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Effect of genetic and pharmacological blockade of GABA receptors on the 5-HT$_{2C}$ receptor function during stress

Cédric B. P. Martin[1,2,3], **Martin Gassmann**[4], **Caroline Chevarin**[1,2,3], **Michel Hamon**[1,2,3], **Uwe Rudolph**[5], **Bernhard Bettler**[4], **Laurence Lanfumey**[1,2,3], and **Raymond Mongeau**[1,3,6]

[1]Paris Descartes, Univ Paris 05, Paris, France [2]UPMC, Univ Paris 06, Paris, France [3]Inserm UMR S894, Centre de Psychiatrie et Neurosciences, Paris, France [4]Department of Biomedicine, Institute of Physiology, University of Basel, Basel, Switzerland [5]Laboratory of Genetic Neuropharmacology, McLean Hospital, Belmont, MA, USA and Department of Psychiatry, Harvard Medical School, Boston, MA, USA [6]EA4475 – Pharmacologie de la circulation cérébrale, Paris, France

Abstract

5-HT$_{2C}$ receptors play a role in psychoaffective disorders and often contribute to the antidepressant and anxiolytic effects of psychotropic drugs. During stress, activation of these receptors exerts a negative feedback on serotonin (5-HT) release, probably by increasing the activity of GABAergic interneurons. However, to date, the GABA receptor types that mediate the 5-HT$_{2C}$ receptor-induced feedback inhibition are still unknown. To address this question, we assessed the inhibition of 5-HT turnover by a 5-HT$_{2C}$ receptor agonist (RO 60-0175) at the hippocampal level and under conditions of stress, after pharmacological or genetic inactivation of either GABA-A or GABA-B receptors in mice. Neither the GABA-B receptor antagonist phaclofen nor the specific genetic ablation of either GABA-B1a or GABA-B1b subunits altered the inhibitory effect of RO 60-0175, although 5-HT turnover was markedly decreased in GABA-B1a knock-out mice in both basal and stress conditions. In contrast, the 5-HT$_{2C}$ receptor-mediated inhibition of 5-HT turnover was reduced by the GABA-A receptor antagonist bicuculline. Because a significant effect of 5-HT$_{2C}$ receptor activation persisted in mutant mice deficient in the α_3 subunit of GABA-A receptors, it can be inferred that non α_3 subunit-containing GABA-A receptors, but not GABA-B receptors, mediate the 5-HT$_{2C}$-induced inhibition of stress-induced increase of hippocampal 5-HT turnover in mice.

Correspondence should be sent to: Cédric Martin, Ph.D., Current Address: Unit "Signalling, neurobiology and cancer", Institut Curie - CNRS UMR3306 - INSERM U1005, 91405 ORSAY – FRANCE, Tel : +33 1 69 86 31 55, cedric.martin@inserm.fr.

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Keywords

bicuculline; phaclofen; GABA-A α3 subunit; GABA-B; knockout

Introduction

5-HT_{2C} receptors are thought to play a key role in stress-related disorders (for a review see Martin et al., 2014), not only through their modulatory role on the corticotropin releasing hormone (CRH) neurons in the paraventricular nucleus of the hypothalamus (PVN) that regulates glucocorticoid secretion (Heisler et al., 2007), but also by modulating GABAergic neurotransmission. Indeed, several studies argue for a close interaction between excitatory 5-HT_{2C} receptors, linked to Gαq proteins and phospholipase C, and the inhibitory GABAergic system. On the one hand, 5-HT_{2C} receptors are expressed by GABA interneurons in various brain areas, including the raphe nuclei (Serrats et al., 2005; Boothman et al., 2006; Invernizzi et al., 2007; Liu et al., 2007; Quérée et al., 2009; Bubar et al., 2011). As expected, 5-HT_{2C} receptor agonists increase the activity of GABA neurons in these nuclei (Boothman et al., 2006). Thus, under conditions of elevated 5-HT tone, such as after high doses of selective serotonin reuptake inhibitors (SSRIs; Cremers et al., 2007) or during restraint-stress (Mongeau et al., 2010), 5-HT_{2C} receptor stimulation inhibits 5-HT release and turnover in the hippocampus, probably by activating GABA interneurons. Indeed, SSRIs increase extracellular GABA while 5-HT_{2C} receptor stimulation decreases extracellular 5-HT in the dorsal raphe (Calcagno and Invernizzi, 2010). In keeping with this view, local infusion of either GABA-A or GABA-B receptor agonists into either the median or the dorsal raphe nuclei was found to decrease extracellular 5-HT concentration in the forebrain (e.g. the nucleus accumbens), but GABA-A receptor ligands appeared the most effective in this respect (Tao et al., 1996). Conversely, Cremers et al. (2007) showed that a GABA-B receptor antagonist, similarly to a 5-HT_{2C} receptor antagonist, potentiated SSRI-induced 5-HT outflow in the hippocampus whereas a GABA-A receptor antagonist enhanced basal 5-HT outflow. Thus, it appears that both GABA-A and GABA-B receptors participate in the inhibitory control of 5-HT neurotransmission mediated by GABA. However, the data also suggest that the indirect negative feedback signal triggered by 5-HT_{2C} receptor activation under elevated 5-HT tone (under SSRI treatment or stress conditions) is likely to be dependent on GABA-B rather than GABA-A receptors.

GABA-A receptors are ionotropic receptors composed of different subunits (α1–6, β1–3, γ1–3, δ, ε, θ, π and ρ1–3). Various alpha subunits are differentially expressed among brain regions, with the α3 subunit being the prominent in monoaminergic neurons (Fritschy et al., 1992). GABA-B receptors are metabotropic receptors composed of GABA-B1 and GABA-B2 subunits. The GABA-B1 subunit exists in two abundant isoforms, GABA-B1a and GABA-B1b that localize to pre- and post-synaptic elements, respectively (Gassmann and Bettler, 2012).

Inhibition of 5-HT release and 5-HT turnover by 5-HT_{2C} receptor activation was previously observed to occur exclusively in a stress condition and in particular in the hippocampus, a brain area most relevant to stress disorders, and in which 5-HT_{2C} receptor activation with

the 5-$HT_{2C/2B}$ receptor agonist RO 60-0175 was previously shown to exert a clear-cut inhibition of 5-HT turnover during stress (Mongeau et al., 2010). The action of RO 60-0175 at 5-HT_{2C} receptors was demonstrated in hippocampal and other areas with the selective 5-HT_{2C} receptor antagonist SB 242,084, which also has an effect by itself during stress in the hippocampus, but not other areas (Mongeau et al., 2010).

Here we investigated whether specific subtypes of GABA-A and GABA-B receptors are involved in this modulation during stress. To this end, we used the following two strategies: (i) individual genetic ablation of either the GABA-B1a, GABA-B1b or the GABA-Aα3 subunit and (ii) pharmacological blockade, using either the GABA-B antagonist phaclofen or the GABA-A antagonist bicuculline.

Materials and methods

Animals

Animals were housed 3–6 per cage (33 × 15 × 13 cm) under standard laboratory conditions (12h light-dark cycle, lights on at 7:00 h, room temperature 21±1°C) with free access to food and water for at least one week before any treatment. All procedures implicating animals were conducted in strict agreement with the institutional guidelines for use of animals and their care, in compliance with national and international laws and policies (council directive no. 87-848, October 19, 1987, Ministère de l'Agriculture et de la Forêt, Service Vétérinaire de la Santé et de la Protection Animale, permission no. 75-977 to L.L.).

Neurochemical experiments with GABA-A receptor inactivation were performed on 2–4 month-old male mice of the C57BL/6J strain from a breeding center (CER Janvier, Le-Genest-St-Isle, France) and GABA-Aα3 subunit deficient mutants and wild-type littermates of the same C57BL/6J genetic background (Yee et al., 2005), raised in Paris. Neurochemical experiments with GABA-B receptor inactivation were performed on 2–4 month-old male mice of the BALB/c strain (CER Janvier) and mutant mice of the same genetic background but deficient in GABA-B1a or GABA-B1b subunits, and their wild-type littermates (Vigot et al., 2006).

Stress procedure

Mice were stressed by physical constraint for 45 minutes inside a 50-ml tube, perforated at the tip to allow breathing, as previously described (Mongeau et al., 2010). They were sacrificed immediately after the stress session and the hippocampi were rapidly dissected on ice for biochemical determinations. Previous studies showed that this restraint-stress enhances 5-HT release as well as 5-HT turnover. The increase in 5-HT turnover is exclusively due to increased 5-hydroxyindoleacetic acid (5-HIAA) tissue levels as 5-HT catabolism increases with 5-HT utilization (Mongeau et al., 2010).

Measurements of 5-HT and 5-HIAA

Tissue levels of 5-HT and its metabolite 5-HIAA were determined using high-pressure liquid chromatography (HPLC) with electrochemical detection (ED). Dissected hippocampi were weighed and immediately homogenized by sonication for 15 s in 250 μL of an

extraction solution at 0–4°C (perchloric acid 0.1 M; EDTA 1.34 mM; sodium metabisulfite 0.05% w/v). Homogenates were centrifuged at 30,000 g for 20 min at 4°C and the supernatants were stored at −80°C. Samples were then thawed on ice, neutralized using 2 M KH_2PO_4/K_2HPO_4, pH 7.4, and endogenous ascorbic acid was removed using ascorbate oxidase (0.01 mg/ml; Boehringer-Mannheim, Germany). After centrifugation at 20,000 g for 10 min at 4°C, supernatants were collected and aliquots (10 µl) were automatically injected into the HPLC system. 5-HT and 5-HIAA were separated on a Beckman Coulter Ultrasphere 5 µm C18 (250 × 4.6 mm) column, protected by a Brownlee (3 cm, 5 µm) pre-column, using a mobile phase (K_2HPO_4 70 mM, triethylamine 3.1 mM, EDTA 0.1 mM, methanol 16 % v/v, octane sulphonate 1.05 mM, pH 3) at a flow rate of 1 ml/min. Compounds were oxidized by a coulometric electrochemical detector (ESA, Bedford, MA, USA) with an analytical cell (Model 5011) equipped with two electrodes set at +50 mV and +350 mV. The gain of the detector was set at 100 nA. The signal was sent to a computer and analyzed by an acquisition program (Empower 2, Waters, France). Results are expressed as ng 5-HT or 5-HIAA/g of fresh tissue and 5-HIAA/5-HT ratios (the turnover index which normally increases with utilization as extracellular 5-HT is metabolized into 5-HIAA).

Drugs

The 5-HT_{2C} receptor agonist RO 60-0175 [(S-2-chloro-5-fluoro-indol-1-yl)-1-methylethylamine fumarate] (3 mg/kg; Tocris, Bristol, UK), the GABA-B receptor antagonist phaclofen (2 mg/kg; Sigma-Aldrich, France) and GABA-A receptor antagonist bicuculline (8 mg/kg; Sigma-Aldrich, France) were freshly prepared in saline (0.9% NaCl) solution with a brief sonication, and administered intraperitoneally (i.p.) 30 minutes before initiating the restraint-stress period. The doses chosen were previously shown to be effective at the respective drug targets (Zarrindast and Farahvash, 1994; Dalvi and Rodgers, 1996). We have assessed 5-HT_{2C} receptor function after blockade or deletion of GABA receptors using a 3 mg/kg dose of RO 60-0175 previously shown to act on 5-HT_{2C} receptors, using the selective 5-HT_{2C} receptor antagonist SB 242,084 in neurochemical and behavioural assays (Mongeau et al. 2010, Kennett et al., 2000). Note that a dose of 6 mg/kg of RO 60-0175 inhibits totally the stress-induced increase of 5-HT turnover in C57BL/6J mice (Mongeau et al., 2010). However, in most brain areas, beside the dorsal raphe, RO 60-0175 is without effect by itself on extracellular 5-HT or 5-HT turnover in the basal conditions, that is - in absence of either stress or SSRI administration (Mongeau et al., 2010; Millan et al., 1998; Gobert et al., 2000; Calcagno and Invernizzi, 2010).

Statistical calculations

Most data were compared in pairs using the two-tailed Student's *t*-test. Once the inhibitory effect of RO 60-0175 on 5-HT turnover had been verified in each mouse strain (the C57BL/6J and the BALB/c strains being necessary to study the effects of GABA-A and GABA-B receptors inactivation, respectively), data were represented as percentages of respective controls and compared using the one-tailed Student's *t*-test.

Results

Effects of stress and 5-HT$_{2C}$ receptor activation on 5-HT turnover in BALB/c *vs* C57BL/6J mice

The 5-HT$_{2C}$ receptor-mediated inhibition of stress-induced increase in 5-HT turnover was evaluated in the hippocampus of both BALB/c and C57BL/6J mice, because both strains were used to investigate the effects of genetic ablation of either GABA-A or GABA-B receptors on the 5-HT$_{2C}$-evoked response. Studies with selective GABA-A and GABA-B receptor antagonists were also performed in these mouse strains.

As shown in Fig. 1A, restraint-stress induced a similar increase in 5-HT turnover in C57BL/6J mice (+20±4 % in 5-HIAA/5-HT ratio, compared to naive mice) and BALB/c mice (+28±4 %; means ± S.E.M., n= 14–17 and 20–29, respectively). As expected from our previous study (Mongeau et al., 2010), RO 60-0175 at 3 mg/kg induced a significant decrease (−15±2%; $p<0.001$) of the overall 5-HIAA/5-HT ratio in C57BL6/J mice. A similar reduction (−17±2%; $p<0.001$) was observed in BALB/c mice (Fig. 1B). These reductions may appear small, but it is important to consider that only the enhancement of 5-HT turnover by stress can be modulated by RO 60-0175 (Mongeau et al., 2010). We have estimated this stress-induced increase in 5-HT turnover (i.e. the absolute increase in 5-HIAA/5-HT ratio caused by stress over baseline value from naïve mice). We found it to be similarly inhibited (by nearly 70%) in both strains following RO 60-0175 administration (C57BL6/J: saline = 0.200±0.035; RO = 0.065±0.033 n= 14–16; p<0.01; BALB/c: saline = 0.217±0.022; RO = 0.052±0.021; n= 22–28; p<0.0001). The 3 mg/kg dose of RO 60-0175 was thus effective in reducing 5-HT turnover without inducing maximal inhibitory effects in either strains of mice.

Effects of GABA-B and/or GABA-A receptors inactivation on the 5-HT$_{2C}$ receptor-mediated inhibition of 5-HT turnover in stressed mice

In naive animals, the basal value of 5-HIAA/5-HT ratio did not significantly differ in mutant mice lacking the GABA-Aα3 subunit and littermate WT mice of the C57BL/6J strain (GABA-Aα3$^{-/-}$= 1.41±0.05, n= 4; WT = 1.35±0.05, n= 4, n.s.). Similarly, genetic ablation of the GABA-B1b subunit had no effect on basal 5-HT turnover in the hippocampus of BALB/c mice (GABA-B1b$^{-/-}$= 0.78±0.05, WT= 0.72±0.05, means ± S.E.M., n=6–7; n.s.). In contrast, mutants with genetic ablation of the GABA-B1a subunit displayed a significant decrease of 5-HT turnover compared to BALB/c WT mice (GABA-B1a$^{-/-}$= 0.60±0.04, WT= 0.80±0.04, means ± S.E.M., n= 6–9; $p<0.01$, Student's t-test)

As shown in Fig. 2, in the stress conditions, administration of the 5-HT$_{2C}$ receptor agonist RO 60-0175 reduced 5-HT turnover by about 20% in both GABA-B1a$^{-/-}$ and GABA-B1b$^{-/-}$ mice, similarly to the effect normally observed in BALB/c mice (Fig. 1B). Furthermore, a similar significant reduction by RO 60-0175 of 5-HT turnover was observed after pharmacological blockade of GABA-B receptors with phaclofen in stressed BALB/c WT mice (Fig. 2). In contrast, although RO 60-0715 no longer significantly decreased 5-HT turnover when GABA-A receptors were blocked by bicuculline, an inhibitory effect of the

5-HT_{2C} receptor agonist occurred in stressed GABA-A deficient mice of the GABA-A$\alpha_3^{-/-}$ genotype (−12%; p=0.05; Fig. 2) as in C57BL6/J wild-type mice (Fig. 1B).

Discussion

As previously stated, the inhibition of stress-induced increase in 5-HT turnover by 5-HT_{2C} receptor activation is most likely mediated by the GABAergic rather than the CRH system (Mongeau et al., 2010). Indeed, one would expect an increase rather than a reduction of the stress-induced enhancement of 5-HT release in response to 5-HT_{2C} receptor activation by RO 60-0175 if the 5-HT_{2C} receptor effect was to occur through CRH, a neurohormone that increase 5-HT neurons activity (Mo et al., 2008; Martin et al., 2014). Moreover, an indirect negative feedback control of monoaminergic neurotransmission has been shown to occur via GABAergic interneurons. More specifically, an inhibitory effect of 5-$HT_{2A/2C}$ receptor agonists on 5-HT neurotransmission through GABAergic neurotransmission has been demonstrated using electrophysiological approaches (Boothman et al., 2006; Invernizzi et al., 2007; Quérée et al., 2009). Because Cremers and colleagues (2007) found that the GABA-B receptor antagonist phaclofen, like the 5-HT_{2C} receptor antagonists, potentiates the effect of the SSRI citalopram on extracellular 5-HT levels in the ventral hippocampus, it was hypothesized that GABA-B receptors were involved in the 5-HT_{2C} receptor-mediated inhibition of stress-induced increase of 5-HT turnover (Mongeau et al., 2010). However, our present results clearly show that neither the administration of phaclofen nor the specific genetic ablation of GABA-B1a or GABA-B1b subunits prevented this 5-HT_{2C} receptor-mediated inhibition. This suggests that the 5-HT_{2C} receptors that control 5-HT turnover during stress are different from those that regulate 5-HT outflow during acute SSRI treatment.

Interestingly, however, GABA-B receptors do appear to regulate 5-HT turnover under basal conditions. Mice lacking the GABA-B1a subunit (but not those lacking GABA-B1b) have a significant decrease in 5-HT turnover compared to WT littermates. Glutamatergic terminals mainly express the GABA-B1a subunit (Vigot et al., 2006) and cortical glutamatergic neurons project to inhibitory GABAergic interneurons in the raphe area (Jankowski and Sesack, 2004). The reduction in 5-HT turnover observed in GABA-B1a$^{-/-}$ mice might thus be explained by a stronger excitatory glutamatergic input onto GABAergic interneurons inhibiting 5-HT neurons in raphe nuclei, resulting from a lack of negative feedback on glutamatergic terminals via GABA-B heteroreceptors (Fig. 3). In keeping with this view, previous studies have shown increased 5-HT neurotransmission following glutamatergic receptor blockade (Lopez-Gil et al., 2007).

In contrast to phaclofen, the GABA-A receptor antagonist bicuculline prevented the inhibitory effect of RO 60-0175 in the stress condition, indicating that GABA-A receptors were involved in this 5-HT_{2C} receptor-mediated response. Previous pharmacological studies in behaving mice also suggested that GABA-A receptor involvement in 5-HT_{2C} responses (Donatti and Leite-Panissi, 2009; de Oliveira Sergio et al., 2011). The GABA-Aα3 subunit is densely expressed in serotonergic neurons (Fritschy et al., 1992) and is potentially involved in the regulation of stress and anxiety (Dias et al., 2005; Judge et al., 2006; Marowsky et al., 2012). We therefore hypothesized that genetic ablation of the GABA-Aα3

subunit should mimic the effect of bicuculline. However, a significant effect of RO 60-0175 on the stress-induced increase of 5-HT turnover was still observed in mice lacking the GABA-Aα3 subunit. 5-HT neurons in the dorsal raphe nucleus express other alpha subunit isoforms (Pirker et al., 2000). Therefore our results do not exclude that GABA-A receptors on 5-HT neurons mediate the inhibitory response to 5-HT_{2C} receptor activation (Fig. 3). Alternatively, it is possible that GABA-A receptors on GABAergic interneurons expressing the GABA-Aα1 subunit (Gao et al., 1993) contribute to the inhibitory response. Considering that bicuculline is not specific for any of the GABA-A receptor subtypes, our data show that the α3 subunit is, at the very least, not indispensable to the 5-HT_{2C} receptor-mediated response. Because of the multiple subunits constituting the GABA-A receptors throughout the brain (Rudolph and Möhler, 2006), it will remain difficult to determine precisely which GABA-A receptor subtype(s) actually mediate(s) the 5-HT_{2C} receptor-induced negative feedback control of 5-HT neurotransmission in stressed mice.

To conclude, during stress, in addition to the 5-HT_{2C} receptor activation that increases CRH release and produces CRH2 receptor-mediated excitation of 5-HT neurons (Day et al., 2004; Martin et al., 2014), activation of 5-HT_{2C} receptors on GABAergic neurons can trigger a negative-feedback control of serotonergic transmission mainly through GABA-A receptors on 5-HT neurons.

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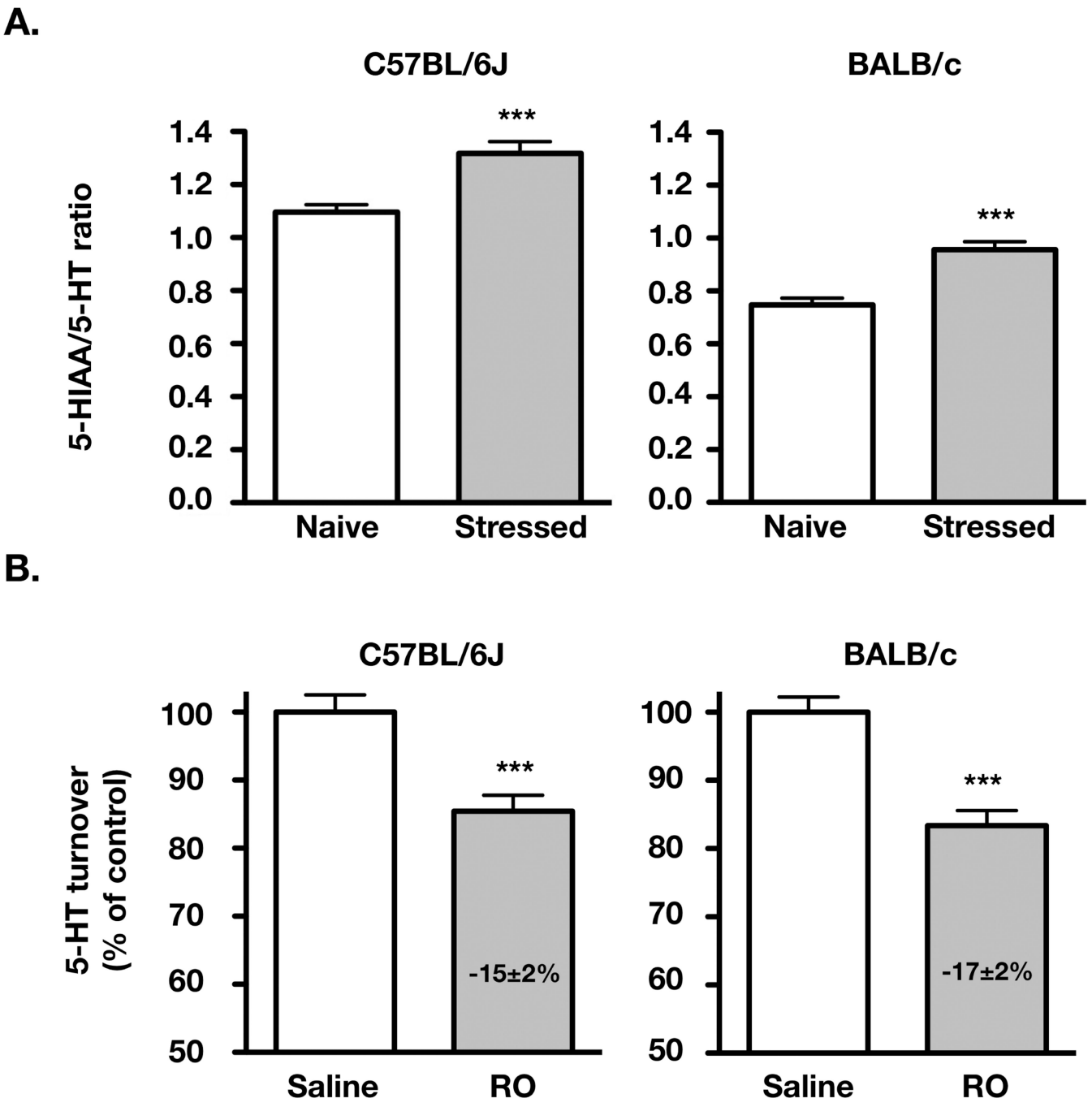

Figure 1. 5-HT$_{2C}$ receptor-mediated inhibition by RO 60-0175 of the stress-induced increase in 5-HT turnover in BALB/c and C57BL/6J mice

A) Effect of restraint stress on the 5-HT turnover [5-HIAA/5-HT ratio; means ± S.E.M.] in the hippocampus of C57BL/6J (n= 14–17) and BALB/c (n= 20–29) WT mice. **B)** The effects of RO 60-0175 (RO; 3 mg/kg i.p.) are presented as a percentage (means ± S.E.M.) of the 5-HIAA/5-HT ratio of saline-treated mice of the C57BL/6J (n=14–16) and BALB/c (n=22–31) strain. ***p<0.001, two-tail Student's t-test.

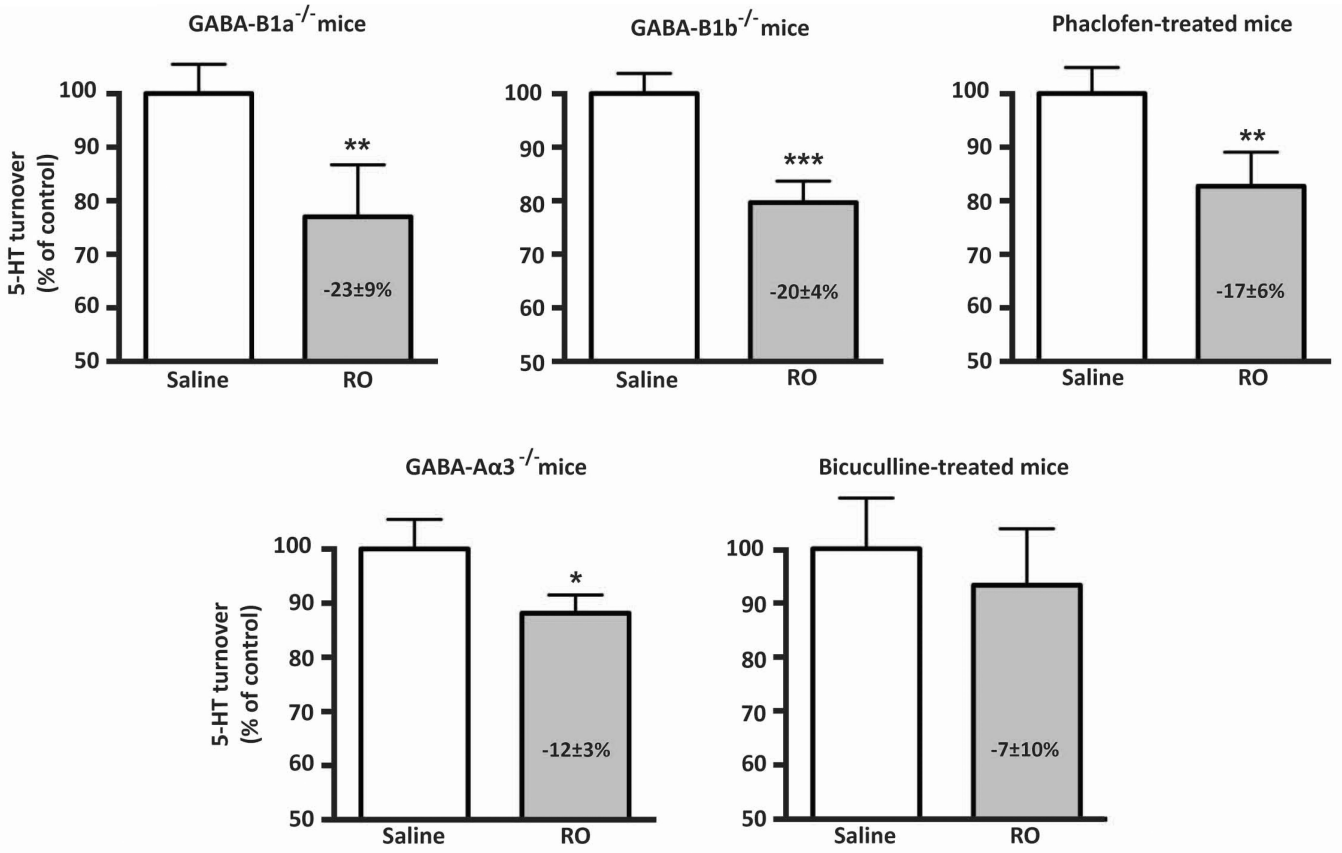

Figure 2. Consequences of pharmacological or genetic inactivation of specific GABA receptors on 5-HT$_{2C}$ receptor-mediated inhibition of 5-HT turnover in stressed mice

The effect of RO 60-0175 (RO; 3 mg/kg, i.p.) on 5-HT turnover under conditions of stress was determined in mice with genetic ablation of either GABA-B1a, GABA-B1b or GABA-Aα_3 receptor subunit and in WT mice after pharmacological blockade of GABA-A or GABA-B receptors with bicuculline (8 mg/kg, i.p.) or phaclofen (2 mg/kg, i.p.), respectively. Results are presented as a percentage of the 5-HIAA/5-HT ratio in saline-treated mice of the same genotype (means ± S.E.M.; n= 4–8). * $p \leq 0.05$, ** $p < 0.01$, one-tail Student's *t*-test.

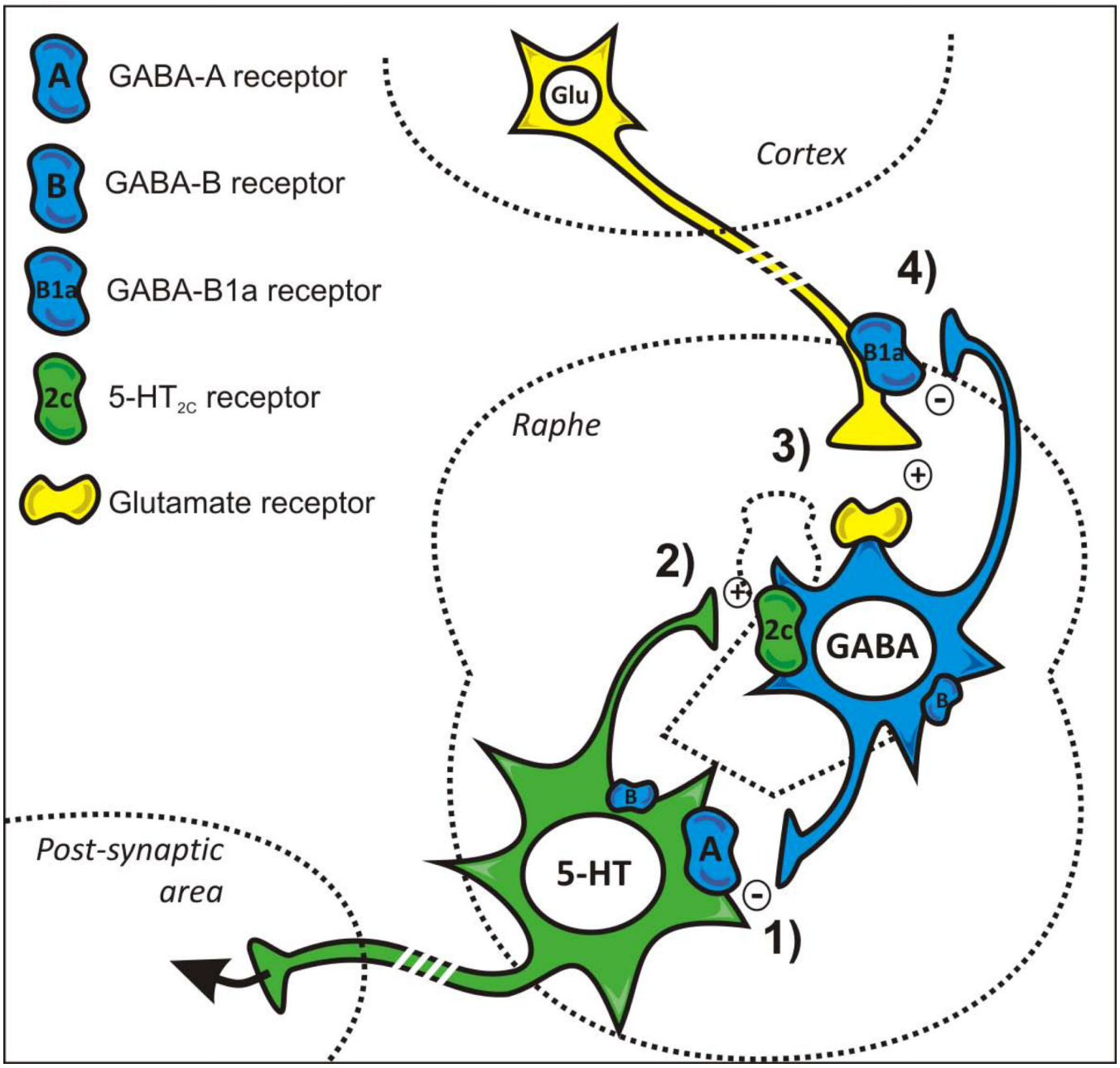

Figure 3. Schematic representation of GABAergic interneuron-mediated inhibition of serotonergic neuron activity

GABAergic interneurons are known to inhibit 5-HT neuronal activity via GABA-A receptors (1). Because of 5-HT release during stress, 5-HT_{2C} receptors expressed by GABAergic interneurons are activated (2), which leads to a GABA-mediated negative feedback control of 5-HT neuron during stress (Mongeau et al., 2010). Glutamatergic (Glu) projections from forebrain (cortex) areas also excite GABAergic interneurons (3), but inhibitory terminal GABA-B1a subunit containing heteroreceptors exert a presynaptic modulation on these projections (4). Accordingly, the excitatory influence of cortical

glutamatergic projections onto GABA interneurons is larger in GABA-B1a$^{-/-}$ mice, which would account for a reduced 5-HT turnover in these mutants.