

## RESEARCH ARTICLE

# Deletion of GIRK2 Subunit of GIRK Channels Alters the 5-HT<sub>1A</sub> Receptor-Mediated Signaling and Results in a Depression-Resistant Behavior

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## Abstract

**Background:** Targeting dorsal raphe 5-HT<sub>1A</sub> receptors, which are coupled to G-protein inwardly rectifying potassium (GIRK) channels, has revealed their contribution not only to behavioral and functional aspects of depression but also to the clinical response to its treatment. Although GIRK channels containing GIRK2 subunits play an important role controlling excitability of several brain areas, their impact on the dorsal raphe activity is still unknown. Thus, the goal of the present study was to investigate the involvement of GIRK2 subunit-containing GIRK channels in depression-related behaviors and physiology of serotonergic neurotransmission.

**Methods:** Behavioral, functional, including in vivo extracellular recordings of dorsal raphe neurons, and neurogenesis studies were carried out in wild-type and GIRK2 mutant mice.

**Results:** Deletion of the GIRK2 subunit promoted a depression-resistant phenotype and determined the behavioral response to the antidepressant citalopram without altering hippocampal neurogenesis. In dorsal raphe neurons of GIRK2 knockout mice, and also using GIRK channel blocker tertiapin-Q, the basal firing rate was higher than that obtained in wild-type animals, although no differences were observed in other firing parameters. 5-HT<sub>1A</sub> receptors were desensitized in GIRK2 knockout mice, as demonstrated by a lower sensitivity of dorsal raphe neurons to the inhibitory effect of the 5-HT<sub>1A</sub> receptor agonist, 8-OH-DPAT, and the antidepressant citalopram.

**Conclusions:** Our results indicate that GIRK channels formed by GIRK2 subunits determine depression-related behaviors as well as basal and 5-HT<sub>1A</sub> receptor-mediated dorsal raphe neuronal activity, becoming alternative therapeutic targets for psychiatric diseases underlying dysfunctional serotonin transmission.

**Keywords:** Dorsal raphe, GIRK, 5-HT<sub>1A</sub>, electrophysiology, citalopram

## Introduction

Dysfunctional serotonin (5-HT) transmission plays a key role in the etiology and treatment of depression. Increasing 5-HT by selectively blocking its reuptake is the main pharmacological

strategy to treat it (Krishnan and Nestler, 2008). The major source of 5-HT in the brain is the dorsal raphe (DR) nucleus (Dahlstroem and Fuxe, 1964), and it is highly regulated by the

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5-HT<sub>1A</sub> autoreceptors, which play a critical role in the development, modulation, and treatment of depression. A functional polymorphism in the promoter region of the human *Htr1a* gene, which regulates 5-HT<sub>1A</sub> receptor levels, is linked to predisposition to mental illness as well as anxiety- and depression-related behaviors and response to antidepressants (Strobel et al., 2003; Lemonde et al., 2004; Lesch and Gutknecht, 2004; Le Francois et al., 2008). Diverse genetic manipulations of 5-HT<sub>1A</sub> receptor levels have confirmed the role of these receptors in anxiety- and depression-like phenotypes in mice (Heisler et al., 1998; Parks et al., 1998; Richardson-Jones et al., 2010; Ferres-Coy et al., 2013). It is widely accepted that the slow onset of the response to antidepressants is related to the progressive desensitization of the inhibitory effects mediated by activation of 5-HT<sub>1A</sub> receptors onto 5-HT neurotransmission (Blier and de Montigny, 1994; Artigas et al., 1996). Also, the behavioral response to antidepressants has been linked to increased neurogenesis, which is mediated by stimulation of 5-HT<sub>1A</sub> receptors (Santarelli et al., 2003). The G protein-coupled inwardly rectifying potassium (GIRK) channels are the main inhibitory effectors of 5-HT<sub>1A</sub> receptors (Williams et al., 1988), and therefore they could be alternative candidates for the study of depression and antidepressant responses involving the 5-HT<sub>1A</sub> receptor-mediated signaling.

Neuronal GIRK channels are tetramers mainly formed by GIRK1-3 subunits, since the expression of GIRK4 subunits is limited in the brain (Karschin et al., 1996). Specifically, the GIRK2 subunit plays a relevant role in GIRK channel function, given that the predominant form of GIRK channels is a heterotetramer containing GIRK1 and GIRK2 subunits (Liao et al., 1996), and it is responsible for the generation of G-protein coupled receptor-mediated GIRK currents in several brain areas, including the locus coeruleus (LC) and the hippocampus (HPP) (Luscher et al., 1997; Slesinger et al., 1997; Torrecilla et al., 2002; Labouebe et al., 2007; Cruz et al., 2008). Moreover, mutation of GIRK2 subunits causes a GIRK1 protein downregulation (Signorini et al., 1997; Torrecilla et al., 2002), and the constitutive activity of GIRK2 subunit-containing GIRK channels reduces neuronal excitability in vitro (Luscher et al., 1997; Torrecilla et al., 2002). Recently, it has been demonstrated that the maintenance of the tonic noradrenergic activity, which is another important neurotransmission system widely implicated in mood disorders, is under the control of GIRK2 subunit-containing GIRK channels (Torrecilla et al., 2013). Mice lacking GIRK2 subunits exhibit a reduced anxiety-like phenotype (Blednov et al., 2001; Pravetoni and Wickman, 2008), while mice lacking 5-HT<sub>1A</sub> receptors display increased anxiety-related behavior (Heisler et al., 1998; Parks et al., 1998; Ramboz et al., 1998). Given the involvement of these receptors in the etiology and treatment of depression and their functional relationship with GIRK channels, the aim of our study was to investigate the role of GIRK2 subunit-containing GIRK channels in depression-related behaviors and adult neurogenesis as well as basal and 5-HT<sub>1A</sub> receptor-mediated electrophysiological activity in DR neurons.

## Methods

### Animals

We used C57BL6/J wild-type (WT), GIRK2 heterozygous (GIRK2<sup>+/−</sup>), and GIRK2 homozygous (GIRK2<sup>−/−</sup>) mice (3 months old) derived from heterozygote crossing (Signorini et al., 1997). In the electrophysiological, behavioral, and locomotor activity experiments, male and female mice were used; data were pooled given the lack of gender differences in any parameter obtained from these studies. Immunohistochemistry and hypothermic studies were

performed in male mice. Animals were maintained at 22 ± 2°C in a 12-h-light/-dark cycle, with food and water provided ad libitum. All procedures were conducted in accordance with the European Community Council Directive The Protection of Animals Used for Experimental and Other Scientific Purposes (86/609/EEC) and the Spanish Law for the care and use of laboratory animals (RD 1201/2005). Experimental protocols were reviewed and approved by the Local Committee for Animal Experimentation at the University of the Basque Country.

### Behavioral Tests

All tests were performed between 9:00 AM and 1:00 PM. Mice were transferred to a noise-free and temperature-controlled testing room at least 1 hour before the experiments. No more than 1 test was performed in each mouse.

#### Novelty Suppressed Feeding Test

The novelty suppressed feeding test (NSFT) was performed as previously described (Santarelli et al., 2003). Animals were food deprived 24 hours prior to the test (water ad libitum). The testing apparatus consisted of a plastic box (45 × 45 × 20 cm) with a wooden bedding-covered floor illuminated by a 70W lamp and a white paper platform with a food pellet in the center. The test was carried out for 10 minutes and the latency to eat was timed. Afterwards, animals were transferred to their home cages, and the amount of food consumed by each mouse in the subsequent 5 minutes was measured.

#### Tail Suspension Test

In the tail suspension test (TST), mice were suspended 60 cm from the surface with adhesive tape placed approximately 2 cm from the tip of the tail. Animals were monitored (6 minutes) by video camera for subsequent blind analysis. Mice were considered immobile when they stood completely motionless or hung passively. Intraperitoneal administration of citalopram (10 mg/kg) was carried out 30 minutes prior to the experiments.

#### Locomotor Activity

Locomotor activity was assessed in an open-field (20 × 30 × 30 cm) bar system. The floor consisted of a stainless-steel grid conformed by 28 bars of 3 mm each, associated to a detector of changes in electric resistance. The activity was monitored for 30 minutes and data were collected for horizontal activity.

### In Vivo Electrophysiological Procedures

Mice were anesthetized with chloral hydrate (400 mg/kg, i.p.), and supplementary doses were administered as needed. Single-barreled glass micropipettes (1–2 μm tip diameter) were lowered into the DR (relative to bregma: AP -4.5 mm, ML -1.0 mm, DV -2.5 to -4.0 mm) with a lateral angle of 20° to avoid damaging the sagittal sinus. DR neurons were identified using established criteria, which included slow (0.5–2.5 Hz) and regular firing rate, and a long duration (0.8–1.5 ms) positive action potential. A burst was defined according to Gartside et al. (2000), and burst-firing neurons in mice were identified as previously described (Gobbi et al., 2007), including a first interspike interval of ≤20 ms and a termination interval ≥160 ms.

Firing pattern analyses were performed using Spike2 software (Cambridge Electronic Design). The following parameters were analyzed offline: firing rate, coefficient of variation, percentage of cells exhibiting burst firing, firing rate of burst firing neurons, number of burst per cell, percentage of spikes in burst,

mean spikes per burst, and the response to drug administration. Basal firing rate was recorded for at least 3 minutes before drug administration. One cell per animal was recorded when any drug was administered. All the recorded neurons met the previously mentioned established criteria and were located within the DR.

Intracerebroventricular administrations were performed 10 minutes prior to the recordings using a microsyringe (5  $\mu$ L, Hamilton, Bonaduz, Switzerland) connected to a 30-gauge needle that was inserted into the right lateral ventricle (relative to bregma: AP -0.5 mm, ML -1.0 mm, DV -2.0 mm). A volume of 1  $\mu$ L of tertiapin-Q (100 pmol, dissolved in artificial cerebrospinal fluid [ACSF]), or ACSF (used as a control group) was injected directly into the right lateral ventricle.

### 8-OH-DPAT-Induced Hypothermia

Body temperature was assessed rectally, inserting a lubricated probe approximately 2 cm and monitored with a digital thermometer. Ten minutes after baseline measurements, animals received 8-OH-DPAT (0.5 mg/kg i.p.) or 0.9% saline (i.p.), and body temperature was measured the subsequent 10, 20, 30, and 60 minutes.

### Immunohistochemistry Procedures

#### Fixation and Tissue Processing

Mice were anaesthetized with sodium pentobarbital (200 mg/kg, i.p.). Animals were perfused through the aortic arch with 3.75% acrolein (25 mL, TAAB) in a solution of 2% paraformaldehyde and 0.1 M phosphate buffer (PB), pH 7.4, followed by 2% paraformaldehyde (75 mL). Coronal brain slices were cut into 40- $\mu$ m thickness using a vibratome microtome. Sections at levels -1.34 mm/-2.30 mm posterior to bregma and 1.18 mm/0.26 mm anterior to bregma from HPP and subventricular zone (SVZ), respectively, were selected for immunohistochemistry according to the stereotaxic mouse brain atlas of [Franklin and Paxinos \(1997\)](#).

#### Antibodies

A polyclonal affinity-purified rabbit antibody raised against phosphorylated Histone H3 (HH3) and a monoclonal mouse antiserum generated against glial fibrillary acidic protein (GFAP) were used for the determination of proliferating cells and glia. We determined the number of proliferating cells by the presence of phosphorylated HH3 that were not colocalized with GFAP to exclude glial phenotype. Omission of primary and/or secondary antibodies resulted in a total absence of target labeling ([Rodriguez et al., 2008, 2009](#)).

#### Immunohistochemistry

This procedure was performed as previously described ([Rodriguez et al., 2008, 2009](#)). Sections were first incubated for 30 minutes in 30% methanol in 0.1 M PB and 30% H<sub>2</sub>O<sub>2</sub> and then rinsed with 0.1 M PB for 5 minutes and placed in 1% sodium borohydride for 30 minutes. After incubating brain sections in 0.5% bovine serum albumin, in 0.1 M TS and 0.25% Triton X-100 for 30 minutes, they were incubated for 68 hours at room temperature in 0.1% bovine serum albumin in 0.1 M TS and 0.25% Triton X-100 containing rabbit polyclonal antiserum for HH3 (1:1000) and mouse monoclonal antiserum for GFAP (1:6000). For HH3 labeling, sections were placed in 0.1 M TS and 0.25% Triton X-100 containing 1:400 dilutions of biotinylated donkey anti-rabbit IgG (Jackson ImmunoResearch, Stratech Scientific Ltd., Soham, UK) for 1 hour at room temperature and avidin-biotin peroxidase complex for 30 minutes at room temperature. The peroxidase reaction product was visualized in a solution

prepared from SGZ kits for 2 to 3 minutes. For GFAP labeling, sections were incubated in 0.1 M TS and 0.25% Triton X-100 containing 1:400 dilution of biotinylated horse anti-mouse IgG (Vector Laboratories, Peterborough, UK) for 1 hour at room temperature and avidin-biotin peroxidase complex for 30 minutes at room temperature. The GFAP peroxidase reaction was observed by incubation in a solution containing 0.022% 3,3'-diaminobenzidine and 0.003% H<sub>2</sub>O<sub>2</sub> for 1 to 2 minutes. With this procedure, the GFAP labeling was seen in brown, allowing us to differentiate it from the HH3-labeled cells (blue). Sections were mounted onto gelatinized slides and allowed to dry overnight and then dehydrated in increasing concentrations of ethanol (50%, 70%, 80%, 90%, 95%, and 100%) and xylene. Coverslips were applied using Entellan and were left overnight before counting.

#### Cell Quantification

The number of HH3-immunoreactive neurons and HH3/GFAP colocalized cells was determined by counting the labeled cells in both hemispheres in sections of dentate gyrus (DG) of the HPP and of the SVZ at levels previously mentioned. Cells were counted using light microscopy. The number of HH3-positive cells and the area measurements of the DG and SVZ bounded by the lateral ventricles, corpus callosum, and caudate-putamen nucleus were determined blindly to ensure consistency and reproducibility.

#### Drugs

Chloral hydrate, 8-OH-DPAT hydrobromide, and WAY100635 maleate were obtained from Sigma-Aldrich. Citalopram hydrobromide and tertiapin-Q were from Tocris Bioscience. Tertiapin-Q was prepared in ACSF. Chloral hydrate, 8-OH-DPAT, WAY100635, and citalopram were prepared in 0.9% saline.

#### Statistical Analysis

Data obtained from the TST, locomotor activity, and immunohistochemistry studies were compared through genotypes by 1-way ANOVA followed by Newman-Keuls posthoc test. For the 8-OH-DPAT hypothermic response analysis, 2-way ANOVA followed by Bonferroni posthoc test was used. In the NSFT, data were analyzed using Kaplan-Meier survival analysis and Mantel-Cox log-rank test.

Changes in firing rate induced by 8-OH-DPAT and citalopram are expressed as percentages of the baseline firing rate (mean firing rate during 3 minutes prior to drug administration). Data compiled from dose-response curves were analyzed for the best simple nonlinear fit to the 3-parameter logistic equation ([Parker and Waud, 1971](#)) using GraphPad Prism Software (v5.01; GraphPad Software Inc). The following equation was used:

$$E = E_{\max} [A]^n / (ED_{50}^n + [A]^n),$$

where [A] is the concentration of the drug, E is the effect on the firing rate induced by A, E<sub>max</sub> is the maximal effect, ED<sub>50</sub> is the effective dose for eliciting 50% of the E<sub>max</sub>, and n is the slope factor of the dose-response curve. Extra sum-of-squares F test (GraphPad Prism Software) was used for statistical comparison of the response to a drug in dose-response curves and for comparison of ED<sub>50</sub> among groups.

Spontaneous firing rate and coefficient of variation were analyzed in selected pair comparisons using unpaired 2-tailed t test. Two-sided  $\chi^2$  analysis of contingency tables was used to evaluate differences in the percentage of neurons presenting burst firing.

Other parameters derived from burst pattern were analyzed by the nonparametric Kruskal-Wallis test followed by Dunn's post-hoc test. The level of significance was considered  $P < .05$ .

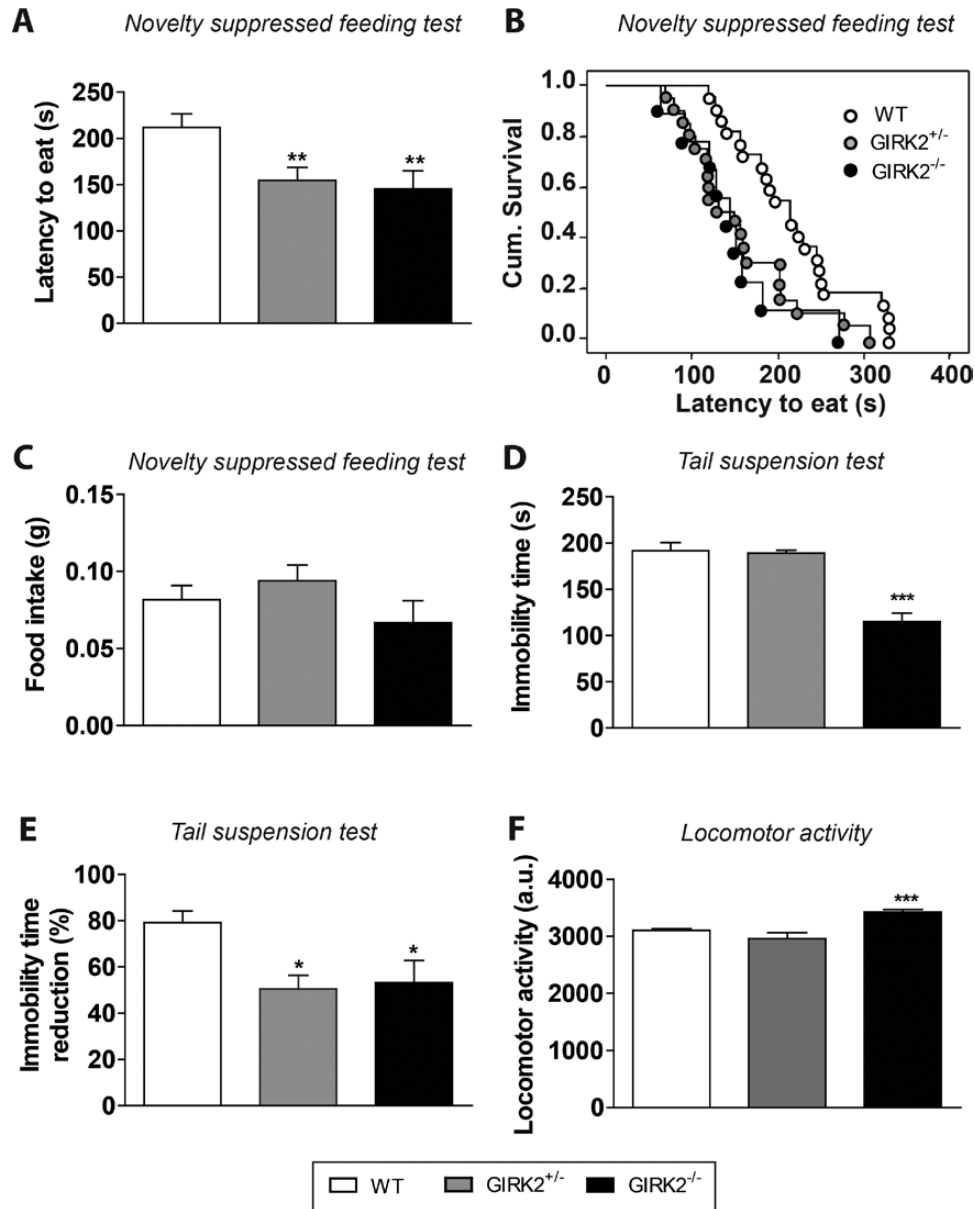
## Results

### Behavioral Characterization of GIRK2 Mutant Mice and the Response to Citalopram

To evaluate whether GIRK2 subunit ablation impacts depressive-related behaviors, GIRK2 mutant mice were evaluated using 2 well-established behavioral models of antidepressant activity,

the NSFT and TST (Santarelli et al., 2003; Cryan et al., 2005; Heurteaux et al., 2006). In the NSFT, both GIRK2<sup>-/-</sup> and GIRK2<sup>+/-</sup> mice showed a decreased latency to eat compared with WT mice (GIRK2<sup>-/-</sup>: 145.60 ± 19.63 seconds, n=9; GIRK2<sup>+/-</sup>: 154.60 ± 14.30 s, n=20; WT: 212.00 ± 14.85 seconds, n=22,  $P < .01$ , Kaplan-Meier survival analysis, Mantel-Cox log-rank test) (Figure 1a-b). This decrease in the latency to eat in the mutant groups was not due to an increased appetite (Figure 1c).

When evaluating the behavioral characteristics in the TST, GIRK2<sup>-/-</sup> mice showed a lower immobility time than GIRK2<sup>+/-</sup> and WT mice (GIRK2<sup>-/-</sup>: 115.10 ± 9.08 seconds, n=16; GIRK2<sup>+/-</sup>: 189.10 ± 3.24 seconds, n=16; WT: 191.80 ± 8.87 seconds, n=14,



**Figure 1.** Behavioral characterization of G-protein inwardly rectifying potassium (GIRK)2 mutant mice. (A-B) In the novelty suppressed feeding test (NSFT), both GIRK2<sup>+/-</sup> and GIRK2<sup>-/-</sup> mice displayed a lower latency to eat compared with wild-type (WT) mice. Results are expressed as mean latency to eat (seconds) (A) or cumulative survival (B) (n=9–22 mice/group; \*\* $P < .01$ , Kaplan-Meier survival analysis, Mantel-Cox log-rank test). (C) No changes in the amount of food consumed after the NSFT were observed among groups (n=9–22 mice/group). (D) In the tail suspension test (TST), GIRK2<sup>-/-</sup> mice displayed a lower immobility time compared with GIRK2<sup>+/-</sup> and WT mice (n=14–16 mice/group; \*\*\* $P < .001$  vs WT, 1-way ANOVA followed by Newman-Keuls test). (E) In the TST, 30 minutes after the citalopram injection (10 mg/kg, i.p.), both GIRK2<sup>+/-</sup> and GIRK2<sup>-/-</sup> mice showed a lower reduction of the immobility time compared with WT mice (n=7–10 mice/group; \* $P < .05$  vs WT mice, 1-way ANOVA followed by Newman-Keuls test). (F) GIRK2<sup>-/-</sup> mice showed an increased locomotor activity compared with GIRK2<sup>+/-</sup> and WT mice (n=12–15 mice/group; \*\*\* $P < .0001$ , 1-way ANOVA followed by Newman-Keuls test). Bars represent mean ± SEM of n animals.

$P < .0001$ , 1-way ANOVA followed by Newman-Keuls test) (Figure 1d). Next, we examined the effect of citalopram on the immobility time in the TST. In all groups, the administration of citalopram (10 mg/kg, i.p.) caused a reduction of the immobility time (Figure 1e). However, in  $GIRK2^{-/-}$  and  $GIRK2^{+/-}$  mice, this reduction was significantly lower than that observed in WT mice ( $GIRK2^{-/-}$ :  $53.2 \pm 9.5\%$ ,  $n=7$ ;  $GIRK2^{+/-}$ :  $50.5 \pm 5.9\%$ ,  $n=7$ ; WT:  $79.2 \pm 5.1\%$ ,  $n=10$ ,  $P < .05$ , 1-way ANOVA followed by Newman-Keuls test). We next tested the locomotor activity of  $GIRK2$  mutant mice. As expected (Blednov et al., 2001; Pravetoni and Wickman., 2008; Arora et al., 2010),  $GIRK2^{-/-}$  mice showed an elevated motor activity compared with  $GIRK2^{+/-}$  and WT mice ( $GIRK2^{-/-}$ :  $3429 \pm 43.65$ ,  $n=14$ ;  $GIRK2^{+/-}$ :  $2962 \pm 104.90$ ,  $n=12$ ; WT:  $3105 \pm 25.39$ ,  $n=15$ ,  $P < .0001$ , 1-way ANOVA followed by Newman-Keuls test) (Figure 1f).

### Electrophysiological Properties of DR Neurons of $GIRK2$ Mutant Mice

To examine the contribution of  $GIRK2$  subunit-containing  $GIRK$  channels to the basal activity of DR neurons, firing rate, coefficient of variation, and burst activity were studied in WT and  $GIRK2$  mutant mice in vivo.

All neurons recorded from WT ( $n=86$ ),  $GIRK2^{+/-}$  ( $n=79$ ), and  $GIRK2^{-/-}$  ( $n=45$ ) mice fit the standard criteria (see Methods).

Spontaneous firing rate of DR neurons in  $GIRK2^{-/-}$  mice was higher than in WT mice ( $1.99 \pm 0.15$  Hz,  $n=45$ ;  $1.68 \pm 0.08$  Hz,  $n=86$ , for  $GIRK2^{-/-}$  and WT mice, respectively).  $P < .05$ , unpaired 2-tailed t test). No changes were seen among groups in either the coefficient of variation or the burst activity. A more extended study of the burst pattern (mean firing rate, number of bursts, percentage of spikes firing in bursts, and mean spikes in burst) showed no differences among groups (Table 1).

Next, the effect of the high-affinity  $GIRK$  channel blocker, tertiapin-Q (100 pmol, i.c.v.) (Jin and Lu, 1998; Kanjhan et al., 2005), on the electrophysiological properties of DR neurons was assessed. The firing rate of DR neurons in tertiapin-Q-injected WT mice (WT TPN-Q:  $2.32 \pm 0.27$  Hz,  $n=15$ ; WT:  $1.68 \pm 0.08$  Hz,  $n=86$ ,  $P < .001$ ; unpaired 2-tailed t test). However, no significant changes were seen in either the coefficient of variation or the burst parameters compared with WT mice. No differences were observed in firing rate ( $1.80 \pm 0.27$  Hz,  $n=7$ ), coefficient of variation ( $35.13 \pm 4.42\%$ ,  $n=7$ ), or number of burst firing neurons (14%) in the intracerebroventricular ACSF-injected WT group compared with WT mice (data not shown).

### Effect of $Girk2$ Gene Deletion on the 5-HT<sub>1A</sub> Receptor-Mediated Inhibition of DR Neuronal Activity

To investigate the role of  $GIRK2$  subunits in the 5-HT<sub>1A</sub> receptor-mediated transmission, we compared the inhibitory effect of cumulative increasing doses of the 5-HT<sub>1A</sub> receptor agonist, 8-OH-DPAT (12.5–300  $\mu$ g/kg, i.p.), on the firing rate of DR neurons of WT and  $GIRK2$  mutant mice. In all groups, 8-OH-DPAT caused a progressive and dose-dependent inhibition of the firing rate (Figure 2a-b). In  $GIRK2^{+/-}$  mice, the dose-response curve shifted to the right, so that the ED<sub>50</sub> mean value was significantly higher than that obtained in WT mice (ED<sub>50</sub>:  $42.15 \pm 2.89$   $\mu$ g/kg,  $n=8$ ; ED<sub>50</sub>:  $34.93 \pm 3.41$   $\mu$ g/kg,  $n=15$ , for  $GIRK2^{+/-}$  and WT mice, respectively.  $P < .05$ , nonlinear fit analysis, extra sum-of-squares F test). In  $GIRK2^{-/-}$  mice, the shift in the dose-response curve was even greater, and so the ED<sub>50</sub> mean value increased 2-fold compared with WT mice (ED<sub>50</sub>:  $69.50 \pm 8.57$   $\mu$ g/kg,  $n=5$ ,  $P < .01$ , nonlinear fit analysis, extra sum-of-squares F test) (Figure 2c). Subsequent administration of the 5-HT<sub>1A</sub> receptor antagonist, WAY100635 (1–1.5 mg/kg, i.p.), completely recovered the firing activity in the 3 groups (WT:  $86.22 \pm 7.04\%$ ,  $n=13$ ;  $GIRK2^{+/-}$ :  $92.54 \pm 12.98\%$ ,  $n=6$ ;  $GIRK2^{-/-}$ :  $122.20 \pm 23.27\%$ ,  $n=3$ ). No significant differences were found among groups.

Next, the sensitivity of the 5-HT<sub>1A</sub> receptor to endogenous 5-HT was tested by using the selective serotonin reuptake inhibitor citalopram (0.5–3 mg/kg, i.p.), which increases 5-HT levels in the synaptic cleft and indirectly activates these receptors (Pineyro and Blier, 1999). For this purpose, citalopram dose-response curves were performed in WT and  $GIRK2$  mutant mice. In all the groups, cumulative doses of citalopram caused a progressive and dose-dependent inhibition of the firing rate. However, citalopram showed less potency inhibiting DR neurons in  $GIRK2^{-/-}$  mice, so that the ED<sub>50</sub> mean value for this group was significantly greater than the values obtained for  $GIRK2^{+/-}$  and WT mice (ED<sub>50</sub>:  $1.36 \pm 0.04$  mg/kg,  $n=6$ ; ED<sub>50</sub>:  $1.04 \pm 0.04$  mg/kg,  $n=7$ ; ED<sub>50</sub>:  $0.97 \pm 0.03$  mg/kg,  $n=13$ , for  $GIRK2^{-/-}$ ,  $GIRK2^{+/-}$ , and WT mice, respectively.  $P < .0001$ , nonlinear fit analysis, extra sum-of-squares F test) (Figure 2d).

### Effect of Pharmacological Blockade of $GIRK$ Channels on the Citalopram-Induced Inhibition of DR Neuronal Activity

To further investigate the 5-HT<sub>1A</sub>- $GIRK$  signaling pathway, we performed a pharmacological blocking of  $GIRK$  channels with tertiapin-Q, and the sensitivity of the 5-HT<sub>1A</sub> receptor to the effect of citalopram was evaluated in WT TPN-Q mice and a WT control group (ACSF-injected group, WT control). In both groups,

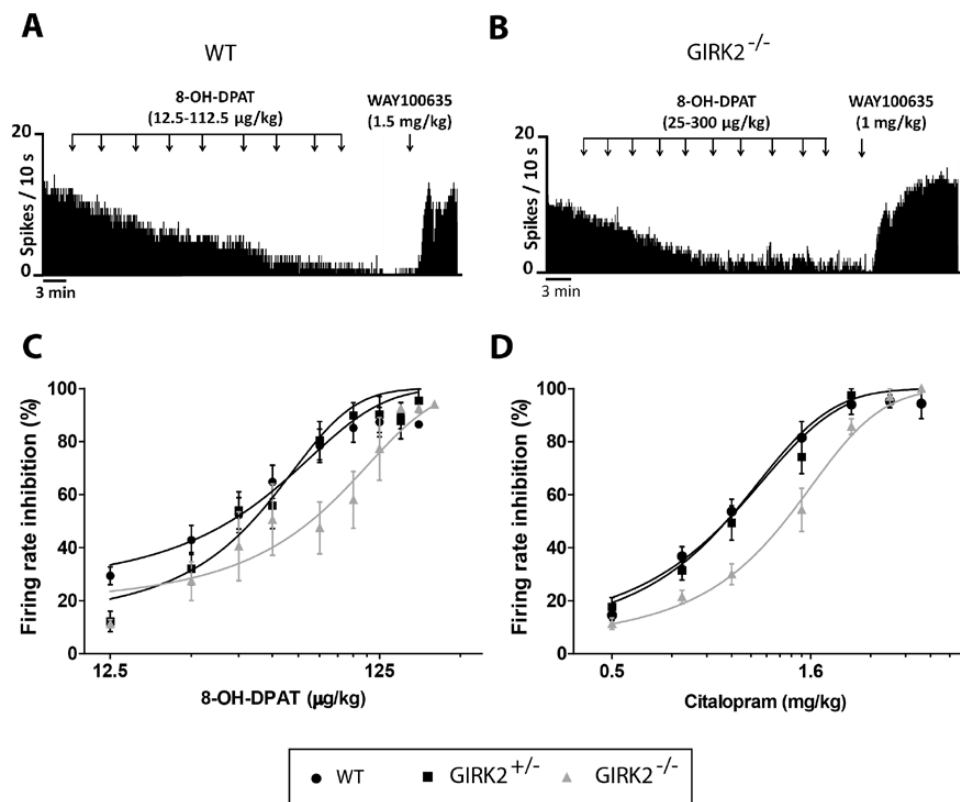
**Table 1.** In Vivo Electrophysiological Properties of Dorsal Raphe (DR) Neurons Recorded under Basal Conditions in Wild-Type (WT) and G-Protein Inwardly Rectifying Potassium ( $GIRK2$ ) Mutant Mice

	WT (n=86)	$GIRK2^{+/-}$ (n=79)	$GIRK2^{-/-}$ (n=45)	WT TPN-Q (n=15)
Firing rate (Hz)	$1.68 \pm 0.08$	$1.88 \pm 0.09$	$1.99 \pm 0.15^*$	$2.33 \pm 0.27^{**}$
Coefficient of variation (%)	$37.64 \pm 1.21$	$36.57 \pm 1.21$	$35.21 \pm 1.54$	$40.38 \pm 3.25$
Burst firing neurons (%)	7	5	2	13
- Firing rate (Hz)	$2.22 \pm 0.36$	$2.01 \pm 0.37$	4.03	$1.49 \pm 0.38$
- Number of bursts	$1.33 \pm 0.21$	$4.33 \pm 1.85$	1.00	$2.5 \pm 1.50$
- Spikes in burst (%)	$1.15 \pm 0.46$	$1.01 \pm 0.87$	0.41	$2.8 \pm 1.32$
- Mean spikes per burst	$3.00 \pm 0.81$	2.00	2.00	$3.50 \pm 0.50$

Abbreviations:  $GIRK2^{+/-}$ ,  $GIRK2$  heterozygous mice;  $GIRK2^{-/-}$ ,  $GIRK2$  homozygous mice; WT, wild type; WT TPN-Q, tertiapin-Q-injected wild-type mice.

\* $P < .05$  and \*\* $P < .01$  vs WT. Unpaired 2-tailed t-test.

Each cell was recorded for at least 3 minutes (180 seconds were taken for subsequent analysis). All data are presented as the mean  $\pm$  SEM of n experiments.



**Figure 2.** Inhibitory effect of the 5-HT<sub>1A</sub> receptor agonist 8-OH-DPAT and citalopram on the firing rate of dorsal raphe (DR) neurons in wild-type (WT) and G-protein inwardly rectifying potassium (GIRK2) mutant mice. (A-B) Representative firing rate histograms illustrate the inhibitory effect of 8-OH-DPAT (12.5–300 µg/kg, i.p.) on DR basal activity in WT (A) and GIRK2<sup>-/-</sup> mice (B). Subsequent administration of the 5-HT<sub>1A</sub> antagonist, WAY100635 (1–1.5 mg/kg, i.p.), completely reversed the 8-OH-DPAT-induced inhibitory effect. (C-D) Dose-response curves for 8-OHDPAT (C) and citalopram (0.5–3 mg/kg, i.p.) (D) on DR firing rate in WT, GIRK2<sup>+/-</sup>, and GIRK2<sup>-/-</sup> mice. Each point represents the mean ± SEM of *n* experiments (*n* = 5–15 mice/group).

citalopram (0.5–3 mg/kg, i.p.) caused a progressive and dose-dependent inhibition of the firing rate (Figure 3a-b). However, it showed less potency inhibiting DR neurons in WT TPN-Q, so that the ED<sub>50</sub> mean value for this group was significantly greater than the value obtained for the WT control (ED<sub>50</sub>: 1.25 ± 0.04 mg/kg, *n* = 5; ED<sub>50</sub>: 0.99 ± 0.06 mg/kg, *n* = 4, for WT TPN-Q and the WT control, respectively. *P* < .0001, nonlinear fit analysis, extra sum-of-squares F test) (Figure 3c). ED<sub>50</sub> mean values were similar between GIRK2<sup>-/-</sup> mice and WT TPN-Q, indicating that citalopram had the same potency in both groups (ED<sub>50</sub>: 1.25 ± 0.04 mg/kg, *n* = 5; ED<sub>50</sub>: 1.36 ± 0.04 mg/kg, *n* = 6, for WT TPN-Q and GIRK2<sup>-/-</sup> mice, respectively). As expected, the WT control group showed similar ED<sub>50</sub> values to the WT mice.

### Characterization of 8-OH-DPAT-Induced Hypothermia in GIRK2 Mutant Mice

To further study the functional status of 5-HT<sub>1A</sub> receptors in GIRK2 mutant mice, we evaluated the 8-OH-DPAT-induced hypothermic response. In all groups, 8-OH-DPAT (0.5 mg/kg, i.p.) caused a decrease in temperature (*n* = 6–7/group). GIRK2<sup>-/-</sup> mice showed a reduced 8-OH-DPAT-induced hypothermia in every different time point compared with WT mice. A 2-way ANOVA revealed that in minute 30, the temperature decrease induced by 8-OH-DPAT was significantly smaller in GIRK2<sup>-/-</sup> mice compared with WT mice ([*F*(2,68) = 10.30, *P* < .05]) (Figure 4). In addition, 3 other groups of GIRK2<sup>+/-</sup>, GIRK2<sup>-/-</sup>, and WT mice were injected with 0.9 % saline (saline groups, i.p.) to check if the manipulation of the animals or the intraperitoneal injection was causing

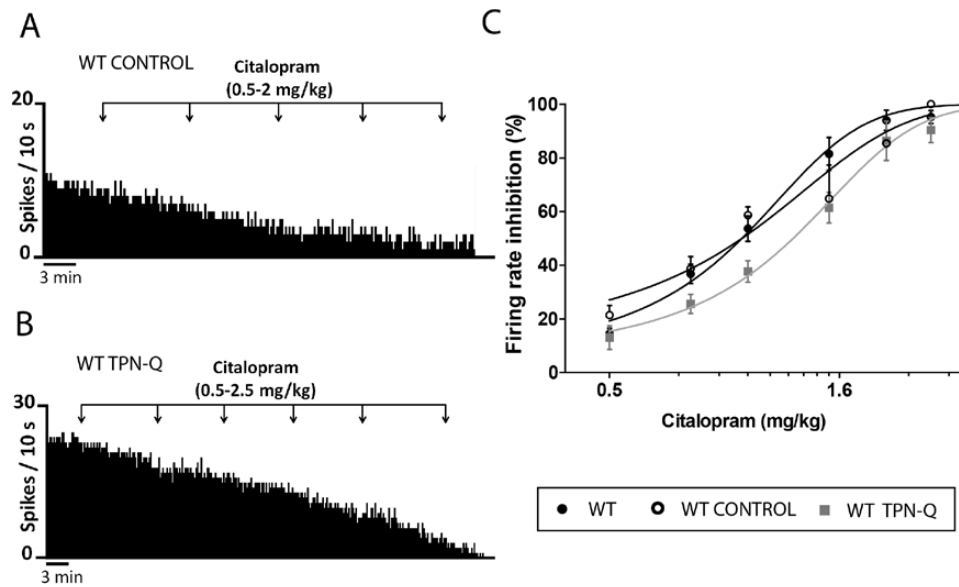
an effect on the response. No significant changes in temperature were observed in these groups.

### Basal Neurogenesis in GIRK2 Mutant Mice

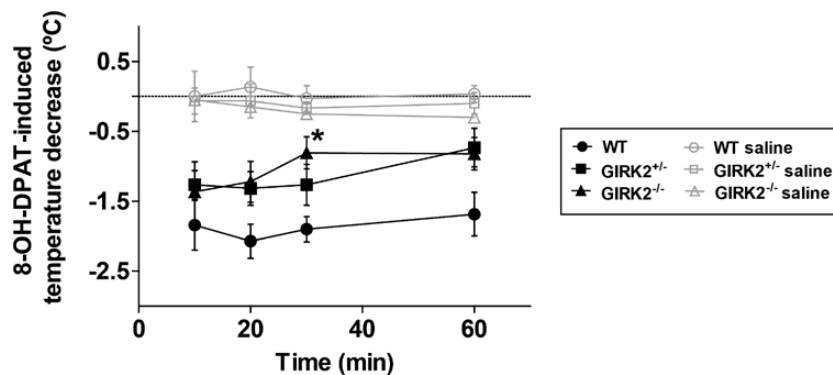
Finally, we investigated whether GIRK2 subunits are involved in basal adult neurogenesis measuring the area density (#cells/mm<sup>2</sup>, Sv) of new proliferating cells in the DG of the HPP and in the SVZ of WT and GIRK2 mutant mice (*n* = 6–10/group). In both regions of all groups, newly generated cells were observed, as indicated by HH3 immunoreactivity (HH3-IR) (Figure 5a-b, d-e). Quantitative analysis of the Sv of HH3-IR cells showed no differences among genotypes in either the DG (WT: 17.18 ± 1.89; GIRK2<sup>+/-</sup>: 16.69 ± 1.36; GIRK2<sup>-/-</sup>: 14.71 ± 1.51) (Figure 5c), or in the SVZ (WT: 152.43 ± 8.81; GIRK2<sup>+/-</sup>: 154.34 ± 6.10; GIRK2<sup>-/-</sup>: 139.99 ± 16.00) (Figure 5f). Although GFAP immunoreactivity was detected throughout both areas, <2% of HH3-IR cells expressed GFAP in the DG (WT: 1.54%; GIRK2<sup>+/-</sup>: 1.92%; GIRK2<sup>-/-</sup>: 1.61%) and the SVZ (WT: 0.48%; GIRK2<sup>+/-</sup>: 0.36%; GIRK2<sup>-/-</sup>: 0.50%), showing no statistical differences among genotypes. The low number of HH3-IR cells that colocalize with GFAP found in this study is consistent with other reports and suggests that the proliferating cells are of neural lineage (Rodriguez et al., 2008, 2009, 2011; Fiol-deRoque et al., 2013).

### Discussion

The goal of our study was to determine the role that GIRK2 subunit-containing GIRK channels play in depression-related behaviors as well as in the control of 5-HT<sub>1A</sub>-mediated inhibitory



**Figure 3.** Inhibitory effect of citalopram on the firing rate of dorsal raphe (DR) neurons in wild-type (WT) and tertiapin-Q injected mice. (A-B) Representative firing rate histograms illustrate the inhibitory effect of citalopram (0.5–2.5 mg/kg, i.p.) on DR basal activity in artificial cerebrospinal fluid (ACSF)-injected mice (WT control, i.c.v.) (A) and tertiapin-Q injected mice (WT TPN-Q, 100 pmol, i.c.v.) (B). (C) Dose-response curves for citalopram (0.5–3 mg/kg, i.p.) on DR firing rate in WT, WT control, and WT TPN-Q. Each point represents the mean  $\pm$  SEM of *n* experiments (*n* = 4–5 mice/group).

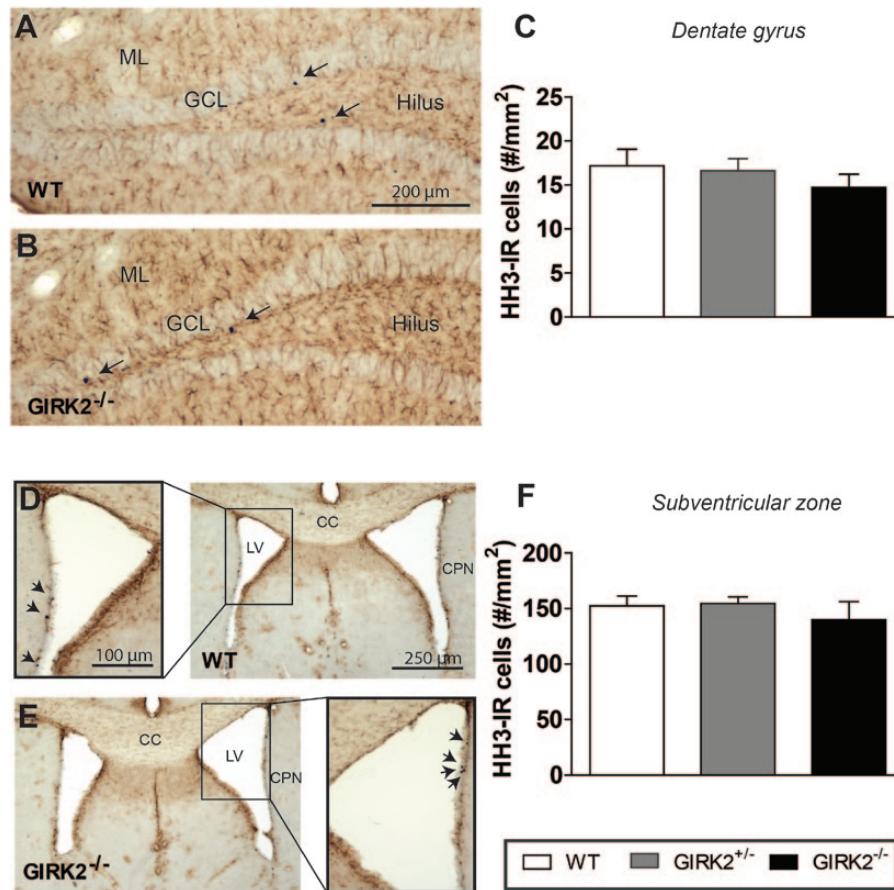


**Figure 4.** Hypothermic response induced by 8-OH-DPAT in wild-type (WT) and G-protein inwardly rectifying potassium (GIRK)2 mutant mice. The temperature decrease induced by 8-OH-DPAT (0.5 mg/kg, i.p.) was significantly lower in GIRK2<sup>-/-</sup> mice relative to WT mice in minute 30. Mice of each genotype injected with 0.9% saline (saline groups) were used as controls. Each point represents the mean  $\pm$  SEM of *n* animals (*n* = 6–7 mice/group; \**P* < .05 vs WT, 2-way ANOVA followed by Bonferroni test).

effects. We found that mice lacking GIRK2 subunits of GIRK channels display a depression-resistant phenotype combined with a reduced behavioral response to citalopram, an increase in the firing rate of DR neurons, and a reduction of 5-HT<sub>1A</sub> receptor-mediated responses. In addition, GIRK2 subunit deletion does not affect basal adult neurogenesis.

Our findings suggest that *Girk2* gene deletion promotes a depression-resistant behavior and determines the response to the antidepressant citalopram. Thus, GIRK2<sup>-/-</sup> mice showed a marked decrease in the immobility time in the TST, and both GIRK2<sup>+/+</sup> and GIRK2<sup>-/-</sup> mice presented a lower latency to eat in the NSFT that was not correlated with an increase in appetite. It is important to remark that general locomotor activity status is a confusing factor in these tests (Blednov et al., 2001). Here we observed that GIRK2<sup>-/-</sup> mice, but not GIRK2<sup>+/+</sup> mice, have signs of hyperactivity, as reported by other studies (Blednov et al., 2001; Pravetoni and Wickman, 2008; Arora et al., 2010). This has been attributed to D1 receptor activation (Blednov et al., 2002). Given that GIRK2<sup>+/+</sup> mice do not have hyperactivity but showed depression-resistant phenotype in the NSFT, these results might be attributable to a less depressive-like

behavior rather than to a more active state. We suggest that the 5-HT<sub>1A</sub>-GIRK signaling may be mediating this depression-resistant phenotype: first, studies in 5-HT<sub>1A</sub> receptor knockout mice show that they display an antidepressant-like phenotype under baseline conditions (Heisler et al., 1998; Mayorga et al., 2001), as we observed in GIRK2 mutant mice. Secondly, citalopram was less potent here in reducing the immobility in the TST. Similarly, it has been reported that the expression of the antidepressant-like behavioral response of selective serotonin reuptake inhibitors in the TST requires the presence of functional 5-HT<sub>1A</sub> receptors (Mayorga et al., 2001). Additionally, our observations are in line with those showing that chronic administration of fluoxetine exerts a beneficial influence on a rodent model of depression through suppression of GIRK-dependent signaling in the DR (Cornelisse et al., 2007). Interestingly, the depression-resistant phenotype that we observed here is combined with the previously reported reduced anxiety-like behavior of GIRK2 mutant mice (Blednov et al., 2001; Pravetoni and Wickman, 2008). Therefore, this phenotype represents a great advance over the classic depression-resistant phenotype of 5-HT<sub>1A</sub> knockout mice, since they also



**Figure 5.** Brightfield micrographs showing phosphorylated Histone H3 (HH3, a proliferating mitotic marker) labelled cells within the dentate gyrus (DG) and subventricular zone (SVZ) of wild-type (WT) and G-protein inwardly rectifying potassium (GIRK2) mutant mice. (A-B) Dual labeling of HH3 positive cells (arrows) and glial cells (glial fibrillary acidic protein [GFAP], brown) in the hippocampal DG of WT (A) and GIRK2<sup>-/-</sup> mice (B). (C) Bar graph showing no differences among groups in the area density of HH3 positive cells within the DG (all layers included). (D-E) Dual labeling of HH3 positive cells (arrows) and glial cells (GFAP, brown) in the SVZ of WT (D) and GIRK2<sup>-/-</sup> mice (E). (F) Bar graph showing no differences among groups in the area density of HH3 positive cells within the SVZ. Bars represent mean  $\pm$  SEM of *n* animals (*n* = 6–10 mice/group). CC, corpus callosum; CPN, caudate-putamen nucleus; LV, lateral ventricle; GCL, granular cell layer; ML, molecular layer.

display a robust anxiety-like behavior (Heisler et al., 1998; Parks et al., 1998; Ramboz et al., 1998). Given the constitutive and global nature of the *Girk2* gene deletion, it is not possible to completely identify the circuit(s) or neurotransmitter system(s) that explain this depression-resistant phenotype. It is conceivable that GABA<sub>B</sub> receptor-mediated transmission is also affected by the GIRK2 mutation, and this could account for some of the effects seen in this study. However, there are some key points in our work that make this assumption unlikely. First, DR neuronal activity is mainly regulated by 5-HT<sub>1A</sub> receptors (Sprouse and Aghajanian, 1987), and drugs used in the present work act directly or indirectly on 5-HT<sub>1A</sub> receptors. Despite the presence of GABA<sub>B</sub> receptors in DR neurons (Bischoff et al., 1999), there is no evidence of a 5-HT<sub>1A</sub>-GABA<sub>B</sub> receptor interaction in DR neurons. Therefore, the responses to 8-OH-DPAT and citalopram observed in this study do not involve the activation of the GABA<sub>B</sub> receptor-mediated transmission and have to be mediated by the 5-HT<sub>1A</sub>-GIRK2 signaling. Also, the combined anxiety- and depression-resistant phenotype of GIRK2 mutant mice is contrary to the phenotype of GABA<sub>B</sub> mutant mice, which show an anxiety-like behavior and behave similarly to control mice in the TST (Mombereau et al., 2004). Overall, although GABA<sub>B</sub>-GIRK signaling is probably impacted by the mutation, the direct mediation by GABA<sub>B</sub> receptors of the effects seen in this study is unlikely.

The implication of GIRK2 subunit-containing GIRK channels in the 5-HT<sub>1A</sub> receptor-mediated neurotransmission is also supported by our *in vivo* electrophysiological findings. First, we observed that the lack of GIRK2 subunits induces an increase in the firing rate of DR neurons. This increase was also observed by the total blocking of GIRK channels with tertiapin-Q. On one hand, these results suggest that the intrinsic activity of GIRK2 subunit-containing GIRK channels controls the transmission in the DR. Similar findings have been obtained in LC neurons, where it has been observed that GIRK2 subunit-containing GIRK channels regulate the tonic activity of LC neurons *in vivo* (Torrecilla et al., 2013). *In vitro*, LC neurons of GIRK2/3<sup>-/-</sup> mice show an increased firing rate (Cruz et al., 2008). In addition, the contribution of GIRK2 subunits to the resting membrane potential has been reported in different type of neurons, including LC and HPP neurons (Luscher et al., 1997; Torrecilla et al., 2002; Chen and Johnston, 2005; Koyrakh et al., 2005). Nevertheless, in layer 5/6 pyramidal neurons of the prefrontal cortex of GIRK2<sup>-/-</sup> mice, intrinsic electrophysiological properties remained unaltered compared with WT mice (Hearing et al., 2013). Taken together, the contribution of GIRK2 subunits to neuronal excitability seems to vary across cell types and brain regions. On the other hand, the complete blockade of GIRK channels with tertiapin-Q results in a similar increase in the firing rate of DR neurons to that observed in GIRK2<sup>-/-</sup> mice. This suggests that GIRK2



subunits, though their expression is low in the DR (Saenz del Burgo et al., 2008), are forming important populations of GIRK channels that control the neuronal tonic activity. In the DR, the most abundant neuronal populations, which are the GABAergic and 5-HT neurons, express GABA<sub>B</sub> and 5-HT<sub>1A</sub> receptors coupled to GIRK channels (Bowery, 1987; Innis and Aghajanian, 1987; Waldmeier et al., 1988; Beck et al., 2004; Day et al., 2004). In fact, it is widely accepted that in 5-HT neurons, GABA<sub>B</sub> and 5-HT<sub>1A</sub> receptors are coupled to a common pool of G proteins (Innis and Aghajanian, 1987; Williams et al., 1988). However, the specific distribution of GIRK2 subunit-containing GIRK channels in these cells remains unknown. Therefore, further studies are needed to determine the subunit composition of GIRK channels coupled to each particular receptor in specific cell types and their role in the physiology of these cells. Alterations in neurotransmitter levels could also affect the firing pattern of DR neurons in GIRK2 mutant mice, since there are decreased and increased levels of 5-HT and 5-HIAA, respectively (Torrecilla et al., 2013).

Second, our study also reveals that DR 5-HT<sub>1A</sub> receptors, both endogenously and exogenously activated, are affected by the deletion of GIRK2 subunits, which is shown by reduced receptor functionality. Similarly,  $\mu$  and  $\alpha_2$  receptors of LC neurons in GIRK2 mutant mice were desensitized in vivo (Torrecilla et al., 2013). Our results agree with this study, indicating that in the DR, ablation of the *Girk2* gene produces loss of function of the 5-HT<sub>1A</sub>-GIRK signaling pathway. It is important to remark that the reduced function of 5-HT<sub>1A</sub> autoreceptors has been proposed as a key consequence of chronic antidepressant treatments, which would explain the delayed onset effect of antidepressants (Artigas et al., 1996; Blier et al., 1998). In fact, new pharmacological or genetic strategies to faster desensitize 5-HT<sub>1A</sub> and  $\alpha_2$  autoreceptors have been of great interest in the study of depression treatment (Sanacora et al., 2004; Richardson-Jones et al., 2010; Portella et al., 2011). The observation that 8-OH-DPAT and citalopram preserved their maximal inhibitory efficacy is consistent with other studies. In LC neurons in GIRK2 mutant mice, the potency of morphine and clonidine was reduced, yet the inhibitory efficacy remained unaltered in vivo (Torrecilla et al., 2013). In hippocampal neurons in GIRK2<sup>-/-</sup> mice, in vitro postsynaptic GIRK currents induced by stimulation of the 5-HT<sub>1A</sub> receptor were markedly reduced but not absent (Luscher et al., 1997). Studies conducted to determine the analgesic properties of morphine showed that while the potency of morphine was reduced, its efficacy was preserved in GIRK2<sup>-/-</sup> and GIRK2/3<sup>-/-</sup> mice (Mitrovic et al., 2003; Cruz et al., 2008). Also, the pharmacological blockade of GIRK channels caused a reduction in the potency but not in the inhibitory efficacy of citalopram, supporting the primary role of GIRK2 subunit-containing GIRK channels in the inhibitory response of 5-HT<sub>1A</sub> receptors. The tertiapin-Q dose used was selected according to a previous study, where doses >100 pmol caused dangerous effects (Marker et al., 2004). By testing the 8-OH-DPAT-induced hypothermia, which reflects the sensitivity of 5-HT<sub>1A</sub> autoreceptors in mice (Goodwin et al., 1985; Richardson-Jones et al., 2011), we confirmed that GIRK2 subunits regulate the functionality of 5-HT<sub>1A</sub> autoreceptors, as previously reported (Costa et al., 2005).

Our findings show that in basal conditions, *Girk2* gene ablation promotes a depression-resistant phenotype without modifying adult neurogenesis in the DG of the HPP or in the SVZ. In line with this, it has also been observed that deletion of the background potassium channel TREK-1 does not alter the adult neurogenesis but results in a depression-resistant phenotype (Heurteaux et al., 2006). Although the role of neurogenesis in the behavioral effects induced by antidepressant drugs

remains controversial under certain conditions (see Hanson et al., 2011), compelling work suggests that the increment of hippocampal neurogenesis induced by chronic treatment with fluoxetine reverses some behavioral dysfunctions in animal models of anxiety/depression and control animals (Malberg et al., 2000; Santarelli et al., 2003; Airan et al., 2007; David et al., 2007, 2009; Surget et al., 2008; Wang et al., 2008). Furthermore, specific neurogenic and behavioral effects of fluoxetine require the activation of 5-HT<sub>1A</sub> receptors (Santarelli et al., 2003). Based on the wide literature on the primary role of GIRK2 subunits in generating GIRK currents by the activation G protein coupled receptors throughout the brain, including the CA1 layer of the HPP (reviewed in Luscher and Slesinger, 2010), and the fact that 5-HT<sub>1A</sub> receptors in the DR are desensitized, we hypothesize that genetic ablation of GIRK2 subunits could compromise the activity of 5-HT<sub>1A</sub> receptors also in the DG and therefore the neurogenic effects of fluoxetine. Nevertheless, it could be feasible that the deletion of the GIRK2 subunit strongly determines this behavioral phenotype, and therefore it would limit the importance of neurogenesis mediating the behavioral responses of chronic fluoxetine treatment. In line with this, it has been reported that the complex behavioral phenotype that 5-HT<sub>1A</sub> knockout mice show is developmentally determined and neurogenesis independent (Santarelli et al., 2003).

In conclusion, our results show the specific role of GIRK2 subunit-containing GIRK channels in the promotion of a depression-resistant phenotype as well as their control of the tonic neuronal activity and mediation of the 5-HT<sub>1A</sub> receptor inhibitory responses. New strategies targeting the 5-HT<sub>1A</sub>-GIRK2 pathway could be of great therapeutic interest for the study of pathologies related to an altered 5-HT transmission, such as depression and development of alternative treatments.

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## Statement of Interest

None.

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