



The Hallucinogenic Serotonin_{2A} Receptor Agonist, 2,5-Dimethoxy-4-Iodoamphetamine, Promotes cAMP Response Element Binding Protein-Dependent Gene Expression of Specific Plasticity-Associated Genes in the Rodent Neocortex

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Psychedelic compounds that target the 5-HT_{2A} receptor are reported to evoke psychoplastic effects, including enhanced dendritic arborization and synaptogenesis. Transcriptional regulation of neuronal plasticity-associated genes is implicated in the cytoarchitectural effects of serotonergic psychedelics, however, the transcription factors that drive this regulation are poorly elucidated. Here, we addressed the contribution of the transcription factor cyclic adenosine monophosphate (cAMP)-response element binding protein (CREB) in the regulation of neuronal plasticity-associated genes by the hallucinogenic 5-HT_{2A} receptor agonist, 2,5-dimethoxy-4-iodoamphetamine (DOI). *In vitro* studies with rat cortical neurons indicated that DOI enhances the phosphorylation of CREB (pCREB) through mitogen-activated protein (MAP) kinase and calcium/calmodulin dependent kinase II (CaMKII) pathways, with both cascades contributing to the DOI-evoked upregulation of *Arc*, *Bdnf1*, *Cebpb*, and *Egr2* expression, whilst the upregulation of *Egr1* and *cFos* mRNA involved the MAP kinase and CaMKII pathway respectively. We observed a robust DOI-evoked increase in the expression of several neuronal plasticity-associated genes in the rat neocortex *in vivo*. This DOI-evoked upregulation of neuronal plasticity-associated genes was completely blocked by the 5-HT_{2A} receptor antagonist MDL100,907 *in vitro* and was also abrogated in the neocortex of 5-HT_{2A} receptor deficient mice. Further, 5-HT_{2A} receptor stimulation enhanced pCREB enrichment at putative cAMP response element (CRE) binding sites in the *Arc*, *Bdnf1*, *Cebpb*, *cFos*, but not *Egr1* and *Egr2*, promoters in the rodent neocortex. The DOI-mediated transcriptional induction of *Arc*, *cFos*

and *Cebpb* was significantly attenuated in the neocortex of CREB deficient/knockout (CREB $\alpha\delta$ KO) mice. Collectively, these results indicate that the hallucinogenic 5-HT_{2A} receptor agonist DOI leads to a rapid transcriptional upregulation of several neuronal plasticity-associated genes, with a subset of them exhibiting a CREB-dependent regulation. Our findings raise the intriguing possibility that similar to slow-acting classical antidepressants, rapid-action serotonergic psychedelics that target the 5-HT_{2A} receptor may also recruit the transcription factor CREB to enhance the expression of neuronal plasticity-associated genes in the neocortex, which could in turn contribute to the rapid psychoplastogenic changes evoked by these compounds.

Keywords: 5-HT_{2A} receptor, cAMP response element binding protein, immediate early gene, BDNF, *Arc*, serotonergic psychedelic, cortical neuron, CREB deficient mice

INTRODUCTION

There has been a renewal of interest in serotonergic psychedelics as potential rapid-acting antidepressants for the treatment of anxiety and depression (Nutt et al., 2020; Vollenweider and Preller, 2020; Banks et al., 2021; De Gregorio et al., 2021). Most serotonergic psychedelics target the serotonin_{2A} (5-HT_{2A}) receptor, and agonist action at the 5-HT_{2A} receptor is implicated in the molecular, cytoarchitectural, hallucinogenic and mood-related behavioral effects of serotonergic psychedelics (Vollenweider et al., 1998; González-Maeso et al., 2003, 2007; Martin and Nichols, 2016; López-Giménez and González-Maeso, 2018; Preller et al., 2018; Madsen et al., 2019; Slocum et al., 2021). Diverse serotonergic psychedelics, as well as the hallucinogenic 5-HT_{2A} receptor agonist, 2,5-dimethoxy-4-iodoamphetamine (DOI), can elicit both unique and distinctive molecular, behavioral and electrophysiological effects (Aghajanian and Marek, 1999; González-Maeso et al., 2003, 2007; Marek, 2017; Banerjee and Vaidya, 2020; Savino and Nichols, 2021). Common to all of these 5-HT_{2A} receptor agonists is a regulation of neuronal structural plasticity in the neocortex; from increased dendritic complexity to enhanced synaptogenesis (Ly et al., 2018, 2020; Savalia et al., 2021; Shao et al., 2021; Vargas et al., 2021). The rapid transcriptional effects evoked *via* agonistic action at the 5-HT_{2A} receptor, in particular the enhanced expression of neuronal plasticity-associated gene expression, are implicated in contributing to these psychoplastogenic effects (Nichols et al., 2003; Olson, 2018; Berthoux et al., 2019; Artin et al., 2021; de Vos et al., 2021; Jepsen et al., 2021).

5-HT_{2A} receptors are G-protein coupled receptors that drive Gq-signaling to activate phospholipase C beta (PLC β) - mediated cleavage of phosphatidylinositol bisphosphate (PIP₂) into inositol triphosphate (IP₃) and diacylglycerol (DAG) (Raymond et al., 2001; Sharp and Barnes, 2020). IP₃ mobilizes release of calcium from intracellular endoplasmic reticulum stores, which activates calcium/calmodulin dependent kinase II (CaMKII), while DAG activates protein kinase C (PKC) that phosphorylates and activates mitogen-activated protein (MAP) kinase (MAPK) signaling (Banerjee and Vaidya, 2020). These kinases in turn are reported to enhance the phosphorylation of the transcription factor, cyclic adenosine monophosphate (cAMP)-response-element binding protein (CREB), that has

been previously shown to regulate the expression of several neuronal plasticity-associated genes (Lonze and Ginty, 2002; Sakamoto et al., 2011; Belgacem and Borodinsky, 2017). CREB is also a central target for diverse classes of antidepressant treatments, contributes to the neurotrophic, neurogenic and behavioral effects of antidepressants (Nibuya et al., 1996; Duman and Nibuya, 1997; Thome et al., 2000; Chen et al., 2001; Nair and Vaidya, 2006), and is dysregulated in both animal models of depression (Carlezon et al., 2005; Duman et al., 2005; Krishnan and Nestler, 2008, 2010) and in major depressive disorder patients (Blendy et al., 1996; Koch et al., 2009). Here, we sought to address whether CREB plays an important role in the regulation of neuronal plasticity-associated gene expression within the neocortex that arise in response to treatment with the hallucinogenic 5-HT_{2A} receptor agonist, DOI. Using both *in vitro* and *in vivo* approaches, as well as a CREB-deficient mouse line, we demonstrate that CREB contributes to the DOI-mediated regulation of a subset of neuronal plasticity-associated genes within the neocortex. This raises the intriguing possibility that similar to classical antidepressants, serotonergic psychedelics that target the 5-HT_{2A} receptor may also recruit CREB-mediated transcriptional regulation of specific neuronal plasticity-associated genes in the neocortex, thus contributing to the effects on neuronal structural plasticity and mood-related behavior.

MATERIALS AND METHODS

Animal Treatments

Male Sprague-Dawley rats (2–3 months), serotonin_{2A} receptor knockout (5-HT_{2A}^{-/-}) mice (Weisstaub et al., 2006) and wild-type (WT) littermate controls (4–5 months) maintained on a 129S6/SvEv background were bred in the Tata Institute of Fundamental Research (TIFR) animal facility and CREB deficient/knockout (CREB $\alpha\delta$ KO) mice were bred in the University of Pennsylvania animal facility and used for all experiments. For experiments using CREB deficient mice, the hypomorphic CREB $\alpha\delta$ knockout mouse line that lacks the α and δ isoforms of CREB (CREB $\alpha\delta$ KO) were used (Blendy et al., 1996). CREB $\alpha\delta$ KO mice were generated as previously described (Walters and Blendy, 2001) and were maintained as F1 hybrids

of 129SvEvTac:C57BL/6. For all experiments, CREB $\alpha\delta$ KO mice and the WT controls were generated *via* crossing heterozygote CREB $\alpha\delta$ 129SvEvTac with heterozygote CREB $\alpha\delta$ C57BL/6 mice, allowing for a uniform genetic background in the experimental cohort. For the establishment of *in vitro* cortical cultures, rat embryos were derived at embryonic day 17.5 (E17.5) from timed pregnant Sprague-Dawley dams. All animal procedures using Sprague-Dawley rats and serotonin_{2A} receptor knockout (5-HT_{2A}^{-/-}) mice were carried out in accordance with the Committee for Care and Supervision of Experimental Animals (CPCSEA) and approved by the TIFR Institutional Animal Ethics Committee. All experiments with the CREB $\alpha\delta$ KO mouse line were carried out in accordance with the NIH guideline for the care and use of laboratory animals and were approved by the University of Pennsylvania Animal Care and Use Committee. Animals were group housed and maintained on a 12 h light–dark cycle (lights on at 7 am) with access to food and water *ad libitum*.

Sprague-Dawley rats, CREB $\alpha\delta$ KO and litter-matched WT mice, received intraperitoneal injections of the 5-HT_{2A} agonist, 2,5-dimethoxy-4-iodoamphetamine (DOI, 8 mg/kg, Sigma-Aldrich, United States) or vehicle (0.9% NaCl) (Vaidya et al., 1997). 5-HT_{2A}^{-/-} and litter-matched WT mice received intraperitoneal administration of DOI (2 mg/kg) or vehicle (0.9% saline) and animals were sacrificed 2 h post drug administration (Vaidya et al., 1997; Benekareddy et al., 2012).

To make comparisons with a rapid-action antidepressant treatment, we also subjected a cohort of Sprague Dawley rats to electroconvulsive seizure (ECS) treatment *via* ear-clip electrodes (ECS unit, UGO Basile, Comerio, Italy) (frequency: 100 pulses/s; pulse width: 0.9 ms; pulse duration: 0.5 s; current: 80 mA). ECS animals were subjected to a single ECS treatment while sham-treated control animals underwent the application and removal of ear-clip electrodes without electrical stimulation, and were sacrificed 2 h post treatment. Animals were decapitated immediately, neocortex was dissected and snap frozen in liquid nitrogen.

Cortical Neuron Cultures

Primary cortical neuron cultures were established from E17.5 rat embryos as described previously (Desouza et al., 2011; Fanibunda et al., 2019). Rat embryonic cortices were dissected and treated with trypsin-EDTA for 10 min, prior to dissociation in culture medium - Neurobasal medium supplemented with 2% B27 supplement, 0.5 mM L-glutamine, 5 U/ml penicillin and 5 U/ml streptomycin (Invitrogen, United States). Cells were plated on poly-D-lysine (Sigma-Aldrich, United States) coated 35 mm dishes at a density of 10⁶ cells/dish. Following attachment and neurite extension *in vitro* for a period of 7 days, neurons were treated with DOI (10 μ M) or vehicle (DMSO) for 2 h on day *in vitro* (DIV) 10. In experiments to delineate signaling events downstream of 5-HT_{2A} receptor activation, neurons were treated with DOI (10 μ M) for 2 h in the presence of the 5-HT_{2A} receptor antagonist, MDL100,907 (10 μ M) and specific signaling pathway inhibitors, namely the phospholipase C (PLC) inhibitor U73122 (5 μ M), the mitogen activated protein kinase kinase (MAPKK) inhibitor U0126 (50 μ M) and the CaM kinase II (CaMKII) inhibitor KN-62 (10 μ M) (Tocris Bioscience, United Kingdom).

The inhibitors were added to the cultures 30 min prior to DOI treatment, and were present throughout the duration of DOI exposure. Following treatments, cortical neurons were processed for immunofluorescence, RNA extraction for qPCR analysis, or western blot analysis.

Immunofluorescence

Immunofluorescence staining was performed as described previously (Fanibunda et al., 2019). In brief, cortical neurons were fixed in 4% paraformaldehyde, followed by blocking in 10% horse serum and incubated with primary antibodies, rabbit anti-pCREB (1:1000; Cell Signaling Technology, MA, United States) or goat anti-5-HT_{2A} receptor (1:500, Santa Cruz Biotechnologies, United States) along with the pan-neuronal marker, mouse anti-MAP2 (1:1000, Sigma-Aldrich, United States) overnight at 4°C. This was followed by incubation with secondary antibodies, Alexa 488 conjugated anti-goat (1:500; Molecular probes, CA, United States) or Alexa 488 conjugated anti-rabbit (1:500; Molecular Probes, CA, United States) or biotinylated horse anti-mouse (1:500, Roche Applied Science, Switzerland) with subsequent incubation with streptavidin-conjugated Alexa 568 (1:500, Molecular Probes, CA, United States) for 2 h. Following secondary antibody incubation and serial washes, cortical neurons were mounted in Vectashield (Vector Laboratories, CA, United States), and images were captured on the Zeiss LSM5 Exciter laser scanning microscope.

Western Blot Analysis

Rat cortical neurons were lysed in Laemmli sample buffer (2% SDS, 10% glycerol, 60 mM Tris-Cl, 0.01% bromophenol blue) and proteins were resolved *via* sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), followed by transfer onto polyvinylidene fluoride (PVDF, GE Healthcare, United Kingdom) membranes. Blots were blocked in 5% fat-free milk in 0.05% Tris Buffered Saline-Tween 20 (TBS-T) and incubated in primary antibodies in 0.05% TBS-T, overnight at 4°C. Primary antibodies included rabbit anti-pCREB (1:500; Cell Signaling Technology, MA, United States) and rabbit anti-CREB (1:1000, Cell Signaling Technology). Blots were washed 3–5 times and incubated with a 1:5000 dilution of horseradish peroxidase-conjugated goat anti-rabbit antibody (GE Healthcare, United Kingdom) for 1 h. Protein-antibody complexes were detected on X-ray films following addition of Enhanced Chemiluminescence (ECL) substrate (GE Healthcare, United Kingdom). The relative density of the pCREB and CREB bands was quantitated using ImageJ software (NIH, United States), and was represented as a pCREB/CREB ratio.

Quantitative PCR Analysis

RNA was isolated using Tri Reagent (Sigma-Aldrich, United States), according to the manufacturer's protocols. Two μ g of RNA per sample was reverse transcribed using a complementary DNA (cDNA) synthesis kit (QuantiTect reverse transcription kit, Qiagen, Germany). Quantitative PCR (qPCR) was performed in a Mastercycler[®] ep realplex real-time PCR system (Eppendorf, Germany). cDNA was amplified using a SYBR Green kit (Applied Biosystems, CA, United States), with

primers for the genes of interest (**Supplementary Table 1**). Hypoxanthine phosphoribosyl transferase (*Hprt*) was used as an endogenous housekeeping gene control for normalization. The relative expression levels between control and treated samples were computed by the comparative Ct method, as described previously (Schmittgen and Livak, 2008). Data are represented as fold change \pm SEM as compared to control.

Chromatin Immunoprecipitation

Chromatin immunoprecipitation (ChIP) was carried out as described previously (Desouza et al., 2011). Briefly, bilateral frontal neocortices were dissected and fixed to crosslink the DNA with the bound proteins. The tissue was placed in a pre-chilled Dounce homogenizer, sonicated and immunoprecipitated using a phosphorylated CREB (pCREB) antibody (1 μ g; Cell Signaling Technology, MA, United States). Following reverse crosslinking and chromatin precipitation, qPCR analysis was performed for upstream sequences of the *Arc*, *Bdnf1*, *Cebpb*, *cFos*, *Egr1*, and *Egr2* promoters that contained putative CRE sites as predicted using AliBaba 2.1¹. For each sample the results were normalized to the input chromatin from the same sample. (Primer sequences used in ChIP experiments: **Supplementary Table 2**).

Dimethoxy-4-Iodoamphetamine-Mediated Head Twitch Behavior

Administration of DOI results in a stereotypical head twitch behavior, characterized by rapid radial movements of the head (Canal and Morgan, 2012; Halberstadt and Geyer, 2013). This behavior was videotaped in the home cage for a total duration of 20 min, commencing 20 min following administration of the DOI or vehicle (saline) treatment. The total number of head twitches in this time window were counted by an experimenter blind to the experimental treatment groups.

In situ Hybridization

CREB $\alpha\delta$ KO and litter-matched WT mice were administered DOI and vehicle (Veh) resulting in four groups: WT + Veh, WT + DOI, CREB $\alpha\delta$ KO + Veh, CREB $\alpha\delta$ KO + DOI. Mice were anesthetized with sodium thiopentone and transcardially perfused with 4% paraformaldehyde (PFA). The brains were postfixed in 4% PFA overnight and cryoprotected in 30% sucrose in 4% PFA prior to being shipped to TIFR, India. Coronal sections of 30 μ M thickness were cut on the freezing microtome (Leica Biosystems, Germany), fixed, blocked and acetylated. The floating sections were incubated for 20 h at 60°C in a hybridization buffer (50% formamide, 1xSSC, 25xDenhardt's solution, 40 mM dithiothreitol, 150 μ g/ml yeast tRNA, 10% dextran sulfate, 400 μ g/ml salmon sperm DNA) containing ³⁵S-UTP labeled antisense riboprobes for *Arc* mRNA at a concentration of 1×10^6 cpm/300 μ l. Antisense riboprobes to *Arc* mRNA were generated from a transcription-competent plasmid kindly provided by Dr. Oswald Steward (University of California, Irvine, CA, United States). Following hybridization,

all sections were washed in ribonuclease A (20 mg/ml; USB corporation, United States), followed by stringent washes in decreasing concentrations of SSC, mounted on slides, air dried and exposed to Hyper film β -max (GE Healthcare, United States) for 7 days. Levels of *Arc* mRNA were quantified using Scion Image (Scion, United States) and calibrated using ¹⁴C standards to correct for non-linearity. Equivalent areas of the somatosensory and prefrontal cortex were outlined and optical density measurements were determined (3–4 sections/animal).

Statistical Analysis

Results were subjected to statistical analysis using Student's unpaired *t*-test for experiments with two groups (GraphPad InStat) and one-way ANOVA (GraphPad, Prism 8) for experiments using signaling pathway inhibitors, followed by Tukey's *post hoc* test for group comparisons. For four group experiments statistical analysis was performed using two-way ANOVA (GraphPad, Prism 8). Tukey's *post hoc* test for group comparisons was applied only when there was a significant two-way ANOVA interaction observed between the two variables of DOI treatment and 5-HT_{2A}^{-/-} KO genotype or DOI treatment and CREB $\alpha\delta$ KO genotype. Statistical significance was determined at $p < 0.05$.

RESULTS

Acute Treatment With the 5-HT_{2A} Receptor Agonist, Dimethoxy-4-Iodoamphetamine, Regulates Neuronal Plasticity-Associated Gene Expression via the Mitogen-Activated Protein Kinase and CaMKII Signaling Pathways and Enhances Phosphorylated cAMP Response Element Binding Protein Expression *in vitro*

The 5-HT_{2A} receptor agonist DOI, is a potent hallucinogen that is known to evoke an increase in the expression of several neuronal plasticity-associated genes (González-Maeso et al., 2003). Following ligand binding, the Gq-coupled 5-HT_{2A} receptor can differentially recruit multiple signaling pathways to bring about distinct signaling responses. We sought to address the contribution of the 5-HT_{2A} receptor, the Gq-coupled phospholipase C (PLC) signaling pathway, the MAP kinase and CaM Kinase II (CaMKII) signaling pathways and the transcription factor CREB to the DOI-evoked induction of neuronal plasticity-associated gene expression. We stimulated primary rat cortical neurons *in vitro* with DOI, in the presence or absence of the 5-HT_{2A} receptor antagonist, MDL100,907; PLC inhibitor, U73122; CaMKII inhibitor, KN-62 or the MAPKK inhibitor, U0126 (**Figure 1A**). We observed an increase in transcript expression of *Arc*, *Bdnf1*, *Cebpb*, *cFos*, *Egr1*, and *Egr2* mRNA levels following DOI treatment, which was inhibited by

¹<http://www.gene-regulation.com/pub/programs.html>

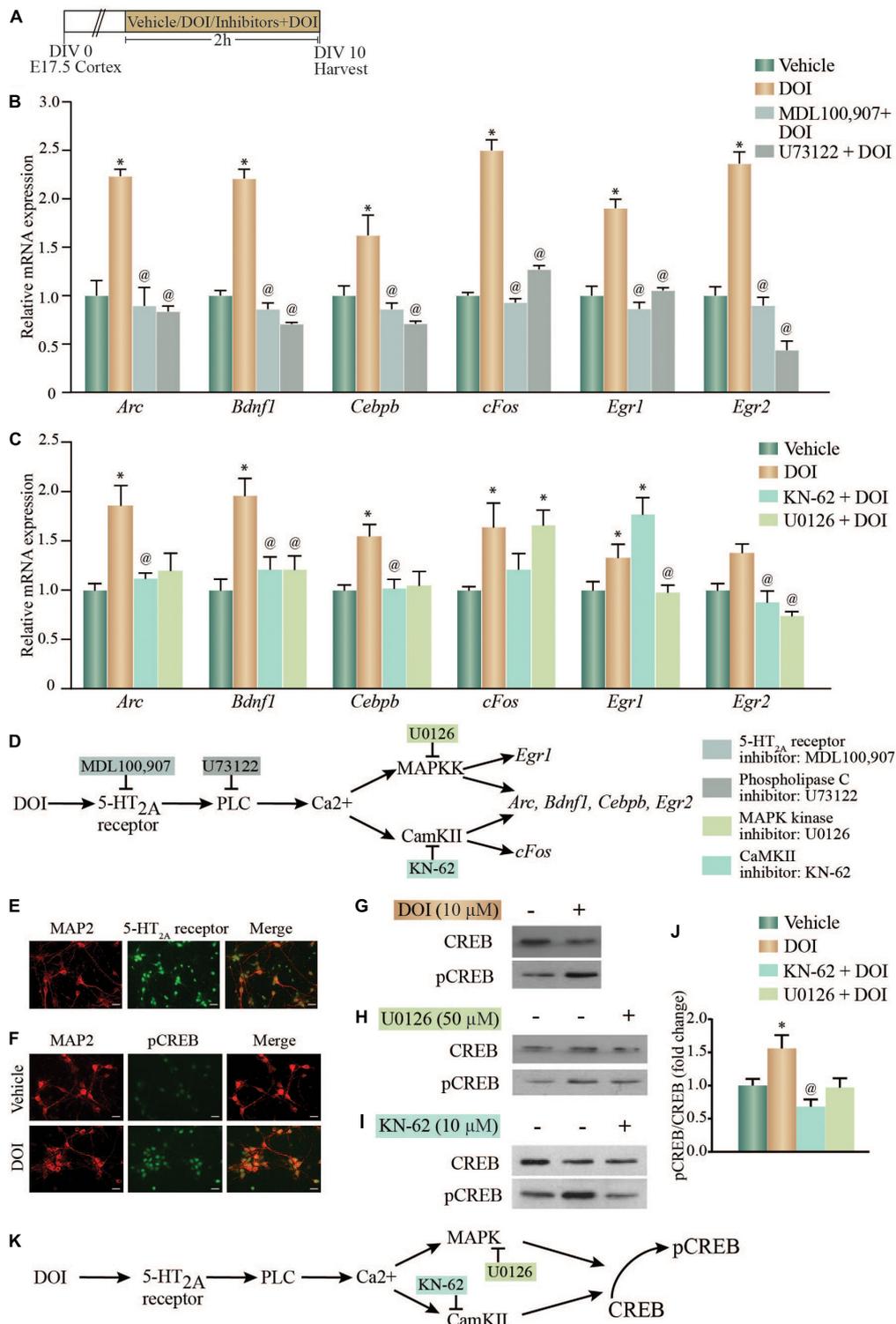
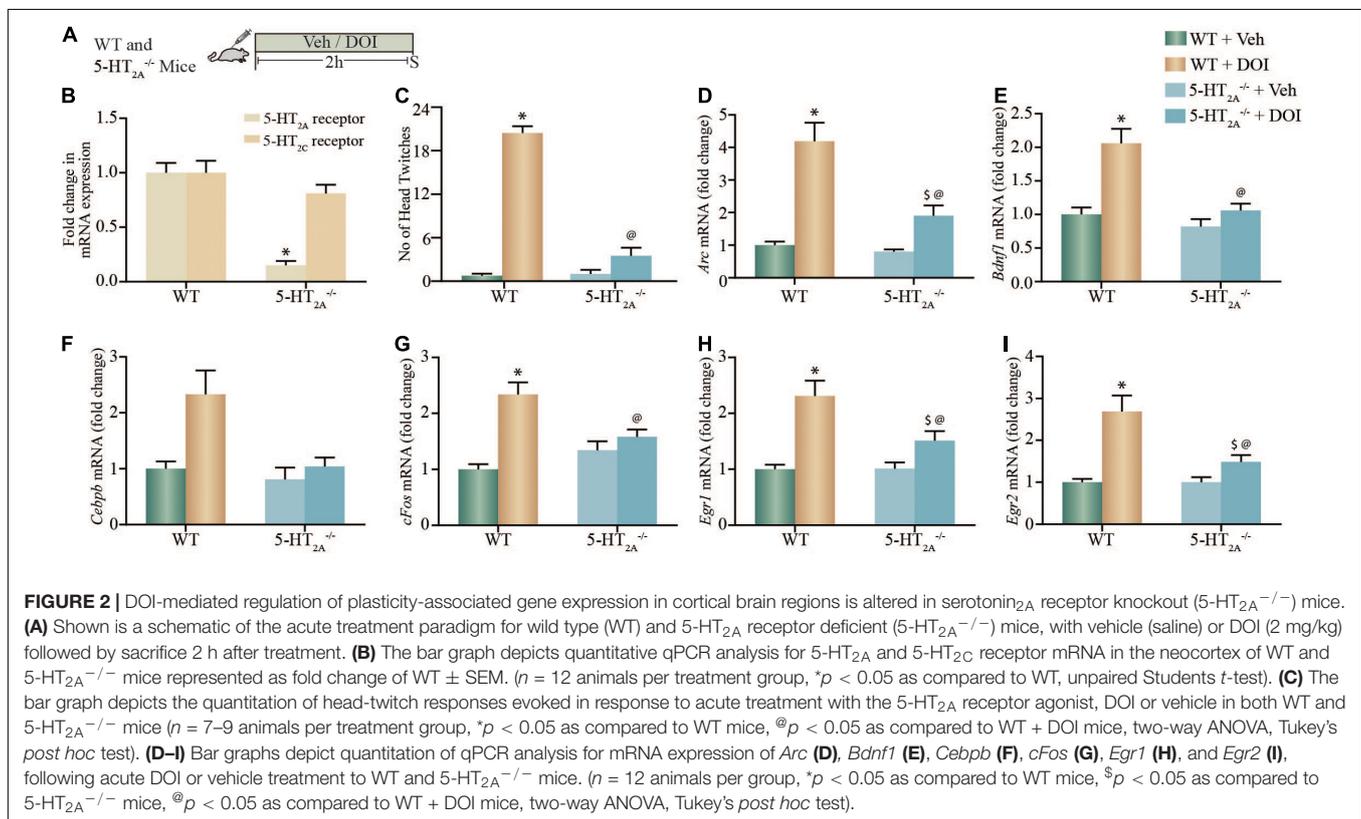


FIGURE 1 | Acute treatment with the 5-HT_{2A} receptor agonist, DOI regulates neuronal plasticity-associated gene expression via the MAP kinase and CaMKII signaling pathways and enhances phosphorylated CREB (pCREB) expression *in vitro*. **(A)** Shown is a schematic of the treatment paradigm for cortical neurons derived from E17.5 rat embryos, allowed to differentiate till day *in vitro* (DIV) 10, following which neurons were treated with vehicle (DMSO) or the 5-HT_{2A} receptor agonist, DOI (10 μM), in the presence or absence of CaMKII and MAP kinase signaling pathway inhibitors (CaMKII inhibitor: KN-62; MAPK inhibitor: U0126). **(B)** Shown is the relative mRNA expression for plasticity-associated genes following DOI treatment in the presence or absence of the 5-HT_{2A} receptor antagonist, MDL100,907 or PLC inhibitor, U73122, represented as fold change of vehicle ± SEM. (Representative results from $n = 4$ wells per treatment group/ $N = 2$, * $p < 0.05$ (Continued)

FIGURE 1 | as compared to vehicle, $^{\circ}p < 0.05$ as compared to DOI, one-way ANOVA, Tukey's *post hoc* test). **(C)** Shown is the relative mRNA expression for plasticity-associated genes following DOI treatment in the presence or absence of MAP kinase and CaMKII signaling pathway inhibitors, represented as fold change of vehicle \pm SEM. (Representative results from $n = 3-5$ wells per treatment group/ $N = 3$, $^{\circ}p < 0.05$ as compared to vehicle, $^{\circ}p < 0.05$ as compared to DOI, one-way ANOVA, Tukey's *post hoc* test). **(D)** Shown is a schematic summarizing the putative signaling pathways that may contribute to DOI-induced gene expression. The CaMKII inhibitor, KN-62 and the MAPKK inhibitor, U0126 inhibit the CaMKII and MAP kinase signaling pathways respectively. The DOI-mediated upregulation of *Arc*, *Bdnf1*, *Cebpb*, and *Egr2* mRNA levels was blocked by both the MAPKK and CaMKII inhibitors, whereas the increase in *cFos* mRNA was blocked by the CaMKII, not the MAPKK, inhibitor and the upregulation of *Egr1* mRNA was blocked by the MAPKK, not the CaMKII, inhibitor. **(E)** Shown are representative immunofluorescence images of rat cortical neurons *in vitro* with double staining for the neuronal marker MAP2 (red) and 5-HT_{2A} receptor (green). Scale bar: 30 μ m. Magnification: 20X. **(F)** Shown are representative immunofluorescence images of rat cortical neurons with double staining for pCREB (green) and the neuronal marker MAP2 (red) – upper panel: Vehicle; lower panel: DOI. Scale bar: 30 μ m. Magnification: 20X. **(G–J)** Shown are representative immunoblots for pCREB and CREB protein levels in rat cortical neurons treated with DOI **(G)** or with DOI in the presence or absence of the MAPKK inhibitor U0126 **(H)** or the CaMKII inhibitor KN-62 **(I)**. **(J)** Quantitative densitometric analysis of pCREB/CREB levels in rat cortical neurons treated with DOI in the presence or absence of U0126 or KN-62. Results are expressed as fold change of vehicle \pm SEM. (Representative results from $n = 3-5$ wells per treatment group/ $N = 3$, $^{\circ}p < 0.05$ as compared to vehicle, $^{\circ}p < 0.05$ as compared to DOI, one-way ANOVA, Tukey's *post hoc* test). **(K)** Shown is a schematic depicting the putative pathway via which pCREB levels are enhanced following DOI administration, indicative of a role for the MAP kinase and CaMKII signaling pathways.



the 5-HT_{2A} receptor antagonist MDL100,907, as well as the PLC signaling pathway inhibitor, U73122 **(Figure 1B)** {one-way ANOVA: *Arc*: [$F_{(3,12)} = 11.23$, $p = 0.0008$]; *Bdnf1*: [$F_{(3,12)} = 90.59$, $p < 0.0001$]; *Cebpb*: [$F_{(3,12)} = 14.78$, $p = 0.0002$], *cFos*: [$F_{(3,12)} = 135.6$, $p < 0.0001$], *Egr1*: [$F_{(3,12)} = 26.17$, $p < 0.0001$], *Egr2*: [$F_{(3,12)} = 28.45$, $p < 0.0001$]}. These observations indicate that the 5-HT_{2A} receptor mediates the DOI-evoked induction in neuronal plasticity-associated genes, *via* recruiting the Gq-coupled PLC pathway. We next sought to delineate the contribution of the downstream MAP kinase and CaM Kinase II (CaMKII) signaling pathways to the DOI-evoked upregulation of neuronal plasticity-associated genes. We observed an upregulation of *Arc*, *Bdnf1*, *Cebpb*, and *Egr2* mRNA levels following

DOI treatment, which was inhibited by both the CaMKII inhibitor KN-62 and the MAPKK inhibitor U0126 **(Figure 1C)** {one-way ANOVA: *Arc*: [$F_{(3,9)} = 7.551$, $p = 0.008$]; *Bdnf1*: [$F_{(3,11)} = 6.436$, $p = 0.009$]; *Cebpb*: [$F_{(3,8)} = 5.293$, $p = 0.027$], *Egr2*: [$F_{(3,10)} = 7.685$, $p = 0.006$]}. In contrast, the DOI-mediated upregulation of *cFos* mRNA levels was prevented by only the CaMKII inhibitor KN-62, while the increase in the *Egr1* transcript levels was abrogated exclusively by the MAPKK inhibitor U0126 **(Figure 1C)** {one-way ANOVA: *cFos*: [$F_{(3,11)} = 5.563$, $p = 0.014$], *Egr1*: [$F_{(3,11)} = 14.02$, $p = 0.0004$]}. This indicates a differential involvement of the MAP kinase and the CaMKII pathway in the 5-HT_{2A} receptor-mediated transcriptional regulation of specific neuronal plasticity-associated genes **(Figure 1D)**.

We next sought to address the possible role of the transcription factor CREB in mediating the signaling events evoked by DOI, downstream of the 5-HT_{2A} receptor. The DOI regulated genes, *Arc*, *Bdnf1*, *Cebpb*, *cFos*, *Egr1*, and *Egr2* were found to contain putative cAMP response element (CRE) (TGACG/CGTCA/TGACGTCA) sites in the upstream promoter regions, suggestive of the possibility of a CREB-dependent transcriptional regulation. We first confirmed 5-HT_{2A} receptor expression in cortical neurons, by immunofluorescence staining (Figure 1E). Immunostaining with the phosphorylated CREB (pCREB) antibody demonstrated an increase in pCREB immunofluorescence intensity in DOI-treated rat cortical neurons as compared to vehicle-treated control neurons (Figure 1F). Immunoblotting to detect pCREB levels, also demonstrated a robust increase in pCREB/CREB levels in DOI-treated cortical neurons (Figure 1G), further corroborating that DOI treatment enhances pCREB levels. Further, the MAPKK inhibitor (Figure 1H) and the CaMKII inhibitor (Figure 1I), both prevented the DOI-evoked increase in pCREB/CREB levels, demonstrating a role for the MAP kinase pathway and CaMKII pathway in mediating the DOI-evoked increase in pCREB levels (Figure 1J) [pCREB/CREB: $F_{(3,17)} = 5.561$, $p = 0.008$]. This data collectively suggests that DOI, a hallucinogenic ligand of the Gq-coupled 5-HT_{2A} receptor, recruits the PLC pathway driving MAP kinase and CaMKII signaling to enhance the phosphorylation of the transcription factor CREB in rat cortical neurons (Figure 1K).

Dimethoxy-4-Iodoamphetamine-Mediated Regulation of Plasticity-Associated Gene Expression in the Neocortex Is Altered in 5-HT_{2A}^{-/-} Receptor Deficient Mice

Given that the hallucinogen DOI, in addition to exhibiting agonist action at the 5-HT_{2A} receptor, also binds to the 5-HT_{2C} receptor with lower affinity (Titeler et al., 1988), we further sought to evaluate the contribution of the 5-HT_{2A} receptor to the DOI-mediated induction of neuronal plasticity-associated genes in 5-HT_{2A}^{-/-} receptor deficient mice (Figure 2A). We confirmed that 5-HT_{2A}^{-/-} mice exhibit both a robust reduction in cortical 5-HT_{2A}, but not in 5-HT_{2C}, mRNA expression (Figure 2B), accompanied by a significant decline in a stereotypical head twitch response (HTR) behavior evoked by the 5-HT_{2A} receptor agonist DOI (Canal and Morgan, 2012; Halberstadt and Geyer, 2013). Two-way ANOVA analysis for HTR events (Figure 2C) indicated a significant DOI by 5-HT_{2A}^{-/-} genotype interaction [$F_{(1,29)} = 113.4$, $p < 0.0001$], as well as significant main effects of DOI [$F_{(1,29)} = 189.1$, $p < 0.0001$] and 5-HT_{2A}^{-/-} genotype [$F_{(1,29)} = 107.6$, $p < 0.0001$].

We next performed qPCR analysis to assess whether the DOI-evoked upregulation of specific neuronal plasticity-associated genes *Arc*, *Bdnf1*, *Cebpb*, *cFos*, *Egr1*, and *Egr2* mRNA expression observed *in vitro*, was altered in the neocortex of WT and 5-HT_{2A}^{-/-} mice following acute DOI administration (Figures 2D–I). We noted significant two-way

ANOVA interactions of DOI by 5-HT_{2A}^{-/-} genotype for the neocortical mRNA expression of *Arc* [$F_{(1,44)} = 4.920$, $p = 0.0318$], *Bdnf1* [$F_{(1,44)} = 4.45$, $p = 0.04$], *cFos* [$F_{(1,44)} = 12.59$, $p = 0.0009$], *Egr1* [$F_{(1,44)} = 4.257$, $p = 0.045$] and *Egr2* [$F_{(1,44)} = 1118$, $p < 0.0001$], but not for *Cebpb* mRNA expression [$F_{(1,44)} = 2.49$, $p = 0.12$]. We also noted significant main effects of DOI for *Arc* [$F_{(1,44)} = 81.38$, $p < 0.0001$], *Bdnf1* [$F_{(1,44)} = 20.37$, $p < 0.0001$], *Cebpb* [$F_{(1,44)} = 8.518$, $p = 0.0055$], *cFos* [$F_{(1,44)} = 26.72$, $p < 0.0001$], *Egr1* [$F_{(1,44)} = 34.58$, $p < 0.0001$], and *Egr2* [$F_{(1,44)} = 1392$, $p < 0.0001$] mRNA expression in the neocortex. Significant main effects of 5-HT_{2A}^{-/-} genotype were observed for neocortical mRNA levels of *Arc* [$F_{(1,44)} = 15.79$, $p = 0.0003$], *Bdnf1* [$F_{(1,44)} = 15.77$, $p = 0.0003$], *Cebpb* [$F_{(1,44)} = 7.399$, $p = 0.009$], and *Egr2* [$F_{(1,44)} = 1122$, $p < 0.0001$], but not for *cFos* and *Egr1* mRNA expression. *Post hoc* Tukey comparison analysis revealed that the DOI-evoked upregulation of *Bdnf1* and *cFos* expression noted in the neocortex of WT mice was completely abrogated in 5-HT_{2A}^{-/-} receptor deficient mice, and the DOI-evoked increase in *Arc*, *Egr1*, and *Egr2* neocortical mRNA levels noted in WT mice was significantly attenuated in the 5-HT_{2A}^{-/-} receptor deficient genotype. Collectively, studies with pharmacological blockade of the 5-HT_{2A} receptor *in vitro*, and using 5-HT_{2A}^{-/-} mice *in vivo*, indicate the critical contribution of the 5-HT_{2A} receptor to the DOI-mediated regulation of a subset of neuronal plasticity-associated genes in the neocortex.

Acute Treatment With Dimethoxy-4-Iodoamphetamine Enhances Both the Expression of Putative cAMP Response Element-Containing Plasticity-Associated Genes and the Enrichment of Phosphorylation of cAMP Response Element Binding Protein Within the Promoter Regions of Specific Plasticity-Associated Genes in the Neocortex of Adult Rats

Given that we observed that the 5-HT_{2A} receptor agonist, DOI, induced a robust upregulation of neuronal plasticity-associated genes in rat cortical neurons in culture, as well as in WT but not 5-HT_{2A}^{-/-} mice, we next examined whether DOI administration to Sprague-Dawley male rats could evoke similar alterations in neuronal plasticity-associated genes in the neocortex. We systemically administered DOI (8 mg/kg) and evaluated gene expression in the neocortex at a 2 h time-point post treatment (Figure 3A). DOI administration evoked a robust increase in the gene expression of *Arc*, *Atf3*, *Atf4*, *Bdnf1*, *Cebpb*, *Cebpd*, *Egr1*, *Egr2*, *Egr3*, *Egr4*, *cFos*, *JunB*, and *Nfkb1a* in the rat neocortex (Figure 3B).

We next examined if a subset of the genes upregulated by DOI treatment, that are known to contain putative CRE binding sites in their promoter regions based on *in silico* analysis, also exhibit enrichment for pCREB within their promoters (Figure 3C). Enhanced expression of *Arc*, *Bdnf1*, *Cebpb*, and

cFos in the neocortex of DOI-treated animals was accompanied by a significant enrichment of pCREB at the promoters of *Arc*, *Bdnf1*, *Cebpb*, and *cFos* (Figure 3D). In contrast, the enhanced gene expression of *Egr1* and *Egr2* was not associated with any significant change in pCREB enrichment at putative CRE sites within their promoter regions (Figure 3D). Taken together, these results indicate that *in vivo* administration of the 5-HT_{2A} receptor agonist DOI, evokes a robust upregulation of several neuronal plasticity-associated genes in the neocortex, a subset of which exhibit significant pCREB enrichment in their promoter regions.

To draw a comparison of the nature and magnitude of regulation of this subset of neuronal plasticity-associated genes in the neocortex by the serotonergic hallucinogen, DOI, with that evoked by a rapid-action antidepressant treatment, electroconvulsive seizure (ECS) treatment, neocortices derived from acute ECS or sham treated rats were subjected to qPCR analysis (Supplementary Figure 1). We noted a robust increase in *Arc*, *Bdnf1*, *Cebpb*, *cFos*, and *Egr2* but not *Egr1* mRNA levels following acute ECS treatment (Supplementary Figure 1), with the nature and scale of upregulation comparable to that following DOI treatment.

Dimethoxy-4-Iodoamphetamine-Mediated Regulation of Plasticity-Associated Gene Expression in Cortical Brain Regions Is Perturbed in CREB $\alpha\delta$ Knockout (CREB $\alpha\delta$ KO) Mice

Given the evidence both *in vitro* and *in vivo*, that the 5-HT_{2A} receptor agonist, DOI induces (1) a robust increase in pCREB levels in rat cortical neurons, (2) a significant enrichment of pCREB at the promoters of specific plasticity-associated genes that are robustly enhanced by DOI treatment, we next sought to address the contribution of CREB to the regulation of these transcripts. We used hypomorphic CREB $\alpha\delta$ KO mice (Blendy et al., 1996) that are reported to have a greater than 90% reduction in CREB binding activity to consensus CRE target sites (Walters and Blendy, 2001). We treated WT and CREB $\alpha\delta$ KO mice with vehicle or DOI (8 mg/kg), and assessed transcript expression of specific neuronal plasticity-associated genes at a 2 h time-point post treatment (Figure 4A). To rule out the possibility that CREB may indirectly regulate 5-HT_{2A} or 5-HT_{2C} receptors, we first assessed whether the baseline expression of these receptors was altered in CREB $\alpha\delta$ KO mice as compared to their WT controls, and observed no change in 5-HT_{2A} or 5-HT_{2C} receptor mRNA levels within the neocortex (Figure 4B). Further to assess whether CREB deficient mice exhibit any change in 5-HT_{2A} receptor evoked behavioral responses, we evaluated the HTR behavior evoked by DOI. Behavioral analysis to quantify the number of HTR events in WT and CREB $\alpha\delta$ KO mice, indicated that the number of HTR responses evoked by DOI were unaltered in CREB $\alpha\delta$ KO mice (Figure 4C). While we noted no significant two-way ANOVA interaction of DOI and CREB $\alpha\delta$ KO genotype, we observed a significant main effect of DOI [$F_{(1,12)} = 58.64$, $p < 0.0001$]. This indicates that the loss of CREB $\alpha\delta$ isoforms does not alter either the expression of the 5-HT_{2A} receptor, or the

behavioral HTRs evoked by the 5-HT_{2A} receptor agonist DOI, that are critically dependent on the cortical 5-HT_{2A} receptor.

We next performed *in situ* hybridization and qPCR analysis to address whether the regulation of specific neuronal plasticity-associated genes that exhibit pCREB enrichment at their promoters following DOI treatment, were altered in the neocortex of CREB $\alpha\delta$ KO mice. Radioactive *in situ* hybridization analysis indicated that the DOI-evoked robust induction of *Arc* mRNA levels both in the somatosensory cortex and in the prefrontal cortex was significantly attenuated in CREB $\alpha\delta$ KO mice (Figure 4D). Two-way ANOVA analysis for *Arc* mRNA levels in the somatosensory cortex (Figure 4E) indicated a significant DOI by CREB $\alpha\delta$ KO genotype interaction [$F_{(1,12)} = 8.39$, $p = 0.013$], as well as significant main effects of DOI [$F_{(1,12)} = 16.81$, $p = 0.002$] and CREB $\alpha\delta$ KO genotype [$F_{(1,12)} = 24.39$, $p = 0.0003$]. The robust DOI-evoked upregulation of *Arc* mRNA in the somatosensory cortex of WT control mice was completely lost in the CREB $\alpha\delta$ KO mice. Two-way ANOVA analysis for *Arc* mRNA levels in the prefrontal cortex (Figure 4F) indicated a significant DOI by CREB $\alpha\delta$ KO genotype interaction [$F_{(1,11)} = 15.73$, $p = 0.002$], as well as significant main effects of DOI [$F_{(1,11)} = 78.83$, $p = 0.0001$] and CREB $\alpha\delta$ KO genotype [$F_{(1,11)} = 14.75$, $p = 0.003$]. The robust DOI-evoked upregulation of *Arc* mRNA in the prefrontal cortex of WT control mice was significantly attenuated in the CREB $\alpha\delta$ KO mice. No change was observed in the basal expression of *Arc* mRNA in either the somatosensory or prefrontal of CREB $\alpha\delta$ KO mice, which did not differ from vehicle-treated WT controls.

qPCR analysis was carried out to assess whether the DOI-evoked upregulation of *Bdnf1*, *Cebpb*, *cFos*, and *Egr2* mRNA expression was altered in the neocortex of CREB $\alpha\delta$ KO mice following acute DOI administration (Figures 4G–J). Two-way ANOVA analysis for *Bdnf1* mRNA levels in the neocortex indicated no significant DOI by CREB $\alpha\delta$ KO genotype interaction (Figure 4G), however we did observe a significant main effect of DOI [$F_{(1,10)} = 10.19$, $p = 0.01$] and no main effect of CREB $\alpha\delta$ KO genotype. Two-way ANOVA analysis for *Cebpb* mRNA levels in the neocortex (Figure 4H) indicated a significant DOI by CREB $\alpha\delta$ KO genotype interaction [$F_{(1,12)} = 6.413$, $p = 0.026$], as well as a trend toward a main effect of DOI [$F_{(1,12)} = 3.388$, $p = 0.09$] and a significant main effect of CREB $\alpha\delta$ KO genotype [$F_{(1,12)} = 50.49$, $p = 0.0001$]. Tukey's *post hoc* group comparisons indicated that the CREB $\alpha\delta$ KO mice exhibited a significant baseline decrease ($p = 0.03$) in *Cebpb* mRNA levels in the neocortex. Further, while DOI-evoked a robust and significant upregulation of *Cebpb* mRNA levels in WT animals ($p = 0.04$), this was completely lost in the CREB $\alpha\delta$ KO mice. *Cebpb* mRNA levels in the DOI-treated WT cohort differed significantly from the DOI-treated CREB $\alpha\delta$ KO mice ($p < 0.0001$). Two-way ANOVA analysis for *cFos* mRNA levels in the neocortex (Figure 4I) indicated a significant DOI by CREB $\alpha\delta$ KO genotype interaction [$F_{(1,12)} = 8.966$, $p = 0.011$], as well as a trend toward a main effect of DOI [$F_{(1,12)} = 4.493$, $p = 0.056$] and a significant main effect of CREB $\alpha\delta$ KO genotype [$F_{(1,12)} = 102.5$, $p < 0.0001$]. Tukey's *post hoc* group comparisons indicated that the CREB $\alpha\delta$ KO mice exhibited a significant baseline reduction ($p = 0.001$) in expression of *cFos* mRNA in

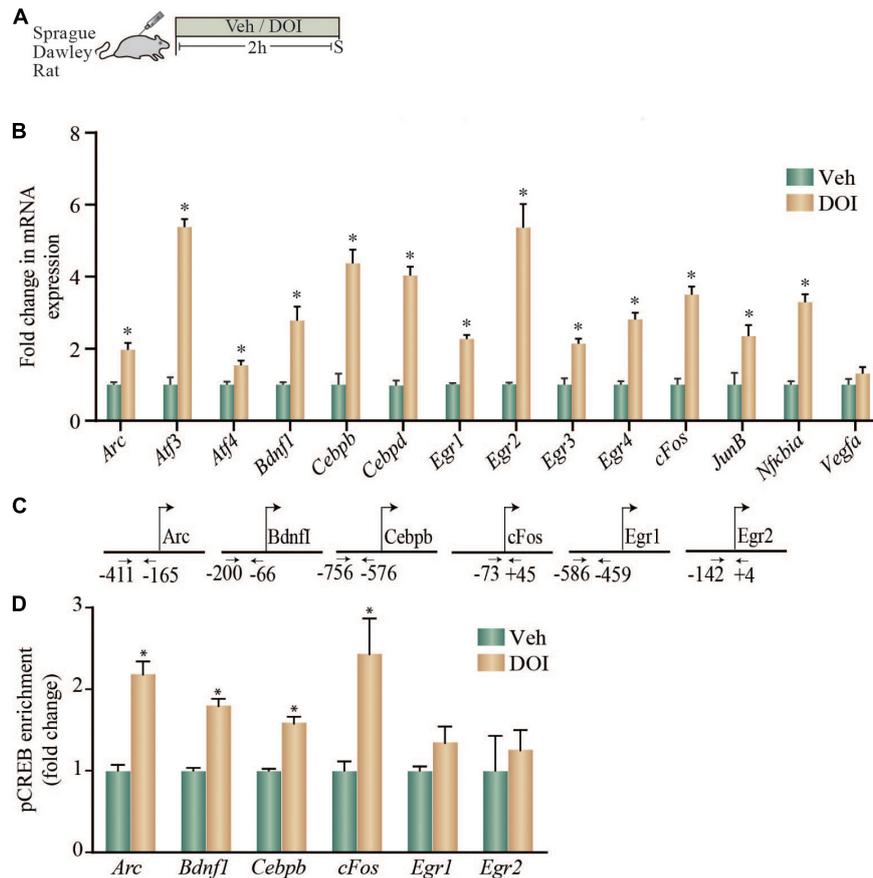


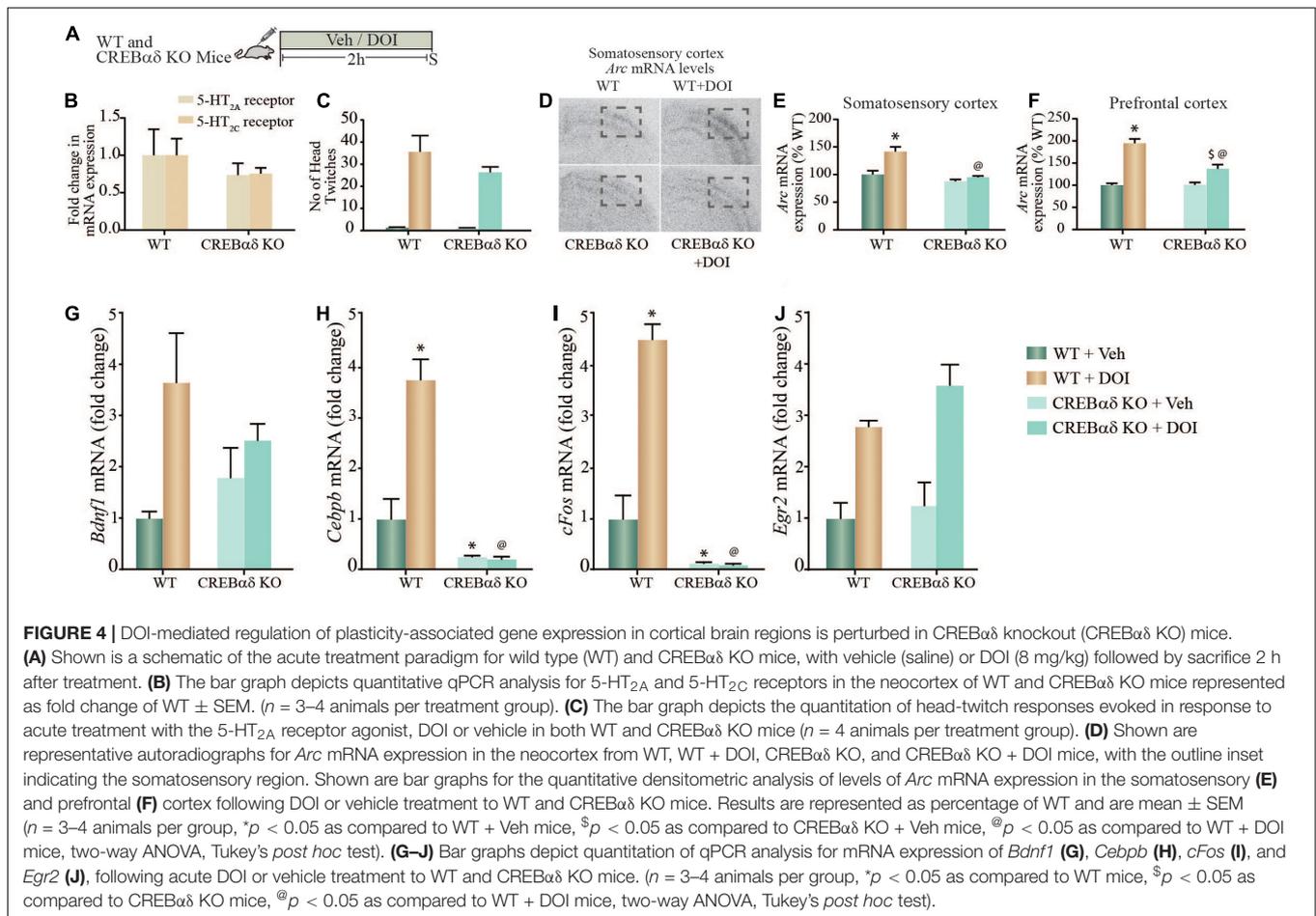
FIGURE 3 | Acute treatment with DOI enhances both the expression of putative CRE-containing plasticity-associated genes and the enrichment of pCREB within the promoter regions of specific plasticity-associated genes in the neocortex of adult rats. **(A)** Shown is a schematic of the treatment paradigm wherein adult Sprague-Dawley rats were injected with vehicle or DOI (8 mg/kg) and were sacrificed 2 h following treatment. **(B)** The bar graph indicates the fold change in mRNA expression of specific plasticity-associated genes in the neocortex of vehicle and DOI-treated rats represented as fold change of vehicle \pm SEM ($n = 4-6$ per treatment group, $*p < 0.05$ as compared to vehicle, unpaired Students t -test). **(C)** Shown are the chromatin immunoprecipitation (ChIP) PCR amplicons with primer locations spanning putative CRE sequences in the upstream gene regulatory sequences for *Arc*, *Bdnf1*, *Cebpb*, *cFos*, *Egr1*, and *Egr2*. **(D)** Shown is a bar graph for pCREB enrichment at the *Arc*, *Bdnf1*, *Cebpb*, *cFos*, *Egr1*, and *Egr2* promoters based on ChIP analysis performed on tissue derived from the neocortex of vehicle and DOI treated adult rats. Results are expressed as the fold change of vehicle \pm SEM. ($n = 7-10$ animals per treatment group, $*p < 0.05$ as compared to vehicle, unpaired Students t -test).

the neocortex, and while DOI-evoked a robust and significant upregulation of *cFos* mRNA levels in WT animals ($p = 0.02$) this was completely lost in the CREB $\alpha\delta$ KO mice. Neocortical *c-Fos* mRNA levels differed significantly between DOI-treated WT mice and the DOI-treated CREB $\alpha\delta$ KO cohort ($p < 0.0001$). In contrast, we observed no significant DOI by CREB $\alpha\delta$ KO genotype interaction for *Egr2* mRNA levels within the neocortex (**Figure 4J**). Further, while we did note a significant main effect of DOI for *Egr2* expression [$F_{(1,11)} = 10.19$, $p = 0.003$] we observed no main effect of CREB $\alpha\delta$ KO genotype. Collectively, these observations indicate that CREB contributes to the acute DOI-evoked upregulation of *Arc*, *Cebpb*, and *cFos* mRNA within the neocortex, but not to the DOI-induced neocortical increase in *Bdnf1* and *Egr2* mRNA expression. Furthermore, baseline expression of *Cebpb* and *cFos* mRNA, but not *Arc* mRNA is also significantly attenuated in the CREB $\alpha\delta$ KO mice. These results demonstrate that the transcription factor CREB contributes to

the regulation of a subset of neuronal plasticity-associated genes that are regulated by the hallucinogenic 5-HT $_2A$ receptor agonist, DOI in the neocortex.

DISCUSSION

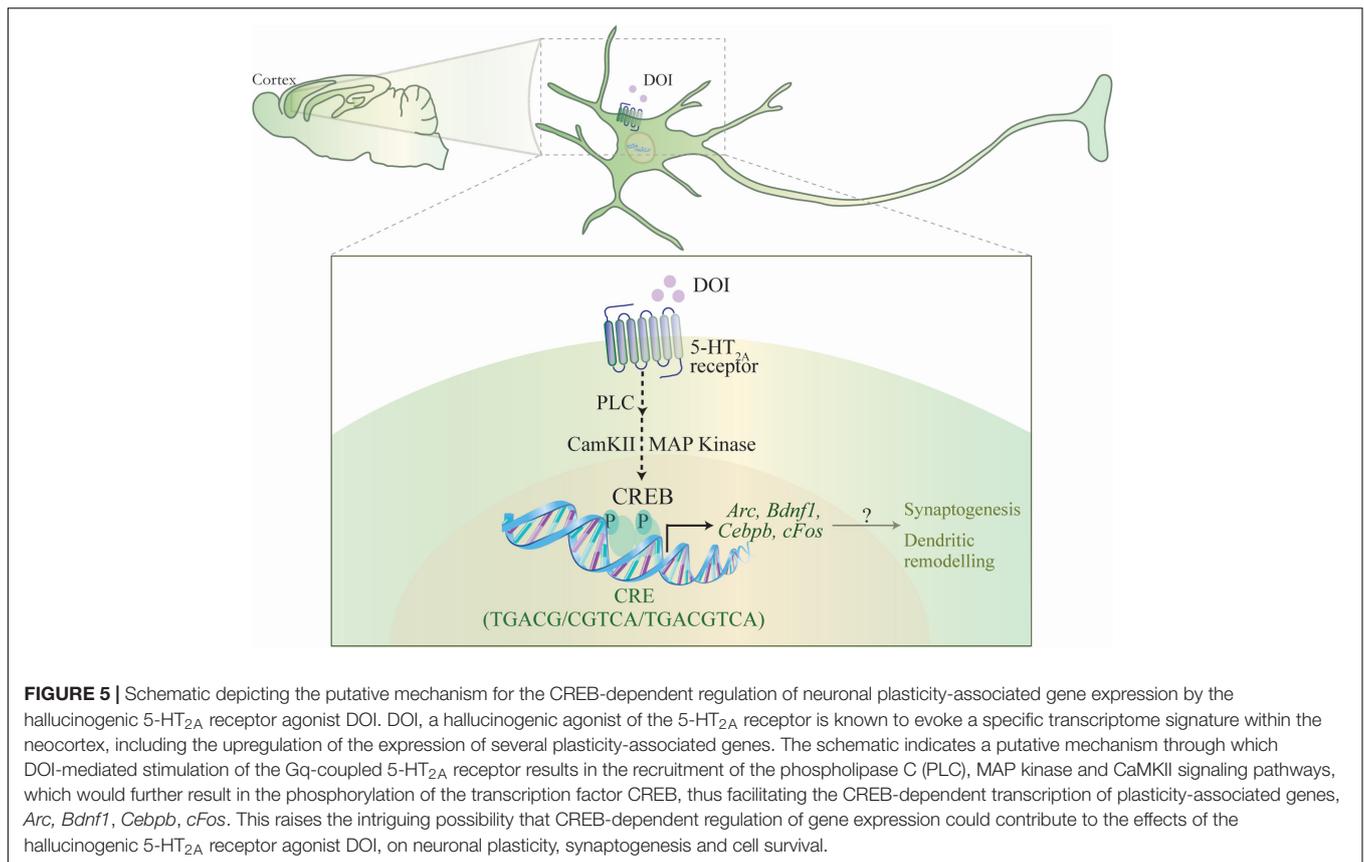
Here, we show that DOI, a hallucinogenic ligand of the Gq-coupled 5-HT $_2A$ receptor, recruits the nuclear transcription factor CREB to influence the expression of a specific subset of neuronal plasticity-associated genes in the rodent neocortex. DOI mediated stimulation of the 5-HT $_2A$ receptor in rat cortical neurons recruits the PLC, MAP kinase and CaMKII signaling pathways to rapidly increase the phosphorylation of CREB. 5-HT $_2A$ receptor stimulation by DOI, results in the upregulation of *Arc*, *Bdnf1*, *Cebpb*, and *Egr2* mRNA in cortical neurons, with a role for both the MAP kinase and CaMKII signaling pathways.



We also noted that while the regulation of *Egr1* is dependent on the MAP kinase pathway, the regulation of *cFos* expression by DOI is dependent on the CaMKII pathway. Further, we show recruitment of pCREB at CRE binding sites within the promoters of a subset of DOI-regulated target genes (*Arc*, *Bdnf1*, *Cebpb*, *cFos*) in the neocortex. In contrast, there are also specific DOI-regulated plasticity-associated genes (*Egr1*, *Egr2*) wherein we did not observe pCREB enrichment at putative CRE sites in their promoters. These findings collectively indicate that 5-HT $_{2A}$ receptor stimulation enhances pCREB enrichment at the promoters of a subset of DOI regulated target genes, suggestive of an upregulation of neuronal plasticity-associated genes in both a CREB-dependent and independent manner. This is further supported by evidence that the DOI-evoked transcriptional upregulation of specific neuronal plasticity-associated genes (*Arc*, *cFos*, and *Cebpb*) is attenuated in the neocortex of CREB $\alpha\delta$ KO mice (Figure 5).

Prior literature indicates that ligands at the 5-HT $_{2A}$ receptor can differentially recruit unique transcriptome fingerprints in the neocortex (González-Maeso et al., 2003), with the hallucinogenic compounds DOI and LSD enhancing the expression of specific transcripts, including *Egr1* and *Egr2* (González-Maeso et al., 2003, 2007). It is interesting that the nature and magnitude of regulation of specific neuronal

plasticity-associated genes is comparable to that noted with rapid-action antidepressant treatments such as ECS. Despite the knowledge that hallucinogenic agonists of the 5-HT $_{2A}$ receptor, such as DOI, induce the expression of multiple neuronal plasticity-associated genes, the contribution of specific signaling pathways and transcription factors to the 5-HT $_{2A}$ receptor regulated cortical gene expression remains poorly elucidated. DOI-mediated 5-HT $_{2A}$ receptor stimulation recruits the phospholipase C (PLC)-PKC-MAP kinase cascade and the PLC-CaMKII pathways that are known to also target the transcription factor CREB (Banerjee and Vaidya, 2020; Sharp and Barnes, 2020). The beta-arrestin-receptor complex also acts as a scaffold to activate the Raf-MEK-MAP kinase cascade (Schmid et al., 2008; McCorvy and Roth, 2015). Following 5-HT $_{2A}$ receptor activation by DOI, we observe that both the MAP kinase and CaMKII pathways, contribute to the DOI-evoked transcriptional increase of *Arc*, *Bdnf1*, *Cebpb*, and *Egr2* expression in rat cortical neurons, whilst the CaMKII pathway and the MAP kinase pathway contribute to the DOI-mediated regulation of *cFos* and *Egr1* expression respectively. This highlights the recruitment of distinct signaling pathways by the 5-HT $_{2A}$ receptor agonist, DOI (Banerjee and Vaidya, 2020), several of which converge on the transcription factor CREB (Shaywitz and Greenberg, 1999), placing it as a potential key 'hub' regulator



of the transcriptional changes that arise in response to DOI treatment. Interestingly, many of the genes we find to be robustly enhanced in expression following DOI treatment in cortical neurons *Arc*, *Bdnf1*, *Cebpb*, *cFos*, *Egr1*, and *Egr2*, are reported to contain putative CRE sites (TGACG/CGTCA/TGACGTCA) (Sheng et al., 1990; Niehof et al., 1997; Ahn et al., 1998; Walters and Blendy, 2001; Fukuchi et al., 2005; Bilbao et al., 2014; Duclot and Kabbaj, 2017; Ly et al., 2020). Our *in vitro* studies clearly indicate a robust induction in pCREB levels at the Ser133 site, with phosphorylation *via* the MAP kinase and CaMKII pathways likely contributing to this induction. Our experiments motivate further characterization of the target sites of CREB phosphorylation (Kornhauser et al., 2002), as certain phosphorylation signatures like Ser142 (Sun et al., 1994) can also serve to evoke an inhibitory effect on CREB mediated transcription.

A previous microarray study from our lab has reported several genes to be upregulated by DOI in the rodent neocortex (Benekareddy et al., 2010). We performed *in silico* analysis on the published array results, and noted that several of the genes upregulated by DOI have CRE elements in their upstream regulatory promoter regions, suggestive of a putative role for CREB in the regulation of multiple DOI-evoked transcripts. DOI-evoked a robust increase in the transcript expression of several CRE-containing genes, namely *Arc*, *Atf3*, *Atf4*, *Bdnf1*, *Cebpb*, *Cebpd*, *Egr1*, *Egr2*, *Egr3*, *Egr4*, *cFos*, *JunB*, and *Nfkb1a* in the rat neocortex. Interestingly, we observed pCREB enrichment at

the promoters of specific DOI-regulated genes. The expression of *Arc*, *Bdnf1*, *Cebpb*, and *cFos* genes but not *Egr1* and *Egr2* expression was accompanied by a significant enrichment of pCREB at their promoters in the neocortex of DOI-treated animals. In this regard, it is interesting to note that the genes, *Egr1* and *Egr2* which are part of the reported DOI-evoked hallucinogenic fingerprint (González-Maeso et al., 2003) do not appear to show pCREB enrichment at their promoters. While we have restricted our analysis of pCREB recruitment to CRE sites close to the promoter region, we cannot preclude the possibility of CREB binding at remote CRE sites either in distal enhancer regions, or within introns as we have not scanned pCREB enrichment at these loci. It is also important to keep in mind that the differential regulation of target genes by CREB may further be influenced by CRE sequence composition, location of the CRE and distance from the transcription start site (Mayr and Montminy, 2001; Altarejos and Montminy, 2011; Davis et al., 2020). Thus, the presence of a CRE site does not necessarily predict the recruitment of pCREB, and other transcription factors besides CREB are also likely to be recruited by DOI and by neuronal activity-dependent mechanisms, to evoke transcriptional increase of target genes. Our findings provide impetus for a broader genome-wide analysis of pCREB enrichment to get a sense of the span of pCREB-mediated regulation of transcription by DOI, and an understanding of recruitment of pCREB at both canonical and non-canonical CREs. It is also of interest to note that the

target genes regulated by DOI are known to exhibit substantial signaling crosstalk, and could also exert further feedback effects on CREB-dependent transcriptional regulation (Wu et al., 2001; Deisseroth and Tsien, 2002; Wiegert and Bading, 2011; Belgacem and Borodinsky, 2017). *Bdnf*, a DOI-regulated target gene, also contributes to the transcriptional regulation of *Arc* mRNA expression (Bramham et al., 2008; Benekareddy et al., 2012), and could further *via* regulation of the MAP kinase cascade impinge on pCREB mediated gene regulation (Finkbeiner et al., 1997). This raises the intriguing possibility that reciprocal interactions between BDNF and CREB could serve to amplify the regulation of several neuronal plasticity-associated genes (Finkbeiner et al., 1997; Shieh and Ghosh, 1999; Nair and Vaidya, 2006). These include the transcriptional regulation of *Arc* and *c-Fos* which are reported to play a significant role in coupling experience-dependent transcriptional regulation to synaptic plasticity (Duman et al., 2005; Bramham et al., 2008; Flavell and Greenberg, 2008; Shepherd and Bear, 2011; Minatohara et al., 2015). Fos may also function at enhancer elements to coordinate global activity-dependent gene transcription (Malik et al., 2014; Joo et al., 2016). Further, following DOI-evoked stimulation of the 5-HT_{2A} receptor, membrane depolarization and enhanced firing could also recruit additional activity-dependent mechanisms that drive this transcriptional program. This then suggests that stimulation of the 5-HT_{2A} receptor, sets into play a coordinated transcriptional program that involves crosstalk of diverse signaling cascades and transcription factors that drive the expression of several neuronal plasticity-associated genes (*Arc*, *Bdnf*, *cFos*), with CREB being amongst the key hub transcriptional factors that contributes to an important component of the DOI-evoked gene regulation pattern.

We have capitalized on the use of the CREB $\alpha\delta$ KO mouse line, which is deficient for the α and δ CREB isoforms leading to a robust reduction in CREB binding to consensus CRE target sites (Walters and Blendy, 2001), to evaluate the contribution of CREB to the effects of DOI on neuronal plasticity-associated gene expression. It is important to note that the CREB $\alpha\delta$ KO mice do not exhibit alterations in either the baseline expression of the 5-HT_{2A} receptor, or the stereotypical HTR behavior evoked by the 5-HT_{2A} receptor agonist DOI, that is known to be dependent on the cortical 5-HT_{2A} receptor. This would suggest that alterations in DOI-evoked gene expression in CREB $\alpha\delta$ KO mice are unlikely to arise due to a change at the level of 5-HT_{2A} receptor expression and coupling given that the CREB $\alpha\delta$ KO mice exhibit a robust DOI-evoked HTR response no different from WT controls and show no change in 5-HT_{2A} receptor expression. Interestingly, the DOI-evoked induction in cortical *Arc* mRNA expression was lost in CREB $\alpha\delta$ KO mice. Similar to this observation, the DOI-evoked increase in *Cebpb* and *cFos* mRNA levels were also abolished in the neocortex of CREB $\alpha\delta$ KO mice. Further, in CREB deficient mice the baseline expression of *Cebpb* and *cFos*, but not *Arc* mRNA was also reduced, thus supporting a role for CREB in regulation of basal expression of *Cebpb* and *cFos*. The DOI-evoked induction in *Egr2* mRNA was not altered in the neocortex of CREB $\alpha\delta$ KO mice, which is consistent with the evidence that pCREB was not found to be enriched at the *Egr2* promoter following DOI treatment. The

DOI-evoked increase in *Bdnf* transcript variant levels was also not significantly attenuated in CREB $\alpha\delta$ KO mice, and further we did not observe a change in basal *Bdnf* transcript expression either. We have focused on *Bdnf* which has been reported to contain CRE elements at its promoter, and exhibit CREB-mediated regulation in other contexts (Tabuchi et al., 2002; Esvald et al., 2020). *BdnfIII* and *BdnfIV* promoters are also known to contain CRE elements, as is the *Bdnf* coding exon, and it will be important to systematically address the contribution of CREB to the DOI-mediated regulation of multiple *Bdnf* transcript variants (Tao et al., 1998; Esvald et al., 2020). Indeed, prior reports clearly indicate several conditions in which CREB-dependent regulation of *Bdnf* gene expression contributes to neuroplasticity, in particular in the context of neuronal activity-dependent transcriptional coupling that drives structural and synaptic plasticity (Shieh and Ghosh, 1999; Vogt et al., 2014; Yan et al., 2016; Rafa-Zabłocka et al., 2018). While the CREB deficient mice provide a valuable tool to address the contribution of CREB to the DOI-mediated regulation of plasticity-associated gene expression, they come with a caveat of a constitutive, developmental onset loss-of-function of CREB which could in turn disrupt several key signaling pathways. In addition, we have not extensively profiled the consequence of loss of CREB $\alpha\delta$ subunits on the composition of the transcription factors that now occupy CRE sites in the absence of CREB $\alpha\delta$, which could itself substantially influence CRE-mediated transcriptional outcomes. Further experiments are warranted to systematically evaluate the contribution of CREB to the effects of DOI on neuronal plasticity-associated genes using approaches that allow for a more targeted strategy of adult onset, neuronal circuit-specific loss of function of CREB.

Our findings highlight a key role for CREB in contributing to the DOI-mediated regulation of specific neuronal plasticity-associated genes in the neocortex. This raises the intriguing possibility that similar to both slow-onset and rapid action antidepressants that recruit CREB to drive transcriptional changes in neurotrophic and plasticity-associated genes (Nibuya et al., 1996; Duman and Nibuya, 1997; Thome et al., 2000; Chen et al., 2001), serotonergic psychedelics that target the 5-HT_{2A} receptor may also recruit CREB to drive a plasticity-associated transcriptional program. These observations encourage further investigation into the role of CREB in regulating the transcription of plasticity-associated genes evoked by hallucinogenic 5-HT_{2A} receptor agonists, thus creating a conducive milieu for the psychoplastogenic actions of serotonergic psychedelics on dendritic plasticity and synaptogenesis in the neocortex.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by the University of Pennsylvania Animal Care and Use Committee. All animal

procedures using Sprague-Dawley rats and serotonin_{2A} receptor knockout (5-HT_{2A}^{-/-}) mice were carried out in accordance with the Committee for Care and Supervision of Experimental Animals (CPCSEA) and approved by the TIFR Institutional Animal Ethics Committee. All experiments with the CREB $\alpha\delta$ KO mouse line were carried out in accordance with the NIH guideline for the care and use of laboratory animals.

AUTHOR CONTRIBUTIONS

LD, MB, SF, FM, BJ, UG, and TG performed the experiments and analyzed the data. JB and VV designed the experiments and analyzed the data. SF and VV wrote the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fnmol.2021.790213/full#supplementary-material>

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