

EFFECT OF GppNHp ON GIRK CURRENTS IN DORSAL RAPHE NUCLEUS NEURONS FROM 5HTT^{-/-} MICE

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Abstract. The selective serotonin reuptake inhibitors (SSRIs) determine a reduced efficacy of serotonin (5-HT) in inhibiting serotonergic neurons of the dorsal raphe nucleus (DRN) because they trigger a functional desensitization of these receptors, either by 5-HT_{1A} autoreceptors internalization or by the uncoupling between 5-HT_{1A} receptors and their G-proteins. In our study, we have used knock-out mice devoid of the serotonin transporter (5-HTT^{-/-}) that is considered a good animal model for depression. Using patch-clamp whole-cell recordings, we found that the effect of 5-HT_{1A} agonists can be mimicked by 5'-guanylyl-imido-diphosphate (GppNHp; 400 μM). The 5-HTT^{-/-} knock-out mutation reduced the current density induced by GppNHp in females but not in males. These data may suggest that the decreased response of 5-HT_{1A} receptors to agonists in 5-HTT^{-/-} mutants reflects notably alteration in the coupling between G-proteins and GIRK channels in females but not in males.

Key words: 5-HT_{1A}, GppNHp, dorsal raphe nucleus, 5-HTT mutation.

INTRODUCTION

Many studies suggest that the central serotonergic system is involved in the regulation of mood, notably in depressed patients [23]. Furthermore, the serotonin transporter (5-HTT) is the target of the most widely used antidepressants, i.e. the selective serotonin reuptake inhibitors (SSRIs) [9]. A 5-HT transporter knockout (5-HTT^{-/-}) mouse is considered as a model of whole-life treatment with selective serotonin reuptake inhibitors (SSRIs) [3].

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Serotonin (5-HT) exerts a negative feed-back control on the activity of 5-HT synthesizing neurons through the stimulation of 5-HT_{1A} autoreceptors (Fig. 1). This effect is caused by 5-HT_{1A}-evoked hyperpolarization, leading to inhibition of 5-HT neurons firing. This hyperpolarization involves G_i proteins whose activation induces an increase in G protein-dependent inward rectifying potassium (GIRK) current [1] and a decrease in calcium conductance. The selective serotonin reuptake inhibitors (SSRIs) determine a reduced efficacy of 5-HT in inhibiting serotonergic neurons of the dorsal raphe nucleus (DRN) because they trigger a functional desensitization of these receptors, either by 5-HT_{1A} autoreceptors internalization [17], or by the uncoupling between 5-HT_{1A} receptors and their G-proteins [16]. Disruption of the 5-HTT (serotonin transporter) gene induces an increase in the 5-HT extracellular concentration because of lack of 5-HT reuptake. 5-HT_{1A} autoreceptor tonic activation, which results from the 5-HT extracellular rate increase, causes a functional desensitization of these autoreceptors, as observed after chronic treatment with SSRIs [15].

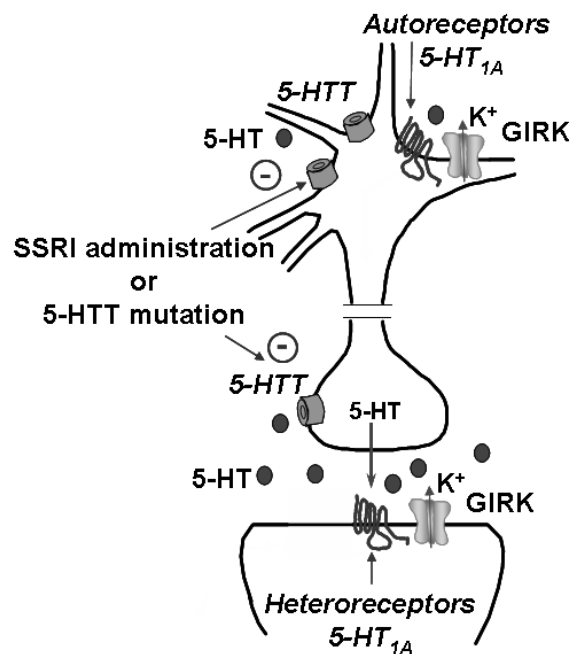


Fig. 1. Effect of SSRI administration or 5-HTT mutation on the signaling pathways of 5-HT_{1A} receptors.

In order to test the effect of 5HTT mutation on coupling between the G_i-proteins and the inwardly rectifying currents (GIRK), GTP was replaced by the non-hydrolyzable GTP analogue, 5'-guanylyl-imido-diphosphate (GppNHp).

Using the whole cell patch-clamp technique on brainstem slices, we investigated the physiological properties of somatodendritic 5-HT_{1A} autoreceptors, in different neuron subtypes from wild-type (5-HTT^{+/+}) and knockout mice deficient in 5-HTT (5-HTT^{-/-}) in the presence of GppNHp.

MATERIALS AND METHODS

CHEMICALS

NaCl, KCl, NaH₂PO₄, MgCl₂·6H₂O, CaCl₂·2H₂O, NaHCO₃, D-glucose, sucrose, 5-carboxamidotryptamine [5-CT], (±)-2-dipropylamino-8-hydroxy-1,2,3,4-tetrahydronaphthalene hydrobromide [8-OH-DPAT] and 5'-guanylylimido-diphosphate [GppNHp] were purchased from Sigma-Aldrich USA. GppNHp was used into the pipette and the 5-HT_{1A} agonists were added into the bath solution.

ANIMALS

Experiments were performed using females and males of wild-type 5-HTT^{+/+} and homozygous 5-HTT^{-/-} genotype with C57Bl/6 genetic background obtained from heterozygous and homozygous breeding. Genotyping was performed as described by Bengel *et al.* [3]. After weaning and sexing, males and females were housed in groups of 6 animals per cage and maintained under standard laboratory conditions (22 ± 1°C, 60% relative humidity, 12–12 hours light-dark cycle, food and water *ad libitum*). Animals were used when 2 months old. The endogenous estrogen fluctuation in females was taken into account, the samples being collected at all periods of the oestrus and the data pooled together. Procedures involving animals and their care conformed to institutional guidelines and complied with national and international laws and policies.

PREPARATION OF BRAINSTEM SLICES AND NEURONAL RECORDING

Mice were decapitated and the brains were rapidly removed and immersed in an ice-cold (≈4 °C) artificial cerebrospinal fluid (ACSF) of the following composition (in mM): NaCl 128, KCl 3.5, NaH₂PO₄ 1.2, MgCl₂ 1.3, CaCl₂ 2, NaHCO₃ 25, D-glucose 11, sucrose 17, maintained at pH 7.4 by continuous bubbling with an O₂/CO₂ mixture (95%/5%). D-glucose, sucrose were freshly added before recording.

A block of tissue containing the DRN was affixed to the stage of a vibratome (TPI, series 1000) with cyanoacrylate glue, submerged in ice-cold and bubbled ACSF and cut into coronal sections of 250 μm thickness. Brainstem slices were

collected in gassed ACSF (continuous bubbling with 95% O₂ / 5% CO₂ mixture, pH 7.4) and maintained at room temperature (22–25 °C). A slice was then transferred into a small recording chamber (2 mL) and covered with a nylon mesh, while continuously perfused with gassed ACSF (22–25 °C) at a constant flow rate of 3 mL/min. Recording electrodes (4–6 MΩ) were pulled from borosilicate glass tubes (Phymep, France) on a PP-830 micropipette puller (Narishige, Japan), filled with internal solution containing (in mM): KH₂PO₄ 108, CaCl₂ 1, MgCl₂ 1, EGTA 11, Hepes 10, Mg-ATP 4, Na-GTP 0.4, adjusted to pH 7.4 with 36 mM KOH (final K⁺ concentration 144 mM), and kept in ice until use. Electrodes were advanced towards DRN neurons under visual guidance, using an upright microscope (Nikon E600FN, Japan). Pipette voltage offset was neutralized prior to the formation of a gigaohm seal and was not further corrected. Patch-clamp recordings were performed in whole-cell configuration and signals were amplified with an Axopatch 200B amplifier (Axon Instruments, Union City, CA, USA) using fast current-clamp and voltage-clamp modes. Signals were low-pass filtered at 2 kHz, digitized at 15 kHz and analyzed with a computer using a Digidata 1200 interface. Data were stored and analysed by the pCLAMP 9.2 software (Axon Instruments, Union City, CA, USA). Input resistance (R_m), series resistance (R_s) and membrane capacitance (C_m) were determined from current transients elicited by a 5 mV depolarizing step from a holding potential of –60 mV. Criteria for cell inclusion [1] in the study were as follows: $R_m > 100$ MΩ and stable R_s (≤ 20 MΩ), C_m (30–55 pF) and spike amplitude (≥ 55 mV). It was also measured the time constant of the membrane voltage response to a 45–50 pA hyperpolarizing current pulse (hyperpolarization voltage time constant – HV time constant). The neurons were considered healthy when maintaining the same cellular characteristics, except drug-induced modifications. Liquid junction potential errors [20] were calculated using pCLAMP 9.2 at a value of –13.9 mV and the results were corrected considering these errors. In current clamp mode, spike discharge frequency (f : Hz) was regularly monitored and determined for at least 2 min at various epochs of recordings. Starting from a holding potential of –60 mV and using voltage-clamp mode, voltage steps from –30 mV to –125 mV, with 5 mV increment and 1.2 s duration, were applied in order to hyperpolarize the neuronal membrane, which in turn activates the G-protein-dependent inwardly rectifying potassium current. Steady-state currents were plotted against voltage (I/V curves). In order to compare and quantify data between cells, current density (d : pA/pF) was computed using the steady-state current induced by voltage steps down to –125 mV.

During the first 5 min of recording (whole cell configuration) the initial f and d values (baseline values: f_0 and d_0) were measured. After 20 – 30 min of GppNHp diffusion from the pipette into the cell the f and d values (f_{GppNHp} and d_{GppNHp}) were again measured. The same protocol was run in the presence of 5-HT_{1A} agonists (5-CT and 8-OH-DPAT) resulting the f and d values: f_{agonist} and d_{agonist} . The variations of f ($\Delta f_{\text{agonist}} = f_{\text{agonist}} - f_0$ or $\Delta f_{\text{GppNHp}} = f_{\text{GppNHp}} - f_0$, Hz, respectively) and d ($\Delta d_{\text{agonist}} =$

$d_{\text{agonist}} - d_0$ or $\Delta d_{\text{GppNHp}} = d_{\text{GppNHp}} - d_0$, pA/pF, respectively) were calculated. Thresholds for ‘GppNHp-induced inhibition’ were considered when ratios after ~15–30 min GppNHp diffusion into cells (a reasonable amount of time to observe GppNHp effects, [10]) to baseline values were as follows: $f_{\text{GppNHp}}/f_0 \leq 0.33$ and $d_{\text{GppNHp}}/d_0 \geq 1.5$.

STATISTICAL ANALYSIS

Descriptive statistics displayed frequencies for qualitative data and means and standard errors, for quantitative ones. The relationships between qualitative variables were tested using logistic regression analyses when comparing a binary criterion and several potential factors, and Fisher’s exact tests [8] when comparing two variables. With samples of small size and, consequently, normality hypotheses difficult to assess, we adopted a non parametric approach. Therefore, the relationships between quantitative variables and qualitative ones were tested using Kruskal-Wallis tests for three or four-valued qualitative variables and Wilcoxon two-sample tests for binary ones (comparisons of two groups). No correction for multiple comparisons was performed. All the tests were two-tailed, and p -values lower than 0.05 were considered as statistically significant. Computations were performed using the SAS V8 statistical software.

RESULTS

GppNHp-INDUCED INHIBITION OF DRN NEURONS IN FEMALES AND MALES OF WILD-TYPE AND 5-HTT^{-/-} GENOTYPE

Inhibition similar to that induced by 5-HT_{1A} agonist superfusion was observed, within 15 min after the start of whole cell recordings in the presence of GppNHp, in a certain proportion of neurons from 5-HTT^{+/+} (5/25 in females and 11/19 in males, Fig. 2) and 5-HTT^{-/-} (8/18 in females and 7/14 in males, Fig. 3) mice. As in the case of 5-HT_{1A} agonists, the proportions of GppNHp-inhibited neurons were not significantly different between females and males and between wild-type and mutant mice (logistic regression analysis, see Table 1). Details of the observed effects are presented in the following sections.

GppNHp-SENSITIVE CELLS

GppNHp produced a strong inhibition, with hyperpolarization and dramatic decrease or arrest of spontaneous firing (Figs. 2A, 3A), using 3.5 mM of external K⁺ and current clamp mode. Furthermore, a basal outward potassium current appeared. The inward current was elicited by hyperpolarizing voltage steps and increased in amplitude (Figs. 2B, 3B) in voltage clamp mode.

In all cells sensitive to GppNHp diffusion, current density increased irreversibly as a function of time to reach a maximum (Figs. 2C, 3C). However, this was blocked by barium (2 mM), a GIRK channel blocker, both in mice of 5-HTT^{+/+} (Fig. 2) and 5-HTT^{-/-} (Fig. 3) genotypes.

Table 1

Proportions of inhibited neurons in the DRN for females and males of wild-type and 5-HTT^{-/-} genotype during GppNHp diffusion or 5-HT_{1A} agonist superfusion. GppNHp was applied at 400 μ M. 5-CT and 8-OH-DPAT were applied at 50 nM – 3 μ M and 300 nM – 30 μ M, respectively. The highest concentrations were used to ascertain the absence of neuronal sensitivity. Numbers are indicated versus the total number of recorded cells in each group of mice. *: $p < 0.001$, different from 5-HT_{1A} agonist-sensitive cells in wild-type females

	5-HTT ^{+/+}		5-HTT ^{-/-}	
	♀	♂	♀	♂
GppNHp	5/25	11/19	8/18	7/14
%	20%*	58%	44%	50%
5-CT	6/9	3/7	3/6	5/8
8-OH-DPAT	7/7	1/4	7/9	7/13
Total	13/16	4/11	10/15	12/21
%	81%	36%	66%	57%

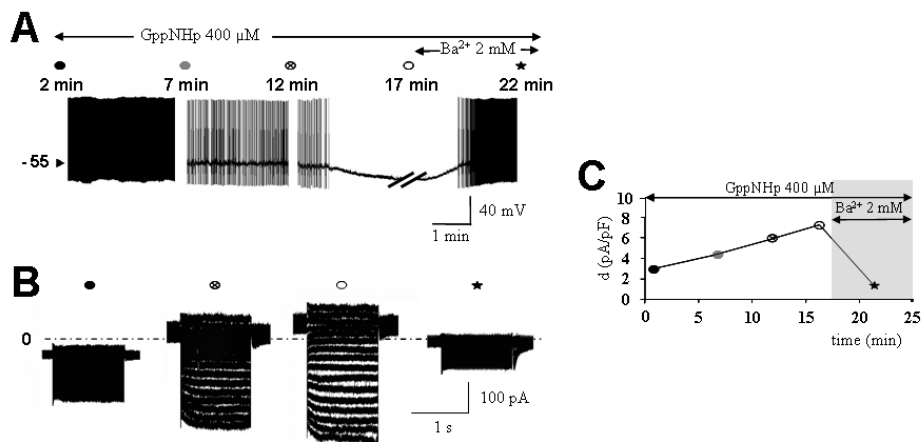


Fig. 2. GppNHp effect on GIRK current in a DRN neuron from a 5-HTT^{+/+} mice. A. GppNHp (400 μ M) induces a progressive inhibition with hyperpolarization and cessation of spike firing in current clamp mode. Barium (2 mM) reverses GppNHp-induced changes in membrane potential and firing of the cell. B. GppNHp (400 μ M) increases progressively the amplitude of the current evoked by 5 mV increment voltage steps between potentials from -45 mV to -145 mV, from a holding potential of -60 mV, as shown at the beginning (\bullet) and after 12 min (\otimes) and 17 min (\circ) of the recording shown in A. Barium (\blacksquare , 2 mM) suppresses the GppNHp effect. Current traces at each voltage step are an average of 3 sweeps. C. Current density (bottom) as a function of time for the recordings shown in A and B, respectively.

Barium could depolarize the cells with recovery of spike discharge, in current clamp recordings, and decrease of GppNHp-induced current, in voltage clamp recordings, ruling out run-up artifacts during GppNHp diffusion into cells. Interestingly, barium also could block a component of the control current (Fig. 2B). On the contrary, ZD 7288 (0.1 mM), an organic I_h blocker [4], had no effect on the GppNHp-induced inhibition ($n = 3$, data not shown). Therefore, this barium sensitivity suggests that the GppNHp difference current observed in DRN neurons is compatible with activation of a G-protein-gated inwardly rectifying current [24].

In similar recordings for one hour duration, but with GTP instead of GppNHp in the pipette, action potential discharge and current density remained stable as did resting membrane potential, membrane resistance and action potential amplitude ($n = 5$, data not shown).

GppNHp-INDUCED CHANGES OF SPIKE DISCHARGE FREQUENCY AND CURRENT DENSITY WITH GENDER AND GENOTYPE

Six parameters (f_0 , f_{GppNHp} , Δf_{GppNHp} , d_0 , d_{GppNHp} and Δd_{GppNHp}) have been compared between sex by genotype and between genotype by sex using Wilcoxon two-sample tests. Results are displayed in Table 2. f_0 and d_0 were not significantly different between all groups of mice (~ 10 Hz and ~ 5 pA/pF in females and males of wild-type and 5-HTT^{-/-} genotype, respectively) but f_{GppNHp} was significantly larger in 5-HTT^{-/-} females than in wild-type counterparts ($p = 0.017$), while there were no significant differences between 5-HTT^{-/-} and wild-type males. In addition, d_{GppNHp} and Δd_{GppNHp} (pA/pF) were significantly smaller in 5-HTT^{-/-} females than in wild-type counterparts ($p = 0.040$ and $p = 0.019$, respectively). In contrast, these variables were not significantly different in males between 5-HTT^{-/-} and wild-type genotypes. Thus, GppNHp effects decreased in females and did not change in males with the 5-HTT^{-/-} mutation. Further comparison between females and males indicates that f_{GppNHp} and Δf_{GppNHp} (Hz) are significantly different in mutant mice, with f_{GppNHp} larger in females than in males ($p = 0.020$) and Δf_{GppNHp} smaller in females than in males ($p = 0.011$).

COMPARISON BETWEEN THE PROPORTIONS OF GppNHp- AND 5-HT_{1A} AGONIST-SENSITIVE CELLS

In a logistic regression analysis including three factors (drug type: GppNHp or 5-HT_{1A} agonists, with the proportions of 5-CT- and 8-OH-DPAT-sensitive cells pooled together; gender; and genotype), the only significant factor was the type of drug ($p = 0.013$). When considering interactions, the interaction between drug and gender was almost significant ($p = 0.051$). Hence in wild-type females, the percentage of GppNHp-sensitive cells was significantly smaller than that of 5-HT_{1A} agonist-sensitive cells ($p = 0.0002$, see Table 2). On the contrary, no significant differences between GppNHp- and 5-HT_{1A} agonist-sensitive cells were observed in other groups (mutant females and wild-type and mutant males).

Table 2

Spike discharge frequency and current density in GppNHp-inhibited neurons in females and males of wild-type and 5-HTT^{-/-} genotype. For each column, significant differences between pairs, with the Wilcoxon two-sample tests, are highlighted by bold print

		f_0	f_{GppNHp}	Δf_{GppNHp}	d_0	d_{GppNHp}	Δd_{GppNHp}
♀	5-HTT ^{+/+} <i>n</i> = 5	9.4 ± 1.3	0 ± 0	-9.4 ± 1.3	6.7 ± 0.7	11.6 ± 1.5	5.0 ± 0.1
	5-HTT ^{-/-} <i>n</i> = 8	9.4 ± 1.2	2.1 ± 0.2 ^{a, b}	-7.3 ± 1.4 ^b	4.5 ± 0.5	6.9 ± 0.9 ^a	2.4 ± 0.2 ^a
♂	5-HTT ^{+/+} <i>n</i> = 11	9.6 ± 0.3	0.80 ± 0.01	-8.8 ± 1.4	4.0 ± 0.3	8.3 ± 0.7	4.3 ± 0.9
	5-HTT ^{-/-} <i>n</i> = 7	12.9 ± 0.9	0.10 ± 0.01	-12.7 ± 1.1	4.1 ± 0.1	7.5 ± 0.6	3.4 ± 0.6

^a $p < 0.05$, different from wild-type mice of the same gender. ^b $p < 0.05$, different from male mice of the same genotype

COMPARISON BETWEEN THE EFFECTS OF GppNHp AND 5-CT ON GIRK CURRENT

The mean values of current density variation for 5-CT (2–3 μM , $\Delta d_{5\text{-CT}} = d_{5\text{-CT}} - d_0$ pA/pF) and GppNHp were similar for each group of mice (females and males of wild-type and 5-HTT^{-/-} genotype).

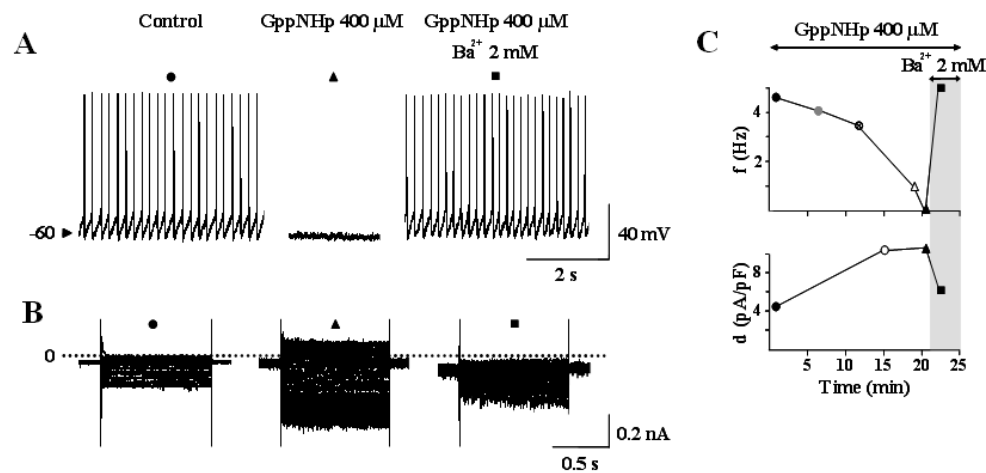


Fig. 3. GppNHp effect on the GIRK current in a DRN neuron from a 5-HTT^{-/-} mice. A. GppNHp (400 μM) produces hyperpolarization and cessation of spike firing after 20 min (\blacktriangle). Barium (2 mM, \blacksquare) reverses this effect. B. GppNHp increases the amplitude of the current evoked by hyperpolarization steps. Current traces at each voltage step are an average of 3 sweeps. C. Spike discharge frequency (top) and current density (bottom) as a function of time for the recordings shown in A and B, respectively.

Δd_{5-CT} was 4.9 ± 0.6 pA/pF ($n = 6$) and 2.2 ± 0.3 pA/pF ($n = 3$) in females of wild-type and 5-HTT^{-/-} genotype, respectively, and 4.5 ± 0.7 pA/pF ($n = 3$) and 3.1 ± 0.1 pA/pF ($n = 5$) in males of wild-type and 5-HTT^{-/-} genotype, respectively, not very different from Δd_{GppNHp} respective values (see Table 2).

GppNHp NON-SENSITIVE CELLS

Some cells in 5-HTT^{+/+} (20/25 in females and 8/19 in males) and 5-HTT^{-/-} (10/18 in females and 7/14 in males) mice, exhibited little or no change in spike discharge frequency or the current elicited by hyperpolarizing voltage steps after ~20–30 min GppNHp diffusion. These neurons were considered as GppNHp non-sensitive cells (Fig. 4).

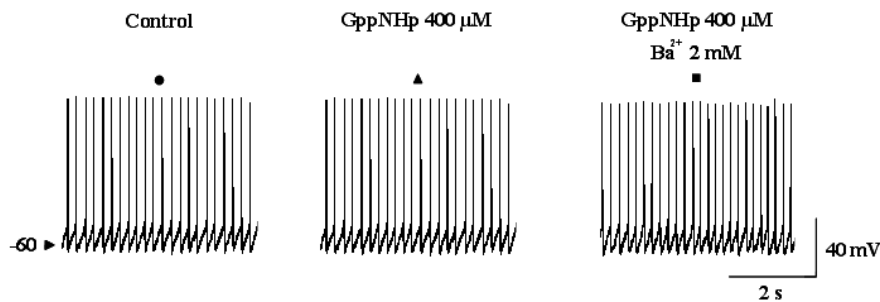


Fig. 4. GppNHp non-sensitive neuron in DRN from a 5-HTT^{-/-} mice. No effect of GppNHp was recorded after 20 min (▲).

Their spike discharge frequency and current density did not differ significantly from the corresponding baseline values for GppNHp-sensitive cells in all groups of mice (~ 10 Hz and ~ 5 pA/pF in females and males of wild-type and 5-HTT^{-/-} genotype, respectively). Furthermore, superfusion of 5-CT (30 μM) did not change spike discharge frequency and the current elicited by hyperpolarizing voltage steps in neurons exhibiting no changes in these parameters after ~ 20–30 min GppNHp diffusion (7 cells in 5-HTT^{+/+} and 4 cells in 5-HTT^{-/-} mice, not shown).

DISCUSSION

In GppNHp-inhibited neurons of the DRN, spike discharge frequency and current density at the beginning of the recordings were not significantly different among all groups of mice (females and males of wild-type and 5-HTT^{-/-} genotype), nor from those of GppNHp non-sensitive DRN neurons in these various groups of mice. This suggests that an enhanced active G-protein concentration, and hence

GIRK current-activation, which may have resulted from a large concentration of extracellular serotonin caused by serotonin transporter deficiency, does not occur under baseline conditions in 5-HTT^{-/-} mice. On the other hand, as reported in the literature, extracellular serotonin does not increase in DRN of chronically SSRI-treated rats, another model of serotonin transporter blockade [2]. Therefore, GppNHp-induced effects observed in our experiments are very probably due to G-protein coupling to GIRK channels in the plasma membrane of DRN neurons, and not to increased basal serotonin binding to receptors.

Surprisingly, no GppNHp-induced depolarization was observed, as would be expected from G-protein-coupled excitatory receptors in the DRN. This can be explained by more powerful G-protein-mediated inhibitions than excitations, even within a cell. For instance, 5-HT₂ receptor-mediated excitations are detectable only after 5-HT_{1A} receptor blockade in the DRN [7]. Furthermore, in wild-type females, the percentage of sensitive cells was lower during GppNHp diffusion (20%) than during 5-HT_{1A} agonists superfusion (81%), although the only described transduction mechanism in the literature for 5-HT_{1A} receptor gating of the GIRK current involves G proteins [11], while no differences were observed in other groups of wild-type males and mutant females and males. Such a discrepancy in wild-type females is puzzling, even though data obtained here with 5-HT_{1A} agonists are reported for the sake of qualitative-, but not quantitative comparison with GppNHp effects. This could result from the use of a single dose of GppNHp, i.e. 0.4 mM, which may not have been maximal to trigger GIRK current while 5-HT_{1A} agonists were used at maximal concentrations. Also, several G-protein related mechanisms triggering GIRK current may be involved, including the 5-HT_{1A} receptor-dependent process. A large number of accessory proteins involved in signal processing through G proteins have been described [19] and may be regulated differently during 5-HT_{1A} receptors activation. For example, in the hypothalamus of female mice, it has been shown that estrogens can block a GIRK current through G-proteins [13]. Hence, in neurons in the DRN of female mice where estrogen receptors have been identified [21], the estrogen transduction pathway may counteract G protein-GIRK current coupling triggered by GppNHp diffusion, more in wild-type than in 5-HTT^{-/-} mice [5].

The slow and fast inward relaxations observed in the GppNHp-difference current suggest involvement of different GIRK subunits in DRN neurons, consistent with the localization of several GIRK subunits, namely GIRK1, GIRK2 and GIRK3, but not GIRK4, subunits in the DRN of rats and mice [6, 12]. The slow relaxation may reflect the involvement of a GIRK1 subunit, which possesses slow activation properties [24]. The barium-blocked component of the control current observed in our preparation may correspond to a basal activity of GIRK channels [18]. Heterogeneity of GIRK multimeric complexes could underlie involvement of different GIRK currents triggered by various physiological states of the animals. Identification of these GIRK subunits in wild-type and 5-HTT^{-/-} mice will need further experiments such as single cell RT-PCR investigations.

We observed 58% (11 out of 19 recorded cells) of GppNHp-sensitive neurons in male wild-type mice, a result that can be compared to data in the literature where most studies are performed in males. Knowing that 60% of DRN neurons exhibit 5-HT_{1A} autoreceptors in mice [14] and rats [22], it can be hypothesized that in males, the pool of G-proteins linked to 5-HT_{1A} is similar to the pool of G-proteins present within a cell and/or within the DRN to trigger GIRK current. This is confirmed by the fact that 5-HT_{1A} agonists had no additive effects on the GppNHp difference current and GppNHp non-sensitive cells were also non-sensitive to 5-HT_{1A} agonists in our preparation.

CONCLUSIONS

In conclusion, our study demonstrated that the 5-HTT^{-/-} knock-out mutation reduces the GppNHp-induced coupling between G-proteins and GIRK channels in females but not in males. These characteristics might be relevant to gender-dependent molecular adaptive mechanisms affecting 5-HT neurotransmission during chronic 5-HT reuptake blockade by SSRI antidepressants.

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