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Hallucinogens Recruit Specific Cortical 5-HT$_{2A}$ Receptor-Mediated Signaling Pathways to Affect Behavior

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SUMMARY

Hallucinogens, including mescaline, psilocybin, and lysergic acid diethylamide (LSD), profoundly affect perception, cognition, and mood. All known drugs of this class are 5-HT$_{2A}$ receptor (2AR) agonists, yet closely related 2AR agonists such as lisuride lack comparable psychoactive properties. Why only certain 2AR agonists are hallucinogens and which neural circuits mediate their effects are poorly understood. By genetically expressing 2AR only in cortex, we show that 2AR-regulated pathways on cortical neurons are sufficient to mediate the signaling pattern and behavioral response to hallucinogens. Hallucinogenic and nonhallucinogenic 2AR agonists both regulate signaling in the same 2AR-expressing cortical neurons. However, the signaling and behavioral responses to the hallucinogens are distinct. While lisuride and LSD both act at 2AR expressed by cortex neurons to regulate phospholipase C, LSD responses also involve pertussis toxin-sensitive heterotrimeric G$_{i/o}$ proteins and Src. These studies identify the long-elusive neural and signaling mechanisms responsible for the unique effects of hallucinogens.

INTRODUCTION

Throughout history, naturally occurring hallucinogenic substances such as psilocybin and mescaline have been recognized for their capacity to alter perception, emotion, and cognition (Nichols, 2004). All such hallucinogenic compounds (HCs) exhibit high affinity for 5-HT$_{2A}$ receptors (2AR) (Gonzalez-Maeso and Sealfon, 2006; Roth et al., 1998). Genetic or pharmacological inactivation of 2AR signaling blocks the behavioral effects of HCs in a variety of species, including mice, rats, and humans (Fiorella et al., 1995; Gonzalez-Maeso et al., 2003; Vollenweider et al., 1998). Taken together, these findings indicate that 2AR activation is necessary for the psychoactive effects of HCs.

The demonstration that HCs elicit their psychoactive effects via 2AR activation has not resolved the fundamental paradox that 2AR activation is a universally shared property of HCs and that 2AR activation is required for HC effects, but yet not all 2AR agonists exhibit hallucinogenic activity. Indeed, nonhallucinogenic compounds (NHCs) such as lisuride and ergotamine share significant structural similarities and comparable agonist activities at 2AR (Egan et al., 1998), but lack psychoactive properties (Pieri et al., 1978).

2AR is expressed widely in the central nervous system (CNS) and is expressed in structures involved in psychosis—the ventral striatum and ventral tegmental area (Li et al., 2004; Lopez-Gimenez et al., 1997, 2001; Nocjar et al., 2002; Pazos et al., 1985). Several of these structures have also been implicated in HC effects (Nielsen and Scheel-Kruger, 1986; Vetulani et al., 1979; Willins and Meltzer, 1997). However, the neuronal substrates that mediate hallucinogen effects remain obscure.

Our previous study of two HCs, lysergic acid diethylamide (LSD) and 1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane (DOI), suggests the hypothesis that HCs induce a characteristic, 2AR-dependent regulation of gene expression in mouse somatosensory cortex (SSC) (Gonzalez-Maeso et al., 2003). By extending our previous biochemical studies to a large group of diverse chemicals, we provide a basis for predicting hallucinogenic potential from effects in mouse. We find that HC and NHC 2AR agonists differ in their regulation of signaling and physiology in the same neurons in vivo and in vitro. By using

a genetic strategy to restore specifically 2AR-signaling capacity to cortical neurons in *htr2A*$^{-/-}$ mice, we show that the unique signaling and neurobehavioral effects of HCs do not result from their previously proposed regulation of subcortical-cortical circuits, but are intrinsic to 2AR-expressing cortical pyramidal neurons. We formulate a new model for the mechanism of action of HCs.

RESULTS

HCs Are 2AR Ligands in the Mouse

The responses to HC and NHC 2AR agonists have predominantly been studied in rats, primates, and humans. The effects of these drugs in mice have been less well studied. Because hallucinogens require 2AR activation, we sought to verify that HCs and NHCs exhibit affinities to mouse 2AR that are comparable with those seen in other experimental models. When assayed by displacement of [^{3}H]ketanserin from membranes prepared from mouse cortex (see Figure S1 in the Supplemental Data), all agonists displayed affinities that were consistent with rat and human values (Hoyer et al., 1994). Thus, the 2AR signaling system in mice presents a tractable model system to study the effects of HCs.

Acute Behavioral Responses Induced by HCs

Behavioral animal models cannot capture the perturbations of perception, cognition, and mood produced by HCs in humans. However, rodents might exhibit behavioral proxies of human hallucinogenic effects. Systemic administration of HCs in rats and nonhuman primates elicits several unconditioned effects: changes in exploratory behavior (Adams and Geyer, 1985a), grooming (Trulson and Howell, 1984), interruption of operant responding (Mokler and Rech, 1984), head twitch response (HTR), ear scratch response (ESR), and hyperthermia (Corne and Pickering, 1967; Darmani et al., 1990; Maj et al., 1977; Silva and Calil, 1975).

To identify a mouse model of acute hallucinogenic potential, we examined a variety of responses produced by several HCs and NHCs (Figure S2). Among the behavioral measures assayed, only two were elicited by HCs and not NHCs: the ESR and HTR. Other measures such as locomotion, rearing, grooming, and basal body temperature changes were inconsistently affected by HCs and NHCs (Figure S2 and Tables S1 and S2 in the Supplemental Data).

The HTR and ESR were both dependent upon the presence of 2AR signaling capacity, even at a high dose of DOI (Figure S2). However, the ESR was not elicited by every HC and was not normally distributed among individual mice—proving to be an "all or nothing" response. In contrast, HTR was reliably and robustly elicited by all HCs tested. Furthermore, HTR was not produced by NHCs, and was absent in *htr2A*$^{-/-}$ mice.

All 2AR agonists studied [DOI, 1-(2,5-dimethoxy-4-methylphenyl)-2-aminopropane (DOM), 1-(2,5-dimethoxy-4-bromophenyl)-2-aminopropane DOB (DOB), psilocin, mescaline, LSD, ergotamine, R-lisuride, and S-lisuride] penetrate the CNS after systemic administration. However, only the drugs with hallucinogenic potential in humans (Nichols, 2004) activated a significant HTR in *htr2A*$^{+/+}$ mice, but not in *htr2A*$^{-/-}$ mice (Figure 1A). The NHC 2AR agonists (ergotamine, R-lisuride, and S-lisuride) were inactive in both genotypes (Figure 1A). Thus, the HTR was used as a mouse behavioral proxy of human hallucinogenic potential in subsequent studies.

HCs Elicit a 2AR Signaling Response Distinct from NHCs

Consistent with psychoactive effects of certain 2AR agonists in humans, the HTR was produced only by the HCs, a subset of drugs with 2AR agonist properties. To explain this finding, we reasoned that HCs might interact with 2AR to recruit specific signaling pathways not activated by NHCs.

To test this hypothesis, we compared HC- and NHC-activation of multiple signal transduction pathways. Because signal transduction cascades ultimately regulate gene transcription (Ruf and Sealfon, 2004), we assessed HC and NHC signaling by measuring the "downstream" transcriptome responses elicited by HCs and NHCs in mouse SSC, a responsive and reliable tissue for assessing the cellular responses of HCs (Gonzalez-Maeso et al., 2003; Scruggs et al., 2000). We quantified the in vivo induction of 19 transcripts regulated by HCs and NHCs in *htr2A*$^{+/+}$ and *htr2A*$^{-/-}$ mice (Tables S3 and S4). Each agonist elicited a differential and reproducible response. To assess the predictive value of these gene induction responses on behavior, we used principal components analysis (PCA) to reduce a 19-dimensional signaling space (reflected by gene transcripts) to two axes (Figure 1B). Plotted in this way, the transcriptome responses of HCs and NHCs predicted their behavioral effects in *htr2A*$^{+/+}$ and *htr2A*$^{-/-}$ mice and their hallucinogenic potential in human (Figure 1B). We selected the most informative signaling response markers for distinguishing 2AR activation, as well as HC from NHC by statistical significance (Tables S5 and S6). Regulation of *c-fos* tracked with agonist activity at 2AR, and induction of *egr-2* and *egr-1* most robustly predicted behavioral activity of HCs in mouse (Figure 1C and Tables S7 and S8).

Activation of Transcriptome Response in Neurons that Express 2AR

The effects of HCs and NHCs on transcriptome activation indicated that these compounds interact with 2AR to activate distinct signaling pathways. This interpretation implies that the genes induced by HCs and NHCs should be regulated in neurons expressing 2AR. To test this idea, we prepared primary cultures of cortical neurons from *htr2A*$^{+/+}$ and *htr2A*$^{-/-}$ mouse embryos. Using fluorescent in situ hybridization (FISH), we found that both LSD and R-lisuride induced *c-fos* expression in neurons expressing 2AR mRNA, while only LSD (but not R-lisuride) induced *egr-2* expression in 2AR-expressing neurons (Figures 2A and 2B). FISH studies of primary cultures derived from

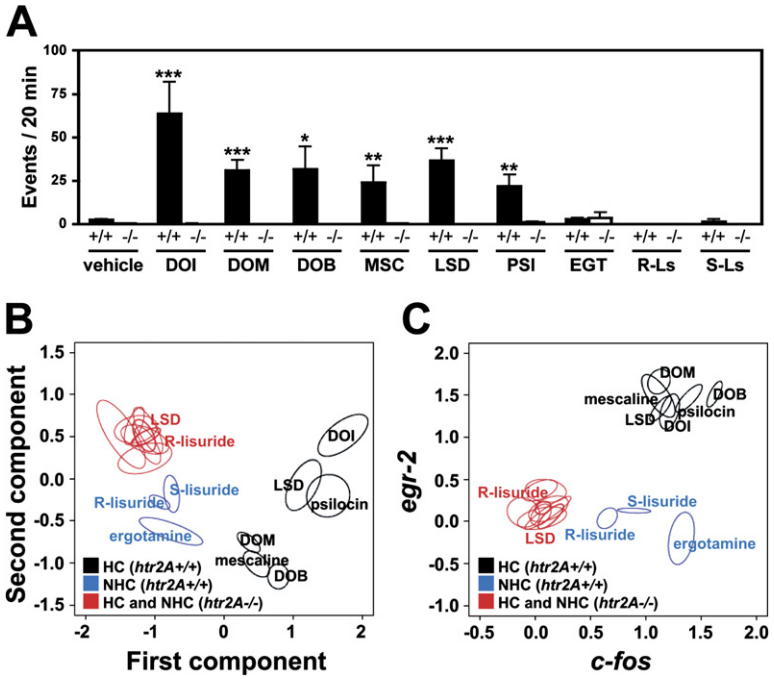

Figure 1. Specific Response of Hallucinogenic Drugs

(A) Behavioral response to HCs and NHCs. *htr2A*$^{+/+}$ (+/+) and *htr2A*$^{-/-}$ (−/−) littermates were injected (i.p.) with the tested compound, and HTR was scored (n = 6 per treatment group). Data are means ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001; Bonferroni's post hoc test of two-factor ANOVA. MSC, mescaline; PSI, psilocin; EGT, ergotamine; R-Ls, R-lisuride; S-Ls, S-lisuride.

(B) PCA in the quantified signaling reporters. The responses of the first two PCA axes differentiated the cellular effects induced by HCs and NHCs in *htr2A*$^{+/+}$ (n = 6–19 mice per group; see Table S3) and *htr2A*$^{-/-}$ (n = 5–10 mice per group; see Table S4) determined by qRT-PCR assays. Only the legends for LSD and R-lisuride are shown in *htr2A*$^{-/-}$ mice (thicker ellipses). The ellipse's center is the mean of the samples for a particular chemical, and the shape of the ellipse describes two-dimensional sample variance of the sample mean, which is the same as SEM for one-dimensional data.

(C) Comparison of the changes of expression (log_2) in the two most informative reporters, *c-fos* and *egr-2,* for HCs and NHCs in *htr2A*$^{+/+}$ and *htr2A*$^{-/-}$ mice. Only the legends for LSD and R-lisuride are shown in *htr2A*$^{-/-}$ mice (thicker ellipses). The ellipse's center is the mean of the samples, and the shape of the ellipse describes variance of the mean along each axis.

htr2A$^{-/-}$ mice showed no induction of these transcripts by LSD or R-lisuride.

We also studied the role of neuronal action potentials in HC signaling by determining transcriptome responses in the presence of tetrodotoxin (TTX). The suppression of cell firing by TTX did not affect the activation of gene transcripts in cortical primary culture, thus indicating that neuronal circuitry could not be involved in the induction of *egr-1* and *egr-2* mRNAs. This result indicates that LSD activation of *egr-1* and *egr-2* is intrinsic to neurons with 2AR signaling capacity (Figure S3).

We next used FISH to study whether the different HC and NHC 2AR-mediated responses also occurred in the same neurons in vivo. Consistent with other studies (Lopez-Gimenez et al., 2001), 2AR expression was most intense in layer V (Figures 2C and 2D), and was also observed in layers II/III (Figures S4 and S5). The 2AR agonist marker *c-fos* was induced in layers II/III and V by both LSD and R-lisuride. Nearly all *c-fos*-positive cells expressed 2AR mRNA (Figure 2C and Figure S5). LSD induced the HC-specific marker *egr-2* in layer V 2AR-positive neurons (Figure 2D), but not in layers II/III (data not shown). R-lisuride did not induce *egr-2* in SSC. No induction of *c-fos* or *egr-2* was observed in *htr2A*$^{-/-}$ mice injected with either LSD or R-lisuride (Figure S6).

Effect of Drug Dose on Transcriptome Response

To establish that the different response patterns obtained with HCs and NHCs could not be explained by differences in drug efficacy, we examined the induction of several transcripts in vitro and in vivo in response to a range of LSD and R-lisuride doses. As seen in Figure 3, R-lisuride induced *c-fos* at doses ranging from 1 to 10 μM in vitro and 0.4 to 0.8 mg/kg in vivo. R-lisuride did not induce *egr-1* or *egr-2* at any dose tested. In contrast, LSD induced *c-fos*, *egr-1*, and *egr-2* at doses ranging from 1 to 10 μM in vitro and from 0.24 to 0.96 mg/kg in vivo. These data show that R-lisuride cannot induce *egr-1* or *egr-2* at drug doses up to 10-fold greater than those sufficient to induce *c-fos*. Transcriptome regulation was absent at all doses tested in *htr2A*$^{-/-}$ mice and primary cortical cultures from *htr2A*$^{-/-}$ mice.

Electrophysiological Response to HCs and NHCs

To determine whether the HC-signaling signature reflects distinct changes in neuronal physiology, we examined the HC and NHC effects on voltage-gated currents in SSC slices prepared from *htr2A*$^{+/+}$ and *htr2A*$^{-/-}$ mice. We used standard whole-cell patch-clamp recordings on individual layer V pyramidal neurons to study the voltage-gated currents in the pyramidal neurons in the presence of TTX. A voltage ramp from −110 mV to −30 mV activated a wide range of ionic currents in these cells that was altered by the application of HCs in most of the cells from *htr2A*$^{+/+}$ and *htr2A*$^{-/-}$ mice. Since the changes in current were heterogeneous, we subtracted the HC responses from baseline responses in the same neuron (Figure 4B and Figure S7). The resulting HC-induced

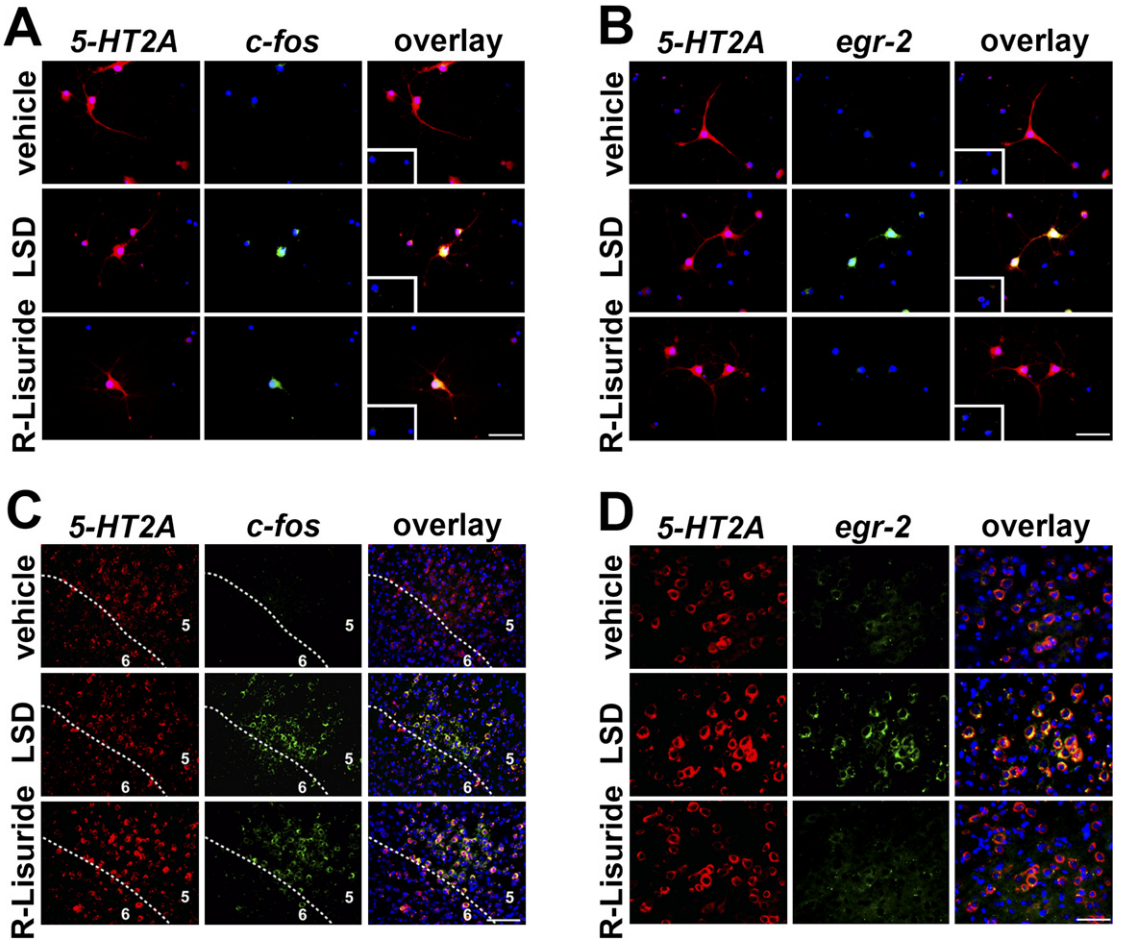

Figure 2. Colocalization of *2AR* and the Genomic Markers *c-fos* and *egr-2* in Mouse SSC and Cortical Primary Cultures

(A and B) Double-label FISH in cortical primary cultures treated for 45 min with vehicle, LSD (10 μM), or R-lisuride (10 μM). (Insets) *htr2A*$^{-/-}$ mice cortical primary cultures. Red, green, and blue colors indicate *2AR*, *c-fos* (A) or *egr-2* (B), and nucleus (DAPI), respectively. (C and B) Double-label FISH was performed in SSC cortex in mice treated (i.p.) with vehicle, LSD, or R-lisuride. Red, green, and blue colors indicate *2AR*, *c-fos* (C) or *egr-2* (D), and nucleus (DAPI), respectively. (C) shows layers 5 and 6; (D), layer 5. Scale bars: (A, B, and D), 40 μm; (C), 60 μm.

responses were divided into three groups based on their magnitude: small (including no change), medium, and large (see Table S9). Small and medium currents were observed in cells from both *htr2A*$^{+/+}$ and *htr2A*$^{-/-}$ mice, indicating that they were 2AR-independent. However, large currents were only observed in cells from *htr2A*$^{+/+}$ mice treated with LSD (Figures 4B and 4D and Table S9). These LSD-induced large currents were seen in approximately 25% of the cells tested, and exhibited an average charge of 334 ± 97 C and a reversal potential of −67.5 ± 2.4 mV, suggesting that they represented the sum of several different currents. This large current was never observed with R-lisuride (Figures 4C and 4D).

Lisuride and LSD Compete for the Same Receptor Target

Our findings demonstrate that both LSD and R-lisuride require a functional 2AR to generate their signaling profiles. R-lisuride displaces [^{125}I]LSD with high affinity from 2AR-expressing membrane preparations (Figure 4A), suggesting that they compete for the same binding site. However, we reasoned that it is possible that LSD and R-lisuride might interact with receptor targets other than 2AR, which would explain their distinct effects on signaling and behavior. To test this possibility, we first studied whether R-lisuride demonstrated competitive and/or additive effects with LSD in different measures. We found that R-lisuride blocked the LSD-induced large currents in SSC neurons (Figures 4C and 4D). Furthermore, we observed that pretreatment of mice with R-lisuride prevented LSD-mediated induction of *egr-1* and *egr-2* in vivo, whereas the induction of *c-fos* was unaffected (Figure 4E). The combined effects of LSD and R-lisuride administration on *c-fos* induction were not additive, a result incompatible with a model where each drug stimulates *c-fos* through different receptors. Furthermore, LSD-induced HTR (Figure 4F) was prevented when the animals were first pretreated with R-lisuride. These

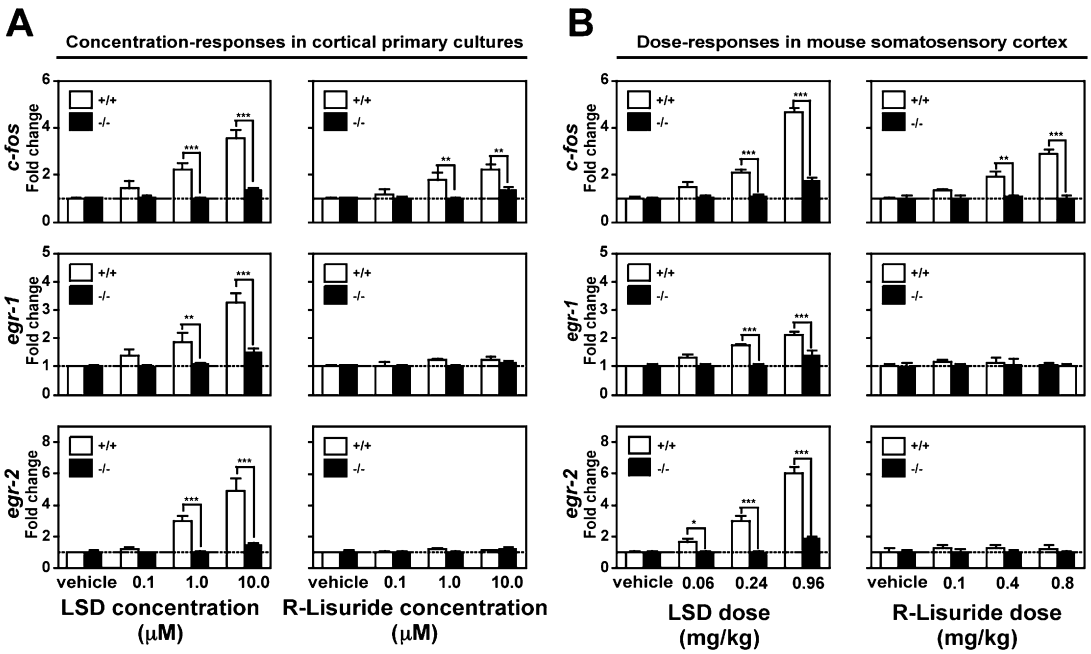

Figure 3. Comparable 2AR-Dependent Signaling Responses In Vivo and in Cortical Primary Cultures

(A) Concentration-response curves of LSD- or R-lisuride-induced cellular response in cortical primary culture assayed by qRT-PCR. *htr2A*$^{+/+}$ (+/+) or *htr2A*$^{-/-}$ (−/−) mouse cortical primary cultures were treated for 45 min with the indicated concentration (n = 4–12 per group).

(B) Dose-response curves of LSD- or R-lisuride-induced cellular response in mouse SSC cortex assayed by qRT-PCR. *htr2A*$^{+/+}$ (+/+) or *htr2A*$^{-/-}$ (−/−) mice were injected (i.p.) with the doses indicated (n = 4–8 mice per group). Drug-induced changes in expression levels are reported as fold change over vehicle. Data are means ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001; Bonferroni's post hoc test of two-factor ANOVA.

pharmacological data suggest that R-lisuride and LSD target the same population of 2AR to induce distinct effects on electrophysiology, intracellular signaling, and behavior.

Lisuride is not simply a 2AR agonist, but also interacts with a variety of central monoamine receptors, including dopamine and 5-HT$_{1A}$ receptors. These other receptors have been proposed as candidates targeted by lisuride that may integrate with 2AR signaling to prevent the induction of hallucinogenic response (Callahan and Appel, 1990; Marona-Lewicka et al., 2002).

In order to evaluate the potential role of dopaminergic receptors in the modulation of the responses to LSD elicited by R-lisuride, we tested the cellular response in SSC in vivo and the behavioral response induced by LSD after preinjection with SKF82958 or quinpirole, D$_1$-like and D$_2$-like receptor agonists, respectively. Neither of the dopaminergic agonists alone induced *egr-1* or *egr-2* in mouse cortex (Figure 5A), nor did they affect HTR (Figure 5B). The expression of *c-fos* was unaffected by quinpirole, whereas *c-fos* was induced by SKF82958 (Figure 5A). The combined effects of SKF82958 and LSD administration on *c-fos* induction were additive, which was opposite to the effects of coadministration of R-lisuride and LSD (see above). When combined with LSD, neither SKF82958 nor quinpirole affected the induction of the HC-specific *egr-1* or *egr-2* genes (Figure 5A). Furthermore, the LSD-induced HTR was not affected by either SKF82958 or quinpirole (Figure 5B). These results suggest that the effects of LSD and lisuride arise from interaction with the same 2AR target, and that their effects do not result from activity at dopamine receptors.

Lisuride is a full agonist in modulation of [^{35}S]GTPγS binding in membranes of CHO cells expressing 5-HT$_{1A}$ receptors (McLoughlin and Strange, 2000). Several behavioral assays have also confirmed the high efficacy of lisuride at 5-HT$_{1A}$ receptors (Millan et al., 1991; Rex et al., 1998). It has been suggested that coactivation of 5-HT$_{1A}$ receptors (*htr1A*) and 2AR by lisuride could explain why lisuride acts as a 2AR agonist, yet lacks hallucinogenic properties (Marona-Lewicka et al., 2002). We tested this hypothesis by examining the behavioral effects of LSD and R-lisuride in *htr1A*$^{+/+}$ and *htr1A*$^{-/-}$ mice. Because both of these compounds are 2AR agonists, coactivation of 5-HT$_{1A}$ receptors by LSD or R-lisuride should attenuate their hallucinogenic properties. Thus, the behavioral responses to each compound should be augmented in the *htr1A*$^{-/-}$ mice. As shown in Figure 5C, this was not the case. There was no significant effect of genotype on the behavioral response to either R-lisuride or LSD. Taken together, our results suggest that neither dopamine nor 5-HT$_{1A}$ receptor activation functionally antagonize 2AR-dependent effects mediated by R-lisuride, and indicate that R-lisuride and LSD interact with the same molecular target to induce distinct effects on electrophysiology, intracellular signaling, and behavior.

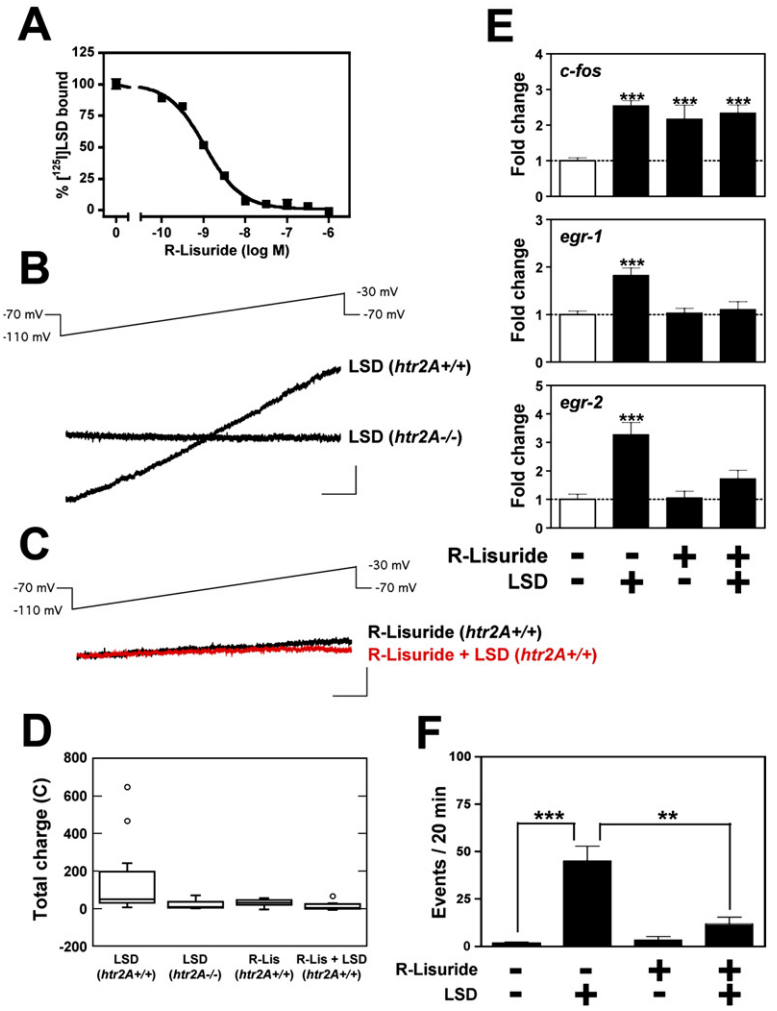

Figure 4. LSD and R-Lisuride Target the Same Population of 2AR

(A) [^{125}I]LSD competition curve by R-lisuride. R-lisuride displaced [^{125}I]LSD in membrane preparations from HEK293 cells stably expressing human 2AR.

(B) Effect of LSD on voltage ramp-activated ionic currents. Residual LSD-evoked currents were observed in single neurons in *htr2A*$^{+/+}$ and *htr2A*$^{-/-}$ mice, after subtraction of current prior to LSD (1 μM) application (holding potential [Vh] = −70 mV, ramp from −110 to −30 mV in 3.5 s).

(C) R-lisuride (5 μM) blocked the effect of LSD on currents elicited by voltage ramp. Application of R-lisuride did not alter the baseline responses in neurons from *htr2A*$^{+/+}$ mice, but abolished the effect of LSD. Scale bars, 100 pA, 500 ms.

(D) Summary of currents elicited by voltage ramp in box plots (n = 11–19). Responses with large amplitude seen in neurons from *htr2A*$^{+/+}$ mice were absent in neurons after preincubation with R-lisuride ($p < 0.05$), or in neurons from *htr2A*$^{-/-}$ mice ($p < 0.05$).

(E) Cellular response was determined in SSC in mice injected with LSD 15 min after being preinjected with a high dose of R-lisuride (0.8 mg/kg; n = 5–8 per group). Data are means ± SEM. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

(F) HTR was determined in mice injected with LSD 15 min after being preinjected with a high dose of R-lisuride (0.8 mg/kg; n = 3–6 per group). R-lisuride blocks the LSD-inducted behavioral response. Data are means ± SEM. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$; ANOVA with Bonferroni's post hoc test.

Receptor Signaling Response to HCs and NHCs

HCs and NHCs induce different transcriptome responses in cortical neurons that express 2AR. We next investigated the signaling cascade responsible for the unique transcriptional responses elicited by HCs. 2AR activates phospholipase C-β (PLC-β) via heterotrimeric $G_{q/11}$ proteins, leading to increased accumulation of inositol phosphates and intracellular calcium mobilization (Barnes and Sharp, 1999). We first examined the role of this pathway in the HC-specific responses in primary cortical cultures by studying the gene responses after pretreatment with the PLC-β inhibitor U73122. Inhibition of PLC-β eliminated the gene response obtained by either LSD or R-lisuride (Figure 6A). These results suggest that the classical $G_{q/11}$-PLC-β 2AR signaling pathway is required for the response to HCs, but is not unique and specific to HCs.

We previously discovered that the neuroprotective effects of some agonists acting at the dopamine D_2 receptor result from the capacity of these agonists to regulate a signaling pathway in addition to the classical $G_{i/o}$ protein pathway that is known to couple with that receptor (Nair and Sealfon, 2003). The classical PLC-β response is regulated by both HCs and NHCs acting at 2ARs. HCs elicit unique 2AR-dependent gene, signaling pattern, and behavioral responses (see above). We speculated that the HC 2AR complexes might cause regulation of an additional signaling pathway that is not affected by NHC 2AR complexes. Some reports have implicated pertussis toxin- (PTX-) sensitive $G_{i/o}$ proteins in the cellular responses mediated by 2AR (Kurrasch-Orbaugh et al., 2003; Raymond et al., 2001). To explore the consequences of $G_{i/o}$ protein inhibition on the 2AR-induced transcriptome changes, the cortical primary cultures were pretreated with PTX. While PTX did not affect the gene responses to R-lisuride, it greatly attenuated the responses elicited by LSD (Figure 6B). These results suggest that the gene response elicited by LSD requires a coactivation of $G_{i/o}$ and $G_{q/11}$ proteins that is unique to HCs.

In several cell types, $G_{i/o}$ protein-regulated phosphorylation networks involve $G_{\beta\gamma}$ subunit-mediated activation of Src (Banes et al., 1999; Crespo et al., 1994; Gutkind, 2000; Quinn et al., 2002). Therefore, we tested the effects of Src inhibition on the 2AR-mediated gene responses

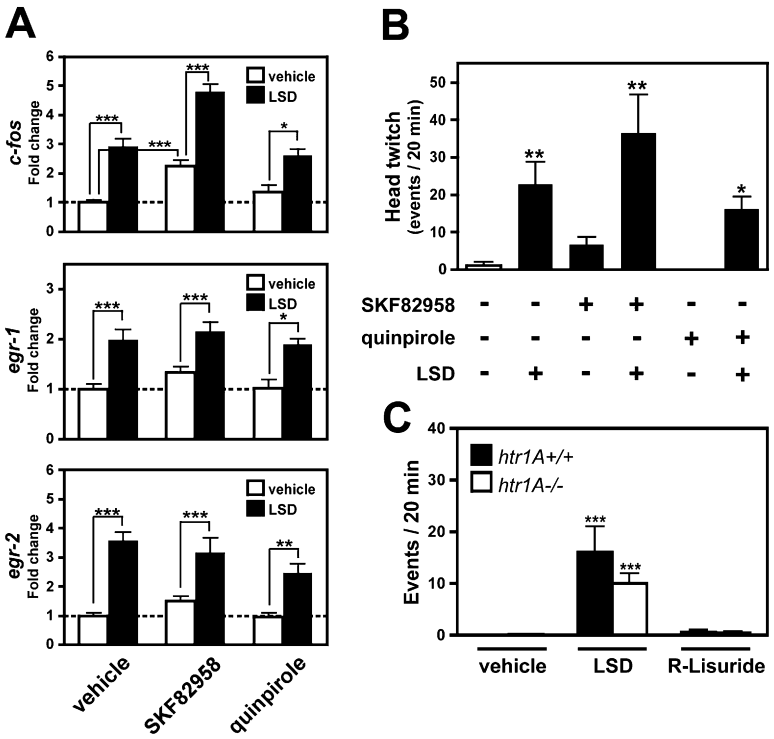

Figure 5. Effect of D_1-Like, D_2-Like, and 5-HT_{1A} Receptor Signaling on the Responses to LSD

(A) Cellular response was determined in SSC in mice injected with vehicle or LSD 15 min after being preinjected with vehicle, SKF82958 (1 mg/kg), or quinpirole (1 mg/kg). Data are means ± SEM (n = 6–8 per group). *p < 0.05, **p < 0.01, ***p < 0.001; ANOVA with Bonferroni's post hoc test.

(B) HTR was determined in mice injected with vehicle or LSD 15 min after being preinjected with vehicle, SKF82958 (1 mg/kg), or quinpirole (1 mg/kg). Data are means ± SEM (n = 7–11 per group). *p < 0.05, **p < 0.01, ***p < 0.001; ANOVA with Bonferroni's post hoc test.

(C) Behavioral response (HTR) of *htr1A*$^{+/+}$ and *htr1A*$^{-/-}$ mice to LSD (0.24 mg/kg) and R-lisuride (0.4 mg/kg). Data are means ± SEM. n = 4 to 7 per genotype. ***p < 0.001 for drug versus vehicle; not significant for genotype effects.

induced by LSD and lisuride. The gene response pattern to R-lisuride was unaffected by inhibition of Src. However, the response to LSD in the presence of Src inhibitor was dramatically affected so as to be indistinguishable from the response to R-lisuride (Figure 6B). G protein-coupled receptors (GPCRs) may also activate phosphatidylinositol-3 kinases (PI-3-K) via $G_{\beta\gamma}$ subunits (Gutkind, 2000). Pretreatment with the PI-3-K inhibitor LY294002 had no

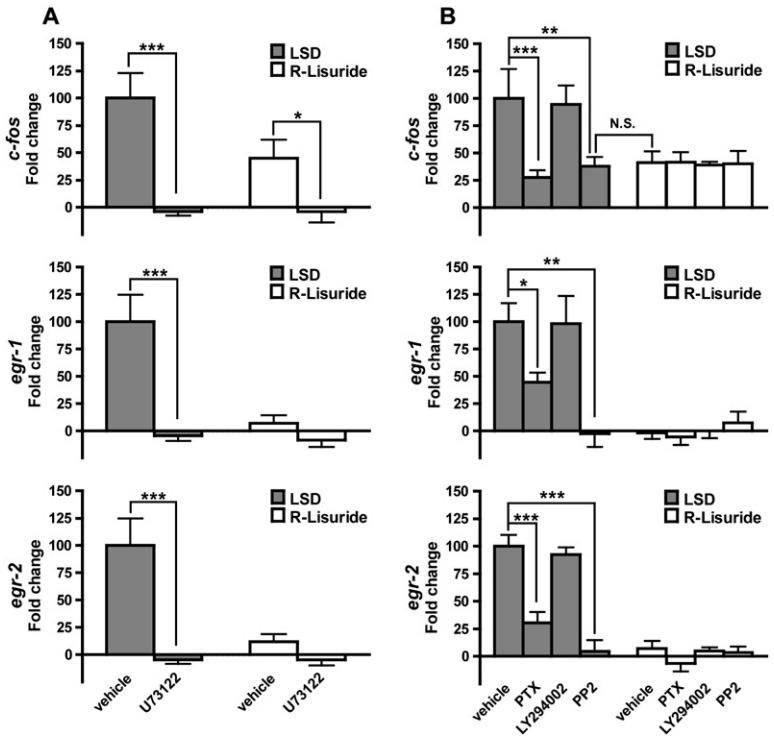

Figure 6. LSD-Specific Signaling in Primary Cortical Neurons

(A) Gene regulation by both LSD and R-lisuride is eliminated by inhibition of PLC-β with U73122 (10 μM; 30 min). (B) The gene induction pattern induced by LSD is specifically attenuated by inhibition of $G_{i/o}$ protein with PTX (100 ng/ml, overnight), and by inhibition of Src with PP2 (10 μM; 30 min). The responses to both drugs are not affected by inhibition of PI-3-K with LY294002 (10 μM; 30 min). Note that the responses to R-lisuride are unaffected by Src inhibition, and that the responses to LSD and R-lisuride are identical in the absence of Src activity. Data are means ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001; ANOVA with Bonferroni's post hoc test (n = 6–12 per group).

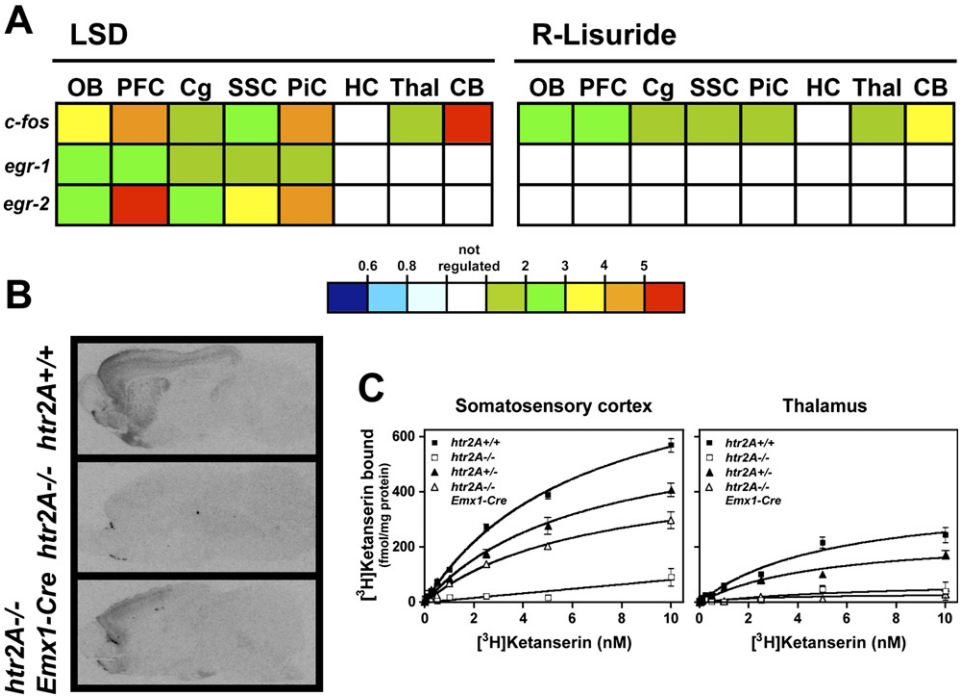

Figure 7. Restoration of Cortical Expression of 2AR Recreates Wild-Type Expression Pattern and Binding Capabilities

(A) Brain mapping of the genomic markers induced by LSD and R-lisuride. OB, olfactory bulb; PFC, prefrontal cortex; Cg, cingulate cortex; SSC, somatosensory cortex; PiC, piriform cortex; HC, hippocampus; Thal, thalamus; CB, cerebellum (n = 6 per group; see Tables S10 and S11).

(B) [^{125}I]DOI autoradiography in *htr2A*$^{+/+}$, *htr2A*$^{-/-}$, and *htr2A*$^{-/-}$*:Emx1-Cre*$^{+/-}$ mice.

(C) [^{3}H]Ketanserin saturation curves in *htr2A*$^{+/+}$, *htr2A*$^{+/-}$, *htr2A*$^{-/-}$, and *htr2A*$^{-/-}$*:Emx1-Cre*$^{+/-}$ mice SSC membranes.

effect on either the LSD- or R-lisuride-mediated transcriptome response. These results indicate that 2AR activation by LSD causes a specific regulation of $G_{i/o}$ protein and Src that is responsible for the unique HC pattern of gene induction.

Neuronal Specificity of HC-Specific Signaling Pathways

Although we used SSC as our experimental model of HC-induced signaling and electrophysiology, it remained unclear whether the HC signaling response could be produced by 2AR expressed on other neuronal populations in the CNS. To address this question, we examined *egr-1*, *egr-2*, and *c-fos* induction by LSD and R-lisuride in several 2AR-expressing brain regions (Figure 7A, Tables S10 and S11). With the exception of the hippocampus, LSD and R-lisuride activated *c-fos* in all brain regions examined. LSD induction of *egr-1* and *egr-2* was observed in cortical structures and the olfactory bulb (Figure 7A, Tables S10 and S11), but not in structures such as the thalamus or cerebellum. In contrast, R-lisuride failed to induce *egr-1* or *egr-2* in any brain region examined. This finding indicates that certain populations of neurons may be unique in their capacity to generate 2AR-mediated HC signaling. Moreover, these findings implicate cortical 2AR signaling as a likely mediator of HC effects on behavior.

Genetic Restoration of 2AR Expression to the Cortex of *htr2A*$^{-/-}$ Mice Rescues HC-Induced Signaling and Behavior

To test the hypothesis that cortical 2AR signaling is integral to the effects of HCs, we used a genetic strategy to selectively restore 2AR expression to the cortex of *htr2A*$^{-/-}$ mice. The *htr2A*$^{-/-}$ mice used in this study contain a transcriptional/translational termination sequence inserted between the promoter and the first coding exon (Figure S8). This so-called "stop" cassette was flanked by lox-P sequences, allowing it to be excised by the bacteriophage P1 recombinase, Cre. To restore 2AR expression to the cortex of *htr2A*$^{-/-}$ mice, we crossed them with a second line of mice expressing Cre recombinase under the control of the *Emx1* promoter. The homeobox gene *Emx1* is expressed in the forebrain during early brain maturation (Boncinelli et al., 1993), and has been used previously to control forebrain gene expression (Iwasato et al., 2000).

Because the *htr2A* coding region remains under the control of its endogenous promoter, rescue of 2AR expression was expected to occur only in tissues where the expression patterns of the *htr2A* and *Emx1* promoters overlap. *Emx1* is expressed in glutamatergic neurons of the forebrain (Gorski et al., 2002); thus, we predicted that *Emx1-Cre* should restore 2AR expression only to cortical glutamatergic neurons (one of the only structures

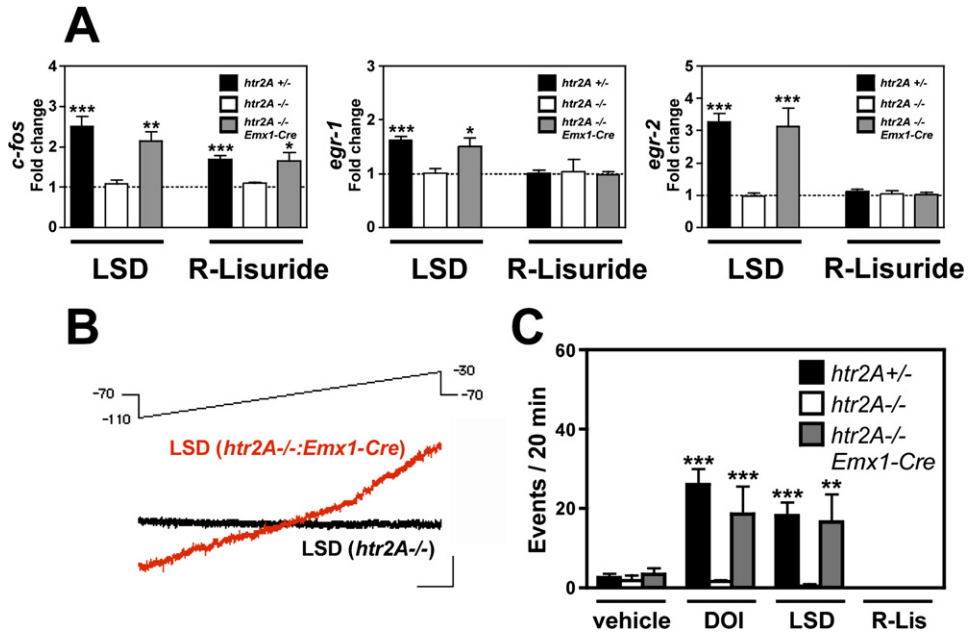

Figure 8. Cortical 2AR Function Mediates Cellular and Behavioral Responses Induced by Hallucinogens

(A) *htr2A*$^{+/+}$, *htr2A*$^{-/-}$, and *htr2A*$^{-/-}$*:Emx1-Cre*$^{+/-}$ mice were injected with vehicle, LSD, or R-lisuride, and the cellular response was determined in SSC by qRT-PCR (n = 4–8 per group). Data are mean ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001.

(B) Effect of LSD on voltage ramp-activated ionic currents in *htr2A*$^{-/-}$ and *htr2A*$^{-/-}$*:Emx1-Cre*$^{+/-}$ mice. Scale bars, 100 pA, 500 ms (n = 16).

(C) *htr2*$^{+/-}$, *htr2A*$^{-/-}$, and *htr2A*$^{-/-}$*:Emx1-Cre*$^{+/-}$ mice were injected with vehicle, LSD, or R-lisuride, and the head twitch behavioral response was scored (n = 4–6 per group). Data are mean ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001; Bonferroni's post hoc test of two-factor ANOVA.

where 2AR and Emx1 are coexpressed). We confirmed the predicted cortical pattern of 2AR expression in the rescue line using [^{125}I]DOI autoradiography and radioligand binding assays (Figure 7). Using FISH, we also determined that expression of 2AR was localized primarily to pyramidal cortical neurons (data not shown). In contrast, 2AR expression was absent in striatum, thalamus, and amygdala—tissues expressing 2AR, but not *Emx1* (Figures 7B and 7C).

Because of the breeding strategy required to generate *htr2A*$^{-/-}$*:Emx1-Cre*$^{+/-}$ mice (see Experimental Procedures), they are heterozygous for 2AR expression in cortex. We found that the level of 2AR expression in the cortex of *htr2A*$^{-/-}$*:Emx1-Cre*$^{+/-}$ mice was comparable to that in *htr2A*$^{+/-}$ mice, indicating a high efficiency of *Cre*-mediated recombination (Figure 7C). We have previously found that loss of a single allele in *htr2A*$^{+/-}$ mice does not interfere with their behavioral response to HCs (data not shown). Therefore, *htr2A*$^{+/-}$ littermates were deemed appropriate controls for testing *htr2A*$^{-/-}$*:Emx1-Cre*$^{+/-}$ mice.

Restoration of 2AR in the cortex of *htr2A*$^{-/-}$*:Emx1-Cre*$^{+/-}$ mice was sufficient to rescue signaling and behavioral responses to LSD (Figure 8). Specifically, LSD activated HTR behavior, electrophysiological response, and the HC-specific markers *egr-1* and *egr-2* in *htr2A*$^{-/-}$*:Emx1-Cre*$^{+/-}$ mice to levels seen in *htr2A*$^{+/-}$ mice. The specificity of the hallucinogen properties was maintained in *htr2A*$^{-/-}$*:Emx1-Cre*$^{+/-}$ mice, as R-lisuride activated neither HTR nor *egr-1/egr-2* expression in cortical rescue or *htr2A*$^{+/-}$ control mice.

DISCUSSION

Our study addresses the mechanisms by which HCs elicit their psychoactive effects. We found that while both HCs and NHCs show agonist activity at cortical 2AR in vivo, the HCs elicit a characteristic and predictive signaling response not shared by NHCs. The HC transcriptome signaling signature was absent in *htr2A*$^{-/-}$ mice, but was produced both in *htr2A*$^{+/+}$ mice and in their cultured cortical neurons. Inhibitor studies in primary neuronal cultures revealed that the HC-characteristic transcriptome response to LSD depends on its specific regulation of $G_{i/o}$ proteins and Src. We also demonstrated that genetic restoration of 2AR signaling capacity to cortical neurons of *htr2A*$^{-/-}$ mice was sufficient to rescue the HC-specific signaling signature and behavioral response.

Implications for Other Hypotheses of HC Effects

In this study, our observations point to a specific HC interaction with cortical 2AR to explain its unique properties. However, other hypotheses have been proposed to account for the different effects of HC and NHC 2AR agonists on behavior. In the course of this study, we have tested and eliminated several of these alternative explanations for our results.

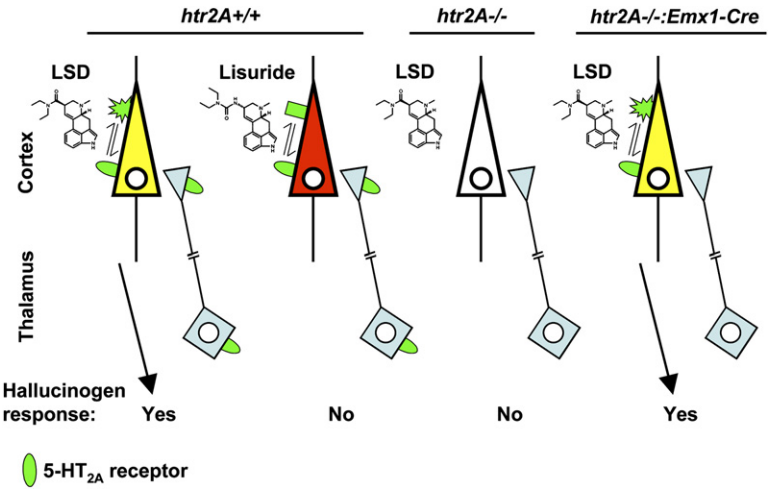

Figure 9. Model of the Mechanism Underlying HC-Specific-Induced Behavioral Response

This schematic presents a model for the mechanism of HCs that is consistent with our experimental results. The two left panels illustrate the responses in *htr2A*$^{+/+}$ mice. Both LSD and lisuride act at receptors expressed by cortical pyramidal neurons. However, the receptor conformation stabilized by LSD is proposed to be distinct, leading to the different pattern of cellular signaling and physiological and neurobehavioral responses observed. The characteristic cellular signaling responses to LSD and lisuride were also obtained in cortical primary cultures (not illustrated). The two right panels show the interpretation of the results obtained in the genetic models studied. The cellular signaling and physiological and behavioral responses to HCs were eliminated in *htr2A*$^{-/-}$ mice, but rescued by genetic restoration of 2AR to cortical pyramidal neurons (*htr2A*$^{-/-}$*:Emx1-Cre*$^{+/-}$ mice). As the effects of HCs are restored in the cortical rescue mouse, the unique effects of HCs cannot involve regulation of subcortical-cortical circuits and must be intrinsic to 2AR-expressing cortical neurons. Thus, HCs and NHCs act at 2AR on the same cortical pyramidal neurons, but only HCs stabilize a receptor conformation that recruits the signaling pathways responsible for their neurobehavioral effects.

Lisuride is well known to have significant agonist activity at dopamine receptors (Millan et al., 2002; Newman-Tancredi et al., 2002). Some researchers have suggested that the differences between LSD and lisuride result from the greater dopamine receptor agonism of lisuride (Adams and Geyer, 1985b; Callahan and Appel, 1990; Horowski and Wachtel, 1976). However, we find that dopaminergic receptor activation does not affect the 2AR-dependent responses selectively induced by LSD (See Figure 5). The finding that the induction of *c-fos* was synergistically induced by LSD and SKF82958, but not by LSD and R-lisuride, suggests that R-lisuride does not modulate the induction of *c-fos* in cortex by targeting D_1-like receptors.

It has also been proposed that 5-HT_{1A} receptor stimulation counteracts 2AR effects on HC-specific behaviors (Marona-Lewicka et al., 2002). We examined this hypothesis by assessing the effect of R-lisuride and LSD in mice lacking 5-HT_{1A} receptors. We found that R-lisuride did not significantly induce HTR in *htr1A*$^{+/+}$ or *htr1A*$^{-/-}$ mice. The activating effects of LSD on HTR behavior were comparable between *htr1A*$^{+/+}$ and *htr1A*$^{-/-}$ mice. These findings indicate that the 5-HT_{1A} receptor signaling properties of HCs and NHCs do not affect their potential to elicit a behavioral response in mice. Overall, these results further support the hypothesis that LSD and R-lisuride target the same population of 2AR in mouse cortex, yielding different responses. Notably, some cellular responses (e.g., $I\kappa B\alpha$ induction in SSC) or behavioral responses (e.g., grooming) induced by R-lisuride in *ht2A*$^{-/-}$ mice might result from activity at dopaminergic and/or 5-HT_{1A} receptors.

Another departure from prior paradigms was our finding that *c-fos* was induced primarily in neurons that express 2AR. Earlier reports have suggested that *c-fos* is induced by HCs mostly in neurons that do not express 2AR, implying an indirect mechanism for HC effects (Gresch et al., 2002; Scruggs et al., 2000, 2003). Here we found that this is not the case. These divergent results may be explicable by our use of FISH, rather than immunohistochemistry, as our primary method of assessing 2AR expression.

Neuronal Target of HCs

Our findings also bear on the hypothesized role of thalamic 2AR in the mechanism of action of HCs. Previous studies have suggested that 2AR expressed on thalamocortical axon terminals activates glutamate release in the cortex (Marek et al., 2001; Scruggs et al., 2000, 2003). Our *htr2A*$^{-/-}$*:Emx1-Cre*$^{+/-}$ rescue line shows a selective restoration of 2AR expression in the cortex. As 2AR expression is not restored in the thalamus, 2AR should not be expressed on the cortical terminals of thalamic neurons in these mice. The expression of 2AR in the cortex neurons alone was sufficient to elicit HC-specific behavioral and signaling effects (Figure 8). In contrast, 2AR restoration exclusively to the thalamus did not restore HC-induced behaviors (data not shown). Although previous studies implied that the effects of HCs require the release of glutamate (see above), our data suggest that the circuit involved in this effect is a cortical-cortical circuit and does not require activation of a thalamocortical pathway. In addition, HC-specific signaling patterns could be obtained in primary cortical cultures in the absence of action potentials and thalamic neurons. The HC-specific gene signature was observed in neurons that express 2AR mRNA both in vivo and in primary culture experiments. Thus, several lines of evidence indicate that cortical pyramidal neurons expressing 2AR are the cellular targets responsible for HC effects (Figure 9).

Implications of Distinct HC Signaling at Cortical 2AR

We found that HCs and NHCs activate the expression of overlapping, but distinct, genes. For example, we

observed that the induction of *c-fos* was a marker of signaling common to all 2AR agonists. On the other hand, *egr-1* and *egr-2* were induced by all HCs used in our study, but not by any NHC tested. However, the regulation of the upstream pathways and not the changes in gene expression are responsible for the physiological and behavioral response to HCs. This view is consistent with our observation that HTR was activated within minutes of drug administration, whereas gene induction often reached maximal levels at about 1 hr (Gonzalez-Maeso et al., 2003). Moreover, mice deficient in *egr-1* exhibit normal behavioral responses to DOI (data not shown).

We took advantage of the consonant differences in the gene expression patterns obtained both in vivo and in vitro by LSD and R-lisuride to identify the pathways responsible for the unique effects of HCs. Inhibition of PLC-β fully abolished the responses to both LSD and R-lisuride. However, PTX only affected the responses to LSD, without affecting the response to R-lisuride. These data indicate that 2AR generates different heterotrimeric G protein-dependent signaling responses when activated by LSD or R-lisuride.

Additional experiments were conducted to explore the HC-mediated neuronal signaling pathways. In the presence of 3-(4-chlorophenyl) 1-(1,1-dimethylethyl)-1H-pyrazolo[3,4-d]pyrimidin-4-amine (PP2), which specifically inhibits Src, the transcriptome response induced by LSD in cortical primary culture was indistinguishable from the one induced by R-lisuride. These data map out a 2AR-dependent signaling cascade specifically activated by HCs, linking $G_{i/o}$ protein and Src. The effects of Src inhibition on the HC-specific component of the transcriptome signaling signature are more complete than the effects of PTX. This observation raises the possibility that the LSD-induced signaling between 2AR and Src may involve parallel pathways in addition to $G_{i/o}$ proteins that are not yet identified. Nonetheless, as discussed below, these findings provide strong support for the hypothesis that the remarkable neuropsychological responses to HCs result from their stabilization of specific 2AR conformational states, leading to distinct regulation of intracellular signaling pathways.

Agonist Trafficking as a Potential Mechanism of Action of HCs

Our results support the hypothesis that HCs and NHCs, while acting at the same 2AR expressed by cortical neurons, elicit different patterns of signaling that are responsible for their different behavioral activities.

The interpretation that different agonists acting at 2AR regulate different signaling pathways is consistent with recent pharmacological theory and experiments. The standard pharmacological model of GPCR activation, the ternary complex model (De Lean et al., 1980), postulates that the efficacy of ligands depends on their ability to shift the equilibrium between two conformational states: inactive and active (Kenakin, 2002). However, both theory and experimental evidence suggest that GPCRs adopt multiple conformations when activated by different agonists (Berg et al., 1998; Ghanouni et al., 2001; Gonzalez-Maeso et al., 2003; Nair and Sealfon, 2003; Prioleau et al., 2002). This expanded version of the ternary complex model posits that different receptor agonists stabilize distinct conformations that preferentially recruit and activate specific signaling pathways (Kenakin, 2003; Urban et al., 2006). Our observations are consistent with such a model as it explains how distinct cellular responses could be produced by HC and NHC interactions with cortical 2AR (Figure 9). As predicted by this model, the activation of HC-specific gene activation patterns was found overwhelmingly in 2AR-expressing neurons, indicating that these signaling pathways were intrinsic to 2AR activation and not the result of indirect activation via action potentials or neuronal circuits.

Concluding Remarks

Our data suggest that cortical neurons may be unique in their capacity to generate the specific signaling pathways of HCs. 2AR is expressed in subcortical brain regions of *htr2A*$^{+/+}$ mice, yet 2AR expression was not rescued in these areas of *htr2A*$^{-/-}$*:Emx1-Cre*$^{+/-}$ mice, indicating that subcortical populations of 2AR are not required for HC actions. 2AR is most highly expressed in layer V pyramidal neurons (Lopez-Gimenez et al., 2001). Because layer V functions as the "output" layer of the cortex (Sapienza et al., 1981), it has been hypothesized that layer V neurons engage in "gating" functions that are of critical importance to the proper balance between cortical-subcortical communications (Barkai and Hasselmo, 1994). It is reasonable to suspect that 2AR-mediated HC signaling may perturb the normal gating functions of layer V cortex, disrupt cognition and sensory processing, and lead to the characteristic HC effects on behavior.

The strategy we developed to elucidate HC action should be applicable to CNS-active compounds, with therapeutic potential in other disorders. Thus, our findings may advance the understanding of neuropsychiatric disorders that have specific pharmacological treatments whose mechanisms of action are not fully understood.

EXPERIMENTAL PROCEDURES

Experimental Animals

Experiments were performed on adult (8–12 weeks old) male 129S6/SvEv mice. For experiments involving genetically modified mice, *htr2A*$^{+/+}$ or *htr2A*$^{+/-}$ littermates were used as controls. Animals were housed in 12 hr light/dark cycle at 23°C with food and water ad libitum. The Institutional Animal Use and Care Committee approved all experimental procedures at Columbia University and Mount Sinai School of Medicine.

Cortical Rescue of 2AR in *htr2A*$^{-/-}$ Mice

Generation of *htr2A*$^{-/-}$ mice has been described previously (Gonzalez-Maeso et al., 2003). To generate *htr2A*$^{-/-}$ mice, a transcriptional termination sequence (NEO-STOP) was placed between the promoter and the coding sequence. Using Emx-Cre-recombinase, this transcriptional termination sequence (flanked by lox-P sites) is excised from

htr2A$^{-/-}$ mice, rescuing receptor expression in brain regions where their expression pattern overlaps (Figure S8).

Drug Administration

DOI, DOM, DOB, dimethyltryptamine (DMT), and mescaline (Sigma-Aldrich) were dissolved in saline and injected i.p. LSD, R-lisuride, S-lisuride, and ergotamine (Sigma-Aldrich) were injected i.p. after suspension in a minimal amount of DMSO and made up to volume with normal saline. Psilocin (Sigma-Aldrich) was dissolved in distilled water supplemented with a minimal amount of acetic acid and injected i.p. The injected doses were DOI, 2 mg/kg; DOM, 4 mg/kg; DOB, 1 mg/kg; DMT, 15 mg/kg; mescaline, 20 mg/kg; LSD, 0.24 mg/kg; psilocin, 2 mg/kg; ergotamine, 0.5 mg/kg; R-lisuride, 0.4 mg/kg; and S-lisuride, 4 mg/kg, unless otherwise indicated. Quinpirole (1 mg/kg; Tocris Bioscience) and SKF82958 (1 mg/kg; Sigma-Aldrich) were dissolved in saline and injected i.p.

Behavioral Studies

Head twitch behavioral response was evaluated as previously described (Gonzalez-Maeso et al., 2003). The following events were scored 15 min following injection with drug or vehicle for a period of 20 min. ESR was scored as previously described (Darmani et al., 1990). Locomotor activity was measured by counting the number of transitions across 6 equally sized squares on the floor of a 30 cm × 15 cm arena. Grooming was assessed as event bouts separated by a minimum of 10 s. Rearing events were scored when a mouse raised itself into a near vertical position on its two hind legs. Temperature change was measured using a Vaseline-lubricated 2 cm thermocouple-measuring device inserted into the mouse rectum prior to drug injection (T_0) and at the end of the 20 min test period (T_1). Temperature change was calculated by subtracting T_1 from T_0.

Mouse Brain Samples

Experiments were performed as previously described, with minor modifications (Gonzalez-Maeso et al., 2003). Drugs (see above) or vehicle was injected i.p., and mice were sacrificed by cervical dislocation 60 min after the injection. The brain was dissected, frozen on dry ice, and stored at −80°C for total RNA isolation.

Quantitative Real-Time PCR

Quantitative real-time PCR (qRT-PCR) experiments were performed as previously reported (Gonzalez-Maeso et al., 2003).

PCA Analysis of qRT-PCR

The fold change ratios for each pair of experimental and control samples for 19 genes from the qRT-PCR (see above) were transformed by $\log_2$ function. PCA was carried out using the open source software Language R function "princomp" (Ihaka and Gentleman, 1996). For each sample, the $\log_2$ fold change ratio of the 19 genes can be projected onto a plane consisting of the first two components (Johnson and Wichern, 1982). Each sample is then represented by one dot on a two-dimensional plane.

[^{125}I]DOI Autoradiography Binding Assay

Fresh frozen brains from were cut into 14 μm sagittal sections, then thaw-mounted to SuperFrost (Fisher Scientific) slides. Sections were preincubated in assay buffer (4 mM $CaCl_2$, 0.1% ascorbic acid, and 50 mM Tris-HCl [pH 7.4]) for 30 min in the presence of a 5-HT_{2C} receptor antagonist (SB242084; 100 nM). Then sections were transferred to assay buffer containing 200 pM of [^{125}I]DOI and incubated for 1 hr at room temperature. Sections were washed with ice-cold assay buffer two times for 10 min and two times for 5 min, and then rinsed with cold distilled water for 1 min. Air-dried sections were exposed to film overnight.

[^{3}H]Ketanserin and [^{125}I]LSD Binding Assays

Brain cortical membrane preparations were performed as previously reported, with minor modifications (Gonzalez-Maeso et al., 2002). [^{3}H]Ketanserin (DuPont-NEN) was used to label 2AR in saturation and competition assays as previously reported (Ebersole et al., 2003). Nonspecific binding was determined in the presence of 10 μM methysergide (Tocris Cookson Inc.).

HEK293 cell membrane preparations were performed as previously reported, with minor modifications (Ebersole et al., 2003). [^{125}I]LSD (PerkinElmer) was used to label 2AR. Binding assays were incubated for 60 min at 37°C in a total volume of 500 μl, 100 pM [^{125}I]LSD in the absence or presence of R-lisuride as displacer. Nonspecific binding was determined in the presence of 1 μM ketanserin (Tocris Cookson Inc.).

Electrophysiology

Coronal sections (400 μm) were obtained from P10–20 mice following decapitation and brain dissection in chilled artificial cerebrospinal fluid (ACSF; 125 mM NaCl, 2.5 mM KCl, 1.25 mM NaH_2PO_4, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 25 mM $NaHCO_3$, and 25 mM glucose), using a Vibratome 1000 (Vibratome, St. Louis). Slices were allowed to recover for 1 hr at room temperature before use. TTX (Sigma-Aldrich), LSD, and R-lisuride were prepared as stock solutions, and diluted in ACSF to final concentrations daily before use. All drugs were applied via bath perfusion. Brain slices were placed in a recording chamber on an upright microscope (Olympus BX51-WI) and continuously perfused with oxygenated ACSF at 30°C. Whole-cell patch-clamp (ruptured) recordings were performed using an Axopatch 700B amplifier (Axon Instruments). Data were acquired and analyzed using pClamp9 software (Axon Instruments, Sunnyvale). Borosilicate glass pipettes (Warner Instrument Corp, 5–8 MΩ) were filled with: 130 mM CsCl, 2 mM $CaCl_2$, 10 mM HEPES, 11 mM EGTA, 3 mM Mg-ATP, 0.4 mM Na-GTP (pH 7.4; 265−275 mOsm/l). Alexa Fluor 594 hydrazide (100 μM; Invitrogen) was added to the internal solution for visualizing the morphology of recorded neurons. Pyramidal neurons were identified based on layer location and morphology. Neurons were voltage-clamped at −70 mV and voltage ramps (from −110 to −30 mV in 3.5 s) were given every 30 s during baseline and drug application. Drugs were applied for 8 min only after stable responses were obtained in five successive trails. Only cells that maintained a stable access resistance throughout the experiment (less than 5% of change) were included in analysis. At the end of each experiment, morphology of the recorded neuron was examined to confirm the cell type.

Fluorescent In Situ Hybridization

Synthesis of modified DNA oligonucleotide probes, probe labeling, and FISH was performed as previously described (Chan et al., 2005). See Table S12 for oligonucleotide probe sequences.

Cortical Primary Culture

Fetuses (E15–17) from pregnant mothers were removed using aseptic techniques. Fetal brains were isolated and placed in Hanks' balanced salt solution supplemented with HEPES (10 mM), penicillin, streptomycin, and fungizone. Dissected cortical tissue was incubated with 0.25% trypsin for 15 min at 37°C and dissociated by repeated trituration. Cell viability was confirmed by trypan blue exclusion. Cells were plated in DMEM and supplemented with fetal bovine serum (10%) at 0.7×10^6 cells/well (qRT-PCR) or 200×10^3 cells/well (FISH) in 6-well dishes coated with poly-l-lysine. After 2–3 hr, the media was changed to neurobasal media, supplemented with B27 and glutamine (0.5 mM), providing cultures comprised of 90%–95% neurons. Cells were maintained for 7–10 days in vitro before use in experiments. The inhibitors U73211, LY294002, and PP2 were obtained from Tocris Cookson Inc. PTX was obtained from Sigma-Aldrich.

Supplemental Data

The Supplemental Data for this article can be found online at http://www.neuron.org/cgi/content/full/53/3/439/DC1/.

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