 mM TRIS-HCl pH 7.5, 150 mM KCl, 0.1% (w/v) DDM (anagrade), 5 mM DTT, and 1 mM EDTA. Fractions eluting at a volume consistent with the GIRK channel tetramer were pooled, concentrated and examined by SDS-PAGE and Coomassie blue staining. We also analyzed the purified protein using tandem mass spectrometry and detected significant peptides for only GIRK2 and the tetramer were pooled, concentrated and examined by SDS-PAGE and Coomassie blue staining. We also analyzed the purified protein using tandem mass spectrometry and detected significant peptides for only GIRK2 and the

**Flux assay.** Liposomes were diluted 1:20 into flux buffer (20 mM Na-HEPES, pH 7.4; 150 mM NaCl; 0.5 mM EDTA) containing 5 μM of the H\(^+\) sensitive dye 9-Amino-6-chloro-2-methoxyacridine (ACMA). Fluorescence was measured using a Flexstation 3 microplate reader (Molecular Devices) with the following parameters: 410 nm excitation, 480 nm emission, 455 nm cutoff, medium PMT sensitivity, and sampling at 2 seconds. After a stable baseline fluorescence (150 s) was obtained, the H\(^+\) ionophore m-chlorophenyl hydrazine (CCCP) was
automatically added (1 μM), then a second addition consisting of different compounds or vehicle was added 150 s later, followed 900 s later by a third addition with the K⁺ ionophore Valinomycin (100 nM), to determine the maximal K⁺ flux. GIRK2 channels are likely arranged in both orientations in the liposomes. However, we expect the channels oriented inside-out to support high K⁺ flux because of high Na⁺ in the flux buffer and high K⁺ in the liposome.

Data Analysis. To compare between different flux assay runs, the fluorescence was either normalized to the baseline fluorescence before CCCP (F₀) and the fluorescence following Valinomycin (Fᵥ), or in the case of acutely adding a compound, the fluorescence after CCCP (F₀) and the fluorescence following Valinomycin (Fᵥ). This normalized fluorescence (Fₙ), defined as ‘relative K⁺ flux’, was calculated according to the equation (Eq. 1):

\[ Fₙ(t) = \frac{F(t) - Fᵥ}{F₀ - Fᵥ} \]  

where \( Fₙ(t) \) is the normalized fluorescence at time \( t \) and \( F(t) \) is the relative fluorescence unit (RFU) as a function of time.

The rate of K⁺ flux \( (1/τ) \) was determined by fitting the normalized fluorescence decay with a single exponential (Eq. 2):

\[ Fₙ(t) = F₀e^{-\frac{t}{τ}} + C \]  

where \( F₀ \) is the amplitude, \( 1/τ \) is the rate, and \( C \) is a constant. In experiments where it was not possible to fit the decay, the amplitude of the fluorescence just prior to adding valinomycin (1190 s) was used to calculate the fractional inhibition of K⁺ flux.

Dose-response curves for diC₄-PIP₂/neomycin were fit to the Hill equation (Eq. 3):

\[ y = \frac{y_{max}}{1 + \left(\frac{[X]}{x_{50}}\right)^h} \]  

where \( y_{max} \) is the maximal flux rate or fractional inhibition, \([X]\) is the concentration, \( h \) is the Hill coefficient, and \( x_{50} \) is the half-maximal effective concentration, i.e. EC₅₀ for PIP₂ or IC₅₀ neomycin. Dose response curves for modulators, e.g., alcohol, β-OG, capsaicin, do not reach saturation and were normalized to the basal flux rate in the absence of ligand. All values are reported as mean ± S.E.M, with statistical significance \((P < 0.05)\) determined using one-way ANOVA followed by Dunnett’s multiple comparison post hoc test unless otherwise noted. All data were analyzed using Excel (Microsoft) and Prism (GraphPad).

References
1. Luscher, C. & Slesinger, P. A. Emerging roles for G protein-gated inwardly rectifying potassium (GIRK) channels in health and disease. Nat Rev Neurosci 11, 301–15 (2010).
2. Hibino, H. et al. Inwardly rectifying potassium channels: their structure, function, and physiological roles. Physiol Rev 90, 291–366 (2010).
3. Mayfield, J., Blednov, Y. A. & Harris, R. A. Behavioral and Genetic Evidence for GIRK Channels in the CNS: Role in Physiology, Pathophysiology, and Drug Addiction. Int Rev Neurobiol 123, 279–313 (2015).
4. Yamada, K. et al. Association study of the KCNJ3 gene as a susceptibility candidate for schizophrenia in the Chinese population. Hum Genet 131, 443–51 (2012).
5. Nishizawa, D. et al. Association between KCNJ6 (GIRK2) gene polymorphism rs2835859 and post-operative analgesia, pain sensitivity, and nicotine dependence. J Pharmacol Sci 126, 253–63 (2014).
6. Clarke, T. K. et al. KCNJ6 is associated with adult alcohol dependence and involved in gene x early life stress interactions in adolescent alcohol drinking. Neuropharmacology 56, 1142–8 (2011).
7. Saccone, S. F. et al. Cholinergic nicotinic receptor genes implicated in a nicotine dependence association study targeting 348 candidate genes with 3713 SNPs. Hum Mol Genet 16, 36–49 (2007).
8. Lotsch, J., Fruss, H., Veh, R. W. & Doehring, A. A KCNJ6 (Kir3.2, GIRK2) gene polymorphism modulates opioid effects on analgesia and addiction but not on pupil size. Pharmacogenet Genomics 20, 291–7 (2010).
9. Masotti, A. et al. Keppen-Lubinsky syndrome is caused by mutations in the inwardly rectifying K⁺ channel encoded by KCNJ6. Am J Hum Genet 96, 295–300 (2015).
10. Wickman, K. D. et al. Recombinant G-protein βγ-subunits activate the muscarinic-gated atrial potassium channel. Nature 368, 255–7 (1994).
11. Reuneny, E. et al. Activation of the cloned muscarinic potassium channel by G protein βγ subunits. Nature 370, 143–6 (1994).
12. Huang, C. L., Feng, S. S. & Hilgemann, D. W. Direct activation of inward rectifier potassium channels by PIP₂ and its stabilization by Gβγ. Nature 391, 803–6 (1998).
13. Logothetis, D. E., Kurachi, Y., Galper, J., Neer, E. J. & Clapham, D. E. The βγ subunits of GTP-binding proteins activate the muscarinic K⁺ channel in heart. Nature 325, 321–6 (1987).
14. Sui, J. L., Petit-Jacques, J. & Logothetis, D. E. Activation of the atrial KATP channel by the βγ subunits of G proteins or intracellular Na⁺-ions depends on the presence of phosphatidylinositol phosphates. Proc Natl Acad Sci USA 95, 1307–12 (1998).
15. Whorton, M. R. & MacKinnon, R. X-ray structure of the mammalian GIRK2 βγ-G-protein complex. Nature 498, 190–7 (2013).
16. Lewohl, J. M. et al. G-protein–coupled inwardly rectifying potassium channels are targets of alcohol action. Nat Neurosci 2, 1084–90 (1999).
17. Kobayashi, T. et al. Ethanol opens G-protein-activated inwardly rectifying K⁺ channels. Nat Neurosci 2, 1091–7 (1999).
18. Aryan, P., Dvir, H., Choe, S. & Slesinger, P. A. A discrete alcohol pocket involved in GIRK channel activation. Nat Neurosci 12, 988–95 (2009).
19. Bodhinathan, K. & Slesinger, P. A. Molecular mechanism underlying ethanol activation of G-protein–gated inwardly rectifying potassium channels. Proc Natl Acad Sci U S A 110, 18309–14 (2013).
20. Yevenes, G. E. et al. Molecular Requirements for Ethanol Differential Allosteric Modulation of Glycine Receptors Based on Selective Gβγ Modulation. Journal of Biological Chemistry 285, 30203–30213 (2010).
21. Ingoldsson, H. I. & Andersen, O. S. Alcohol's effects on lipid bilayer properties. *Biophys J* **101**, 847–55 (2011).

22. Yeagle, P. L. Modulation of membrane function by cholesterol. *Biochimie* **73**, 1303–10 (1991).

23. Deng, W. et al. Hypercholesterolemia induces up-regulation of K<sub>AC</sub>h cardiac channels via a mechanism independent of phosphatidylinositol 4,5-bisphosphate and G<sub)i</sub>α. *J Biol Chem* **287**, 4925–35 (2012).

24. Crowley, J. J., Treistman, S. N. & Doppio, A. M. Cholesterol antagonizes ethanol potentiation of human brain B<sub>K</sub><sup>+</sup> channels reconstituted in phospholipid bilayers. *Mol Pharmacol* **64**, 365–72 (2003).

25. D'Avanzo, N., Hyrc, K., Enkvetchakul, D., Covey, D. F. & Nichols, C. G. Enantiomeric single protein-sterol interactions mediate regulation of both prokaryotic and eukaryotic inward rectifier K<sup>+</sup> channels by cholesterol. *PLoS One* **6**, e19393 (2011).

26. Romanenko, V. G. et al. Cholesterol sensitivity and lipid raft targeting of Kir2.1 channels. *Biophys J* **87**, 3850–61 (2004).

27. Bukiya, A. N. et al. Cholesterol increases the open probability of cardiac K<sub>AC</sub>h currents. *Biochim Biophys Acta* **1848**, 2406–13 (2015).

28. Bukiya, A. N., Belani, J. D., Rychnovsky, S. & Doppio, A. M. Specificity of cholesterol and analogs to modulate BK channels points to direct sterol-channel protein interactions. *J Gen Physiol* **137**, 93–110 (2011).

29. Atwood, B. K., Lopez, J., Wager-Miller, J., Mackie, K. & Straiker, A. Expression of G protein-coupled receptors and related proteins in HEK293, AtT20, BV2, and N18 cell lines as revealed by microarray analysis. *BMC Genomics* **12**, 14 (2011).

30. Weber, W. Ion currents of Xenopus laevis oocytes: state of the art. *Biochim Biophys Acta* **1421**, 213–33 (1999).

31. Lundbaek, J. A. Structural Insights into GIRK Channel Function. *Annu Rev Biophys Biomol Struct* **31**, 151–72 (2002).

32. Wang, W., Whorton, M. R. & MacKinnon, R. Quantitative analysis of mammalian GIRK2 channel regulation by G proteins, the signaling lipid PIP<sub>2</sub>, and Na<sup>+</sup> in a reconstituted system. *Elife* **3**, e03671 (2014).

33. Ho, I. H. & Murrell-Lagnado, R. D. Molecular mechanism for sodium-dependent activation of G protein-gated K<sup>+</sup> channels. *J Physiol* **520**(Pt 3), 645–51 (1999).

34. Ho, I. H. & Murrell-Lagnado, R. D. Molecular determinants for sodium-dependent activation of G protein-gated K<sup>+</sup> channels. *J Biol Chem* **274**, 8639–48 (1999).

35. Guo, Y., Waldron, G. J. & Murrell-Lagnado, R. A role for the middle C terminus of G-protein-activated inward rectifier potassium channels in regulating gating. *J Biol Chem* **277**, 48289–94 (2002).

36. Rohacs, T., Chen, J., Prestwich, G. D. & Logothetis, D. E. Distinct specificities of inwardly rectifying K(+) channels for phosphoinositides. *J Biol Chem* **274**, 36065–72 (1999).

37. Lopes, C. M. et al. Protein kinase A modulates PLC-dependent regulation and PIP<sub>2</sub>-sensitivity of K<sup>+</sup> channels. *Channels (Austin)* **1**, 124–34 (2007).

38. Xie, L. H., John, S. A., Ribalet, B. & Weiss, J. N. Phosphatidylinositol-4,5-bisphosphate (PIP2) regulation of strong inward rectifier potassium channels (GIRK). *J Biol Chem* **272**, 12448–55 (1997).

39. Lopes, C. M. et al. GIRK channel and gating regulation by G proteins, the signaling lipid PIP<sub>2</sub>, and Na<sup>+</sup> in a reconstituted system. *Elife* **3**, e03671 (2014).

40. Aranda, F. J., Villalain, J. & Gomez-Fernandez, J. C. Capsaicin affects the structure and phase organization of phospholipid membranes. *Biochim Biophys Acta* **1234**, 225–34 (1995).

41. Bukiya, A. N., Durdagi, S., Noskov, S. & Rosemounen-Dantsker, A. Cholesterol Upr regulates Neuronal G Protein-Gated Inwardly Rectifying Potassium (GIRK) Channel Activity in the Hippocampus. *J Biol Chem* **292**, 6135–6147 (2017).

42. Lobos, T., Washiyama, K. & Ikeda, K. Inhibition of G-protein-activated inward rectifying K<sup>+</sup> channels by various antidepressant drugs. *Neuropsychopharmacology* **29**, 1841–51 (2004).

43. Zhou, W., Arrabitt, C., Choe, S. & Slesinger, P. A. Mechanism underlying bipapacaine inhibition of G protein-gated inwardly rectifying K<sup>+</sup> channels. *Proc Natl Acad Sci U S A* **98**, 6482–7 (2001).

44. Kobayashi, T., Washiyama, K. & Ikeda, K. Inhibition of G protein-activated inwardly rectifying K<sup>+</sup> channels by different classes of antidepressants. *PLoS One* **6**, e22808 (2011).

45. Kobayashi, T., Nishizawa, D., Iwamura, T. & Ikeda, K. Inhibition by cocaine of G protein-activated inwardly rectifying K<sup>+</sup> channels expressed in Xenopus oocytes. *Toxicol In Vitro* **21**, 656–64 (2007).

46. Kobayashi, T., Ikeda, K. & Kumanishi, T. Inhibition by various antipsychotic drugs of the G-protein-activated inwardly rectifying K(+) (GIRK) channels expressed in Xenopus oocytes. *Br J Pharmacol* **129**, 1716–22 (2000).

47. Kaufmann, K. et al. ML297 (VU0456810), the first potent and selective activator of the GIRK potassium channel, displays antiepileptic properties in mice. *ACS Chem Neurosci* **4**, 1278–86 (2013).

48. Su, Z., Brown, E. C., Wang, W. & MacKinnon, R. Novel cell-free high-throughput screening method for pharmacological tools targeting K<sup>+</sup> channels. *Proc Natl Acad Sci U S A* **113**, 5748–53 (2016).

49. Pegan, S., Arrabitt, C., Slesinger, P. A. & Choe, S. Andersen's syndrome mutation effects on the structure and assembly of the cytoplasmic domains of Kir2.1. *Biochemistry* **45**, 8599–606 (2006).

50. Harris, R. A., Trudell, J. R. & Mitic, S. J. Ehanol's molecular targets, *Sci Signal* **1**, re7 (2008).

51. Hajdu, P., Varga, Z., Pieri, C., Panyi, G. & Gaspar, R. Jr. Cholesterol modifies the gating of Kv1.3 in human T lymphocytes. *Pflugers Arch* **445**, 674–82 (2003).

52. Lundbaek, J. A. et al. Regulation of sodium channel function by bilayer elasticity: the importance of hydrophobic docking. Effects of Micelle-forming amphiphiles and cholesterol. *J Gen Physiol* **123**, 599–621 (2004).

53. Walters, R. J., Hadley, S. H., Morris, K. D. & Amin, J. Benzoazepines act on GABA<sub>A</sub> receptors via two distinct and separable mechanisms. *Nat Neurosci* **3**, 1274–81 (2000).

54. Zhang, H., He, C., Yan, X., Mirshahi, T. & Logothetis, D. E. Activation of inwardly rectifying K<sup>+</sup> channels by distinct PtdIns(4,5)P<sub>2</sub> interactions. *Nat Cell Biol* **1**, 183–8 (1999).

55. Wang, W., Whorton, M. R. & MacKinnon, R. Novel cell-free high-throughput screening method for pharmacological tools targeting K<sup>+</sup> channels. *Proc Natl Acad Sci U S A* **113**, 6564–72 (2016).

56. Forrest, O., Nichols, C. G., Lamoureux, G. & D'Avanzo, N. Identification of a cholesterol-binding pocket in inward rectifier K(+) (Kir) channels. *Biophys J* **107**, 2786–94 (2014).

57. Ribato, A., Noskov, S., Durdagi, S., Logothetis, D. E. & Levitan, I. Identification of novel cholesterol-binding regions in Kir2 channels. *J Biol Chem* **288**, 31154–64 (2013).

58. Vacek, J. E. Dysregulation of cholesterol balance in the brain: contribution to neurodegenerative diseases. *Dis Model Mech* **5**, 746–55 (2012).

59. Dietschy, J. M. & Turley, S. D. Cholesterol metabolism in the brain. *Curr Opin Lipidol* **12**, 105–12 (2001).

60. Wang, D. & Schreurs, B. G. Dietary cholesterol modulates the excitability of rabbit hippocampal CA1 pyramidal neurons. *Neurosci Lett* **479**, 327–31 (2010).

61. Koyarah, L. et al. Molecular and cellular diversity of neuronal G-protein-gated potassium channels. *J Neurosci* **25**, 11468–78 (2005).
66. Delling, M. et al. The neural cell adhesion molecule regulates cell-surface delivery of G-protein-activated inwardly rectifying potassium channels via lipid rafts. *J Neurosci* **22**, 7154–64 (2002).
67. Wood, W. G., Schroeder, E., Hogy, L., Rao, A. M. & Nemecz, G. Asymmetric distribution of a fluorescent sterol in synaptic plasma membranes: effects of chronic ethanol consumption. *Biochim Biophys Acta* **1025**, 243–6 (1990).
68. Omodeo-Sale, F., Pitt, M., Masserini, M. & Palestini, P. Effects of chronic ethanol exposure on cultured cerebellar granule cells. *Mol Chem Neuropathol* **26**, 159–69 (1995).
69. Barcelo-Coblijn, G., Wold, L. E., Ren, J. & Murphy, E. J. Prenatal ethanol exposure increases brain cholesterol content in adult rats. *Lipids* **48**, 1059–68 (2013).
70. Gietz, R. D. & Schiestl, R. H. Transforming yeast with DNA. *Methods in Molecular and Cellular Biology* **5**, 255–269 (1995).
71. Shaya, D. et al. Voltage-gated sodium channel (Na\(_{\text{v}}\)) protein dissection creates a set of functional pore-only proteins. *Proc Natl Acad Sci USA* **108**, 12313–8 (2011).

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**Author Contributions**
I.W.G. conducted the experiments and collected the data. P.A.S. and I.W.G. planned the research, analyzed the data, and prepared the manuscript.

**Additional Information**

**Competing Interests:** The authors declare that they have no competing interests.

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