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Chronic treatment with LY341495 decreases 5-HT$_{2A}$ receptor binding and hallucinogenic effects of LSD in mice

José L. Moreno[a], Terrell Holloway[a], Vinayak Rayannavar[a], Stuart C. Sealfon[b,c,d], Javier González-Maeso[a,b,c,*]

[a] Department of Psychiatry, Mount Sinai School of Medicine, 1425 Madison Avenue, Box 1229, New York, NY 10029, United States
[b] Department of Neurology, Mount Sinai School of Medicine, 1425 Madison Avenue, Box 1229, New York, NY 10029, United States
[c] Friedman Brain Institute, Mount Sinai School of Medicine, 1468 Madison Avenue, Box 1229, New York, NY 10029, United States
[d] Center for Translational Systems Biology, Mount Sinai School of Medicine, 1425 Madison Avenue, Box 1229, New York, NY 10029, United States

HIGHLIGHTS

- Chronic LY341495 down-regulates 5-HT$_{2A}$ binding in mouse somatosensory cortex.
- This effect does not occur in mGlu2-KO mice.
- Chronic LY341495 decreases head-twitch behavior and cellular responses induced by LSD.
- These findings suggest that repeated blockade of mGlu2 reduces 5-HT$_{2A}$-dependent effects of LSD.

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ABSTRACT

Hallucinogenic drugs, such as lysergic acid diethylamide (LSD), mescaline and psilocybin, alter perception and cognitive processes. All hallucinogenic drugs have in common a high affinity for the serotonin 5-HT$_{2A}$ receptor. Metabotropic glutamate 2/3 (mGlu2/3) receptor ligands show efficacy in modulating the cellular and behavioral responses induced by hallucinogenic drugs. Here, we explored the effect of chronic treatment with the mGlu2/3 receptor antagonist 2S-2-amino-2-(1S,2S-2-carboxycyclopropan-1-yl)-3-(xanth-9-yl)-propionic acid (LY341495) on the hallucinogenic-like effects induced by LSD (0.24 mg/kg). Mice were chronically (21 days) treated with LY341495 (1.5 mg/kg), or vehicle, and experiments were carried out one day after the last injection. Chronic treatment with LY341495 down-regulated [^{3}H]ketanserin binding in somatosensory cortex of wild-type, but not mGlu2 knockout (KO), mice. Head-twitch behavior, and expression of *c-fos*, *egr-1* and *egr-2*, which are responses induced by hallucinogenic 5-HT$_{2A}$ agonists, were found to be significantly decreased by chronic treatment with LY341495. These findings suggest that repeated blockade of the mGlu2 receptor by LY341495 results in reduced 5-HT$_{2A}$ receptor-dependent hallucinogenic effects of LSD.

1. Introduction

The serotonin [21] and glutamate [5] receptor systems play an important role in the mechanism of action of hallucinogenic drugs, such as lysergic acid diethylamide (LSD), mescaline or psilocybin. All hallucinogenic drugs have in common a high affinity for the serotonin 5-HT$_{2A}$ receptor [12], and this receptor has been shown to be necessary for some of their behavioral effects in rodents [13] and humans [23]. Metabotropic glutamate 2 and 3 (mGlu2/3) receptors have also been involved in the cellular and behavioral responses induced by hallucinogenic 5-HT$_{2A}$ receptor agonists. Thus, mGlu2/3 receptor orthosteric agonists, such as LY379268 and LY354740, reduce the cellular [10,27], electrophysiological [17] and behavioral [7,10] effects induced by hallucinogenic drugs. Similar findings have been reported for the mGlu2-selective allosteric agonist biphenyl-indanone A (BINA) [3]. Moreover, mice lacking mGlu2 receptor (mGlu2 knockout, KO), display reduced responses to hallucinogenic drugs, such as 1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane (DOI) and LSD [18].

Another point of interest is the effect of chronic administration of 5-HT$_{2A}$ ligands on expression and behavioral function of mGlu2/3 receptors. Chronic treatment with the hallucinogenic 5-HT$_{2A}$ agonist 1-(2,5-dimethoxy-4-bromophenyl)-2-aminopropane (DOB) reduces the behavioral effects induced by the mGlu2/3

* Corresponding author at: Department of Psychiatry, Mount Sinai School of Medicine, 1425 Madison Avenue, Box 1229, New York, NY 10029, United States. Tel.: +1 212 241 8873; fax: +1 212 996 9785.
E-mail address: Javier.Maeso@mssm.edu (J. González-Maeso).

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agonist LY379268 in mice [2]. These include absence of effect of LY379268 on the 5-HT_{2A} receptor-dependent head-twitch behavioral response, and lower effect of LY379268 on the hyperlocomotor behavioral response induced by phenyclidine (PCP). Chronic treatment with the atypical antipsychotic drug clozapine also affects *mGlu2* mRNA expression and mGlu2/3 ligand binding in mouse cortical regions, an effect that is not observed in 5-HT_{2A}-KO mice [10,16]. Together, these findings suggest that chronic treatment with either hallucinogenic or antipsychotic 5-HT_{2A} ligands modulates the expression of mGlu2/3 receptors. In this study, we investigated the effects of chronic treatment with the mGlu2/3 receptor antagonist LY341495 on the 5-HT_{2A} receptor-dependent cellular and behavioral responses induced in mice by LSD. We measured LSD-dependent expression of *c-fos*, *egr-1* and *egr-2* in mouse somatosensory cortex, and head-twitch behavior. These cellular and behavioral responses have been previously shown to require expression of 5-HT_{2A} receptor in cortical neurons [13].

2. Methods

2.1. Animals

Experiments were performed on adult (8–12 weeks old) male 129S6/SvEv mice. Animals were purchased from Taconic (Hudson, NY), and were housed at 12 h light/dark cycle (lights on, 8:00–20:00) at 23 °C with food and water *ad libitum*. Mice were housed for at least 1 week before experimental use. mGlu2-KO mice [26] were obtained from the RIKEN BioResource Center, Japan (see [18] for details). For experiments involving wild-type and mGlu2-KO mice, all subjects were offspring of heterozygote breeding. The Institutional Animal Use and Care Committee at Mount Sinai School of Medicine approved all experimental procedures.

2.2. Radioligand binding

For effect of chronic treatment with LY341495 on [^{3}H]LY341495 and [^{3}H]ketanserin binding in mouse somatosensory cortex, mice were injected (i.p.) daily for 21 days with LY341495 (1.5 mg/kg). This dose was selected based on previous studies in rodents [7,10]. Control mice were chronically (21 days) treated with vehicle (0.9% NaCl). Mice were sacrificed by cervical dislocation one day after the last injection with chronic LY341495, or vehicle, and bilateral somatosensory cortex (bregma 1.40 to −1.90 mm) was dissected and frozen at −80 °C until processed for radioligand binding assays. Mouse somatosensory cortex plasma membrane preparations were obtained as previously reported [18], and stored at −80 °C until assay.

[^{3}H]LY341495 binding assays (0.0625–30 nM; eleven concentrations) were performed as previously reported [16]. Non-specific binding was determined in the presence of 1 mM L-glutamic acid (Tocris Cookson Inc., Minneapolis, MN). [^{3}H]Ketanserin binding assays (0.0625–10 nM; ten concentrations) were performed as previously [16]. Non-specific binding was determined in the presence of 10 μM methysergide (Tocris Cookson Inc., Minneapolis, MN). [^{3}H]Ketanserin binds with high affinity to both 5-HT_{2A} and 5-HT_{2C} receptors. Previous findings demonstrate that under these experimental conditions (*i.e.*, 5-HT_2 receptor ligand methysergide to define non-specific binding), specific [^{3}H]ketanserin binding is abolished in somatosensory [13] and frontal [20] cortex of 5-HT_{2A} knockout mice as compared to wild-type littermates.

[^{3}H]-2S-2-amino-2-(1S,2S-2-carboxycyclopropan-1-yl)-3-(xanth-9-yl)-propionic acid ([^{3}H]LY341495) was purchased from American Radiolabeled Chemicals, Inc. (Saint Louis, MO). [^{3}H]Ketanserin was purchased from Perkin-Elmer Life and Analytical Sciences, Inc. (Waltham, MA). LY341495 was obtained from Tocris Cookson Inc. (Minneapolis, MN). All other chemicals were obtained from standard sources.

2.3. Head-twitch behavior

Head-twitch behavioral response was measured as previously reported (see [18]) one day after the last injection with chronic LY341495. LSD (0.24 mg/kg) was obtained from Sigma–Aldrich (Saint Louis, MO).

2.4. Reverse transcription quantitative PCR (RT-qPCR)

Mice were injected daily for 21 days with LY341495 (1.5 mg/kg), or vehicle (see above). Induction of *c-fos*, *egr-1* and *egr-2* by LSD (0.24 mg/kg) was measured one day after the last injection with chronic LY341495. Reverse transcription quantitative real-time PCR (RT-qPCR) experiments were performed as previously reported [18]. See [13] for primer sequences.

2.5. Statistical analysis

All graphs and statistical analyses were generated using GraphPad Prism 5.0b. Radioligand binding data were analyzed using a nonlinear curve fit. An extra-sum-of-squares (*F*-test) was used to determine statistical differences for simultaneous analyses of binding saturation curves. Differences in the maximum number of binding sites (B_{max}) were assessed by unpaired Student's *t*-test. Statistical significance of experiments involving two or more groups and two or more treatments was assessed by two-way ANOVA followed by Bonferroni's *post hoc* test. Statistical significance of experiments involving three or more groups was assessed by one-way ANOVA followed by Bonferroni's *post hoc* test. Statistical significance of experiments involving two groups was assessed by Student's *t*-test. The level of significance was chosen at $p = 0.05$. All data are presented as mean ± SEM.

3. Results

3.1. Effect of chronic treatment with LY341495 on mGlu2/3 receptor binding

The simultaneous analysis of multiple saturation curves showed a significantly different [^{3}H]LY341495 binding saturation curve in somatosensory cortex of mice chronically treated with LY341495 ($F[2,116] = 99.75$; $p < 0.001$) (Fig. 1A). Analysis of individual maximum number of binding sites (B_{max}) demonstrated a lower density of mGlu2/3 receptors in mice chronically treated with LY341495 ($t = 7.90$, $df = 10$, $p < 0.001$; Student's *t*-test) (Fig. 1B). The affinity (K_D values) of [^{3}H]LY341495 was not affected by treatment with LY341495 (vehicle, 2.43 ± 0.15 nM; chronic LY341495, 2.61 ± 0.14 nM) ($t = 0.87$, $df = 10$, $p > 0.05$; Student's *t*-test).

3.2. Effect of chronic treatment with LY341495 on 5-HT_{2A} receptor binding

The simultaneous analysis of multiple saturation curves showed a significantly different [^{3}H]ketanserin binding saturation curve in somatosensory cortex of wild-type mice ($F[2,104] = 7.96$; $p < 0.001$) (Fig. 2A), but not of mGlu2-KO mice ($F[2,68] = 0.43$; $p > 0.05$) (Fig. 2B), chronically treated with LY341495. Analysis of individual maximum number of binding sites (B_{max}) indicated a significant effect of chronic treatment with LY341495 ($F[1,14] = 5.41$; $p < 0.05$) (Fig. 2C). Interestingly, *post hoc* analysis revealed that the maximum number of binding sites was decreased in wild type ($p < 0.05$), but not in mGlu2-KO ($p > 0.05$), mice (Fig. 2C). The affinity (K_D values) for [^{3}H]ketanserin was not affected by chronic

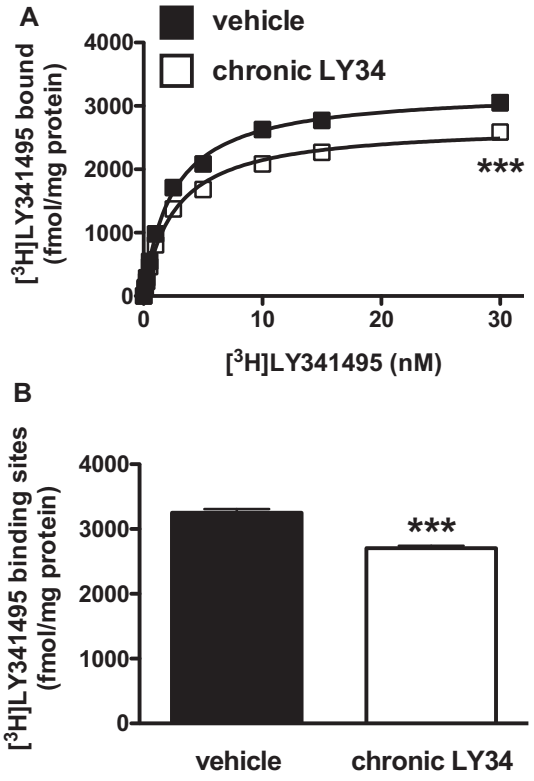

Fig. 1. (A) [^{3}H]LY341495 binding saturation curves in somatosensory cortex of wild-type mice one day after chronic treatment with LY341495 (LY34), or vehicle ($n = 6$). $^{***}p < 0.001$; *F*-test. (B) Maximum number of binding sites (B_{max}) for [^{3}H]LY341495 obtained from individual saturation curves. $^{***}p < 0.001$; Student's *t*-test. Data are mean ± SEM.

treatment with LY341495 (vehicle—wild-type, 4.02 ± 1.43 nM; chronic LY341495—wild-type, 2.66 ± 0.44 nM; vehicle—mGlu2-KO, 3.94 ± 1.49 nM; chronic LY341495—mGlu2-KO, 3.45 ± 0.66) ($F[1,14] = 0.10$; $p > 0.05$).

3.3. Effect of chronic treatment with LY341495 on head-twitch behavior induced by LSD

Head-twitch behavior induced by LSD was decreased in mice chronically treated with LY341495 ($t = 3.88$, $df = 8$, Student's *t*-test) (Fig. 3).

3.4. Effect of chronic treatment with LY341495 on induction of expression of c-fos, egr-1 and egr-2 by LSD

One-way ANOVA indicated that there is a significant effect of chronic treatment with LY341495 on induction of expression of *c-fos* by LSD mouse somatosensory cortex ($F[3,20] = 12.65$, $p < 0.001$) (Fig. 4A). *Post hoc* analysis revealed that a single dose of LSD induces expression of *c-fos* in mice chronically treated with saline ($p < 0.001$), but not in mice chronically treated with LY341495 ($p > 0.05$). *Post hoc* analysis also showed that expression of *c-fos* is not affected in mice chronically treated with LY341495 followed by a single dose of vehicle ($p > 0.05$).

One-way ANOVA indicated that there is a significant effect of chronic treatment with LY341495 on induction of expression of *egr-1* by LSD in mouse somatosensory cortex ($F[3,20] = 5.09$, $p < 0.01$) (Fig. 4B). *Post hoc* analysis revealed that a single dose of LSD induces expression of *egr-1* in mice chronically treated with saline ($p < 0.05$), but not in mice chronically treated with LY341495 ($p > 0.05$). *Post hoc* analysis also showed that expression of *egr-1* is not affected in

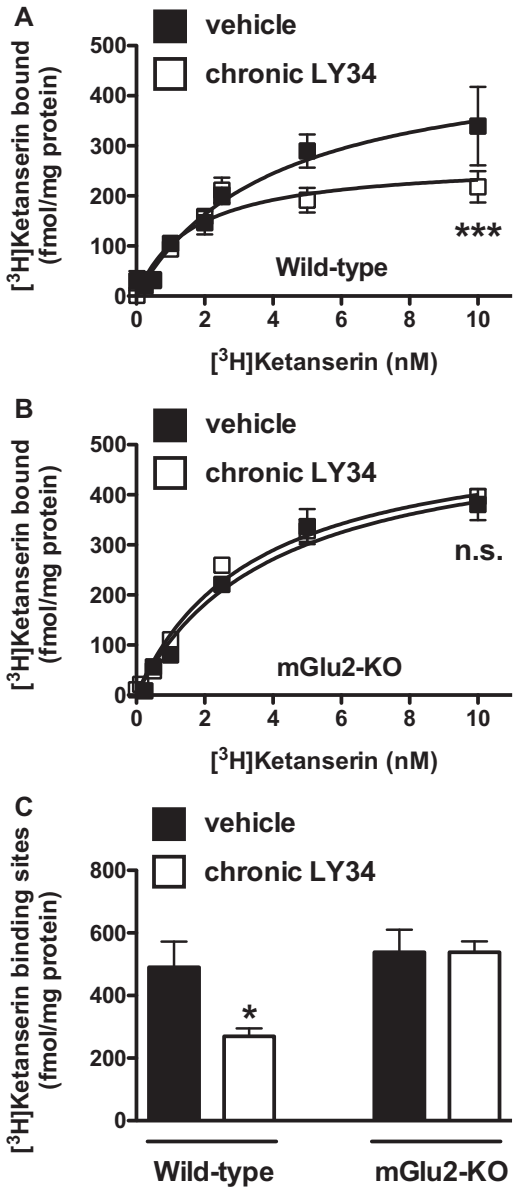

Fig. 2. (A) [^{3}H]Ketanserin binding saturation curves in somatosensory cortex of wild-type mice one day after chronic treatment with LY341495 (LY34), or vehicle ($n = 6$). (B) [^{3}H]Ketanserin binding saturation curves in somatosensory cortex of mGlu2-KO mice one day after chronic treatment with LY341495, or vehicle ($n = 9$). $^{***}p < 0.001$; n.s., not-significant; *F*-test. (C) Maximum number of binding sites (B_{max}) for [^{3}H]ketanserin obtained from individual saturation curves. $^{*}p < 0.05$; $^{***}p < 0.05$; Bonferroni's *post hoc* test of two-way ANOVA. Data are mean ± SEM.

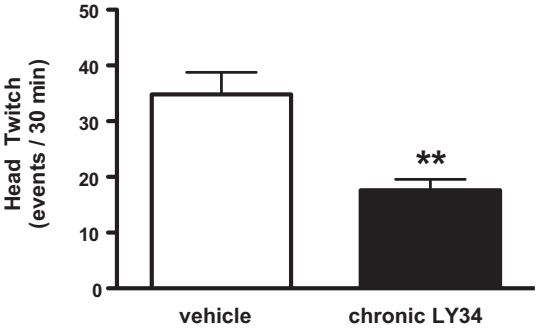

Fig. 3. Head-twitch response induced by LSD one day after chronic LY341495 (LY34), or vehicle ($n = 5$). $^{**}p < 0.01$; Student's *t*-test.

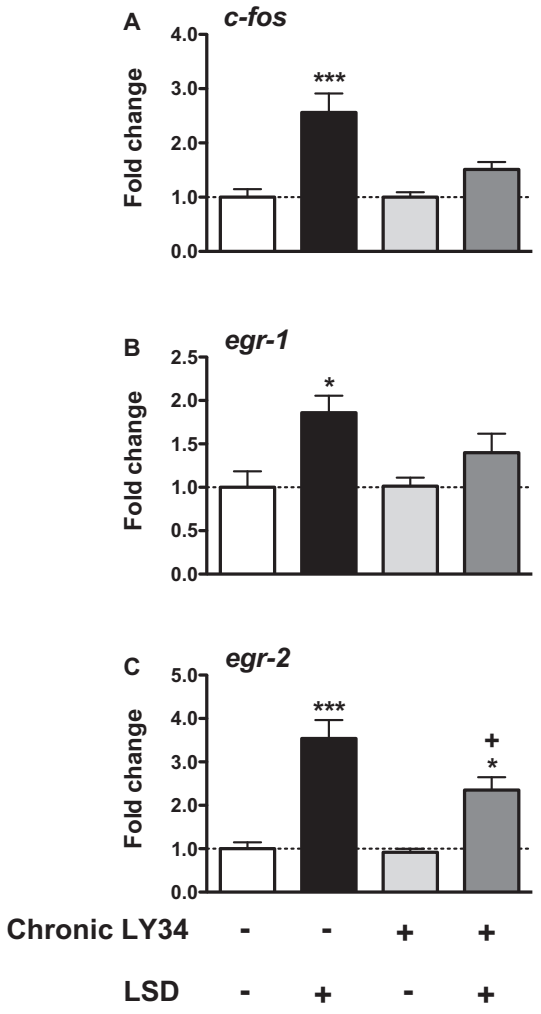

Fig. 4. Cellular response to LSD in mouse somatosensory cortex one day after chronic LY341495 (LY34) assayed by RT-qPCR ($n = 6$). Changes in expression levels of *c-fos* (A), *egr-1* (B), and *egr-2* (C) are reported as fold change over vehicle. $^{*}p < 0.05$; $^{**}p < 0.001$; Bonferroni's *post hoc* test of one-way ANOVA as compared to vehicle. $^{+}p < 0.05$; Bonferroni's *post hoc* test of one-way ANOVA as compared to LSD.

mice chronically treated with LY341495 followed by a single dose of vehicle ($p > 0.05$).

One-way ANOVA indicated that there is a significant effect of chronic treatment with LY341495 on induction of expression of *egr-2* by LSD mouse somatosensory cortex ($F[3,20] = 20.87$, $p < 0.001$) (Fig. 4C). *Post hoc* analysis revealed that a single dose of LSD induces expression of *egr-2* in mice chronically treated with saline ($p < 0.001$), and in mice chronically treated with LY341495 ($p < 0.05$). *Post hoc* analysis also showed that induction of expression of *egr-2* by LSD is lower in mice chronically treated with LY341495 as compared to that obtained in mice chronically treated with vehicle ($p < 0.05$). Expression of *egr-2* is not affected in mice chronically treated with LY341495 followed by a single dose of vehicle ($p > 0.05$).

4. Discussion

This study demonstrates that chronic treatment with the mGlu2/3 receptor antagonist LY341495 down-regulates 5-HT_{2A} receptor binding in mouse somatosensory cortex through a mechanism that requires expression of the mGlu2 receptor, as this effect is not observed in mGlu2-KO mice. The lower density of 5-HT_{2A} receptor binding correlates with changes in hallucinogenic-like behavioral and cellular responses that require expression of 5-HT_{2A} receptor in mouse brain cortex. Thus, chronic treatment with LY341495 decreases LSD-dependent head-twitch behavior, and LSD-dependent induction of expression of *c-fos*, *egr-1* and *egr-2* in mouse somatosensory cortex. Together, these findings suggest that persistent blockade of mGlu2 receptor-dependent signaling results in a lower density of 5-HT_{2A} receptor, which, consequently, decreases the cellular signaling and behavioral responses induced by the hallucinogenic drug LSD.

Hallucinogenic drugs all bind with high affinity to and activate the 5-HT_{2A} receptor [12]. However, closely related 5-HT_{2A} receptor agonists, such a lisuride and ergotamine, do not induce hallucinogenic effects [12]. A potential explanation of why only certain 5-HT_{2A} receptor agonists affect cognition, perception and mood is based on a pharmacological model termed functional selectivity (also known as agonist-trafficking and biased agonism) [11]. Based on this model, agonists stabilize a subset of receptor structural conformations that preferentially activate certain signaling pathways downstream. Previous findings suggest that each 5-HT_{2A} receptor agonist elicits a distinct signal transduction response [13]. Further, it was reported that hallucinogenic drugs [such as DOI, DOB, 1-(2,5-dimethoxy-4-methylphenyl)-2-aminopropane (DOM), mescaline, psilocin and LSD], and non-hallucinogenic drugs (such as R-lisuride, S-lisuride and ergotamine), induce a distinct transcriptome response that predicts their head-twitch behavioral effects in mice [13], and their hallucinogenic potential in human [8]. Among the transcriptome responses induced by hallucinogenic and non-hallucinogenic drugs, regulation of *c-fos* tracks with agonist activity at the 5-HT_{2A} receptor, whereas induction of *egr-1* and *egr-2* predicts head-twitch behavior in mouse [13]. Our findings here suggest that chronic treatment with LY341495 reduces both the 5-HT_{2A} receptor agonist-dependent expression of *c-fos* and the hallucinogenic-dependent expression of *egr-1* and *egr-2*. This effect in mouse somatosensory cortex correlates with the profound decrease of 5-HT_{2A} receptor binding induced by chronic treatment with LY341495.

A potential explanation for the mGlu2 receptor-dependent effect of chronic treatment with the mGlu2/3 receptor antagonist LY341495 on 5-HT_{2A} receptor density in mouse somatosensory cortex is related to expression of 5-HT_{2A} and mGlu2 as a G protein-coupled receptor (GPCR) heterocomplex. GPCRs have traditionally been considered to exist and function as monomeric structural units. However, during the last decade, increasing evidence suggest that GPCRs can form dimers or, potentially, higher-order oligomers [9]. There is also an extensive number of publications suggesting that GPCR heterocomplexes may exist. In this context, previous findings demonstrate that 5-HT_{2A} and mGlu2 receptors interact in close molecular proximity as part of the same GPCR heteromeric complex [6,10,19]. One component of homeostasis in living cells is the functional desensitization with continuous GPCR stimulation. Mechanisms for receptor desensitization include uncoupling from G proteins, receptor endocytosis, and receptor degradation [24]. Agonist-dependent activation is generally required to initiate the molecular mechanisms responsible for GPCR desensitization and endocytosis. Interestingly, previous findings suggest that LY341495 behaves as a mGlu2 receptor inverse agonist, rather than as a neutral antagonist [6]. The heterotrimeric G protein subtypes activated by 5-HT_{2A} and mGlu2 receptors are principally $G_{q/11}$ and $G_{i/o}$, respectively [12]. It has been shown that, in the presence of serotonin, the inverse agonist LY341495 not only decreases $G_{i/o}$-dependent signaling but also potentiates $G_{q/11}$-dependent signaling, and that this signaling crosstalk requires expression of 5-HT_{2A} and mGlu2 as a GPCR heteromeric complex [6]. Together, these findings suggest that the molecular mechanism by which chronic treatment with LY341495 down-regulates 5-HT_{2A} receptor density in mouse somatosensory cortex might be related to

increases in serotonin-dependent 5-HT_{2A} receptor-$G_{q/11}$ protein coupling through the 5-HT_{2A}-mGlu2 receptor complex. However, further work is needed to validate this hypothesis in mice in which 5-HT_{2A} and mGlu2 receptors are co-expressed but do not form a GPCR heteromer.

An interesting finding is the effect of chronic treatment with the mGlu2/3 antagonist LY341495 on [^{3}H]LY341495 binding. A potential explanation would be the presence of LY341495 in the CNS one day after the chronic treatment. However, it is tempting to speculate that this is not the case since LY341495, which is water-soluble, will probably be washed out during the protocol of plasma membrane preparation. In the case of somatostatin and μ-opioid receptors [22], and β_2-adrenergic and κ-opioid receptors [14], GPCR heteromerization regulates the agonist-induced endocytosis and redistribution of the receptors from the cell surface to intracellular compartments, suggesting that molecular proximity between GPCRs plays an important role in the localization and trafficking of these receptors. Based on these findings, the above mentioned crosstalk between 5-HT_{2A} and mGlu2 as a GPCR heteromer with which LY341495 potentiates 5-HT_{2A} receptor-dependent signaling (see [6]) may potentially be involved in the mechanisms responsible for down-regulation of mGlu2/3 receptors by chronic treatment with LY341495. Our findings in mGlu2-KO mice suggest that the mGlu2 receptor is necessary for the effects of chronic treatment with LY341495. However, since LY341495 binds with high affinity to both mGlu2 and mGlu3 receptors [10], further work is needed with mGlu3-KO mice to determine the potential role of the mGlu3 receptor in the effects of chronic treatment with LY341495 on 5-HT_{2A} and mGlu2 receptor densities.

In conclusion, our data support the hypothesis that chronic blockade of mGlu2 receptor-dependent signaling down-regulates 5-HT_{2A} receptor binding in mouse somatosensory cortex and its hallucinogenic-like cellular signaling and behavioral effects. Although hallucinogenic drugs as a model of schizophrenia have limitations [11,12], our findings have potential relevance to develop new methods for modulating 5-HT_{2A} receptor-dependent processes underlying non-drug-induced psychoses. Since the 5-HT_{2A} receptor has been involved in depression and anxiety-related behaviors [25], these results may also have implications with regard to LY341495 use in rodent models of antidepressant responses [1,4,15].

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