RESEARCH ARTICLE

A Quantitative Model of the GIRK1/2 Channel Reveals That Its Basal and Evoked Activities Are Controlled by Unequal Stoichiometry of Gα and Gβγ

Daniel Yakubovich1☯, Shai Berlin1☯¤, Uri Kahanovitch1☯, Moran Rubinstein1, Isabella Farhy-Tselnicker1mb, Boaz Styr1, Tal Keren-Raifman1, Carmen W. Dessauer2, Nathan Dascal1*.

1 Department of Physiology and Pharmacology and Sagol School of Neuroscience, Tel Aviv University, Tel Aviv, Israel, 2 Department of Integrative Biology and Pharmacology, University of Texas Health Science Center, Houston, Texas, United States of America

☯ These authors contributed equally to this work.
¤ Current address: Department of Molecular and Cell Biology, University of California, Berkeley, Berkeley, California, United States of America
mb Current address: Molecular Neurobiology Laboratory, Salk Institute for Biological Studies, La Jolla, California, United States of America
*
dascaln@tauex.tau.ac.il

Abstract

G protein-gated K+ channels (GIRK; Kir3), activated by Gβγ subunits derived from Gαi/o proteins, regulate heartbeat and neuronal excitability and plasticity. Both neurotransmitter-evoked (I_{evoked}) and neurotransmitter-independent basal (I_{basal}) GIRK activities are physiologically important, but mechanisms of I_{basal} and its relation to I_{evoked} are unclear. We have previously shown for heterologously expressed neuronal GIRK1/2, and now show for native GIRK in hippocampal neurons, that I_{basal} and I_{evoked} are interrelated: the extent of activation by neurotransmitter (activation index, R_a) is inversely related to I_{basal}. To unveil the underlying mechanisms, we have developed a quantitative model of GIRK1/2 function. We characterized single-channel and macroscopic GIRK1/2 currents, and surface densities of GIRK1/2 and Gβγ expressed in Xenopus oocytes. Based on experimental results, we constructed a mathematical model of GIRK1/2 activity under steady-state conditions before and after activation by neurotransmitter. Our model accurately recapitulates I_{basal} and I_{evoked} in Xenopus oocytes, HEK293 cells and hippocampal neurons; correctly predicts the dose-dependent activation of GIRK1/2 by coexpressed Gβγ and fully accounts for the inverse I_{basal}·R_a correlation. Modeling indicates that, under all conditions and at different channel expression levels, between 3 and 4 Gβγ dimers are available for each GIRK1/2 channel. In contrast, available Gα_i/o decreases from ~2 to less than one Gα per channel as GIRK1/2’s density increases. The persistent Gβγ/channel (but not Gα-channel) ratio support a strong association of GIRK1/2 with Gβγ, consistent with recruitment to the cell surface of Gβγ, but not Gα, by GIRK1/2. Our analysis suggests a maximal stoichiometry of 4 Gβγ but only 2 Gα_i/o per one GIRK1/2 channel. The unique, unequal association of GIRK1/2 with G protein subunits,
and the cooperative nature of GIRK gating by Gβγ, underlie the complex pattern of basal and agonist-evoked activities and allow GIRK1/2 to act as a sensitive bidirectional detector of both Gβγ and Ga.

Author Summary

Many neurotransmitters and hormones inhibit the electric activity of excitable cells (such as cardiac cells and neurons) by activating a K⁺ channel, GIRK (G protein-gated Inwardly Rectifying K⁺ channel). GIRK channels also possess constitutive “basal” activity which contributes to regulation of neuronal and cardiac excitability and certain disorders, but the mechanism of this activity and its interrelation with the neurotransmitter-evoked activity are poorly understood. In this work we show that key features of basal and neurotransmitter-evoked activities are similar in cultured hippocampal neurons and in two model systems (mammalian HEK293 cells and *Xenopus* oocytes). Using experimental data of the neuronal GIRK1/2 channel function upon changes in GIRK and G protein concentrations, we constructed a mathematical model that quantitatively accounts for basal and evoked activity, and for the inverse correlation between the two. Our analysis suggests a novel and unexpected mechanism of interaction of GIRK1/2 with the G protein subunits, where the tetrameric GIRK channel can assemble with 4 molecules of the Gβγ subunits but only 2 molecules of Ga. GIRK is a prototypical effector of Gβγ, and the unequal stoichiometry of interaction with G protein subunits may have general implications for G protein signaling.

Introduction

G proteins and the linked G protein-coupled receptors (GPCRs) are prominent regulators of excitability, which activate or inhibit ion channels by a variety of mechanisms [1]. This paper focuses on the quantitative analysis of the classical GPCR-initiated signaling cascade that culminates in the activation of GIRK channels (G protein-gated K⁺ channel; Kir3). GIRKs are important transducers of inhibitory neurotransmitter effects in heart and brain. They regulate heartbeat, neuronal excitability and plasticity, analgesia, alcohol and drug effects, and are implicated in a number of disorders such as epilepsy, Down syndrome, bipolar disorder, atrial fibrillation and primary aldosteronism [2,3,4,5,6]. GIRK is also the first-discovered effector of Gβγ [7] and a prototypical model of membrane-delimited G protein signaling. In the now classical scheme, the agonist-bound GPCR catalyzes GDP/GTP exchange at Ga and the separation of GaGTP from Gβγ; Gβγ directly binds to GIRK and triggers channel opening [8,9,10,11].

Mammalian GIRKs are usually heterotetramers of GIRK1 with one of the other subunits (GIRK2, GIRK3 and GIRK4). GIRK1/2 is predominant in mammalian brain, but heterotetrameric GIRK1/3, GIRK2/3 and homotetrameric GIRK2 are also abundant in certain brain regions [2]. A GIRK channel is activated by direct binding of up to 4 molecules of Gβγ, but partial activation is achieved by binding of 1–3 Gβγ molecules [12,13,14,15,16]. NMR studies [17], crystal structure [18] and docking models [19] of GIRK-Gβγ complexes have confirmed the 4:1 Gβγ:GIRK stoichiometry, showing binding of one Gβγ to each interface between adjacent GIRK subunits. Further, a strong association of GIRKs with Gβγ has been suggested by co-immunoprecipitation and Förster/Bioluminescence Resonance Energy Transfer (FRET/BRET, respectively) [20,21,22,23,24]. In support, in *Xenopus* oocytes, GIRK1-containing channels...
recruit Gβγ to the plasma membrane (PM) [25]. GIRK also binds Gαi/o subunits which regulate the channel’s basal activity, specificity and kinetics of signaling [26,27,28,29,30,31,32,33,34], but the mechanisms are poorly understood. No FRET between GIRK subunits and Gαi/o could be detected in the PM [24,35,36]; GIRK1 does not recruit Gα, to the PM [25] and binds Gαi/o in vitro less strongly than Gβγ [36]. The stoichiometry of Gα-GIRK interaction is unknown.

Traditionally, GIRKs have been regarded as inhibitory devices operated exclusively by inhibitory neurotransmitters which elicit the GIRK’s evoked response (Ievoked). However, recent studies revealed that neuronal GIRKs also have a substantial basal activity, Ibasal [37,38,39]. GIRK’s basal activity and the balance between Ibasal and Ievoked are important determinants of neuronal excitability [39,40], bistability of neuronal networks [41], neuronal plasticity [42,43,44], dendritic integration [45], atrial arrhythmia and remodeling [46], and have recently been proposed to be related to effects of Li+, a drug used in the treatment of bipolar disorder [47]. Thus, changes in Ibasal and its relation to Ievoked are physiologically relevant and need to be understood.

The molecular mechanisms of Ibasal and Ievoked have been extensively studied in heterologous model systems, mainly Xenopus oocytes and human embryonic kidney (HEK) cells (e.g. [48,49]). We discovered that, for the neuronal GIRK1/2, Ibasal and Ievoked are coupled. Incremental expression of GIRK1/2 in Xenopus oocytes revealed an inverse correlation between Ibasal and the extent of activation by transmitter. The higher Ibasal, the smaller the index of activation by the transmitter (Ra) and by coexpressed Gβγ (Rβγ) [30]. The Ibasal-Ievoked coupling was regulated by Gαi; coexpression of Gαi3 reduced Ibasal, increased agonist- and Gβγ-induced GIRK currents (a phenomenon we dubbed “priming” by Gαi/o”), and eliminated the inverse correlation between Ibasal and Ra [30,31,50,51]. These findings compelled an unusual explanation of the underlying mechanism. We proposed that Gβγ available for GIRK regulation is in excess over Gαi/o, thus the high Ibasal of GIRK1/2. We suggested that the magnitude of Ibasal and its relation to Ievoked are crucially regulated by the availability of Gαi/o [30,31,50]. Here we demonstrate that cultured hippocampal neurons show the same inverse relation between Ibasal and Ra as previously found in oocytes and HEK cells. This prompted us to further use these heterologous systems to address the coupling between GIRK’s basal and evoked activity.

In the present work we have developed a quantitative model for Ibasal and Ievoked of GIRK1/2, which uses experimentally determined micro- and macroscopic parameters of GIRK1/2 currents and surface densities and accurately simulates and predicts macroscopic GIRK1/2 currents under a variety of conditions. Furthermore, modeling allowed to assess the apparent molar ratios of Gα and Gβγ available for GIRK, which we term “functional stoichiometry”. Our analysis reveals that, in Xenopus oocytes, HEK cells, and hippocampal neurons, 3 to 4 Gβγ molecules are available for the activation of GIRK1/2 channel over a wide range of surface densities of the channel, even when no exogenous Gβγ is coexpressed with GIRK. Calculations in Xenopus oocytes suggest a substantial increase in total concentration of Gβγ in the PM when large amounts of GIRK1/2 are expressed, corroborating the proposed mechanism of recruitment of Gβγ by GIRK1 to the PM [25]. In contrast, modeling shows that at most two Gα molecules are available for channel’s activation, even after overexpression of Gαi3. Furthermore, the Gα/GIRK ratio decreases with increasing channel density. The unequal and variable stoichiometry of GIRK1/2-associated Gα and Gβγ qualitatively and quantitatively explains the inverse Ra-Ibasal relation. Our results indicate a significant extent of association between GIRK1/2 and Gβγ, and support the notion that Gα is a non-obligatory partner in the GIRK-G protein signaling complex [50], but GαGDP plays a crucial role in regulating basal activity and, consequently, the magnitude of agonist response.
Results

Extent of agonist activation is inversely related to $I_{\text{basal}}$ in hippocampal neurons

First, we wanted to characterize the relation between GIRK’s $I_{\text{basal}}$ and $I_{\text{evoked}}$ in hippocampal neurons, known to preferentially express GIRK1/2 [2]. $I_{\text{basal}}$ and $I_{\text{evoked}}$ were measured using standard experimental paradigms ([39,47]; Fig 1A). Baclofen was used to activate the endogenous GABA$_A$ receptor and to generate $I_{\text{evoked}}$ [52]. Net GIRK’s $I_{\text{basal}}$ was revealed as shown in Fig 1A (see also S1 Fig), by adding 100–120 nM tertiapin-Q (TPNQ), which selectively blocks >90% of GIRK currents in hippocampal neurons [39,43,53].

To characterize the relation between $I_{\text{basal}}$ and $I_{\text{evoked}}$, we utilized the activation index $R_a$, defined as $I_{\text{total}}/I_{\text{basal}}$ (where $I_{\text{total}}$ is the total GIRK current; see Fig 1 and S1 Fig) [51]. GIRK currents of cultured hippocampal neurons showed considerable variability: $I_{\text{basal}}$, 0.2–26 pA/pF; $I_{\text{evoked}}$, 1–65 pA/pF ($n = 65$). Strikingly, there was a strong inverse correlation between $R_a$ and $I_{\text{basal}}$ (Fig 1B, closed triangles), which was similar to that observed in oocytes expressing GIRK1/2 (open circles). The strength of the correlation indicates that it may be driven by a distinct molecular mechanism of potential physiological importance. The similarity of this distinctive phenomenon in hippocampal neurons and GIRK1/2-expressing oocytes encouraged us to further investigate it in the *Xenopus* oocyte expression system. The oocyte is particularly suitable for accurate control of protein expression (by titrating the injected RNA) and for current measurements, which are essential for quantitative modeling of $I_{\text{basal}}$ and $I_{\text{evoked}}$ of GIRK1/2.

Modeling the steady-state gating of GIRK1/2 by G$\beta\gamma$

G$\beta\gamma$ is well-established as the main gating agent for GIRK’s $I_{\text{evoked}}$ [8,9]. This is also true for $I_{\text{basal}}$ of heterologously expressed GIRK1/2, which is suppressed by up to 80–90% by the expression of G$\beta\gamma$-binding proteins such as C-terminus of $\beta$-adrenergic kinase, phosducin or G$\alpha$, both in *Xenopus* oocytes [30,31,50] and HEK293 cells [28]. In this work, we did not manipulate...
cellular levels of phosphatidylinositol diphosphate (PIP_2), and used healthy cells which always showed robust GIRK currents, indicating levels of PIP_2 sufficient for channel activation [54]. Thus, under the conditions used in this work, G_βγ was the main gating factor determining the steady-state macroscopic GIRK current (I).

In a general form, I is described [1] by:

$$ I = I_{\text{single}} \cdot P_o \cdot N $$

where \(I_{\text{single}}\) is the single-channel current, \(N\) is the number of functional channels in the PM, and \(P_o\) is the channel’s open probability. In a heterologous expression system, the channel’s surface density (\(N/S\), where \(S\) is the surface area of the cell) can be experimentally manipulated and measured. \(I_{\text{single}}\) of GIRK channels is an activation-independent parameter; \(P_o\) is the gating parameter that changes as a function of the concentration of G_βγ available for GIRK activation by agonist or added G_βγ [9,11].

We start the development of the model by considering how G_βγ, available for activation of GIRK, can be derived from heterotrimeric G_αβγ (Fig 2A). In the absence of GPCR-activated G protein cycle, a small fraction of G proteins dissociates into free G_αGDP and G_βγ due to finite affinity of their interaction [55,56] (the left branch of the reaction in Fig 2A). This free G_βγ can contribute to \(I_{\text{basal}}\) [57,58]. Addition of agonist activates the GPCR and promotes GDP-GTP exchange at G_α and full or partial separation of G_αGTP from G_βγ (the right branch of the reaction in Fig 2A; [59,60,61]). In our experiments in Xenopus oocytes and HEK293 cells, we coexpressed the muscarinic receptor 2 (m2R) which couples to G_αi/o and used acetylcholine (ACh)

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**Fig 2. Gating of GIRK1/2 by G_βγ.** (A) Sources of G_βγ for GIRK activation. G_αGDP●G_βγ is the undissociated G protein heterotrimer. Note that, in isolated Xenopus oocytes or HEK cells, in the absence of added agonist the right, GPCR-dependent branch of the reaction of Fig 2A does not significantly contribute to \(I_{\text{basal}}\), because there are no known G_αi/o-coupled GPCRs or ambient agonists that can “basally” activate the GTPase cycle (discussed in [51]). (B) The schemes of “concerted”, “graded contribution” and “separate gating transitions” models of channel activation. (C) Graded contribution of the four G_βγ-occupied GIRK states to \(P_o\). Fractional \(P_o\) for each state was calculated by normalizing published \(P_o\) values [13] of each of the four modes (corresponding to 1–4 G_βγ occupied state) to \(P_{o,\text{max}}\) (corresponding to 4 G_βγ occupied channel). Almost identical values have been obtained from fractional activation ratios for engineered GIRK channels having 1 to 4 G_βγ binding sites [14].

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at supramaximal doses [62], 2–10 μM, in order to achieve a complete separation/rearrangement between GαGTP and Gβγ [63,64].

There are two existing models of GIRK gating by Gβγ. The allosteric kinetic model of Kura-chi and colleagues, developed for cardiac GIRK1/4 [65,66,67], adequately describes the kinetics and magnitude of agonist- and GTPγS-evoked currents and the effect of RGS proteins. However, this model does not address Ibasal, does not include an explicit Gβγ binding step (Gβγ acts catalytically) and assumes very high surface densities of G proteins, in the order of 56 mM, which are incompatible with our measurements or those of others (see below and Discussion). It would be difficult to adjust this model for our purposes and to adequately describe Ibasal, or to implement the Gβγ recruitment phenomenon.

The second model, termed here “concerted model”, was previously developed by us to describe the G protein-dependent activation of GIRK by Na+ (Fig 2B) [58]. The model included a description of Ibasal and an explicit Gβγ-GIRK binding step, but not the GαGTP-Gβγ dissociation step or Ievoked. Further, it assumed opening of the channel only when all four Gβγ binding sites are occupied (Fig 2B), which does not concur with the experimental findings that suggest a graded contribution of each bound Gβγ molecule [13,14,68].

Therefore, in the present work, we have developed a “graded contribution” model (Fig 2B and 2C), where each Gβγ-occupied state can contribute to channel opening and thus to P0 [13,14,68]. To date it is not known whether Gβγ binding to GIRK is truly cooperative (i.e. whether Gβγ occupancy at one binding site increases the affinity of Gβγ binding at another site). Therefore, for simplicity, in the graded contribution model we assume that Gβγ binding to GIRK is sequential and the affinity of each Gβγ-binding site is independent of the occupancy of other sites. However, overall the process of gating is cooperative, since occupancy of each additional Gβγ-binding site increases P0 in a more-than-additive manner (Fig 2C). The relative contributions of each Gβγ-occupied channel state to P0 are adopted from published data for the homologous GIRK1/4 channel [13,14]. Thus, one bound Gβγ causes channel opening with a P0 which is 1% of the maximal P0, P0,max; two Gβγ give 6%, three Gβγ 26%, and four Gβγ 100% of P0,max (Fig 2C). This approach is applicable only to steady-state calculations of macroscopic currents since it omits the kinetic details, but it allows to bypass the need to determine (or assume) a large number of unknown parameters: state-dependent changes in channel’s affinity to Gβγ, rates of closed-open transitions from different Gβγ-bound states, and the contributions of several potential open states. Once the channel achieved a state with n Gβγ bound, its fractional P0 is known and does not depend on the pathway by which the channel opens. For simplicity, in calculating the steady-state P0 for each Gβγ-occupied state, all open states (usually 2 are reported for GIRKs; [69,70,71,72]) were pooled into a single one (see Fig 2B).

We have also considered a more general model with 4 separate closed states, in which each closed subunit can open independently of other subunits and the opening is promoted by Gβγ binding, giving rise to four open states (the “separate gating transitions model”, Fig 2B). The scheme shown also describes an alternative case in which subsequent closed states C1-C4, arise from the Gβγ-free closed state C0, the transitions between closed states are driven by Gβγ binding, and there are 5 separate C-O transitions. In both cases, it can be shown that, utilizing the approach based on graded contributions of each Gβγ-occupied channel state to P0, the derivation of P0 converges to the same lead equation (eq 6) as for the graded contribution model (see Supplemental Discussion, S2 Text). Therefore, in this study we implemented the graded contribution model to simplify calculations. Throughout this work we also used an extended version of the concerted model, with the inclusion of the GPCR-induced dissociation of GαGTP from Gβγ, to cross-check the conclusions of the graded contribution model.

Quantitative description and modeling of signaling cascades require the evaluation of amounts, stoichiometry, and affinities of interactions of participating proteins [73,74,75,76,77].
We took an approach that rests as much as possible on experimentally determined parameters. The data necessary for simulation by the model (eqs 5–13 in Methods) are the surface density of GIRKs and G proteins in *Xenopus* oocytes, macroscopic parameters of GIRK1/2 gating (whole-cell *I*\textsubscript{basal}, *I*\textsubscript{evoked} and the current induced by coexpression of Gβγ, *I*\textsubscript{βγ}, see S2 Fig) at different channel densities, and *I*\textsubscript{single} and *P*\textsubscript{o,max}. Other parameters were experimentally determined in other works.

**Single-channel currents and *P*\textsubscript{o,max} of GIRK1/2**

To estimate single-channel current (*I*\textsubscript{single}) and *P*\textsubscript{o} of Gβγ- and agonist-activated GIRK1/2, we expressed the channels at low density with m2R and recorded channel activity in cell-attached patches (Fig 3A and 3B). *I*\textsubscript{single} was determined from amplitude distribution histograms (Fig 3A and 3B; right panels). The Gaussian fits to these histograms showed one main conductance level, suggesting that subconductance states, if any, did not significantly contribute to *P*\textsubscript{o}. The average *I*\textsubscript{single} was identical for ACh and Gβγ activation (Fig 3C), ~2.8 pA.

*P*\textsubscript{o} was estimated from patches containing 1 to 3 channels (see Methods). When channels were activated by Gβγ expressed at a saturating dose with no agonist present, *P*\textsubscript{o} was 0.105 ±0.018 (Fig 3D). The Gβγ RNA dose used (5 ng Gβ RNA, 1 ng Gγ RNA) consistently produced maximal macroscopic activation (see below, for example S8 Fig), and ACh generated negligible whole-cell *I*\textsubscript{evoked} which was ~10% of *I*\textsubscript{basal} (Ra = 1.1 ± 0.02, n = 14). Thus, free endogenous GαGTP produced upon activation of m2R did not substantially affect the GIRK1/2 current evoked by saturating Gβγ. We therefore conclude that the *P*\textsubscript{o}, measured in oocytes expressing saturating Gβγ is the *P*\textsubscript{o,max} of GIRK1/2, within a possible ~10% error. In comparison, when GIRK1/2 was activated via the coexpressed m2R (no Gα or Gβγ were coexpressed) with 2–5 μM ACh in the pipette, *P*\textsubscript{o} was 0.037±0.008, less than half of *P*\textsubscript{o,max} (Fig 3B and 3D). (The actual *P*\textsubscript{o} could be higher because of the desensitization observed with ACh but not with Gβγ; see Methods).

**Initial estimation of functional GIRK1/2:Gβγ stoichiometry from macroscopic currents**

For further analysis and modeling of whole-cell *I*\textsubscript{basal} and *I*\textsubscript{evoked}, we varied the surface density of GIRK1/2. The design was to obtain low, intermediate and high densities of GIRK1/2 by injecting 25, 100–200 or 1000–2000 pg RNA of each subunit per oocyte. The cells expressed 1 or 2 ng of m2R RNA which did not affect *I*\textsubscript{basal} (S2C Fig and ref. [78]) but could always produce the maximal *I*\textsubscript{evoked} [78]. *I*\textsubscript{evoked} was elicited by ACh at 10 μM, a saturating dose. Under these conditions, all Gαo should convert to GαGTP, so that all available Gβγ can bind to the channel and activate it. The data are summarized in Table 1; main findings are also briefly highlighted in Fig 3E and 3F. We measured *I*\textsubscript{basal}, *I*\textsubscript{evoked} and *I*\textsubscript{total} in each oocyte (set 1 in Table 1, S2A Fig). In separate groups of oocytes expressing saturating Gβγ, where channel’s *P*\textsubscript{o} reached *P*\textsubscript{o,max}, we measured *I*\textsubscript{βγ} (set 2 in Table 1, S2B Fig).

It is noteworthy that at all channel densities, *I*\textsubscript{βγ} was 1.6–2 fold greater than *I*\textsubscript{total}, the total GIRK current (*I*\textsubscript{basal} + *I*\textsubscript{evoked}) without coexpressed Gβγ (Table 1). A similar *I*\textsubscript{βγ}/*I*\textsubscript{total} ratio of 1.66 was observed in HEK293 cells (Table 2), where all data have been pooled together (because GIRK1/2 expression levels have not been monitored). Similarly, *I*\textsubscript{βγ}/*I*\textsubscript{total} ratio of ~2.2 for GIRK1/2 expressed in HEK cells can be estimated from the data of Wydeven et al. [79] who activated GIRK with baclofen via GABA\textsubscript{A} receptors (*I*\textsubscript{evoked} ~40 pA/pF, *I*\textsubscript{βγ} ~ 90 pA/pF). The inverse value, *I*\textsubscript{total}/*I*\textsubscript{βγ} ranged 0.5–0.62 at different channel densities (Fig 3F, left panel). Since GIRK1/2 was maximally activated by the doses of Gβγ used in these experiments, *I*\textsubscript{total}/*I*\textsubscript{βγ} is equal to the fraction of maximal activation after GPCR activation, *P*\textsubscript{o}/*P*\textsubscript{o,max}. Note that the
Fig 3. Single channel and whole-cell data reveal incomplete activation of GIRK1/2 by agonist compared to Gβγ. (A) Activity of GIRK1/2 in a cell-attached patch of an oocyte expressing the channel, m2R and Gβγ, without an agonist in the pipette. Right panel shows a 2 minutes segment of record, with zoom (below) on a shorter segment. The amplitude distribution histogram of the same 2 min-segment is shown on the right. Red line shows a two-component Gaussian fit. $I_{\text{single}}$ was determined as the difference between the fitted midpoints ($\mu$) of the GIRK current peak on the right ($\mu_2$) and the left peak which corresponds to noise ($\mu_1$).

(B) Activity of GIRK1/2 channels in a cell-attached patch of an oocyte expressing the channel and m2R and activated by 2 μM ACh present in the patch pipette. (Asterisks denote artifacts produced by capacity discharges of patch clamp headstage). The corresponding amplitude histogram of the 2 min-segment of the record is shown on the right. In A and B, GIRK1/2 was expressed at low densities (GIRK1, 10–50 pg RNA; GIRK2, 7–17 pg RNA) whereas RNAs of m2R (1–2 ng/oocyte) and Gβγ (5:1 ng/oocyte) were chosen to produce saturating concentrations of these proteins. Inward K+ currents are shown as upward deflections from zero level. In the traces shown, acquisition was at 20 KHz with 5 KHz analog filter. Very similar values of $I_{\text{single}}$ were obtained with 2 KHz filtering (not shown).

(C) Single channel currents (left plot) are identical with either ACh or Gβγ. (D) $P_o$ is lower with ACh than with Gβγ ($p = 0.029$). Bars in C and D show mean±SEM, number of patches is shown above the bars.

(E) Summary of whole-cell GIRK1/2 currents at three expression levels (densities). See Table 1 for details.

(F) Left panel shows the $I_{\text{total}}/I_{\beta\gamma}$ ratios at three channel densities, calculated from data of Table 1. The right panel shows the fractional open probabilities of channels occupied by 0–4 Gβγ, same as in Fig 2C but in a simple graphic form. The red dotted lines are drawn to allow direct comparison of the experimental data from the left panel with the estimates of fractional $P_o$ from the right panel.

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single-channel data (Fig 3D) show less than 40% activation with saturating ACh (although, as noted above, this is probably an underestimate because of desensitization). In all, in oocytes and HEK cells, when the channel is activated by an agonist, only 40–60% of maximal Po is achieved.

From the data of Fig 3, one can approximately estimate the amount of Gβγ molecules that are bound to the channel after maximal activation by agonist. This is done by comparing between measured values of Itotal/Iβγ (Fig 3F, left panel) and the expected Po/Po,max [13,14] from Fig 2C. To facilitate the comparison, we have redrawn the plot of Fig 2C in a simple graphic form (Fig 3F, right panel), and projected the values of Itotal/Iβγ onto the Po/Po,max plot (red dashed lines). For 3 bound Gβγ, the expected Po/Po,max is 0.26, and for 4 Gβγ it is 1. Thus, with Itotal/Iβγ of 0.4–0.6, we estimate that, even without coexpression of Gβγ, between 3 and 4 Gβγ are available for activation of a single GIRK channel at all channel densities.

Expression-dependent changes in surface levels of GIRK1/2

For saturating Gβγ, Eq 1 for Ipγ takes the form:

\[ Ipγ = I_{\text{single}} \cdot P_{o,max} \cdot N. \]  

(2)

From here, we calculated the total number of functional channels in the PM (N) and the corresponding channel density per μm² of the PM. As shown in Table 1, our “low,” “intermediate” and “high” expression levels correspond to approximately 2.7, 9.7 and 21.7 channels/μm².

Table 1. Whole-cell currents of GIRK1/2, the calculated surface density and Ipγ/Itotal in Xenopus oocytes.

<table>
<thead>
<tr>
<th>Group (channel density)</th>
<th>ng RNA GIRK1, GIRK2</th>
<th>Set 1: experiments with agonist</th>
<th></th>
<th>Set 2: experiments with no agonist</th>
<th></th>
<th>calculated density (channels/μm²)</th>
<th>Ipγ/Itotal</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>lbasal (μA)</td>
<td>levoked (μA)</td>
<td>ltotal (μA)</td>
<td>No Gβγ lbasal (μA)</td>
<td>Gβγ expressed lγ (μA)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low</td>
<td>0.025</td>
<td>0.73±0.065 (51)</td>
<td>1.19±0.09 (51)</td>
<td>1.92±0.14 (51)</td>
<td>1.06±0.13 (18)</td>
<td>3.49±0.37 (14)</td>
<td>2.74±0.29</td>
</tr>
<tr>
<td>Intermediate</td>
<td>0.1–02</td>
<td>3.9±0.36 (55)</td>
<td>2.3±0.2 (55)</td>
<td>6.2±0.45 (55)</td>
<td>3.77±0.55 (28)</td>
<td>12.34±2.82 (26)</td>
<td>9.7±2.2</td>
</tr>
<tr>
<td>High</td>
<td>1–2</td>
<td>13.36±0.87 (10)</td>
<td>3.84±0.81 (10)</td>
<td>17.2±1.42 (10)</td>
<td>15±0.84 (75)</td>
<td>27.6±1.3 (77)</td>
<td>21.7±1</td>
</tr>
</tbody>
</table>

Data are shown as mean±SEM (except Ipγ/Itotal), number of cells is shown in parentheses. Data for each entry were collected from at least 2 independent experiments. The Table summarizes separate sets of experiments: those where lbasal, levoked and ltotal were measured (in each oocyte); and those where Gβγ was coexpressed and Ipγ was measured. In addition, in Set 2, lbasal was measured in each experiment in a separate group of oocytes not injected with Gβγ RNA. For the low density group in oocytes, there was ~30% difference (p = 0.017) for lbasal between the two sets of experiments, probably because of variability among oocyte batches. In intermediate and high density groups lbasal was not different (p>0.4) for both sets of experiments.

do:10.1371/journal.pcbi.1004598.t001

Table 2. Whole-cell currents of GIRK1/2 and Ipγ/Itotal in HEK293 cells.

<table>
<thead>
<tr>
<th>Set 1: experiments with agonist</th>
<th>Set 2: Gβγ expression</th>
<th>Ipγ/Itotal</th>
</tr>
</thead>
<tbody>
<tr>
<td>lbasal (pA/pF)</td>
<td>levoked (pA/pF)</td>
<td>ltotal (pA/pF)</td>
</tr>
<tr>
<td>19±1.4±4.4 (25)</td>
<td>30.6±6.7 (25)</td>
<td>49.7±10.5 (25)</td>
</tr>
</tbody>
</table>

Data are shown as mean±SEM (except Ipγ/Itotal), number of cells is shown in parentheses. Data for each entry were collected from at least 2 independent experiments, except Ipγ which was measured in one experiment. Raw data of Set 1 were reported in [51].

do:10.1371/journal.pcbi.1004598.t002
respectively. In the following, the data of Table 1 served as the basis for testing the predictions of the model and for calculating $G_{\alpha}$ and $G_{\beta\gamma}$ available for channel activation.

**Surface levels of GIRK1/2 are confirmed by quantitative immunochemistry**

To obtain an independent estimate of the density of GIRK1/2 in the PM, we used quantitative immunochemistry of GIRK1 in cytosol-free, manually separated plasma membranes of *Xenopus* oocytes (Fig 4A and 4B) [80,81]. GIRK1 was coexpressed with GIRK2 at high density, and GIRK1 was probed with a C-terminally directed antibody. Western blots of manually separated PM and of the rest of the cells without the nucleus ("cytosol") showed the presence of two forms of GIRK1. A double band of about 55–58 KDa was always observed, and an additional
higher diffuse band was seen in four out of seven experiments (Fig 4A and 4B). These bands correspond to partly and fully glycosylated channels, respectively [82,83]. Three oocyte batches showed only partly glycosylated bands in the PM. Since oocytes injected with 2 ng RNA always had large GIRK1/2 currents, it is likely that both partly and fully glycosylated channels are functional at the PM, in agreement with [83]. Notably, the main fraction of the channel was found in the cytosolic fraction (most likely endoplasmic reticulum and Golgi), largely in a partly-glycosylated form (Fig 4A). This is not unexpected, because in *Xenopus* oocytes the PM constitutes only a very small fraction of the cell’s total mass [81].

Next, molar amounts of GIRK in the PM fraction were calculated, taking into account the presence of two GIRK1 subunits in each channel. Calibration of antibody-produced signal was done with known amounts of the GST-fused distal C-terminus of GIRK1 which contains the epitope for the antibody. Note that this method yields channel levels in concentration units (e.g. mole/L). Both GIRK and Gβγ are associated with the PM (Gβγ is membrane-anchored by a lipid moiety [84]), and the interaction between GIRK’s cytosolic domain and Gβγ takes place within the submembrane space. Therefore, to compare data with GIRK1/2 surface densities obtained by channel counting from currents (Table 1), and for further modeling, we have converted two-dimensional protein densities to protein concentrations within the interaction space as previously described (e.g. [58,85,86,87]; see Supplemental Discussion, S2 Text), according to

$$C = \frac{N}{(W \cdot S \cdot A)}, \quad (3)$$

where C is the concentration of protein in the submembrane space, N is the number of protein molecules in the membrane, S is a membrane area, A is Avogadro number and W is the width of the interaction space. For calculations, we used $S_{\text{oocyte}} = 2 \times 10^7 \, \mu m^2$ (deduced from an oocyte’s capacitance of 200 nF [88] and specific capacitance of plasma membrane of 1 μF/cm²), and W was assumed to be 10 nm. The latter roughly corresponds to the molecular size of the complex of Gβγ and the cytosolic part of GIRK [18]. The influence of this parameter on the conclusions of the model was tested later (see below, S6 Fig, panels D and E). Consequently, the standard conversion factor between channel density (number of channels/μm²) and channel concentration (nM) is 1 channel/μm² = 166 nM.

Conversion of channel concentrations determined in Fig 4 into surface densities using Eq 3 gave ~12–14 channels/μm² for both partly and fully glycosylated channels in the PM, and the average total amount of GIRK1/2 in PM (with partly + fully glycosylated GIRK1) was 19.1±6.8 channels/μm² (Fig 4B). This is in good agreement with the independent assessment of ~22 channels/μm² obtained from measurements of $I_{\text{pmax}}$ for the high GIRK1/2 expression group (Table 1). To note, the latter was calculated using Eq 2 with $P_{\text{o,max}}$ measured at low channel densities. If $P_{\text{o,max}}$ were different at high GIRK1/2 expression levels, the densities calculated from $I_{\text{pmax}}$ and immunochemistry would not match. The close correspondence between the two independent approaches indicates that $P_{\text{o,max}}$ is preserved at the high expression level.

We conclude that the total surface density of GIRK1/2 channels in the PM can be satisfactorily estimated from whole-cell currents (Eq 2, Table 1). Such measurements are calibration-independent and accurate [1], and therefore GIRK1/2 can be used as a “molecular ruler”. In this procedure, the fluorescently labeled GIRK1/2, with its surface density calculated from $I_{\text{pmax}}$, will serve as a reference for estimating PM densities of other fluorescently labeled proteins. To use GIRK1/2 as a molecular ruler, we expressed YFP-GIRK1 (GIRK1 with Yellow Fluorescent Protein (YFP) fused to the N-terminus). Single channel analysis of Gβγ-activated YFP-GIRK1 coexpressed with GIRK2, YFP-GIRK1/2, showed the same $P_{\text{o,max}}$ and $I_{\text{single}}$ as in wild-type
GIRK1/2 (S3 Fig), allowing the use of this construct for calibration purposes. To obtain high current levels of YFP-GIRK1/2 we usually had to inject 5 ng/oocyte of channel’s RNA.

Surface levels of Gβγ

To estimate the expression levels of Gβγ using YFP-GIRK1/2 as molecular ruler, we expressed YFP-GIRK1/2 and, in separate oocytes of the same batch, Gβγ in which either Gβ or Gγ was labeled with YFP. Expression of YFP was monitored from fluorescence intensity in the PM (Fig 4C). In addition, Iβγ was measured and the surface density of YFP-GIRK1/2 was calculated. The amount of YFP molecules per μm² was calculated assuming a 2:2 GIRK1:GIRK2 stoichiometry in a heterotetramer [89] (See also Supplemental Discussion, S2 Text). The surface density of Gβγ-YFP was then calculated based on intensity ratios of YFP-GIRK1 and Gβγ-YFP. A typical experiment is shown and explained in Fig 4D.

To validate the estimates of Gβγ expression, in four experiments as in Fig 4D we also measured the levels of Gβ-YFP (coexpressed with unlabeled Gγ) by quantitative immunochemistry in manually separated plasma membranes. We used purified Gβγ to calibrate the signal produced by the Gβ antibody (Fig 4E). We also constructed and expressed an YFP-fused construct corresponding to *Xenopus laevis* Gβ1, YFP-Gβ-XL (see Methods). Western blots showed a prominent ~36 KDa band of the endogenous Gβ, and ~70 KDa bands corresponding to the expressed YFP-Gβ or YFP-Gβ-XL (Fig 4E). The surface density of the expressed YFP-Gβγ assessed by the quantitative immunchemical method was 28±6 molecules/μm², close to the estimate of 22.1±8.7 molecules/μm² obtained in the same experiments from measurements of fluorescence using YFP-GIRK1 as “molecular ruler” (Fig 4F; 53 oocytes, n = 4 experiments; P = 0.295). These results demonstrate the feasibility of the molecular ruler methodology and provide a good estimate of the expressed Gβγ-YFP. In several sets of experiments (see also below) we consistently found that, with 5 ng RNA of Gβγ, its surface density ranged between 20 and 30 molecules/μm².

We next utilized YFP-Gβ-XL as a caliper for the endogenous oocyte’s Gβ. Results of 4 experiments showed that, in Western blots, Gβ antibody used here gave similar signal with Gβ-XL as with bovine Gβ (S3 Fig, panels C, D). We then estimated the surface density of the endogenous Gβ (the 37 kDa band in Fig 4E) to be 24±4.6 molecules/μm² (n = 4). We have also estimated the concentrations of total and cytosolic endogenous Gβγ from the 4 experiments of Fig 4E, assuming an oocyte’s water volume of 0.5 μl [88]. The total Gβγ concentration was 173±44 nM, the concentration of Gβγ in the cytosolic fraction was 171±44 nM.

Estimation of functional stoichiometry of GIRK1/2, Gβγ and Ga

We define the molar amounts of proteins physically available for the function of the cascade as functional stoichiometry. It can change depending on availability of a protein, in contrast to limiting (maximal) stoichiometry which reflects the maximal molar ratios of interacting proteins. For example, if one GIRK channel can interact with at most 4 Gβγ and 4 Ga molecules, then the limiting GIRK-Gβγ:Ga stoichiometry is 1:4:4.

Having determined the Pmax and surface densities of GIRK1/2 and endogenous Gβγ, we were now able to simulate macroscopic GIRK currents in oocytes and to assess the functional stoichiometry of GIRK1/2-Ga-Gβγ. We initially assumed that all of the Ibasal in oocytes was Gβγ-dependent. The affinities of GIRK-Gβγ and Ga-Gβγ interactions were adopted from published work: KD = 1.86 nM for Ga GDP-Gβγ binding [56], and KD = 50 nM for the GIRK-Gβγ interaction, as estimated by biochemical methods [90]. Simulations were done using eqs 5–16 as explained in the Methods section. Simulated data were compared to experimental measurements of GIRK1/2 activity for the three GIRK1/2 surface density groups (Table 1 and Fig 3E).
Note that GIRK densities were calculated from $I^\beta\gamma$ but simulations were done for the separately measured $I^\text{basal}$ and $I^\text{evoked}$, avoiding circular reasoning.

We first tried to simulate the experimental data by assuming that only endogenous $G^\alpha\beta\gamma$ is available for the activation of GIRK1/2 (S4A Fig). However, no satisfactory description of data can be obtained under this assumption. Simulations that assumed recruitment of 3–4 $G^\beta\gamma$ with GIRK, without $G^\alpha$, gave a better approximation to the data (S4 Fig, panels B, C).

Next, we turned to a more accurate assessment of functional GIRK1/2:G$^\beta\gamma$:G$^\alpha$ stoichiometry. Our model allows to calculate the amounts of $G^\alpha$ and $G^\beta\gamma$ available for GIRK1/2 without any prior knowledge or assumption about the G protein concentrations in the cell, directly from experimental data. This idea is illustrated graphically in Fig 5 for the high GIRK1/2 surface density group of Table 1. The procedure consists of two steps. First, because all $G^\beta\gamma$ available for GIRK is free to activate the channel after addition of agonist, the total GIRK-available $G^\beta\gamma$ can be calculated from $I_{\text{total}}$, as shown in Fig 5A. Here, the solid green line presents the simulated $I_{\text{total}}$ for a range of $G^\beta\gamma$ surface densities, using the $G^\beta\gamma$ density calculated in (A), and compared with the experimentally observed $I_{\text{total}}$.

(B) Estimation of $G^\alpha$ available for interaction with the channel. Simulated $I_{\text{basal}}$ (red line) was calculated using eqs 5–16 for a range of $G^\alpha$ surface densities, using the $G^\beta\gamma$ density calculated in (A), and compared with the experimentally observed $I_{\text{basal}}$. (C, D) The estimates of $G^\beta\gamma$:GIRK (C) and $G^\alpha$:GIRK (D) ratios are stable in a wide range of GIRK-G$^\beta\gamma$ interaction affinities, from $K_D = 5$ nM to 100 nM. Simulations were done with the graded contribution model separately for the low-, intermediate- and high density groups from Table 1 (2.74, 9.7 and 21.7 channels/μm², respectively).

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doi:10.1371/journal.pcbi.1004598.g005

Excess G$^\beta\gamma$ over G$^\alpha$ Governs GIRK Activity
available Gβγ and Ga for a range of KD from 5 to 100 nM. As shown in Fig 5C and 5D, the estimates of Gβγ:GIRK and Ga:GIRK ratios, and the trend in their changes as a function of channel density, remain highly stable within the examined range of KD. Similar conclusions were attained using the concerted model (Table 3 and S5 Fig). The latter does not involve any assumptions for fractional Gβγ contributions to total Po. Similarity of conclusion of the two models alleviates concerns regarding the use of values for fractional Gβγ contributions, adopted from GIRK1/4 studies, to simulate GIRK1/2.

Table 3 also shows the total Gβγ and Ga available for GIRK in PM, in molecules/μm², calculated for each of the three channel densities. It is easy to see that, at intermediate and high channel densities, the endogenous Gβγ, 24 molecules/μm² (that was present in the PM before the expression of GIRK) cannot account for the observed GIRK1/2 activation. In contrast, estimates of total Ga available for GIRK1/2 remain within the limits of endogenous Ga. As mentioned before, the calculations have been made without any assumption regarding the presence or amount of endogenous Gaβγ, and made no specific a priori assumptions regarding recruitment of Gβγ or Ga. Hence, modeling independently predicts the necessity for GIRK1/2-related increase in PM density of Gβγ, corroborating the experimentally observed recruitment of Gβγ, but not Ga, by GIRK1/2 [25].

Next, we addressed the possible contribution of intrinsic, Gβγ-independent activity to Ibasal. About 10–20% of Ibasal of GIRK1/2 in Xenopus oocytes [30,31,50] and HEK293 cells [28] persists after expression of Gβγ scavengers or Ga. Whereas the residual Ibasal may reflect incomplete Gβγ chelation, a genuine Gβγ-independent fraction of Ibasal cannot be discarded. In the extreme case it may contribute up to 20% of GIRK1/2 Ibasal. This may account for up to 10% of P_o,max (because Ibasal can reach at most half of Iβγ, which is the indicator of P_o,max; Tables 1 and 2).

We have therefore extended the model to include the contribution of a hypothetical intrinsic Gβγ-independent channel activity. We assume that the intrinsic basal Po of a channel (Po,intrinsic) is an inherent, density-independent property of a single channel, best described as a fraction of P_o,max. We thus repeated our calculations of GIRK1/2-available Ga and Gβγ assuming a Po,intrinsic in the range between 1% and 10% of P_o,max (S6 Fig, panels A, B). For these calculations, Eq 6 (Methods) was modified in the following way:

$$I = I_{single} \cdot N \cdot P_{o,max} \cdot \left( \phi \cdot \sum_{i=1}^{4} f_{p,i} \cdot \delta_i + (1 - \phi) \right),$$

where $\phi$ is the fraction of P_o,max which is Gβγ-dependent (see Eqs 6 and 7 in the Methods for definitions of other parameters). In the whole range of Po,intrinsic tested, the estimation of 3–4 Gβγ per channel remained highly stable (S6 Fig). The estimate of less than 2 Ga per channel

Table 3. Calculation of Gβγ and Ga available for channel activation in Xenopus oocytes (without coexpressing Gβγ). KD for channel-Gβγ interaction was taken as 50 nM. For calculations with other KD values, see Fig 5 and S5 Fig.

<table>
<thead>
<tr>
<th>Channel density group</th>
<th>channels/μm² (from Table 1)</th>
<th>total available Ga and Gβγ, molecules/μm²</th>
<th>Gβγ:GIRK and Ga:GIRK ratios</th>
<th>total available Ga and Gβγ, molecules/μm²</th>
<th>Gβγ:GIRK and Ga:GIRK ratios</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low</td>
<td>2.74</td>
<td>10.2</td>
<td>3.75</td>
<td>3.74</td>
<td>1.37</td>
</tr>
<tr>
<td>Intermediate</td>
<td>9.7</td>
<td>31.6</td>
<td>5.4</td>
<td>3.26</td>
<td>0.56</td>
</tr>
<tr>
<td>High</td>
<td>21.7</td>
<td>75.5</td>
<td>7.2</td>
<td>3.48</td>
<td>0.33</td>
</tr>
</tbody>
</table>

doi:10.1371/journal.pcbi.1004598.t003
also persisted except at the highest $P_{o,\text{intrinsic}}$ and low GIRK1/2 density, where $G_{\alpha}$:GIRK ratio slightly exceeded 2 (S6B Fig, low surface density, black bar). Interestingly, for a significant Gβγ-independent intrinsic activity (10% of $P_{o,\max}$), up to ~60% of macroscopic $I_{\text{basal}}$ could be Gβγ-independent, especially at low channel densities which are common in native cells (S6C Fig). This finding may be relevant to some cells. For instance, coexpression of the Gβγ scavenger phosducin did not significantly reduce $I_{\text{basal}}$ in atrial cardiomyocytes [94], where the predominant channel is GIRK1/4.

Finally, we considered the possible impact of variation in the presumed width (W) of the submembrane space within which the GIRK-Gβγ interactions occur. S6D and S6E Fig) shows that the main conclusions regarding the functional stoichiometry of GIRK, Gα and Gβγ remained largely unchanged over a wide range of W, 1–20 nm.

Activation of GIRK1/2 by coexpression of Gβγ: experiment and simulation

We next tested the ability of the model to predict a new result: the dose dependency of activation by Gβγ, using the estimates of available Gα and Gβγ calculated from basal and agonist-evoked currents. We injected increasing amounts of wt-Gβγ mRNA into oocytes expressing GIRK1/2 at a constant density, and monitored both GIRK currents and Gβγ expression.

Relative levels of Gβγ in the PM were directly measured in giant membrane patches of the oocytes [30,95] (Fig 6A) using the anti-Gβ antibody. Absolute surface densities of the expressed Gβγ (X axis in Fig 6C) were calculated assuming that 5 ng mRNA of Gβγ gives 30±4 molecules/μm² (n = 47 oocytes). This density was calculated based on 3 experiments performed during the same period as the experiments of Fig 6 and S8 Fig, with wt-Gβ and YFP-Gγ, and using YFP-GIRK1/2 as the molecular ruler.

As shown in Fig 6B, expression levels of Gβγ in the PM (grey bars) reached maximum at 5 ng RNA/oocyte. $I_{\beta\gamma}$ (red circles) reached maximum already at 1 ng Gβγ RNA. Thus, maximal activation of GIRK1/2 has been attained already at submaximal expression levels of Gβγ (see also S8 Fig). Channel density of 13.75 channels/μm² was calculated based on $I_{\beta\gamma}$ measured after expression of 5 ng RNA of exogenous Gβγ. Gβγ and Gα available to GIRK without the coexpression of exogenous Gβγ were calculated from $I_{\text{basal}}$ and $I_{\text{total}}$ (as in Fig 5), yielding ~43 molecules of Gβγ and 10 molecules Gα per μm². We remind that the high density of available “endogenous” Gβγ in the presence of GIRK1/2 is due to Gβγ recruitment, explaining the high $I_{\text{basal}}$ and the relatively low index of activation of GIRK by Gβγ in a given oocyte, $R_{\beta\gamma}$ ($R_{\beta\gamma}$ was defined as $I_{\beta\gamma}$/[average $I_{\text{basal}}$], where average $I_{\text{basal}}$ was determined in a group of oocytes of the same experiment which expressed the channel without Gβγ. The definitions are as in [51]. See S2 Fig for definition of $R_{\beta\gamma}$.

Using the calculated GIRK1/2 density and the amounts of available Gα and Gβγ before coexpression of Gβγ, we next calculated the predicted $I_{\beta\gamma}$ and $R_{\beta\gamma}$ for a range of doses (surface densities) of exogenously coexpressed Gβγ (Fig 6C). The predicted dose-dependencies of $I_{\beta\gamma}$ and $R_{\beta\gamma}$ (blue lines) are in agreement with experimental data (red circles). Assuming that 5 ng/oocyte of Gβ RNA gives either less (20 Gβγ/μm²) or more (44 Gβγ/μm²) molecules of coexpressed Gβγ instead of 30 Gβγ/μm² produced similar predictions, still in good agreement with experiment (S7 Fig). Thus, the results of the simulations are relatively insensitive to a 50% variation in our estimate of coexpressed Gβγ. Further, very similar results were obtained in a separate experiment using a different experimental design, where we expressed increasing doses of Gβγ-YFP and calibrated Gβγ-YFP density using YFP-GIRK1/2 as molecular ruler (S8 Fig).

We note that, because channel’s density is estimated from $I_{\beta\gamma}$ obtained with a saturating dose of Gβγ, the good agreement between measured and predicted $I_{\beta\gamma}$ at this RNA dose might
be expected. However, the densities of $\alpha$ and $\beta\gamma$ available to GIRK before coexpression of exogenous $\beta\gamma$ (0 point on $X$-axis in Fig 6C) are calculated from $I_{\text{basal}}$ and $I_{\text{evoked}}$. Therefore, in both experiments (Fig 6 and S8 Fig), the satisfactory predictions of $I_{\beta\gamma}$ and $R_{\beta\gamma}$ at intermediate $\beta\gamma$ doses, or the shape of the dose-response curves of $I_{\beta\gamma}$ and $R_{\beta\gamma}$ vs. $\beta\gamma$ density, do not result from circular reasoning and are not trivial. This is illustrated by showing simulations that assume equal amounts of endogenous $\alpha$ and $\beta\gamma$ (1, 10 or 24 molecules/μm$^2$) available for GIRK1/2, and no $\beta\gamma$ recruitment. The use of these “classical” assumptions failed to reproduce the experimental data (black, red and green lines in Fig 6C and S7 Fig). In particular, saturation of $R_{\beta\gamma}$ was predicted to happen at much higher doses of coexpressed $\beta\gamma$ than in the experiment, obviously because the presumed initial basal level of $\beta\gamma$ available to the channels was too low, thus requiring expression of more additional $\beta\gamma$.

**Application of the model to the HEK293 expression system and hippocampal neurons**

We next evaluated the model’s applicability to another expression system (HEK293 cells), and also to hippocampal neurons that natively express GIRK1/2 channels.
previously obtained in HEK cells expressing m2R and GIRK1/2 [51] (Table 2), the data from cultured hippocampal neurons (Fig 1), and the data obtained in oocytes, in a uniform manner. To enable direct comparison between the different systems, we arbitrarily segregated all the recordings into 4 groups, based on basal GIRK currents: <3 pA/pF, 3–13 pA/pF, 13–50 pA/pF and >50 pA/pF (Table 4). For modeling, we needed to estimate channel surface densities, which have not been directly measured in HEK293 cells and neurons. To this end, we used $I_{\text{total}}$ to indirectly assess the channel densities. The ratio $I_{\beta\gamma}/I_{\text{total}}$ is fairly consistent in oocytes and HEK cells, ranging between 1.6 and 2.2 (Table 1 and ref. [96]). Thus, for each $I_{\text{basal}}$ range, we calculated $I_{\beta\gamma}$ from $I_{\text{total}}$ assuming $I_{\beta\gamma}/I_{\text{total}} = 2$ (Fig 7). Then we calculated densities using Eq 2 and $P_{\text{o, max}}$ of 0.105, as measured in oocytes.

### Table 4. GIRK currents in mouse hippocampal neurons and in GIRK1/2-expressing Xenopus oocytes and HEK293 cells. Data are presented as mean ± SEM. Current amplitudes in HEK293 cells and neurons were adjusted to 24 mM K⁺ (as in oocytes) as described in Methods.

<table>
<thead>
<tr>
<th>$I_{\text{basal}}$ range, pA/pF</th>
<th>corresponding $I_{\text{basal}}$ in oocytes</th>
<th>cell type</th>
<th>$I_{\text{basal}}$, pA/pF</th>
<th>$I_{\text{evoked}}$, pA/pF</th>
<th>$I_{\text{total}}$, pA/pF</th>
<th>$R_a$</th>
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<tr>
<td>0.5–3 pA/pF</td>
<td>0.1–0.6 μA</td>
<td>neurons</td>
<td>1.8±0.1</td>
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<td>15.3±2.3</td>
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<td></td>
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<td>oocytes</td>
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<td>4.2±0.4</td>
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<td>3–13 pA/pF</td>
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<td>oocytes</td>
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<td>15.6±1</td>
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<td>HEK cells</td>
<td>25.7±3.1</td>
<td>34±7</td>
<td>60±9</td>
<td>2.4±0.2</td>
<td>9</td>
</tr>
<tr>
<td>&gt;50 pA/pF</td>
<td>&gt;10 μA</td>
<td>neurons</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>oocytes</td>
<td>65.9±2.1</td>
<td>84.5±2.7</td>
<td>84.5±2.7</td>
<td>1.3±0.03</td>
<td>41</td>
</tr>
<tr>
<td></td>
<td></td>
<td>HEK cells</td>
<td>67.6±4.6</td>
<td>96±28</td>
<td>164±26</td>
<td>2.5±0.5</td>
<td>3</td>
</tr>
</tbody>
</table>

Fig 7. Estimated densities and calculated functional stoichiometries of the GIRK channel, $G_{\beta\gamma}$ and $G_{\alpha_{i/o}}$ in oocytes, HEK293 cells and neurons. Comparison of cultured mouse hippocampal neurons, and in oocytes and HEK293 cells expressing GIRK1/2. (A) Cells were subdivided into four groups according to the indicated $I_{\text{basal}}$ ranges, and channel densities were estimated assuming $I_{\beta\gamma} = 2I_{\text{total}}$ and $P_{\text{o,max}} = 0.105$. Densities in $G_{\alpha}$ expression experiments in oocytes were estimated from $I_{\text{total}}$ in control groups of oocytes expressing GIRK1/2 and m2R only. (B, C) Estimates of $G_{\beta\gamma}$ and $G_{\alpha}$ available for GIRK activation in the 4 channel density groups. In oocytes and HEK293 cells $I_{\text{evoked}}$ was elicited by ACh via m2R, in neurons—by baclofen acting on GABA$_{B}$ receptors.

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The segregation of GIRK activity by Ibasal yielded relatively similar channel density groups in all cell types; neurons did not appear to express large amounts of GIRK, and the high density group was empty (Fig 7A). Next, using the procedure of Fig 5, we calculated the Gβγ and then Gα available for GIRK in all cases (Fig 7B and 7C). In all three systems, the relationship between G protein subunits and channel density was very similar to that found in oocytes. The most striking result was the persistent, channel density-independent availability of more than 3 Gβγ molecules per channel (Fig 7B). In all cases, the estimated Gα:GIRK ratio was about 2 for the low channel densities, but this number decreased as channel density increased (Fig 7C). Nevertheless, estimates of Gα:GIRK ratio in oocytes were lower than in HEK cells or neurons, indicating that there was a relative shortage of GIRK-associated Gαi/o in oocytes. We have therefore reanalyzed the experiments [30] in which Gα was co-expressed in oocytes in 5–10 fold excess (in terms of RNA quantities) over GIRK1/2 (Fig 7, denoted as "oocytes+Gα").

These doses produced the maximal GIRK-specific "priming" effect of Gαi3: strong reduction in Ibasal and increase in Ievoked without a significant reduction in Itotal (S1 Table). To note, coexpression of Gα3 also produced Rα of ~10 which was comparable to the neurons with the lowest Ibasal and highest Rα (compare S1 Table and Table 4). Calculation of available Gβγ and Gα showed a robust persistence of Gβγ:GIRK ratio of above 3 (Fig 7B). Expectedly, the estimate of the available Gα greatly increased after Gαi3 overexpression, but, remarkably, Gα:GIRK ratio did not exceed 2 Gα molecules/channel (Fig 7C).

Variations in Iβγ/Itotal ratio and Pα,max in different cells could bias our estimates of channel density (Eq 2) and thus also estimates of Gβγ and Gα. Therefore, for neurons, we repeated our calculations for a range of Iβγ/Itotal ratios between 1.5 and 3 (S9A Fig) and Pα,max between 0.05 and 0.2 (S9C Fig). For comparison, a similar range of Iβγ/Itotal ratios was also tested for the oocyte data (S9B Fig). The exact values of Gβγ:GIRK and Gα:GIRK ratios varied, especially with changes in Iβγ/Itotal ratio. Generally, the lowest channel density is most sensitive to perturbations, and, for Iβγ/Itotal = 1.5 (the lowest ratio tested), calculated Gβγ:GIRK and Gα:GIRK ratios exceed our usual estimates. However, this ratio is lower than that observed experimentally (Table 1), likely causing an overestimate of the values of Gβγ and Gα. In all, although the absence of direct measurements of channel densities and Pα,max in HEK cells and neurons introduces an element of uncertainty, our results support the functional stoichiometry of 3–4 Gβγ and 2 or less Gα molecules per GIRK1/2 channel. Importantly, for a wide range of parameters, the main trends persist: available Gβγ is in excess over Gα; Gβγ:GIRK ratio remains high (>3) whereas Gα:GIRK ratio decreases as Ibasal increases.

Changes in functional stoichiometry of GIRK, Gβγ and Gα explain the inverse Rα-Ibasal correlation

The systematic study presented above supports our hypothesis [51] that the inverse Rα-Ibasal relationship for GIRK1/2 reflects a progressive decline in GIRK1/2-associated Gα relative to Gβγ. We could now test this hypothesis quantitatively, and establish whether the calculated changes in GIRK:Gα:Gβγ functional stoichiometry can fully account for the Rα-Ibasal relationship shown in Fig 1. To this end, we simulated the changes in Rα as a function of Ibasal for a range of channel densities. We used channel densities, Ibasal values and Gβγ:GIRK and Gα:GIRK ratios calculated above (Tables 1 and 3 for oocytes, Table 4 for neurons). No free parameters were allowed. The results are shown in Fig 8, for oocytes (grey circles) and hippocampal neurons (black triangles). At this point, the channel density estimates and thus the simulations for oocytes are more reliable than for neurons.

First, for simplicity, we assumed a constant Gβγ:GIRK ratio at all densities (3.5 in oocytes and 3.4 in neurons; see Fig 7). For further simulations, in order to construct continuous curves,
we needed to assign numeric values for G\(\alpha\):GIRK ratios within the full range of channel densities, based on the individual data points calculated for the “density groups” (Fig 8A). Since the reduction in G\(\alpha\):GIRK ratio as a function of channel density is a process of unknown nature, the data points were arbitrarily fitted to a hyperbolic function (Fig 8A, solid lines). Next, we simulated the relation between I\(_{\text{basal}}\) and channel density, by substituting the obtained values of G\(\alpha\):GIRK into eqs 5–15 (Fig 8B). The simulation gave a good match to data of Table 1 (oocytes) and Table 4 (neurons), indicating that the fitting procedure of Fig 8A was satisfactory. Finally, values of G\(\alpha\):GIRK from Fig 8A and I\(_{\text{basal}}\) from Fig 8B were used to simulate the \(R_a\)-I\(_{\text{basal}}\) relationship (Fig 8C, solid black and grey lines), matching well the raw data (triangles and circles). Simulations based on a constant relations of GIRK, G\(\alpha\) and G\(\beta\gamma\) at all channel densities (allowing G\(\beta\gamma\) and G\(\alpha\) recruitment) could not account for the observed trend in \(R_a\) changes. This is exemplified for a 1:4:2 and 1:4:4 GIRK:G\(\beta\gamma\):G\(\alpha\) stoichiometry (Fig 8C, red and green dashed lines, respectively). We conclude that the decrease in G\(\alpha\) available for GIRK activation at higher I\(_{\text{basal}}\) can fully account for the inverse \(R_a\)-I\(_{\text{basal}}\) relationship in both experimental systems.

**Discussion**

**General summary**

In this work we have quantitatively analyzed the GPCR-\(G_{i/o}\)-GIRK1/2 cascade, focusing on basal (I\(_{\text{basal}}\)) and agonist-evoked (I\(_{\text{evoked}}\)) activities, both of which regulate neuronal excitability. We developed a mathematical model which allows quantification and simulation of macroscopic GIRK1/2 currents under steady-state conditions, before and after activation by neurotransmitter or by G\(\beta\gamma\). Our simulations fully rested on experimental data and parameters obtained in this and previous works. The modeling accurately described basal and evoked GIRK1/2 currents in two expression systems and in hippocampal neurons in a wide range of channel’s surface densities, correctly predicted the dose-dependent activation of GIRK1/2 by coexpressed G\(\beta\gamma\) in *Xenopus* oocytes, and fully accounted for the inverse correlation between I\(_{\text{basal}}\) and agonist activation index (\(R_a\)) previously observed in heterologous systems and, as shown here, also in hippocampal neurons. Our experimental findings and the model lay the
basis for further analysis of the GPCR-G_{i/o}-GIRK cascade, for example for GIRKs of different subunit composition, and in different cells.

Importantly, the present quantitative analysis provides novel and often unanticipated insights into the mechanisms of GIRK regulation by G protein subunits, G{βγ} and G{αGDP}. It reveals an unequal and, moreover, variable functional GIRK1/2:G{βγ}:G{α} stoichiometry: 1) Under all conditions tested, between 3 and 4 G{βγ} dimers are available for GIRK1/2; 2) Only two or less G{α} are available per GIRK1/2 channel; 3) Increase of GIRK1/2 surface density is accompanied by a proportional increase in G{βγ} (which is recruited by the channel), but not G{α}. The unequal, effector-dependent G{α}-G{βγ} stoichiometry within the GIRK1/2 signaling cascade is an unexpected departure from classical schemes which usually assume that, prior to GPCR activation, the heterotrimeric G proteins available to the effector exist as stoichiometric complexes of G{α} and G{βγ} [97]. We propose that the unique functional stoichiometry of GIRK1/2 with G protein subunits, and the cooperative nature of GIRK gating by G{βγ}, underlie the complex pattern of basal and agonist-evoked activities and allow GIRK1/2 to act as a sensitive bidirectional detector of both G{βγ} and G{αGDP}.

Our conceptual model of GIRK1/2 regulation (Fig 9) rests on the main findings of this study regarding the GIRK1/2:G{βγ}:G{α} stoichiometry (points 1–3 above) and the notion that, for G{βγ} to activate GIRK, it must have its G{α}-interacting interface exposed and free to contact GIRK [19,92,98,99]. In the resting state, the channel’s environment is enriched in 3–4 molecules of G{βγ} and 1–2 G{α}. In this scenario, between one and three G{βγ} molecules are not associated with G{αGDP} and can bind and activate GIRK, resulting in a basal activity that is between 1 and 26% of total P_{o,max} (see Fig 2C). Because of the gating cooperativity, occupancy of the first two G{βγ}-binding sites yields low I_{basal}. The fewer G{α}, the more “free” G{βγ} remains to occupy the activation sites at rest, yielding higher I_{basal}. After GPCR-induced separation of GαGTP from G{βγ} (lower arm of the scheme), due to gating cooperativity, addition of each G{α}-free G{βγ} ensures a robust 4–6 fold activation (e.g. going from 2 to 3 or 3 to 4 G{βγ}-occupied sites). An even stronger activation takes place with a shift from 2 to 4 G{βγ}-occupied sites (×16); and so on. Overexpression of G{βγ} “sequesters” G{α} and allows full occupancy of all G{βγ} binding sites (middle arm of the scheme). Finally, overexpression of GIRK1/2 recruits G{βγ} but not G{α}, increasing G{βγ}/G{α} ratio (upper arm of the scheme). The balance between available G{αGDP} and G{βγ} yields a continuum of basal activity magnitudes even on the level of a single channel, and sensitively regulates the extent of activation by the agonist.

The scheme emphasizes the important fact that, given the relatively fixed amount of G{βγ} available for GIRK1/2 activation, it is the availability of G{αGDP} that determines the level of basal activity and, consequently, the extent of activation by agonists (as experimentally observed previously; [30,31]). The imbalance between G{βγ} and G{α} renders GIRK1/2 with a sizeable I_{basal}, allowing it to act as a bidirectional, servo-like device [51] where its activity can be regulated not only by positive (G{βγ}, Na+, PIP2) but also negative (G{αGDP}, protein kinase C, G{αqGTP}, PIP2 depletion) stimuli.

Surface levels of GIRK and G proteins in Xenopus oocytes

Surface levels of endogenous G protein subunits and of heterologously expressed channels and G proteins crucially determine the behavior of the reconstituted signaling cascade, but they have never been quantitatively studied in the past in this common model system. We obtained very close estimates of surface levels of GIRK using two independent approaches: quantitative immunochemistry (which measures all channels in the PM) and electrophysiology (which counts only functional channels) (Fig 4). This indicated that the majority of GIRK1/2 channels in the PM of Xenopus oocytes were functional. Further use of GIRK1/2 as a molecular
fluorescent ruler for Gβγ yielded Gβγ density very close to density estimated from quantitative immunochemistry, affirming the "molecular ruler" procedure with GIRK1/2 and lending additional support to the measurements of GIRK1/2 density. We estimated total cellular endogenous Gβγ in the oocyte as ~170 nM, similar to other cell types, 200–800 nM [100] and to the recent high-precision mass spectrometry measurement of ~200 nM Gβ in Xenopus eggs [101]. Notably, examination of data reported in the latter work [101] suggests a total concentration of all Gα in Xenopus eggs of ~350 nM. Thus, total oocyte’s Gβγ is not in excess over Gα, supporting our assumption (S4 Fig) that endogenous heterotrimeric G proteins are in the Gαβγ form before activation by GPCRs or coexpression of GIRK.
Our estimate of 24 molecules/μm² of endogenous PM-associated Gβγ, presumably as Gαβγ, is comparable to the ~40 molecules/μm² evaluated in HEK cells [102]. In terms of concentration, 24 molecules/μm² corresponds to ~4 μM, much higher than the cytosolic level of ~0.2 μM. Such enrichment of G proteins at the PM is expected, because of the lipid modification of both Gα and Gγ [84,103]. It is probable that a substantial fraction of the PM-associated endogenous Gβγ is not available for GIRK activation, being associated with Gαs or Gαq, rather than Gαi/o, or located in separate PM compartments, or associated with other effectors such as adenylyl cyclase [104]. Simulations showed that the main conclusions of our study are not affected by assuming a wide range of endogenous Gαi/o proteins available for GIRK, from 1 to 24 molecules/μm².

In this work, we varied the levels of heterologously expressed GIRK1/2 and Gβγ. For GIRK1/2, the surface densities ranged from about 1 to 30 molecules/μm² in oocytes and about 1–60 channels/μm² in HEK293 cells (Table 4 and Fig 7). Densities of <20 channels/μm² were estimated in cultured hippocampal neurons (Fig 7A). This is comparable to 9–10 channels/μm² found in spines of cerebellar Purkinje neurons by quantitative electron microscopy [105]. Thus, the "low" and "intermediate" densities of expressed GIRK1/2 and Gβγ in oocytes and HEK293 cells may be the most physiologically relevant.

Modeling GIRK1/2 regulation by Gβγ in basal and agonist-activated states

For our description of GIRK currents, we have considered several models of GIRK channel gating by Gβγ (Fig 2B): the previously developed "concerted activation" model [58], the "graded contribution" model, and a more general model that includes 4 to 5 closed-open transitions. We were able to show that the latter two models converge to the same form of description of macroscopic steady-state P, based on fractional contributions of channels occupied by 1 to 4 Gβγ molecules, experimentally demonstrated for GIRK1/4 [13,14] (see Results and Supplemental Discussion S2 Text for a detailed discussion). We have therefore chosen the graded contribution model as our main tool to simulate and predict GIRK1/2 currents. Despite its relative simplicity, this model incorporates several complex properties of GIRK gating, and provides a strong computational tool for the analysis of the G protein-GIRK signaling. First, in this model we implemented the gating cooperativity of GIRK, by including the graded contribution of each bound Gβγ to channel opening (Fig 2C). Second, to our knowledge, this is the first model to describe both basal and agonist-evoked GIRK activity. Third, the model allows to estimate the amount of G protein subunits available for channel activation without any a priori assumptions regarding the levels of endogenous Gα or Gβγ; the GIRK-available Gα and Gβγ are calculated from experimental data (Fig 5). Finally, our method of calculating the functional stoichiometry of GIRK and Gβγ applies even if Ibasal is partly due to the presence of a GPCR activated by low dose of an ambient neurotransmitter. In this case, the available Gα calculated using the method of Fig 5 will represent only that fraction of GIRK-coupled Gα that is still in its GDP-bound form.

In this work we left aside auxiliary/modulatory proteins such as RGS, focusing on the minimal essential composition of the cascade. We also have not addressed the impact of direct GIRK-Gα interaction. We and others did not find significant direct effects of GαGDP on GIRK1/2 gating; the main function of GαGDP is the prevention of basal activation of the channel by ambient Gβγ and the release of Gβγ for channel activation by agonist/GPCR [31,34,36,106]. This function is fully implemented in our model. As for GαGTP, it regulates the kinetics of Ievoked of GIRK1/2 but barely affects the steady-state amplitude [29,36]. Our present results suggest that, for GIRK1/2, effects of activated GαGTP on GIRK1/2 current amplitude are negligible,
within a possible ~10% error. These considerations justify the omission of Gα-GIRK binding reactions from model’s equations. It remains possible that certain GαGTP may differently regulate GIRK channels of other subunit compositions or under certain conditions [34,107].

Both graded contribution and concerted models showed that Ibasal and Ievoked of the expressed GIRK1/2, and their changes with channel surface density, cannot be accurately described unless there is a recruitment of Gβγ to the PM by the channel. Recruitment of Gα is probably negligible (S4 Fig, Table 3). The prediction of preferential availability of Gβγ over Gα for GIRK1/2 concurs with the experimental findings [25], supporting the model’s validity. Further validation came from predicting the system’s response to perturbation in the form of dose-dependent response to coexpression of Gβγ, producing a satisfactory simulation of both Iβγ and Rβγ (Fig 6, S7 and S8 Figs). Finally, on the basis of experimental data and the calculated molar ratios of GIRK:Gβγ:Gα for different channel densities, the model fully accounted for the inverse correlation between Rα and Ibasal in GIRK1/2 (Fig 8), which was the starting point of this endeavor.

We also made the first steps to extend the model to hippocampal neurons. The analysis of GIRK behavior in HEK cells and hippocampal neurons yielded estimates of Gβγ:GIRK and Gα:GIRK ratios close to those obtained in oocytes (3–4 Gβγ and ≤ 2 Gα), and a satisfactory simulation of the inverse Rα-Ibasal relation observed in the neurons (Fig 8). Uncertainties remain, because calculation of channel density in neurons relied on oocyte and partly HEK cell data for Iβγ/Itotal ratios and P o,max. A variety of GIRK compositions and distinct localization and density in cellular compartments further complicate the picture. Future quantitative studies are warranted for a more accurate description of GIRK activity in various neurons.

**Functional stoichiometry of GIRK and G protein subunits**

Our analysis provides two major insights into functional stoichiometry of GIRK1/2 vs. the G protein subunits. First, the molar ratios are both uneven and can change as a function of channels’ density in the PM. The stoichiometry of more than 3 Gβγ per GIRK1/2 is practically invariable, whereas the amount of available Gα/i/o is lower than Gβγ and further drops sharply as the level of expression of the channel increases. These estimates of Gβγ and Gα availability remained remarkably stable under a wide range of potentially variable parameters, such as KD of GIRK-Gβγ binding, the extent of Gβγ-independent basal activity, the size of submembrane reaction space, the Itotal/Iβγ ratio and P o,max in neurons, etc. Second, unexpectedly, the limiting stoichiometry for Gβγ:Gα:GIRK is 4:2:1.

The uneven Gβγ:Gα stoichiometry and the decrease in Gβγ:Gα ratio were suggested by previous qualitative findings [50,51]. Our new results support this hypothesis and provide new insights into the underlying mechanism. Assumptions of pre-assembly of GIRK1 with 1, 2, 3 or 4 Gαβγ heterotrimers failed to recapitulate the observed macroscopic currents and Rα (S4 Fig). The decrease in Gα/GIRK ratio as channel’s levels increase is consistent with total PM concentration of Gα/i/o being relatively constant at all GIRK1/2 densities (Table 3). This is in agreement with little or no recruitment of Gα to the PM by GIRK1/2 [25,50]. On the other hand, the total amount of GIRK-available Gβγ in the PM increases as more GIRK1/2 channels are expressed, substantially exceeding the “basal” concentration of Gβγ of the naïve oocytes. The conspicuous persistence of Gβ:GIRK stoichiometry is best demonstrated in the oocytes, where it rests on a full quantitative analysis of experimental data. The identical estimates obtained in HEK cells and neurons (Fig 7B), though based on partial data, provide further support. The ability of GIRK1/2 to sustain a steady Gβγ-enriched environment strongly argues for a strong association between Gβγ and GIRK1/2, in line with the proposed high-affinity “anchoring” and recruitment of Gβγ by GIRK1 [25]. The mechanism of Gβγ recruitment is
unknown but may be due to co-trafficking from the endoplasmic reticulum [22] or "kinetic scaffolding" and similar mechanisms [63,108,109], as discussed in [25]. At present we cannot rule out that the participation of another Gβγ-binding PM protein, such as a GPCR, or unknown scaffolding proteins, is important for the enrichment of Gβγ.

It is widely accepted that the GIRK signaling cascade occurs within signaling complexes of GIRK channels with subunits of Gβγ, heterotrimeric proteins and some GPCRs (reviewed in [4,61,110,111,112]). The uneven and variable stoichiometry within the GIRK1/2-Gβγ-Gαi signaling complex revealed by this study is compatible with a high-affinity, dynamic complex, where the channel and the G protein subunits are allowed to dissociate and reassociate in the PM [113]. These considerations justify our use of standard kinetic formalism for modeling. Finite affinity and reversibility of GIRK-Gβγ interaction is also supported by the demonstration of competition between two Gβγ effectors, GIRK and voltage-gated calcium channels, for available Gβγ in sympathetic neurons [114], and by recent GIRK2 reconstitution studies in lipid bilayers [106].

In contrast to Gβγ, our present analysis supports the notion [36,51,106] that, at least in heterologous or artificial systems, GαGDP is not an obligatory partner in the complex. Notably, in neurons and HEK cells the calculated Gα:GIRK ratios are higher than in oocytes (Fig 7 and Table 4); thus the higher Ra in these cells. The greater availability of Gα in HEK cells and neurons may reflect the presence of scaffolding or trafficking aids absent in the oocytes. However, the inverse Ra-Imax correlation and the (calculated) reduction in Gα at high channel densities are maintained in neurons and HEK cells, supporting the expendability of Gα.

The apparent limiting stoichiometry of 4 Gβγ molecules per channel is not surprising, since the model explicitly includes 4 Gβγ-binding sites per channel. However, the calculated availability of two or less GαGDP, under most conditions examined, was unexpected. The limit of 2 Gα per channel was only slightly exceeded in simulations of lowest channel densities and when allowing substantial deviations from our standard assumptions (S9 Fig). Most conspicuously, overexpression of Gα3, which reduced Imax by 75–80% and elevated the activation index Ra to about 10, increased the calculated Gα:GIRK ratio from less than 0.5 to 2—but no more—Gα molecules per channel (Fig 7, S1 Table). Taken together, our results point to a limiting stoichiometry of 2 Gα molecules available for a GIRK1/2 channel. It is not clear what limits the amount of available Gα, but it is tempting to speculate that this limit reflects the maximal number of Gα molecules that can interact with GIRK1/2. The actual stoichiometry of this interaction is unknown, but the NMR study of Shimada and colleagues [115] indicates that the interacting surface of GαGTP requires two GIRK1 subunits of a GIRK1 tetramer for full contact. One GIRK1 subunit interacts with the helical domain of Gα and the other one with the GTPase domain [115].

We emphasize that our conclusions are valid for GIRK1/2 but may not be so for other GIRK channels. Thus, homomeric GIRK2 channels, with their low Imax and very high response to Gβγ, do not recruit Gβγ to the PM [25] and probably do not show pre-association with 3–4 Gβγ, or may have more Gα. Excess of Gβγ over Gα has been observed in the phototransduction cascade [116,117] though it has not been linked to any effector. We speculate that effector-dependent changes in the balance of Gα and Gβγ may take place with effectors other than GIRK, playing a role in their regulation.

**Materials and Methods**

**Ethics statement**

All experiments were approved by Tel Aviv University Committee for Animal Use and Care (permits M-08-081 and M-13-002 for Xenopus frogs and M-12-061 for mice).
Animals and oocyte culture

Female frogs, maintained at 20±2°C on 10 h light/14 h dark cycle, were anaesthetized in a 0.17% solution of procaine methanesulphonate (MS222), and portions of ovary were removed through a small incision on the abdomen. The incision was sutured, and the animal was held in a separate tank until it had fully recovered from the anesthesia and then returned to the tank. The animals did not show any signs of postoperative distress and were allowed to recover for at least 3 months until the next surgery. Following the final collection of oocytes, anaesthetized frogs were killed by decapitation and double pithing. *Xenopus* oocytes were injected with RNA, and incubated in for 3–4 days at 20–22°C in NDE-96 solution (in mM: 96 NaCl, 2 KCl, 1 CaCl₂, 1 MgCl₂, 2.5 Na-pyruvate, 50 μg/ml gentamycin, 5 mM HEPES/NaOH, pH 7.5). All experiments with nerve cells derived from newborn mice, that have been analyzed in this paper, have been performed previously [47], and no additional mice have been used.

Antibodies, cDNA constructs, proteins and RNAs

The anti-GIRK1 polyclonal antibody was from Alomone Labs (Jerusalem), #APC-005. This antibody was raised against the distal C-terminal residues 437–501 of mouse GIRK1. 3 μg/mL were used for Western blots. The anti-Gβ polyclonal antibody (T-20, from Santa Cruz. #sc-378) is directed against the last 50 amino acids (a.a.) of mouse Gβ. 0.4 μg/mL were used for Western blots.

Most DNA constructs were as reported previously: bovine Gβ₁, bovine Gγ₂, human muscarinic type 2 receptor (M2R), human Goα3, rat GIRK1, mouse GIRK2, YFP-GIRK1, GIRK2-HA, Gβ₁-YFP, Gγ₂-YFP and Gγ₁-CFP [36,51,57]. To compare antibody labeling efficiency of bovine vs. *Xenopus* Gβ, we created the YFP-Gβ-XL construct in which the last 50 amino acids of the C-terminus in bovine Gβ were made identical to those of *Xenopus* Gβ₁, by mutating 7 a.a.: V296I, A299C, A302R, D303E, A305V, A309S and D322S by standard PCR protocols. Preparation and storage of Gβ₁γ₂ and of the GST-fused distal C-terminus of GIRK1, GST-dCT (a. a.365-501) used for calibrations of Fig 4Awere done as described previously [69,118]. RNA was synthesized *in vitro* [57]. Amounts of injected RNA are indicated in the text, Tables 1, S1 and in Figure legends.

Confocal imaging and calculation of surface density of Gβγ-YFP

Fluorescence levels of the expressed YFP (yellow fluorescent protein) and cerulean (termed here CFP, cyan fluorescent protein) were measured in intact *Xenopus* oocytes essentially as described [25,32]. Both YFP and CFP carried mutations that increase stability and reduce dimerization [32]. Briefly, oocytes were imaged in ND96 solution in a 0.7 mm glass-bottom dish using Zeiss 510META confocal microscope with a 20× air objective. Images were acquired in the spectral mode. CFP was excited at 405 nm and emission was measured at 481–492 nm. YFP was excited at 514 nm and emission was measured at 535–546 nm. Fluorescent signals were averaged from 3 regions of interest (ROI) using Zeiss LSM Image Browser, and averaged background measured at an area outside the cell was subtracted. The average signal from un.injected oocytes was subtracted for final analysis. Saturation of emission measurement was strictly avoided to ensure that the readout of the confocal microscope was linear within the range of measurement. All measurements were made in the linear range of the recording apparatus.

In calculating Gβγ surface density through comparing fluorescent signals from YFP-GIRK and Gβγ-YFP, the fluorescent intensities of the two proteins were compared directly from oocytes of the same batch on the same day, as described in Fig 4D. No correction for non-fluorescent (improperly folded) YFP [119] was needed (assuming that the percent of misfolding...
was similar in all YFP fusion proteins used here), because the ionic current \( I_{\beta\gamma} \) (a fluorescence-independent parameter) was used as the basis for GIRK density estimates.

Giant membrane patches of oocyte membrane were prepared and imaged as described [25,95]. For imaging, fixed membranes were immunostained with the anti-G\( \beta \) antibody at 1:200 dilution. Cy3-conjugated anti-rabbit secondary antibody (Jackson ImmunoResearch) was used for imaging with the 543 nm laser, and excitation was measured at 566–577 nm. Background fluorescence from an area outside the giant patch was subtracted. For final analysis shown in Fig 6, the signal from uninjected oocytes was subtracted from all groups.

**Biochemistry**

Manual separation of plasma membranes from the rest of the oocyte (“cytosol”) was performed as described [81], with modifications. Plasma membranes together with the vitelline membranes (extracellular collagen-like matrix) were removed manually with fine forceps after a 5–15 min incubation in a low osmolarity solution (5 mM NaCl, 5 mM HEPES, and protease inhibitors (Roche Complete Protease Inhibitors Cocktail, 1 tablet/50 ml), pH = 7.5). The remainder of cell (cytosols) was processed separately. First, the nuclei were separated by centri-fuge for 10 min at 700xg at 4°C. Plasma membranes and cytosols were solubilized in 35 \( \mu \)l running buffer (2% SDS, 10% glycerol, 5% \( \beta \)-mercaptoethanol, 0.05% Bromophenol Blue, 62.5 mM Tris-HCl pH 6.8) and heated to 65°C for 5 min. Samples were electrophoresed on 12% polyacrylamide-SDS gel, and transferred to nitrocellulose membranes for Western blotting with the various antisera. The signals were visualized using the SuperSignal kit (Thermo) and quantitated using ImageJ software (National Institutes of Health, USA).

**Electrophysiology**

**Macroscopic current recording in neurons.** Raw data from primary cultures of mouse hippocampal neurons used in the analysis of Figs 1 and 7 were from whole-cell patch clamp experiments. A subset of data shown (40 neurons out of 60) had been reported previously [47] but the \( I_{\text{basal}}-I_{\text{evoked}} \) relation has not been analyzed. The batch solution contained the low-K\( ^+ \) bath solution (in mM: 145 NaCl, 4 KCl, 1.8 CaCl\_2, 1 MgCl\_2, 5.5 D-glucose, 5 HEPES/NaOH; pH 7.4), which was replaced to the high-K\( ^+ \) solution for GIRK current measurement (in mM: 120 NaCl, 25 KCl, 1.8 CaCl\_2, 1 MgCl\_2, 5.5 D-glucose, 5 HEPES/NaOH; pH 7.4). Both external solutions contained 0.5 \( \mu \)M TTX and 0.5 mM kynurenic acid. Patch pipettes (3–5 M\Omega) were filled with intracellular solution (in mM: 130 K-Gluconate, 0 or 6 NaCl, 1 EGTA, 1 MgCl\_2, 10 HEPES, 2 MgATP, 0.3 Tris-GTP, 0.01 Tris-GDP, pH 7.3). Baclofen (Sigma) was added at 100 \( \mu \)M, sTertiapin-Q or rTertiapin-Q (TPNQ; Alomone labs, Jerusalem) at 100–120 nM [120]. We refrained from using Ba\(^{2+} \) as GIRK blocker in neurons because of its low specificity [1]. Indeed, 1 mM Ba\(^{2+} \) blocked a much greater fraction of the total inward current in high-K\( ^+ \) solution (S1 Fig), confirming that additional Ba\(^{2+} \)-sensitive channels contribute to total basal conductance in these cells (e.g. [53]). In contrast, we used Ba\(^{2+} \) to block the expressed GIRK channels in Xenopus oocytes and HEK293 cells, which have negligible intrinsic (endogenous) Ba\(^{2+} \)-sensitive basal currents (S2 Fig) [120,121].

Current measurements in neurons were done at -70 mV. To correct for the difference in K\( ^+ \) driving force when comparing whole-cell currents from neurons and oocytes (for Fig 7 and Table 4), correction to a holding potential of -80 mV was done assuming \( E_k = -37 \) mV in the 25 mM K\( ^+ \) solution. 4 cells (out of 65) with \( I_{\text{basal}} < 0.5 \) pA/pF were discarded because the recording was deemed unreliable owing to the low signal-to-noise ratio. One cell was found to be outlier by Grubb’s test using GraphPad outlier calculator [120,121].

http://graphpad.com/quickcalcs/
Macroscopic current recording in Xenopus oocytes. All experiments were done at 20–22°C essentially as described [51]. Data acquisition and analysis were done using pCLAMP (Molecular Devices, Sunnyvale, CA). Whole-cell currents were measured using two electrode voltage clamp in the ND96 (low K⁺) solution and in a high K⁺ solutions (24 mM K⁺, isotonically replacing NaCl in ND96) as shown in S2 Fig. Currents were recorded at −80 mV, filtered at 500 Hz, and sampled at 5 or 10 kHz. Currents in oocytes were converted to densities, in pA/pF, assuming an oocyte’s capacitance of 200 nF [88]. For analysis of correlation between I_{evoked} and R_a and for Table 1, new raw data in the low and high GIRK1/2 density groups (total of 41 cells) were combined with raw data collected for our previous publication [30] (20 cells). I_{basal} was measured after blocking all GIRK currents by 5 mM Ba²⁺ [31].

Patch clamp recordings in Xenopus oocytes. Patch clamp experiments were done using Axopatch 200B (Molecular Devices, Sunnyvale, CA). Currents were recorded at -80 mV, routinely filtered at 2 kHz and sampled at 20 kHz. In some patches we also used filtering at 5 kHz. Patch pipettes had resistances of 1.4–3.5 MΩ. Pipette solution contained, in mM: 144 KCl, 2 NaCl, 1 MgCl₂, 1 CaCl₂, 1 GdCl₃, 10 HEPES/KOH, pH 7.5. GdCl₃ completely inhibited the stretch-activated channels. The bath solution contained, in mM: 144 KCl, 2 MgCl₂, 6 NaCl, 1 EGTA, 10 HEPES/KOH, pH 7.5. To obtain single channel recordings, oocytes were injected with low doses of RNA of GIRK1 (10–50 pg), and RNA of GIRK2 was 1/2 to 1/3 of that (5–17 pg), to avoid the formation of GIRK2 homotetramers. In addition, 50 ng of the antisense oligonucleotide against oocyte’s endogenous GIRK5 was injected to prevent the formation of GIRK1/5 channels [122]. Number of channels was estimated from overlaps of openings during the whole time of recording (at least 5 min). Single channel current (I_{single}) was calculated from all-point histograms of the original records [123], and open probability (P_o) was obtained from event lists generated using idealization procedure based on 50% crossing criterion [124]. P_o was calculated only from records that contained 1, 2 or 3 channels. Each recording lasted for at least 4 min and contained >10,000 openings. Thus, the probability of missing a channel was negligible (p<10⁻²⁴⁸ for 1-channel records). In support, P_o in patches with 2 or 3 channels was similar (0.071±0.02, n = 2, and 0.078±0.021, n = 3, respectively) and even lower than in 1-channel patches (0.15±0.026, n = 3), opposite to what would be expected in the case of underestimation of channel number. GPCR-evoked GIRK1/2 activity was induced via the coexpressed m2R with 2 or 5 μM ACh in the patch pipette. 2 μM ACh is a saturating concentration for GIRK1/2 expressed in Xenopus oocytes [62]. Because a slow reduction of activity over several minutes was observed in some patches, ACh-induced P_o was estimated during the first minute of the record. For channels activated by coexpressed Gβγ, there was no decrease in P_o over >4 minutes, and the P_o was averaged from the first 4 minutes of the record.

Macroscopic current recording in HEK293 cells. Most of the data on GIRK1/2 expressed in HEK293 cells transfected with cDNAs of GIRK1 and GIRK2 and m2R (Tables 2 and 4; Fig 7) are from experiments described previously [51]. Data on I_{βγ} in HEK cells have been obtained in the same series of experiments but have not been reported previously. HEK293 cells were transfected with cDNAs of GIRK1, GIRK2, m2R (0.5 μg each), without or with the edition of DNAs of Gβ1 and Ga3 (0.2 μg each). Whole cell recordings were performed at -80 mV with patch pipette solution containing, in mM: 130 KCl, 1 MgCl₂, 5 EGTA, 3 MgATP, 10 HEPES. Low-K bath solution contained, in mM: 140 NaCl, 4 KCl, 1.8 CaCl₂, 1.2 MgCl₂, 11 glucose, 2 CdCl₂, 5.5 HEPES. High-K bath solution contained 90 mM KCl and 54 mM NaCl, the rest was as in low-K solution. To compare with data from oocytes (for Fig 7 and Table 4), the correction factor to adjust for current amplitude difference in 90 mM K⁺ (HEK293) vs. 24–25 mM K⁺ solution (oocytes, neurons) was determined experimentally to be 3.27±0.14 (n = 7; measured in oocytes).
Modeling of Gβγ activation of GIRK1/2

In general, the macroscopic GIRK1/2 current, I, can be calculated utilizing a modified (Eq 1):

\[
I = I_{\text{single}} \cdot P_o \cdot N / f_{sc}
\]  

where \(I_{\text{single}}\) is a unitary current and \(N\) is the number of channels [1]. \(f_{sc}\) is a solution conversion factor between solutions used for whole-cell (24 mM K\(^+\)) and in cell-attached patches (144 mM K\(^+\)). \(f_{sc}\) was estimated as 4.63±0.26 (n = 6) by measuring GIRK currents in the same oocytes in the two solutions, in whole-cell configuration.

For the graded contribution model, we define \(P_o\) as:

\[
P_o = P_{o,\text{max}} \cdot \sum_{x=1}^{4} f_{p,x} \phi_x
\]  

where \(P_{o,\text{max}}\) is the maximal open probability, \(f_{p,x}\) is the fraction of \(P_o\) contributed by \(x\) Gβγ-occupied channel (\(x\) is an integer between 1 and 4), and the \(\phi_x\) is the fraction of channels in the \(x\) Gβγ-occupied state and can be calculated according to:

\[
\phi_x = \frac{[C_x]}{C_{\text{total}}}
\]  

where \([C_x]\) is the concentration of channels in \(x\) Gβγ-occupied state and \(C_{\text{total}}\) is the total channel concentration in membrane. For the concerted model, Eq 6 is reduced to

\[
P_o = P_{o,\text{max}} \cdot \phi_4
\]  

where \(\phi_4\) is the fraction of channels with four bound molecules of Gβγ [58].

For the graded contribution model, we calculated values of \(f_{p,x}\) based on data described by Ivanova-Nikolova et al. (1998) [13] rendering ~ 0.01, 0.06, 0.26 and 1 values corresponding to 1–4 Gβγ occupied states. Based on mass-action law and Fig 2B, GIRK1/2 channel activity can be described by the following system of eqs (8–12):

\[
[C_0] \cdot [G\beta\gamma] = \frac{1}{4} K_D \cdot [C_1]
\]  

\[
[C_1] \cdot [G\beta\gamma] = \frac{2}{3} K_D \cdot [C_2]
\]  

\[
[C_2] \cdot [G\beta\gamma] = \frac{3}{2} K_D \cdot [C_3]
\]  

\[
[C_3] \cdot [G\beta\gamma] = 4 \cdot K_D \cdot [C_4]
\]  

\[
[C_0] + [C_1] + [C_2] + [C_3] + [C_4] = C_{\text{total}}
\]  

where the \(K_D\) is a dissociation constant of Gβγ and GIRK1/2. For our simulations we routinely used \(K_D = 50\) nM as measured in direct biochemical experiments [90], but a range of other values has also been tested as explained in the Results.

For both concerted and graded contribution models, G protein dissociation reaction required for modeling of GIRK1/2 basal activity according to the schemes described in Fig 2B.
is formulated as:

\[ k_{on} \cdot [G\beta\gamma] \cdot [Gz_{GDP}] = k_{off} \cdot [Gz_{GDP}G\beta\gamma] \]  

(13)

\[ G\beta\gamma_{total} = [G\beta\gamma] + [Gz_{GDP}G\beta\gamma] + [C_1] + 2 \cdot [C_2] + 3 \cdot [C_3] + 4 \cdot [C_4] \]  

(14)

\[ Gz_{total} = [Gz_{GDP}] + [Gz_{GDP}G\beta\gamma] \]  

(15)

where \( G\beta\gamma_{total} \) and \( Gz_{total} \) are total concentrations of corresponding subunits available for interaction with the channel and \( k_{on} \) and \( k_{off} \) are association and dissociation constants of \( Gi \) protein subunits (0.7±10^6 M^-1 s^-1 and 0.0013 s^-1, respectively [56]).

Eqs 8–12, combined with Eqs (13–15) is the most general form of description of a system containing GIRK channel and G proteins. For simulation of agonist-evoked activity, with saturating doses of both GPCR and agonist, we assumed a complete dissociation of G-protein heterotrimer [63,64] and thus Eq 14 is changed to:

\[ G\beta\gamma_{total} = [G\beta\gamma] + [Gz_{GDP}] + 2[C_1] + 3[C_2] + 4[C_3] \]  

(16)

Simulations were performed using Matlab and Berkeley Madonna software. Steady-state simulations used in model development and application, as well as in most Figures, were done utilizing Matlab 6.5 function “solve” which is a part of Symbolic Math Toolbox. This function first looks for analytical solution, and if the former is absent, switches to numerical iterative algorithm (“trust region algorithm”, “quasi-Newton algorithm”). MATLAB routines for the calculation of \( G\beta\gamma \) and \( Ga \) available for GIRK with the graded contribution model are shown in Supplemental Methods (S1 Text).

In several cases we tested a range of arguments to produce continuous curves range of changes in GIRK1/2 currents or their ratios (\( R_a, R_{\beta\gamma} \)) (Fig 6C, S4, S7 and S8 Figs). Here, in order to reduce calculation time, we utilized Berkley Madonna software which implements 4th order Runge-Kutta method for numerical solution of differential equations (see Supplemental Methods, S1 Text). To assure lack of inconsistencies in calculation, we have compared the Matlab and Berkeley Madonna calculation results for a large number of cases and always obtained the same numbers.

Statistics

Imaging data on protein expression have been normalized as described previously [125]. Fluorescence intensity in each oocyte or giant membrane was calculated relative to the average signal in the oocytes of the control group of the same experiment. This procedure yields average normalized intensity as well statistical variability (e.g. SEM) in all treatment groups as well as in the control group. Statistical analysis was performed with SigmaPlot 11 (Systat Software Inc., San Jose, CA, USA). If the data passed the Shapiro-Wilk normality test and the equal variance test, two-group comparisons were performed using t-test. If not, we performed the Mann-Whitney Rank Sum Test. Multiple group comparison was done with one-way ANOVA if the data were normally distributed. ANOVA on ranks was performed whenever the data did not distribute normally. Tukey’s post-hoc test was performed for normally distributed data and Dunn’s post-hoc test otherwise. Unless specified otherwise, the data in the graphs is presented as mean ± SEM. Correlation between two parameters (such as basal current and \( R_a \)) was tested using the Spearman correlation test by running this test on raw data using the statistical module of SigmaPlot 11.
Supporting Information

S1 Text. Supplemental Methods. MATLAB routines for the calculation of Gβγ and Gα available for GIRK with the graded contribution model; Calculations of model predictions for a range of parameters using the Berkeley Madonna software.

S2 Text. Supplemental Discussion. Conversion from channel densities to concentrations; GIRK1/2 stoichiometry; Estimating steady-state open probability with the “separate gating transitions” model.

S1 Table. Effect of coexpression of Gαi3 on GIRK1/2 currents in oocytes. Data are from 2 to 4 experiments, for each group, shown as mean ± SEM. We did not include experiments with extremely large Gαi3 RNA quantities, as expression of higher doses of Gαi3 usually reduced Ito, indicating a general Gβγ scavenging effect rather than priming.

S1 Fig. Block of inward currents in cultured hippocampal neurons by TPNQ and Ba²⁺. (A) Ba²⁺ (1 mM) blocks a greater fraction of the total inward current in high-K⁺ solution, compared to TPNQ (120 nM). The experimental protocol was the same as in Fig 1, with the additional step of Ba²⁺ addition after TPNQ. ΔTPN and ΔBa denote the magnitudes (shown by double-speared arrows) of TPNQ- and Ba-blocked currents, respectively. Note that Ba²⁺ blocked a much greater fraction of the total inward current in high-K⁺ solution, most probably of the block of additional Ba²⁺-sensitive channels present in these neurons. (B) Comparison of average TPNQ- and Ba²⁺-blocked currents in 14 cells of one batch of neurons. Statistical significance (p<0.001) was determined using Wilcoxon Signed Rank test (the data did not pass normality test).

S2 Fig. GIRK1/2 currents in oocytes. Holding potential was -80 mV, low-K⁺ and high-K⁺ solutions contained 2 and 24 mM K⁺, respectively (K⁺ was replaced for Na⁺). Net GIRK currents were determined by subtracting the current remaining after the addition of 5 mM BaCl₂. (A) Ibasal and Ievoked in an oocyte expressing m2R, GIRK1 and GIRK2. Calculation of Rβγ was done in every cell from its own Ibasal and Ievoked. (B) Iβγ in an oocyte expressing m2R, GIRK1, GIRK2 and Gβγ. Note that adding ACh did not evoke a significant additional GIRK current, suggesting full activation by Gβγ. Rβγ was calculated in each cell by dividing its own Iβγ by the average Iβγ from the control group of the same experiment in which no Gβγ was coexpressed. (C) Expression of m2R in a wide range of doses does not affect Ibasal. 5–8 oocytes have been tested in each group. There were no significant differences between treatments as tested by one-way ANOVA.

S3 Fig. Characterization of YFP-labeled GIRK1 and Gββ. (A, B) Single channel parameters of GIRK1/2 and YFP-GIRK1/2 channels are very similar. (A) Cell-attached records of channel activity expressing the channel and Gβγ (5 ng RNA). (B) Comparison of average isingle and Po. Data are from oocytes of the same batch, recorded during a two-day experiment. (C, D) The anti-Gβ antibody similarly recognizes YFP-labeled bovine and Xenopus Gβ subunits in Western blots of manually peeled plasma membranes. Data are from 4 separate experiments. For Western blots, 15 to 20 plasma membranes were pooled. For confocal imaging, groups of 3–16 oocytes were examined, and the average fluorescence level was compared with that of YFP-GIRK1/2 (therefore the statistical significance was calculated using paired t-test).
of the latter was calculated from the measurement of currents as explained in the text. In each experiment, both confocal imaging, current measurement and Western blots of manually peeled membranes were done in oocytes of the same donor. There was a good agreement for surface density estimates of YFP-GβXL from confocal "molecular ruler" measurements and from quantitative Western blots, either in absolute terms as molecules/μm² (C) or in relative terms, normalized to estimates of YFP-Gβ in each experiment (D). YFP fluorescence can be safely assumed to be independent of the species of fused Gβ (mammalian or Xenopus). Therefore, similar estimates of surface density observed from confocal imaging and Western blots suggest that the Gβ antibody used here recognizes the oocyte’s endogenous Gβ in Western blots similarly to the coexpressed mammalian (bovine) Gβ.

S4 Fig. Simulation of density-dependent changes in whole-cell GIRK1/2 activity. Experimental data (from Table 1) are shown as red circles (mean ± SEM). The simulations of currents and Ra were done using the graded contribution model. (A) Testing the hypothesis that the endogenous Gαβγ heterotrimers are the only source of Gβγ for GIRK activation; Ibasal is due to spontaneous dissociation of Gαβγ into GαGDP and Gβγ (see Fig 2A). Simulations were performed assuming that only part (1 or 10 molecules/μm², black and red curves) or all (24 molecules/μm², blue curves) endogenous G proteins can donate Gβγ to activate GIRK1/2. Note that no satisfactory description of data can be obtained under any of these conditions. The simulated Ibasal is too low; for high channel densities, also the full Ievoked could not be obtained even assuming that all endogenous Gαβγ (i.e. all 24 molecules/μm²) could release Gβγ and activate GIRK. (B) Testing the hypothesis that the expressed GIRK1/2 recruits additional endogenous G protein subunits to the PM, e.g. from other cellular compartments. Simulations were done assuming that each GIRK1/2 channel recruits from 1 to 4 Gα/o heterotrimers. The recruited Gα and Gβγ were added to the pre-existing endogenous plasma membrane-attached Gαβγ before Gβγ expression. (C) Testing the hypothesis that the expressed GIRK1/2 recruits additional endogenous Gβγ, but not Gα, to the PM; the rest was done as in B. Calculations in (B) and (C) assumed 24 molecules/μm² of endogenous Gα/o available for GIRK. Similar results were obtained assuming 10 molecules/μm² (data not shown). Simulations as in A-C were also done with the concerted model, yielding similar results (data not shown).

S5 Fig. The concerted activation model supports the unequal stoichiometry estimates of Gβγ and Gα available for GIRK1/2. The plots present the calculated amounts of Gβγ and Gα available for GIRK1/2 using the concerted model for a range of Kd for the GIRK-Gβγ interaction (5–100 nM), for the three channel density groups of Table 1.

S6 Fig. The presence of Gβγ-independent intrinsic activity and the dimensions of submembrane reaction space do not significantly alter the estimates of functional stoichiometry of GIRK1/2-Gβγ-Gα. Calculations were done assuming Kd = 50 nM for the GIRK-Gβγ interaction. (A-C), the impact of Gβγ-independent basal activity. Calculation were done for Gβγ-independent intrinsic activity of a single channel ranging from 1% to 10% of Po,max. Available Gβγ (A), Gα (B) and the Gβγ-independent fraction of Ibasal (C) were calculated for the three channel density groups of Table 1. (D, E) Varying the submembrane space thickness in a wide range, 1–20 nm, does not significantly change the estimates of functional stoichiometry of GIRK1/2-Gβγ-Gα.

S7 Fig. Simulations of the Gβγ dose-response experiment for a range of assumed Gβγ densities. Because in the experiment of Fig 7 the actual density of Gβγ in the PM has not been
directly measured, the calculations of Fig 7C assumed that it was equal to the average density of 30 Gβγ molecules/μm² (with 5 ng RNA), as measured in other 4 experiments done during the same time period. Here, we run simulations as in Fig 7C for 20 or 44 molecules Gβγ/μm² (A, C) and compare the result with that of Fig 7C (shown here again in B for a direct comparison). The color codes are as in Fig 7: the blue line presents the simulation using graded contribution model and amounts of Gα and Gβγ (without coexpressed Gβγ) calculated as explained in Fig 7 legend, and red, black and green lines show simulation with endogenous G proteins only and no Gβγ recruitment allowed.

S8 Fig. Another experiment on dose-dependent activation of GIRK1/2 by coexpressed Gβγ. The presentation is similar to that of Fig 7. Gβ was coexpressed with Gγ-YFP in incremental doses, and with a constant amount (1 ng RNA) of wt GIRK1/2. RNA of Gγ-YFP was always half of that of Gβ RNA, by weight. (A) Gβγ-YFP fluorescence levels (grey bars, left Y-axis) and GIRK currents (red circles, right Y-axis) are shown on the same plot. GIRK1/2 density, calculated from Iβγ of the 17 ng Gβγ-YFP group, was 13 molecules/μm². In addition, we injected YFP-GIRK1/GIRK2 (5 ng GIRK1-YFP) and measured Ibasal which was 8.4± 1.1 μA (n = 11), comparable to Ibasal of unlabeled GIRK1/2 (9.4±0.8 μA). Thus, we assumed the same density of ~13 channels/μm² for labeled and unlabeled channels. Since the YFP-GIRK1/2 gave a fluorescent signal of 1237 ±221 AU (n = 7), this signal was assumed to correspond to 26 YFP molecules/μm². This number was used as the basis of calculations of Gβγ-YFP density for plots shown in B. (B) Comparison of measured Iβγ or Rβγ (red circles) and simulated currents or Rβγ (blue curves). The left and right Y-axes are related to Iβγ and Rβγ, respectively. Available Gα and Gβγ (before Gβγ coexpression) were estimated from Itotal and Ibasal, giving 3.82 and 0.42 molecules/μm² of Gβγ and Gα, respectively.

S9 Fig. Estimated stoichiometries of Gα and Gβγ available for GIRK in neurons and oocytes in a range of Iβγ/Itotal ratios and P_o,max. Whereas for the oocytes the actual Iβγ/Itotal ratio and P_o,max are known, in neurons these parameters are not known. Both parameters affect the calculated channel density and could affect the estimates of stoichiometry. The calculations shown in this Figure demonstrate the same general trend in stoichiometries of GIRK1/2, Gβγ and Gα as we have found in the previous analysis in the oocytes, in a range of Iβγ/Itotal ratios (for neurons and oocytes; A and B) and P_o,max (for neurons; C). The estimates of Gβγ are around 3-4/channel and relatively independent of Ibasal, and those of Gα are below 2 and drop sharply with the increase in Ibasal. Generally, the lowest channel density is most sensitive to perturbations, and, for the lowest simulated Iβγ/Itotal ratio, calculated Gβγ/channel and Gα/channel exceed our usual estimates.

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Author Contributions
Conceived and designed the experiments: DY SB UK MR IFT BS TKR CWD ND. Performed the experiments: DY SB UK MR IFT BS TKR ND. Analyzed the data: DY SB UK MR IFT BS TKR ND. Contributed reagents/materials/analysis tools: CWD. Wrote the paper: DY SB UK CWD ND.
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MATLAB routines for the calculation of $G_{\beta\gamma}$ and $G_\alpha$ available for GIRK with the graded contribution model

**Template for the calculation of $G_{\beta\gamma_{\text{total}}}$**

```matlab
[c0,c1,c2,c3,c4, cf,gbg,gbgt]=
solve ('4*c0*gbg= K_D*c1', '3*c1*gbg=2* K_D *c2', '2*c2*gbg=3 *K_D *c3',
'c3*gbg=4* K_D *c4', 'c0+c1+c2+c3+c4=n*166e-9 ',
'gbg+c1+2*c2+3*c3+4*c4=m',
'.013*c1+.065*c2+.26*c3+c4=cf',
'cf=p /(72836285*.105)')
% Copy the system to worksheet
% Enter the following:
% n – number of channels per μm²
% p – current in μA ($I_{\text{total}}$)
% gbgt - $G_{\beta\gamma_{\text{total}}}$
```

**Template for the calculation of $G_{\alpha_{\text{total}}}$**

```matlab
[c0, c1, c2, c3, c4, cf, ga, gagbg, gat, gbg]=
solve ('c0*gbg= 4*K_D*c1', '3*c1*gbg=2* K_D *c2', '2*c2*gbg=3 *K_D *c3',
'c3*gbg=4* K_D *c4', 'c0+c1+c2+c3+c4=n*166e-9',
'gbg+gagbg+c1+2*c2+3*c3+4*c4=m', 'ga+gagbg=gat', '0.0013*gagbg=0.7e6*ga*gbg'
'.013*c1+.065*c2+.26*c3+c4=cf',
'cf=q /(72836285*.105)')
% Copy the system to worksheet
% Enter the following:
% n – number of channels per μm²
% q – current in μA ($I_{\text{basal}}$)
% m - $G_{\beta\gamma_{\text{total}}}$
% gat - $G_{\alpha_{\text{total}}}$
```
Template for the simulation of a Gβγ overexpression experiment

\[ [c_0, c_1, c_2, c_3, c_4, cf, curr, ga, gagbg, gat, gbg] = \]
\[ \text{solve ('c0*gbg=4*KD*c1', '3*c1*gbg=2*KD*c2', '2*c2*gbg=3*KD*c3',}
\[ 'c3*gbg=4*KD*c4', 'c0+c1+c2+c3+c4=n*166e-9',
\[ 'gbg+gagbg+c1+2*c2+3*c3+4*c4=m', 'ga+gagbg=gat', '0.0013*gagbg=0.7e6*ga*gbg',
\[ '.013*c1+.065*c2+.26*c3+c4=cf',
\[ 'curr=cf*(72836285*.105)') \]
\%

% Copy the system to worksheet
%
% Enter the following:
%
% n – number of channels per μm²
% curr – current in μA
% m – Gβγtotal
% gat – Gαtotal

All results are in M

Calculations of model predictions for a range of parameters using the Berkeley Madonna software.

To simulate macroscopic GIRK1/2 currents or their ratios (Rα, Rβγ) for a range of arguments in order to produce continuous curves (Figs. 5, 7C, S4, S7, S8), we utilized Berkeley Madonna software which implements 4th order Runge-Kutta method for numerical solution of differential equations. In these calculations, channel activation process by Gβγ was described by following system of differential equations:

\[
\frac{d(Gβγ)}{dt} = k_{off} * GαGDPGβγ + k_{−1} * C_1 + 2 * k_{−1} * C_2 + 3 * k_{−1} * C_3 + 4 * k_{−1} * C_4 -
- Gβγ * (k_{on} * GαGDP + 4 * k_1 * C_0 + 3 * k_1 * C_1 + 2 * k_1 * C_2 + k_1 * C_3)
\]

\[
\frac{d(GαGDP)}{dt} = k_{off} * GαGDPGβγ - k_{on} * GαGDP * Gβγ
\]

\[
\frac{d(C_0)}{dt} = k_{−1} * C_1 - 4 * k_1 * C_0 * Gβγ
\]

\[
\frac{d(C_1)}{dt} = 2 * k_{−1} * C_2 + 4 * k_1 * C_0 * Gβγ - C_1 * (k_{−1} + 3 * k_1 * Gβγ)
\]

\[
\frac{d(C_2)}{dt} = 3 * k_{−1} * C_3 + 3 * k_1 * C_1 * Gβγ - C_2 * (2 * k_{−1} + 2 * k_1 * Gβγ)
\]

\[
\frac{d(C_3)}{dt} = 4 * k_{−1} * C_4 + 2 * k_1 * C_2 * Gβγ - C_3 * (3 * k_{−1} + k_1 * Gβγ)
\]

\[
\frac{d(C_4)}{dt} = k_1 * C_3 * Gβγ - 4 * k_{−1} * C_4
\]

where all symbols are defined as in the main text of the paper, and k₁ and k⁻¹ are forward and reverse rate constants of GIRK-Gβγ interaction, respectively. We used k₁ = 10⁷ M⁻¹s⁻¹ and k⁻¹ = 0.5 s⁻¹. These rate constants of Gβγ and GIRK channel interaction are unknown, but these values are in Smoluchowski limit [1] and render KD = 50 nM which we used in main calculations of our model.
The rate constants of interaction between Gβγ and Goi were $k_{\text{off}} = 0.0013 \text{ s}^{-1}$ and $k_{\text{on}} = 0.7 \times 10^6 \text{ M}^{-1}\text{s}^{-1}$ [2].

**Supplemental discussion**

**Conversion from channel densities to concentrations.**

There is no consensus about the way the reaction rates are changed by the reduction of dimensionality [3,4,5]. Nevertheless, for proteins that interact in the submembrane cytosolic space conversion from two-dimensional rate constants and densities into three-dimensional rate constants and concentrations is a well-accepted paradigm. This is particularly useful, and has been used in the past, in calculations involving GPCRs and G proteins or effectors and G proteins (e.g. [6,7,8,9]).

**GIRK1/2 stoichiometry.**

Silverman et al. [10] have shown that GIRK1/4 channels with 2:2 stoichiometry are preferentially expressed in oocytes and contribute the majority of the macroscopic current, but 3:1 or 1:3 stoichiometry is viable (see also [11]). No such data are available for GIRK1/2. Generally speaking, for macroscopic currents, the exact subunit stoichiometry does not matter, because for a constant RNA ratio as used here (equal amounts of RNAs for both subunits), a relatively constant composition of channel population (in terms of stoichiometry) is expected, subject to natural variability. We cannot exclude the possibility that, in some single-channel recordings, we observed channels of unequal stoichiometry. Nevertheless, since simulations are based on average $i_{\text{single}}$ and $P_{o,\text{max}}$ from a rather large sample of patches (since the closing of data collection for this paper, we have recorded more single GIRK1/2 channels and always obtain a similar $P_{o,\text{max}}$ around 0.11; data not shown), we posit that the average values of $P_{o,\text{max}}$ and $i_{\text{single}}$ faithfully represent the population of channels seen in our macroscopic recordings.

**Estimating steady-state open probability with the “separate gating transitions” model.**

For clarity, here we present the separate gating transitions model from Fig. 2B using the notation which will be utilized in the following equations.

$$
\begin{align*}
C_0^c & \leftrightarrow C_1^c & \leftrightarrow C_2^c & \leftrightarrow C_3^c & \leftrightarrow C_4^c \\
C_0^o & \leftrightarrow C_1^o & \leftrightarrow C_2^o & \leftrightarrow C_3^o & \leftrightarrow C_4^o
\end{align*}
$$
where \( C^c_x \) denotes concentration of channels occupied by \( x \) G\( \beta \gamma \) molecules in closed state and \( C^o_x \) is the concentration of channels occupied by \( x \) G\( \beta \gamma \) molecules in open state.

The open probability for the above scheme can be calculated according to:

\[
P_o = \frac{\sum_{x=0}^{x=4} C^o_x}{\sum_{x=0}^{x=4} C^o_x + \sum_{x=0}^{x=4} C^c_x} \quad (S1)
\]

The equilibrium constant \( K_x \) between \( C^c_x \) and \( C^o_x \) is

\[
K_x = \frac{C^o_x}{C^c_x} \quad (S2)
\]

and \( P_{o,x} \) is the open probability of \( x \) G\( \beta \gamma \) molecules occupied channel and can be defined as

\[
P_{o,x} = \frac{C^o_x}{C^o_x + C^c_x} = \frac{K_x}{1 + K_x} \quad (S3)
\]

If \( C_x \) is the concentration of channels occupied by \( x \) G\( \beta \gamma \) molecules, then

\[
C_x = C^c_x + C^o_x \quad (S4)
\]

and if \( C_{total} \) is total channel concentration, then

\[
C_{total} = \sum_{x=0}^{x=4} C_x = \sum_{x=0}^{x=4} C^c_x + \sum_{x=0}^{x=4} C^o_x \quad (S5)
\]

Thus (S1) can be rearranged

\[
P_o = \frac{\sum_{x=0}^{x=4} C^o_x}{C_{total}} \quad (S6)
\]

Solving (S2) and (S4) for \( C^o_x \) renders

\[
C^o_x = \frac{K_x}{1 + K_x} \cdot C_x \quad (S7)
\]

Substituting (S3) into (S7) renders

\[
C^o_x = P_{o,x} \cdot C_x \quad (S8)
\]

Substituting (S8) into (S6) renders
\[
P_O = \frac{\sum_{x=0}^{x=4} P_{O,x} \cdot C_x}{C_{total}}
\]  
(S9)

If \( f_{p,x} \) is the fraction of \( P_{o,max} \) (maximal observable \( P_o \)) and is defined as \( f_{p,x} = P_{o,x}/P_{o,max} \) and \( \phi_x \) is the fraction of channels occupied by \( x \) G\( \beta \gamma \) molecules (defined as \( \phi_x = C_x/C_{total} \)), then substitution of definitions of \( f_{p,x} \) and \( \phi_x \) to (S9) will render

\[
P_O = P_{o,max} \cdot \sum_{x=0}^{x=4} f_{p,x} \cdot \phi_x
\]  
(S10)

This equation is identical to Equation 6 (Methods).

The graded contribution model and the more elaborated independent gating transitions model will predict distinct single channel behavior of GIRK1/2, which may need to be investigated in detail to distinguish between the two models. Nevertheless, as shown above, the description of steady-state \( P_o \) by the two models converges to Equation 6 (Methods), suggesting that this is a general equation which can describe GIRK1/2 macroscopic gating for any number of open states. As a consequence, the two models should produce identical fractional macroscopic steady-state currents (\( P_o/P_{o,max} \), where \( P_{o,max} \) is equivalent to \( I/I_{\beta \gamma} \), where \( I \) is either \( I_{basal} \), \( I_{total} \) or \( I_{evoked} \)) once the channel is occupied by \( x \) G\( \beta \gamma \). In other words, for steady state description of macroscopic currents that we present in our study, the results are expected to be the similar both for the graded contribution model as well as for the more elaborated independent gating transitions model.

**Supplemental References**


**Supplemental Table 1**

**Table S1.** Effect of coexpression of Gαi3 on GIRK1/2 currents in oocytes. Data are from 2 to 4 experiments, for each group, shown as mean ± SEM. We did not include experiments with extremely large Gαi3 RNA quantities, as expression of higher doses of Gαi3 usually reduced Itotal, indicating a general Gβγ scavenging effect rather than priming [12,13].

<table>
<thead>
<tr>
<th>50-200 pg GIRK1/2 (n=10)</th>
<th>50-200 pg GIRK1/2+0.5-2 ng Gαi3 (n=10)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Ibasal (µA)</strong></td>
<td><strong>Ievoked (µA)</strong></td>
</tr>
<tr>
<td>4.8±0.42</td>
<td>4.2±0.76</td>
</tr>
<tr>
<td><strong>0.5-1 ng GIRK1/2 (n=27)</strong></td>
<td><strong>0.5-1 ng GIRK1/2 + 5-10 ng Gαi3 (n=16)</strong></td>
</tr>
<tr>
<td><strong>Ibasal (µA)</strong></td>
<td><strong>Ievoked (µA)</strong></td>
</tr>
<tr>
<td>12.6±0.7</td>
<td>2.5±0.3</td>
</tr>
</tbody>
</table>
Fig. S1. Block of inward currents in cultured hippocampal neurons by TPNQ and Ba²⁺. (A) Ba²⁺ (1 mM) blocks a greater fraction of the total inward current in high-K⁺ solution, compared to TPNQ (120 nM). The experimental protocol was the same as in Fig. 1, with the additional step of Ba²⁺ addition after TPNQ. ΔTPN and ΔBa denote the magnitudes (shown by double-speared arrows) of TPNQ- and Ba-blocked currents, respectively. Note that Ba²⁺ blocked a much greater fraction of the total inward current in high-K⁺ solution, most probably of the block of additional Ba²⁺-sensitive channels present in these neurons. (B) Comparison of average TPNQ- and Ba²⁺-blocked currents in 14 cells of one batch of neurons. Statistical significance (p<0.001) was determined using Wilcoxon Signed Rank test (the data did not pass normality test).
Fig. S2. GIRK1/2 currents in oocytes. Holding potential was -80 mV, low-K⁺ and high-K⁺ solutions contained 2 and 24 mM K⁺, respectively (K⁺ was replaced for Na⁺). Net GIRK currents were determined by subtracting the current remaining after the addition of 5 mM BaCl₂. (A) Ibasal and Ievoked in an oocyte expressing m2R, GIRK1 and GIRK2. Calculation of Rα was done in every cell from its own Ibasal and Ievoked. (B) Iβγ in an oocyte expressing m2R, GIRK1, GIRK2 and Gβγ. Note that adding ACh did not evoke a significant additional GIRK current, suggesting full activation by Gβγ. Rβγ was calculated in each cell by dividing its own Iβγ by the average Iβγ from the control group of the same experiment in which no Gβγ was coexpressed. (C) Expression of m2R in a wide range of doses does not affect Ibasal. 5-8 oocytes have been tested in each group. There were no significant differences between treatments as tested by one-way ANOVA.
Fig. S3. Characterization of YFP-labeled GIRK1 and Gβ. (A, B) Single channel parameters of GIRK1/2 and YFP-GIRK1/2 channels are very similar. (A) Cell-attached records of channel activity expressing the channel and Gβγ (5 ng RNA). (B) Comparison of average $i_{\text{single}}$ and $P_o$. Data are from oocytes of the same batch, recorded during a two-day experiment. (C, D) The anti-Gβ antibody similarly recognizes YFP-labeled bovine and *Xenopus* Gβ subunits in Western blots of manually peeled plasma membranes. Data are from 4 separate experiments. For Western blots, 15 to 20 plasma membranes were pooled. For confocal imaging, groups of 3-16 oocytes were examined, and the average fluorescence level was compared with that of YFP-GIRK1/2 (therefore the statistical significance was calculated using paired t-test). The density of the latter was calculated from the measurement of currents as explained in the text. In each experiment, both confocal imaging, current measurement and Western blots of manually peeled membranes were done in oocytes of the same donor. There was a good agreement for surface density estimates of YFP-Gβ-XL from confocal "molecular ruler" measurements and from quantitative Western blots, either in absolute terms as molecules/µm² (C) or in relative terms, normalized to estimates of YFP-Gβ in each experiment (D). YFP fluorescence can be safely assumed to be independent of the species of fused Gβ (mammalian or *Xenopus*). Therefore, similar estimates of surface density observed from confocal imaging and Western blots suggest that the Gβ antibody used here recognizes the oocyte's endogenous Gβ in Western blots similarly to the coexpressed mammalian (bovine) Gβ₁.
**Fig. S4. Simulation of density-dependent changes in whole-cell GIRK1/2 activity.** Experimental data (from Table 1) are shown as red circles (mean ± SEM). The simulations of currents and Ra were done using the graded contribution model. (A) Testing the hypothesis that the endogenous Gαβγ heterotrimers are the only source of Gβγ for GIRK activation; \(I_{\text{basal}}\) is due to spontaneous dissociation of Gαβγ into GαGDP and Gβγ (see Fig. 2A). Simulations were performed assuming that only part (1 or 10 molecules/µm², black and red curves) or all (24 molecules/µm², blue curves) endogenous G proteins can donate Gβγ to activate GIRK1/2. Note that no satisfactory description of data can be obtained under any of these conditions. The simulated \(I_{\text{basal}}\) is too low; for high channel densities, also the full \(I_{\text{evoked}}\) could not be obtained even assuming that all endogenous Gαβγ (i.e. all 24 molecules/µm²) could release Gβγ and activate GIRK. (B) Testing the hypothesis that the expressed GIRK1/2 recruits additional endogenous G protein subunits to the PM, e.g.
from other cellular compartments. Simulations were done assuming that each GIRK1/2 channel recruits from 1 to 4 G\textsubscript{i/o} heterotrimers. The recruited G\textsubscript{α} and G\textsubscript{βγ} were added to the pre-existing endogenous plasma membrane-attached G\textsubscript{αβγ} before G\textsubscript{βγ} expression. (C) Testing the hypothesis that the expressed GIRK1/2 recruits additional endogenous Gβγ, but not Gα, to the PM; the rest was done as in B. Calculations in (B) and (C) assumed 24 molecules/µm\textsuperscript{2} of endogenous G\textsubscript{i/o} available for GIRK. Similar results were obtained assuming 10 molecules/µm\textsuperscript{2} (data not shown). Simulations as in A-C were also done with the concerted model, yielding similar results (data not shown).

**Fig. S5.** The concerted activation model supports the unequal stoichiometry estimates of Gβγ and Gα available for GIRK1/2. The plots present the calculated amounts of Gβγ and Gα available for GIRK1/2 using the concerted model for a range of K\textsubscript{D} for the GIRK-Gβγ interaction (5-100 nM), for the three channel density groups of Table 1.
Fig. S6. The presence of Gβγ-independent intrinsic activity and the dimensions of submembrane reaction space do not significantly alter the estimates of GIRK1/2-available G proteins subunits. Calculations were done assuming $K_D = 50$ nM for the GIRK-Gβγ interaction. (A-C), the impact of Gβγ-independent basal activity. Calculation were done for Gβγ-independent intrinsic activity of a single channel ranging from 1% to 10% of $P_{o,max}$. Available Gβγ (A), Gα (B) and the Gβγ-independent fraction of $I_{basal}$ (C) were calculated for the three channel density groups of Table 1. (D, E) Varying the submembrane space thickness in a wide range, 1-20 nm, does not significantly change the estimates of functional stoichiometry of GIRK1/2-Gβγ-Gα.
**Fig. S7.** Simulations of the Gβγ dose-response experiment for a range of assumed Gβγ densities.

Because in the experiment of Fig. 7 the actual density of Gβγ in the PM has not been directly measured, the calculations of Fig. 7C assumed that it was equal to the average density of 30 Gβγ molecules/µm² (with 5 ng RNA), as measured in other 4 experiments done during the same time period. Here, we run simulations as in Fig. 7C for 20 or 44 molecules Gβγ/µm² (A, C) and compare the result with that of Fig. 7C (shown here again in B for a direct comparison). The color codes are as in Fig. 7: the blue line presents the simulation using graded contribution model and amounts of Gα and Gβγ (without coexpressed Gβγ) calculated as explained in Fig. 7 legend, and red, black and green lines show simulation with endogenous G proteins only and no Gβγ recruitment allowed.
**Fig. S8.** Another experiment on dose-dependent activation of GIRK1/2 by coexpressed Gβγ. The presentation is similar to that of Fig. 7. Gβ was coexpressed with Gγ-YFP in incremental doses, and with a constant amount (1 ng RNA) of wt GIRK1/2. RNA of Gγ-YFP was always half of that of Gβ RNA, by weight. (A) Gβγ-YFP fluorescence levels (grey bars, left Y-axis) and GIRK currents (red circles, right Y-axis) are shown on the same plot. GIRK1/2 density, calculated from Iβγ of the 17 ng Gβγ-YFP group, was 13 molecules/μm². In addition, we injected YFP-GIRK1/GIRK2 (5 ng GIRK1-YFP) and measured Ibasal which was 8.4± 1.1 µA (n=11), comparable to Ibasal of unlabeled GIRK1/2 (9.4±0.8 µA). Thus, we assumed the same density of ~13 channels/μm² for labeled and unlabeled channels. Since the YFP-GIRK1/2 gave a fluorescent signal of 1237 ±221 AU (n=7), this signal was assumed to correspond to 26 YFP molecules/μm². This number was used as the basis of calculations of Gβγ-YFP density for plots shown in B. (B) Comparison of measured Iβγ or Rβγ (red circles) and simulated currents or Rβγ (blue curves). The left and right Y-axes are related to Iβγ and Rβγ, respectively. Available Gα and Gβγ (before Gβγ coexpression) were estimated from Itotal and Ibasal, giving 3.82 and 0.42 molecules/μm² of Gβγ and Gα, respectively.
Fig. S9. Estimated stoichiometries of Gα and Gβγ available for GIRK in neurons and oocytes in a range of Iβγ/I_{total} ratios and P_{o,max}. Whereas for the oocytes the actual Iβγ/I_{total} ratio and P_{o,max} are known, in neurons these parameters are not known. Both parameters affect the calculated channel density and could affect the estimates of stoichiometry. The calculations shown in this Figure demonstrate the same general trend in stoichiometries of GIRK1/2, Gβγ and Gα as we have found in the previous analysis in the oocytes, in a range of Iβγ/I_{total} ratios (for neurons and oocytes; A and B) and P_{o,max} (for neurons; C). The estimates of Gβγ are around 3-4/channel and relatively independent of I_{basal}, and those of Gα are below 2 and drop sharply with the increase in I_{basal}. Generally, the lowest channel density is most sensitive to perturbations, and, for the lowest simulated Iβγ/I_{total} ratio, calculated Gβγ/channel and Gα/channel exceed our usual estimates.