Enhanced Function of Prefrontal Serotonin 5-HT$_2$ Receptors in a Rat Model of Psychiatric Vulnerability

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Abstract

Prefrontal serotonin 5-HT$_2$ receptors have been linked to the pathogenesis and treatment of affective disorders, yet their function in psychiatric vulnerability is not known. Here, we examine the effects of 5-HT$_2$ receptors in a rat model of psychiatric vulnerability using electrophysiology, gene expression, and behavior. Following the early stress of chronic maternal separation, we found that serotonin has atypical 5-HT$_2$ receptor-mediated excitatory effects in the adult prefrontal cortex that were blocked by the 5-HT$_{2A}$ receptor antagonist MDL 100907. In the absence of a serotonergic agonist, the intrinsic excitability of the prefrontal cortex was not enhanced relative to controls. Yet, in response to stimulation of 5-HT$_2$ receptors, adult animals with a history of early stress exhibit heightened prefrontal network activity *in vitro*, enhanced immediate early gene expression *in vivo*, and potentiated head shake behavior. These changes arise in the absence of any major alteration of prefrontal 5-HT$_{2A/C}$ mRNA expression or 5-HT$_2$ receptor binding. Our microarray results and quantitative PCR validation provide insight into the molecular changes that accompany such enhanced 5-HT$_2$ receptor function in adult animals following early stress. We observed persistent prefrontal transcriptome changes, with significant enrichment of genes involved in cellular developmental processes, regulation of signal transduction, and G-protein signaling. Specific genes regulated by early stress were validated in an independent cohort, and several altered genes were normalized by chronic blockade of 5-HT$_2$ receptors in adulthood. Together, our results demonstrate enhanced prefrontal 5-HT$_2$ receptor function and persistent alterations in prefrontal gene expression in a rat model of psychiatric vulnerability.

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Introduction

Prefrontal serotonin 5-HT$_2$ receptors have long been implicated in mood and anxiety disorders (Naughton et al., 2000; Stockmeier, 2003). However, it remains controversial whether enhanced 5-HT$_2$ receptor binding in the prefrontal cortex (PFC) predicts the presence of a psychiatric illness (D'haenen et al., 1992; Yatham et al., 2000; Shelton et al., 2009) and whether a change in this measure predicts the effectiveness of treatment (Yatham et al., 1999; Meyer et al., 2001; Zanardi et al., 2001). Instead, it has been hypothesized that 5-HT$_2$ signaling efficiency may be a better predictor of vulnerability to psychiatric illness (González-Maeso and Meana, 2006). Patients with mood disorders have increased coupling efficiency of the 5-HT$_2$ receptor with its associated G-protein in the PFC (Friedman and Wang, 1996) and in blood platelets (Parakh et al., 2008). Furthermore, adding a 5-HT$_2$ antagonist to the current standard treatment for mood and anxiety disorders augments its therapeutic efficacy (Marek et al., 2003) and improves the therapeutic response of treatment-resistant patients (Brawman-Mintzer et al., 2005; Marcus et al., 2008; Sato et al., 2009). However, it has not been directly investigated whether vulnerability to mood and anxiety disorders is associated with an increase in prefrontal 5-HT$_2$ receptor electrophysiological effects and downstream signaling cascades.

Here, we have investigated the function of the prefrontal 5-HT$_2$ receptors in a rat model of increased vulnerability to adult psychopathology. We chose the early stress of maternal separation, since it results in a persistent vulnerability to endophenotypes of anxiety and depression in adulthood (Sánchez et al., 2001). Using a combination of whole-cell electrophysiological recordings, 5-HT$_2$-mediated induction of immediate early gene expression, and behavioral output, we demonstrate that exposure to early stress (ES) increases prefrontal 5-HT$_2$ signaling efficacy in adulthood. We further identified alterations in genes associated with cellular development and signal transduction in the PFC of adult animals previously exposed to ES, using microarray analysis with quantitative PCR (qPCR). Collectively, our findings indicate that a life history of ES enhances the function of prefrontal 5-HT$_2$ receptors. Given the importance of the PFC in modulating emotional behavior (Price, 1999), and the ability of cortical 5-HT$_{2A}$ receptors to influence anxiety states (Weisstaub et al., 2006), this neurobiological abnormality may contribute to the genesis of mood and anxiety disorders.

Materials and Methods

Animals

Sprague Dawley rats were used for all experiments (electrophysiological, behavioral, and molecular experiments) and were maintained on a 12 h light/dark cycle (lights on at 7:00 A.M.) with *ad libitum* access to food and water. The electrophysiological experiments were performed at the University of Toronto and approved by the local Animal Care and Use Committee. The molecular and behavioral experiments were performed at the Tata Institute of Fundamental Research (TIFR), and were approved by the TIFR Institutional Animal Ethics Committee. All protocols conformed to the National Institutes of Health Guidelines on the Care and Use of Experimental Animals.

Early stress paradigm

As a model of vulnerability to psychiatric disorders, the ES paradigm of maternal separation (Nair et al., 2007) was used. In brief, pregnant primiparous dams delivered pups within the animal housing facility and on postnatal day (P) 1 litters were randomly assigned to ES or control groups. Pups in the ES litters were separated from their mothers for a period of 3 h at the same time in the morning each day from P2 to P14. Control litters were left undisturbed. All litters were handled briefly at 3–4 d intervals to allow for cage cleaning. Once weaned, all pups were housed in same-sex sibling groups of 2–5 rats.

Electrophysiological experiments

Slice preparation—Sprague Dawley dams (Charles River) delivered pups within the University of Toronto (Toronto, Ontario, Canada) animal housing facility and underwent the ES paradigm described above. The electrophysiological experiments included recordings of 21 rats from 11 control litters and 28 rats from 10 ES litters. In adolescence and adulthood, male and female rats (P21–83) were anesthetized with chloral hydrate (400 mg/kg) and coronal slices (400 μm thick) of the PFC (4.2 to 2.5 mm from bregma) were sliced on a Dosaka linear slicer (SciMedia) and transferred to 32°C oxygenated ACSF (128 m$_M$ NaCl, 10 m$_M$ D-glucose, 24 m$_M$ NaHCO$_3$, 2 m$_M$ CaCl$_2$, 2 m$_M$ MgSO$_4$, 3 m$_M$ KCl, 1.25 m$_M$ NaH$_2$PO$_4$, pH 7.4) in a prechamber (Automate). For recording, slices were placed in a modified superfusion chamber (Warner Instruments) and mounted on the stage of an Olympus BX50WI microscope. Oxygenated ACSF at 31–33°C flowed over the slice at a rate of 3–4 ml/min.

Whole-cell recording—Whole-cell patch electrodes (2–3 MΩ) contained 120 m$_M$ potassium gluconate, 5 m$_M$ KCl, 2 m$_M$ MgCl$_2$, 4 m$_M$ K$_2$-ATP, 0.4 m$_M$ Na$_2$-GTP, 10 m$_M$ Na$_2$-phosphocreatine, and 10 m$_M$ HEPES buffer (adjusted to pH 7.3 with KOH). Layer V pyramidal neurons in PFC (prelimbic and anterior cingulate regions) were patched under visual control using infrared differential interference contrast microscopy. Pyramidal neurons were identified based on their pyramidal shape and the presence of a prominent apical dendrite. Currents were recorded using continuous single-electrode voltage-clamp mode with a Multiclamp 700b amplifier (Molecular Devices), acquired at 20 kHz, and low-pass filtered (3 kHz) using pClamp10.2 and Digidata1440 software (Molecular Devices).

Data collection—After a 1 min period of baseline recording, serotonergic currents were probed by adding 5-hydroxytryptamine creatinine sulfate (5-HT) (10 μ_M, 30 s) in the bath perfusion followed by at least a 5 min washout period. The amplitude of the dominant 5-HT current was measured using Clampfit software (Molecular Devices) by subtracting the mean current at the peak of the 5-HT response (averaged over 10 s) from the mean current at baseline (averaged over 10 s). There were no significant sex differences in the 5-HT currents within the control and ES groups (unpaired t test, $p = 0.9$). When applying DOI (3 μ_M, 15 min) to measure the inward currents elicited by 5-HT$_2$ stimulation, we noticed the spontaneous appearance of network activity or "up" states in almost every recording from the ES animals. These phenomena were identified and quantified based on previous description (Sanchez-Vives and McCormick, 2000).

To measure membrane excitability, neurons were injected with depolarizing current pulses of 500 ms length, increasing in 10 pA increments from 0 to 400 pA and separated by a 1 s interval. Spontaneous postsynaptic currents (sPSCs) were analyzed with MiniAnalysis software (Synaptosoft). Glutamatergic sPSCs were recorded at baseline and during 5-HT application (10 μ_M, 30 s) under the recording conditions described above. Recording of GABAergic sPSCs were performed with patch electrodes that contained 50 m$_M$ K-gluconate, 75 m$_M$ KCl, 2 m$_M$ MgCl$_2$, 4 m$_M$ K$_2$-ATP, 400 μ_M Na$_2$-GTP, 10 m$_M$ Na$_2$-phosphocreatine, and 10 m$_M$ HEPES buffer (adjusted to pH 7.3 with KOH). These recordings were performed in the presence of the AMPA/KA glutamate receptor antagonist 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) (20 μ_M). Under these conditions, GABAergic sPSCs were completely suppressed by application of the GABA$_A$ receptor blocker bicuculline (10 μ_M; 10 min). Analysis of glutamatergic and GABAergic sPSCs was performed using MiniAnalysis software (Synaptosoft).

Pharmacology—In a subset of experiments, pharmacological agents were applied to the slice using oxygenated ACSF: 50 μ_M D(−)-2-amino-5-phosophonopentanoic acid (APV), 10 μ_M bicuculline, 20 μ_M CNQX, 3 μ_M DOI, 2 μ_M ketanserin tartrate, 30 n$_M$ MDL 100907, and 30 n$_M$ WAY 100635. The MDL 100907 was a gift from Dr. George Aghajanian of Yale University (New Haven, CT). All other compounds were obtained from Sigma or Tocris Bioscience. All compounds stored in stock solutions at −20°C before being diluted in oxygenated ACSF.

Statistical analysis—All statistical comparisons were made at a significance level of 0.05 unless noted otherwise. Statistical comparisons between responses from different experimental groups (control vs ES) were determined using two-tailed unpaired *t* tests. Analysis of correlations between experimental parameters (e.g., postnatal age and 5-HT currents) were examined using linear regression. Two sets of analysis were performed for sPSCs. Within-cell analysis of 5-HT-elicited change in sPSCs was examined with Kolmogorov–Smirnov test (significance level of 0.01). The average sPSC frequency by group was assessed with parametric two-way ANOVA and *post hoc w*ith Bonferroni tests. Comparison between proportions of cells displaying a network activity response or 5-HT-elicited sPSC response was analyzed using Fisher's exact test.

Molecular and behavioral experiments

Experimental design—Sprague Dawley rats (bred in the animal facility at TIFR) underwent the ES paradigm as described above, and the males were examined in adulthood (P60–90). Using qPCR and radioligand binding for [^{3}H]ketanserin, the influence of ES on 5-HT$_{2A}$ and 5-HT$_{2C}$ receptor mRNA expression and 5-HT$_2$ binding in the PFC (4.2 to 2.5 mm from bregma) was assessed. To examine the functional status of the prefrontal 5-HT$_2$ receptors, both control and ES animals were intraperitoneally administered the 5-HT$_2$ agonist DOI (8 mg/kg, Sigma) or vehicle (0.9% NaCl). The effect of DOI on the expression of the immediate early gene Arc was examined using *in situ* hybridization. DOI-induced head twitch response, a behavior mediated by prefrontal 5-HT$_{2A}$ receptors (Willins and Meltzer, 1997), was studied in control and ES animals. To determine changes in gene expression that arise in the PFC following a history of ES, a microarray analysis was

performed. Furthermore, to address gene expression changes that arise following 5-HT$_2$ stimulation, the DOI-induced transcriptome in the PFC of control animals was analyzed. Candidate genes observed to be regulated in the microarray studies were validated using qPCR on independent tissue samples. Finally, we addressed whether a component of the prefrontal transcriptome regulated by early stress history can be reversed by systemic treatment with the 5-HT$_2$ receptor antagonist ketanserin (Sigma).

5-HT$_2$ receptor autoradiography—Control ($n = 4$) and ES animals ($n = 7$) were rapidly decapitated and the brains were frozen on dry ice and stored at $-80°C$ before processing for receptor autoradiography. Coronal sections (14 μm thick) were cut on the cryostat (Leica), thaw mounted on Probe-on Plus slides (Electron Microscopy Sciences), and stored at $-80°C$. Receptor autoradiography for [^{3}H]ketanserin (67ci/mmol; PerkinElmer) binding in the PFC of control and ES animals was assessed as described previously (Preece et al., 2004). In brief, two slides from each brain were preincubated in a buffer containing 170 m$_M$ Tris, pH 7.7 (binding buffer), followed by incubation in the same buffer containing 2 n$_M$ [^{3}H]ketanserin for 2 h at room temperature. Prazosin (1 μ_M; Sigma) was added to block binding to α_1 adrenoceptors. Furthermore, 10 μ_M ketanserin, along with 2 n$_M$ [^{3}H]ketanserin, was used as a nonspecific binding control on separate slides. The slides were washed with binding buffer, air dried overnight, and exposed to ^{3}H-sensitive film (Kodak) for 8–10 weeks. The autoradiograms were developed and binding densities were quantitated using Scion Image software (Scion). The binding density of [^{3}H]ketanserin in the PFC region was determined using optical density measurements (average of 6–8 measurements from three or four sections per animal). The calibration was done using ^{14}C standards, and data were expressed as percentage of control. The slides incubated with 10 μ_M ketanserin (nonspecific binding control) did not yield any detectable signal confirming the specificity of [^{3}H]ketanserin binding.

***In situ* hybridization—**To visualize alterations in circuit activity induced by 5-HT$_2$ stimulation with DOI, *in situ* hybridization was performed for the immediate early gene, activity regulated cytoskeletal-associated protein Arc (Temple et al., 2003). Control and ES animals ($n = 4$–5 per group) received an i.p. injection of DOI or vehicle and were killed 2 h later. Animals were rapidly decapitated and the brains were immediately frozen on dry ice. *In situ* hybridization was performed as described previously (Nair et al., 2007). Cryostat-cut (14 μm) coronal sections (4.2 to 2.5 mm from bregma) were fixed, acetylated, and dehydrated before storage at $-70°C$. Antisense riboprobes to Arc mRNA were generated from a transcription competent plasmid kindly provided by Dr. Oswald Steward (Johns Hopkins University, Baltimore, MD). Slides were incubated with the ^{35}S-UTP-labeled riboprobe (1×10^6 cpm/150 μl) in hybridization buffer (50% formamide, 1× SSC, 25× Denhardt's solution, 40 m$_M$ dithiothreitol, 150 μg/ml yeast tRNA, 10% dextran sulfate, 400 μg/ml salmon sperm DNA) for 16 h at 60°C. Following hybridization, all slides were washed in ribonuclease A (20 μg/μl) followed by stringent washes in decreasing concentrations of SSC. Slides were air dried and exposed to Hyperfilm β-max (Kodak) for 5 d. Levels of Arc mRNA were quantified using Scion Image software and calibrated using ^{14}C standards to correct for nonlinearity. Optical density was measured from both hemispheres of the PFC in three or four sections per brain.

DOI-induced head shake behavior—DOI-induced head shakes are a stereotypical behavior elicited by activation of 5-HT$_{2A}$ receptors in the PFC (Willins and Meltzer, 1997). To address whether a history of ES influenced 5-HT$_{2A}$ receptor-mediated behavior, the frequency of head shakes induced in response to DOI administration was examined in control and ES animals. Adult control and ES rats ($n = 4–5$ per group) received an i.p. injection of either DOI or vehicle, and behavior was recorded in their home cages for a duration of 20 min commencing 20 min after the injection of DOI or vehicle. The head shakes were defined as a rapid radial movement of the head and were counted by an experimenter blind to the treatment groups.

Microarray—To understand the mechanistic underpinnings of the altered 5-HT$_2$ function in ES animals, a microarray approach was used to examine the long-lasting transcriptional changes in adult ES animals compared with controls. Furthermore, to assess whether the transcriptome changes observed in adult animals with a history of ES include a component that may be a consequence of altered 5-HT$_2$ receptor signaling, we compared the ES transcriptome to the changes induced in response to 5-HT$_2$ receptor stimulation by DOI. Animals from three experimental groups [control ($n = 3$), ES ($n = 4$), DOI ($n = 4$)] were rapidly decapitated 2 h after either DOI or vehicle injection. The PFC was dissected, frozen in liquid nitrogen, and stored at −80°C. RNA from bilateral prefrontal cortices from each animal was extracted using an Rneasy minikit (Qiagen). The quality and integrity of the RNA was determined using an optical density ratio of 260/280 using the NanoDrop spectrophotometer (NanoDrop Technologies) and bioanalyzer profiles using Agilent 2100 Bioanalyzer (Agilent Technologies). RNA was labeled using the Agilent Quick Amp PLUS kit according to manufacturer's instructions (Agilent Technologies). Agilent standard spike controls were used in all labeling reactions. The labeled RNA (1650 ng) was fragmented and hybridized to a custom rat array 8X15K (Agilent microarray design identifier: 21617) with 15,000 features. The hybridized slides were washed using wash buffers (Agilent Technologies) and scanned using the Agilent microarray scanner G, model G2565BA, at 100% laser power, 30% photomultiplier tube, and 5 μm resolution. Data extraction was performed with Agilent Technologies Feature Extraction software (version 9.1).

Feature extracted data were analyzed using GeneSpring GX, version 7.3.1 software from Agilent Technologies. Normalization of the data was done in GeneSpring GX using the recommended one color per chip, and per gene data were as follows: (1) transformation: set measurements <0.01 to 0.01; (2) per chip: normalize to 50th percentile; (3) per gene: normalize to specific samples (control or ES). Differentially regulated genes were filtered with a cutoff of >1.5 for upregulation and <0.66 for downregulation. Statistical analysis was done using a *t* test with a significance level of 0.05 and corrected for multiple comparisons using the Benjamini and Hochberg method with a false discovery rate of 0.05 for all arrays (Benjamini and Hochberg, 1995). Hierarchical clustering was done based on fold change values for each gene using the GeneSpring software. The microarray data discussed in this manuscript have been deposited in the National Center for Biotechnology Information's Gene Expression Omnibus (GEO accession number: GSE14720). Upregulated and downregulated genes in the ES animals (compared with control) were subjected to functional analysis using DAVID (Database for Annotation, Visualization, and Integrated

Discovery; http://david.abcc.ncifcrf.gov/) functional annotation tools (Dennis et al., 2003; Huang et al., 2009).

Quantitative PCR—To validate candidate genes from our array studies, qPCR was conducted on tissue samples from an independent experimental cohort of animals. qPCR was also performed to determine the influence of ES on 5-HT_{2A} and 5-HT_{2C} receptor mRNA expression. Tissue samples from the PFC were prepared as described above, RNA was extracted, and quality control was performed to confirm RNA integrity using spectroscopic analysis. RNA was then reverse transcribed and subjected to qPCR with TaqMan probes (Applied Biosystems). Quantification was determined using the Ct method as described previously (Bookout and Mangelsdorf, 2003; Tsankova et al., 2004). Data from all groups were normalized to an average of four endogenous housekeeping genes (*18s rRNA*, *Gapdh*, *Hprt*, *β-actin*). Results were compared with the control group and expressed as a fold change ± SEM. See supplemental Table 5 for a list of primers used.

Blockade with the 5-HT_2 antagonist, ketanserin—To address whether chronic blockade of 5-HT_2 receptor signaling is capable of restoring gene expression changes observed in the PFC of ES animals, we treated animals with the 5-HT_2 antagonist ketanserin. Control and ES animals were treated with ketanserin for 6 d, receiving ketanserin (2 mg/kg) (Pei et al., 2000) or vehicle (10% DMSO) via i.p. injections twice daily. On day 6, the animals were killed 1 h after receiving the first injection of the day. The PFC was dissected, frozen in liquid nitrogen, and stored at −80°C. RNA was extracted, subjected to quality control analysis, reverse transcribed, and subjected to qPCR using SYBR Green (Applied Biosystems) as described above. See supplemental Table 6 for a list of primers used.

Statistical analysis—Statistical analysis was performed using the software Prism (Graphpad). Experiments with two groups were analyzed using the unpaired Student's *t* test. Experiments with four groups were subjected to a two-way ANOVA, followed by a Bonferroni *post hoc* test with a significance level of 0.05.

Results

Early stress changes the adult pattern of responses to serotonin in prefrontal neurons

To assess the effects of ES on the response to serotonin in the PFC, we performed whole-cell recordings of layer V pyramidal neurons in the PFC from control and ES rats. In control animals, we found that bath application of 5-HT (10 μM; 30 s) elicited a dominant outward current in almost all layer V pyramidal neurons ($n = 30/35$; 86%), with only a small percentage of neurons displaying no response ($n = 5/35$; 14%). These findings are summarized in Figure 1*A* and are consistent with previous reports showing that the net serotonergic response of adult layer V pyramidal neurons is dominated by an inhibitory 5-HT_{1A}-mediated outward current (Gartside et al., 2000; Béïque et al., 2004; Puig et al., 2005).

In brain slices from ES animals, by contrast, bath application of 5-HT (10 μM; 30 s) elicited a different pattern of responses in layer V pyramidal neurons (Fig. 1*B*). A group of the

neurons responded to 5-HT with dominant outward currents ($n = 27/47$; 58%), one showed no response ($n = 1/47$; 2%), and almost half the neurons displayed dominant excitatory inward currents ($n = 19/47$; 40%), an effect not observed in control neurons. These unexpected, 5-HT-elicited inward currents were observed in ES animals throughout adolescence and adulthood (P21–83) with no significant change with age ($n = 19$; linear regression: $R^2 = 0.09$; $F_{(1,17)} = 1.66$; $p = 0.2$). The existence of the dominant 5-HT inward currents in ES neurons significantly shifted the mean of the 5-HT current toward an excitatory response: 34.2 ± 3.7 pA in controls ($n = 35$) versus 5.9 ± 5.6 pA in ES ($n = 47$; $p = 0.0002$, unpaired t test with Welch's correction) (Fig. 1C).

Adult prefrontal neurons show greater 5-HT$_2$-mediated currents following early stress

Since 5-HT$_{2A}$ receptors are expressed in prefrontal layer V neurons in adulthood (Burnet et al., 1995; Amargós-Bosch et al., 2004; Santana et al., 2004; Weber and Andrade, 2010), we hypothesized that the inward currents in ES animals were mediated by the 5-HT$_{2A}$ receptor. Notably, the excitatory 5-HT$_2$ current is the dominant serotonergic regulator of prefrontal layer V neurons during the first 2 weeks of normal postnatal development (Zhang, 2003; Béïque et al., 2004). In adult ES rats, we found that the unusual, 5-HT-elicited inward currents were completely blocked in the presence of the 5-HT$_2$ antagonist, ketanserin ($2 \mu_M$; 10 min, $n = 5$), as illustrated in Figure 2A. To compare the magnitude of the 5-HT$_2$ current across the groups, we examined neuronal responses to the partial 5-HT$_2$ agonist, DOI ($3 \mu_M$; 15 min). DOI elicited a significantly larger inward current in layer V pyramidal neurons from ES animals: -9.9 ± 2.7 pA in controls ($n = 9$) versus -42.4 ± 6.1 pA in ES ($n = 11$; unpaired t test with Welch's correction; $p = 0.0003$) (Fig. 2B,C). Finally, to determine whether the 5-HT-elicited inward current was mediated by the 5-HT$_{2A}$ or 5-HT$_{2C}$ receptor, we used the selective 5-HT$_{2A}$ antagonist MDL 100907 (30 n$_M$; 10 min). To examine the inward current in both control and ES neurons, we first had to block the 5-HT$_{1A}$ outward current (note that this inhibitory receptor dominates the net response to 5-HT in control neurons and a large portion of the ES neurons) with the 5-HT$_{1A}$ antagonist WAY 100635 (30 n$_M$; 10 min). Under these conditions, 5-HT-elicited inward currents (10–$30 \mu_M$; 30 s) could be observed in both groups and were completely blocked by MDL 100907: ES animals ($100 \pm 0\%$ blockade, $n = 5$, paired t test, $p = 0.01$) (Fig. 2D) and controls ($100 \pm 0\%$ suppression; $n = 4$; paired t test, $p = 0.01$). Together, these findings suggest that the potentiated excitatory responses to 5-HT in the adult ES prefrontal cortex are mediated by 5-HT$_2$ receptors of the 5-HT$_{2A}$ subtype.

Influence of early stress on 5-HT$_2$ receptor binding, 5-HT$_{2A}$ and 5-HT$_{2C}$ mRNA

Next, we investigated potential mechanisms mediating the enhanced 5-HT$_2$ receptor currents in ES animals. Consistent with work in nonhuman primates (Law et al., 2009), qPCR results showed no significant difference in 5-HT$_{2A}$ receptor (ES vs control fold change: 0.84 ± 0.09; $p = 0.3$, unpaired t test; control, $n = 8$; ES, $n = 10$) or 5-HT$_{2C}$ receptor (ES vs control fold change: 0.98 ± 0.10; $p = 0.9$, unpaired t test; control, $n = 4$; ES, $n = 9$) mRNA expression in the PFC of ES animals. However, quantitative receptor autoradiography using [^{3}H]ketanserin revealed a small but significant, increase in 5-HT$_2$ receptor density in the prefrontal cortex of ES animals ($p = 0.02$, unpaired t test; control, $n = 4$; ES, $n = 7$) (Fig. 3A). It is noteworthy that despite substantial changes in 5-HT$_2$-evoked responses observed in

ES animals, these are not accompanied by commensurate alterations in $5\text{-}HT_2$ receptor binding or mRNA expression.

Early stress does not alter the sensitivity of the $5\text{-}HT_{2A}$-mediated inward current

A small increase in density of $5\text{-}HT_2$ receptors could significantly shift the 5-HT concentration–response curve to the left if the increased density reflects an enhanced number of spare $5\text{-}HT_{2A}$ receptors (Bourne and von Zastrow, 2007). Therefore, we compared the $5\text{-}HT_{2A}$ concentration–response curves from control and ES layer V neurons. These recordings were performed in the presence of a $5\text{-}HT_{1A}$ receptor antagonist (30 nM WAY 100635) as well as blockers for ionotropic glutamatergic (20 μM CNQX and 50 μM AP-5) and GABAergic (10 μM bicuculline) neurotransmission. As illustrated in Figure 3*B*, there was no significant difference in the $5\text{-}HT_{2A}$ concentration–response relationships between control (EC_{50}, 5.6 ± 1.9 μM; $n = 5$) and ES neurons (EC_{50}, 5.1 ± 1.9 μM; $n = 5$; unpaired t test, $p = 0.8$). These results suggest that the number of spare receptors and the sensitivity of the $5\text{-}HT_{2A}$ receptor for 5-HT is unaltered by a history of early stress.

Early stress increases spontaneous network activity in adult prefrontal slices exposed to DOI

Surprisingly, following the application of the $5\text{-}HT_2$ receptor partial agonist DOI (3 μM; 15 min), we observed the occurrence of spontaneous network activity in ES animals, as illustrated in Figure 4. These network activity events produced a temporary excited state in the layer V neurons, often accompanied by the discharge of action potentials when observed in current-clamp (Fig. 4*A*). To quantify the amplitude of the network activity, voltage-clamp recordings were performed (Fig. 4*B*; Table 1).We found that the majority of layer V pyramidal neurons from ES animals displayed spontaneous network activity events in the presence of DOI ($n = 12/13$; 92%) (Table 1). In brain slices from control animals, by contrast, the presence of DOI (3 μM; 15 min) rarely produced spontaneous network activity in layer V pyramidal neurons ($n = 2/13$; 15%; $p = 0.0002$, Fisher's exact test). The scarcity of spontaneous network activity in the control slices following DOI is consistent with the results of previous work (Lambe and Aghajanian, 2007). These results illustrated that a history of ES significantly increased the probability of $5\text{-}HT_2$-mediated network activity measured in layer V pyramidal neurons of the PFC.

Early stress does not increase the intrinsic excitability of prefrontal layer V pyramidal neurons

The increased prefrontal $5\text{-}HT_2$-mediated excitability in ES animals occurred without notable change to the intrinsic excitability of the prefrontal layer V neurons. As shown in Figure 5*B*, layer V pyramidal neurons from ES animal did not display altered neuronal excitability, as quantified by the relationship between current injection and the frequency of action potential firing: slope of 5.42 ± 0.07 Hz/100 pA in controls ($n = 15$) versus slope of 5.40 ± 0.08 Hz/100 pA ($n = 15$; unpaired t test, $p = 0.9$) in ES animals. Consistent with these findings, we observed no difference in the input resistance of layer V neurons between control and ES animals (Table 2). However, we did observe a small, albeit significant, depolarization in ES layer V pyramidal neurons (unpaired t test, $p = 0.03$) (Table 2).

Interestingly, when we repeated the input–output experiments after injecting positive or negative current to bring each neuron to a membrane potential of −75 mV, ES neurons displayed a trend toward reduced excitability compared with controls: slope of 5.70 ± 0.08 Hz/100 pA in controls ($n = 14$) versus slope of 5.55 ± 0.05 Hz/100 pA in ES animals ($n = 15$; unpaired t test, $p = 0.054$) (supplemental Fig. 1).

Early stress enhances 5-HT$_2$-elicited glutamatergic sPSCs and baseline GABAergic sPSCs

To examine further the excitability of the prefrontal brain slices, we measured fast glutamatergic synaptic transmission at baseline and in the presence of 5-HT (10 μ_M; 30 s). At baseline, we observed no difference in baseline frequency of glutamatergic sPSCs (Fig. 5*C*). However, ES animals displayed a significantly higher frequency of glutamatergic sPSCs in the presence of 5-HT (10 ± 1 Hz; $n = 21$) than control animals (3 ± 1 Hz; $n=20$). As illustrated in Figure 5*C*, two-way ANOVA revealed a significant interaction between group and the effect of 5-HT ($F_{(2,96)} = 8.0$, $p = 0.0006$). Consistent with previous work demonstrating that cortical 5-HT$_{2A}$ receptors mediate this effect normally (Weisstaub et al., 2006), the 5-HT$_2$ antagonist ketanserin (2 μ_M; 10 min) completely blocked the 5-HT-elicited increase in glutamatergic sPSCs in ES animals (1 ± 1 Hz; $n = 10$, $p = 0.004$). There were no significant group differences in the amplitude of glutamatergic sPSCs at baseline or in the presence of 5-HT, as illustrated in supplemental Figure 2). Interestingly, similar proportions of neurons showed significant 5-HT-elicited increases in glutamatergic sPSCs (Kolmogorov–Smirnov test, $p < 0.01$) in both control and ES animals (supplemental Table 1). Together, these results suggest that ES animals have increased glutamatergic synaptic activity in the presence of 5-HT, but not at baseline.

To compare prefrontal inhibition in control and ES animals, we examined fast GABAergic synaptic transmission at baseline and in the presence of 5-HT (10 μ_M; 30 s). To measure GABAergic sPSCs, we used high Cl$^-$ patch solution (77 m$_M$) in the presence of continuous application of AMPA/KA receptor blocker, CNQX (20 μ_M). Under these recording conditions, ES layer V neurons displayed significantly more frequent GABAergic sPSCs at baseline, compared with control (two-way ANOVA revealed a significant main effect of ES, $p = 0.001$). Bath application of 5-HT at 10 μ_M (30 s) did not significantly alter the frequency of GABAergic sPSCs in either the control or ES animals (two-way ANOVA revealed no main effect of 5-HT application, $p = 0.5$). A higher concentration of 5-HT may be required to increase the frequency of GABAergic sPSCs overall (Zhou and Hablitz, 1999). There was no significant group difference in the amplitude of GABAergic sPSCs at baseline or in the presence of 5-HT, as illustrated in supplemental Figure 2, and the proportions of neurons showing significant 5-HT-elicited increases in GABAergic sPSCs were not different (supplemental Table 1). These results show that the frequency of spontaneous GABAergic synaptic activity is significantly higher at baseline in brain slices of ES animals and that GABAergic synaptic activity is not increased to the same degree as glutamatergic synaptic activity by this concentration of 5-HT.

Early stress enhances *in vivo* DOI-induced Arc expression in the adult prefrontal cortex

Based on our electrophysiological data demonstrating enhanced DOI responses in prefrontal brain slices of ES animals, we sought to determine whether systemic DOI administration *in*

vivo also increased the activity of the PFC circuit. To visualize *in vivo* PFC circuit activity, we examined the expression of the immediate early gene, Arc (Temple et al., 2003), which has been previously shown to be strongly upregulated following systemic DOI administration (Pei et al., 2000). Consistent with the electrophysiological data showing that ES animals do not exhibit intrinsic differences in excitability, we observed no difference of baseline Arc mRNA expression between control and ES animals (Fig. 6). However, we observed that the DOI-elicited Arc mRNA expression in ES animals was significantly potentiated compared with controls (two-way ANOVA revealed a significant ES × DOI interaction; $F_{(1,15)} = 11.39$, $p = 0.004$) (Fig. 6). Collectively, these experiments indicated that a history of ES resulted in enhanced circuit activation in the PFC in response to 5-HT$_2$ stimulation in the absence of changes to baseline PFC excitability.

A history of early stress enhances DOI-induced head shake behavior

Activation of 5-HT$_{2A}$ receptors in the PFC is thought to mediate DOI-induced head shake behavior (Willins and Meltzer, 1997). To address whether the altered electrophysiological and immediate early gene expression responses to DOI in ES animals are also reflected at the level of 5-HT$_2$-mediated behaviors, we measured DOI-induced head shakes. We found that the number of head shakes induced by systemic administration of DOI was significantly increased in ES animals (two-way ANOVA revealed a significant ES × DOI interaction; $F_{(1,14)} = 7.01$, $p = 0.02$) (Fig. 7). Spontaneous head shakes observed in the absence of DOI across the 20 min observation period did not differ between control and ES animals. Our results showed that ES animals exhibit enhanced 5-HT$_2$-mediated behavioral responses in adulthood.

Early stress alters the transcriptome in the prefrontal cortex

To gain an understanding into the molecular mechanisms that accompany enhanced prefrontal 5-HT$_2$ signaling in ES animals, we performed a transcriptome analysis on ES animals in adulthood. A history of ES significantly upregulated 666 genes and downregulated 232 genes in the PFC (ES vs control) (supplemental Table 2). To identify functionally clustered gene sets significantly regulated by ES, we used DAVID (Dennis et al., 2003; Huang et al., 2009). This analysis revealed that in the transcriptome of ES animals there was significant enrichment in functional categories of genes such as stress–response pathways, transmembrane components, cellular development processes, and genes involved in transduction of extracellular signal and ion homeostasis (Fig. 8*A*; supplemental Table 3).

The regulation of the expression of several genes in ES animals was then validated by qPCR in tissue samples from an independent cohort of animals (Table 3), indicating a strong reproducibility of the array expression results. Notably, ion channels such as potassium channels (*Kcna3, Kcnh2, Kcnh7*), which are modulated by Gα_q-protein stimulation (Chung and Schlichter, 1997; Schledermann et al., 2001), were regulated in adult ES animals. This is particularly interesting given that we found altered electrophysiological and network activity responses to 5-HT$_2$ receptor stimulation (Figs. 2, 4; Table 1). In addition, we also found that cellular developmental and neuronal plasticity-related genes, such as the NR2D NMDA receptor subunit (*Grin2d*), neuroligin 1 (*Nlgn1*), CamKinase1γ (*Camk1g*), and calcineurin (*Ppp3r2, Ppp3ca*), are upregulated in adult ES animals. Genes capable of altering signal

transduction, like ADP-ribosyltransferase 5 (*Art5*) and guanine nucleotide binding protein 1 (*Gnb1*), are also regulated by ES, potentially contributing to the altered responses following 5-HT$_2$-receptor stimulation. Further, we also observed regulation of genes that are targets for antidepressants and mood stabilizers, such as the β3 adrenergic receptor (*Adrb3*) (Stemmelin et al., 2008) and inositol monophosphatase-1 (*Impa1*) (Parthasarathy et al., 2003). The fold changes and significance levels for these validated genes are shown in Table 3.

Since our data suggest enhanced 5-HT$_2$ signaling in ES animals, we sought to address whether the ES transcriptome exhibits gene expression changes indicative of enhanced 5-HT$_2$ signaling. To test this hypothesis, we used microarray analysis to compare gene expression changes induced by ES to those that arise following systemic stimulation with the 5-HT$_2$ agonist, DOI. The heat maps highlight the overlap observed between the ES and DOI-induced transcriptomes in the PFC (Fig. 8*B*). We found ~13% overlap, with 95 genes upregulated in common between untreated ES and DOI-treated controls and 26 genes downregulated in common (Fig. 8*C*). Again, we used qPCR in an independent cohort of animals to validate these findings. Similar to the expression differences seen in ES animals at baseline (Table 3), stimulation of the 5-HT$_2$ receptor with DOI in control animals also resulted in a significant increase in the expression of *Nlgn1* (DOI vs control fold change: 2.29 ± 0.19; $p = 0.0001$, unpaired t test; $n = 4$ per group) and *Impa1* (DOI vs control fold change: 1.32 ± 0.09; $p = 0.04$, unpaired t test; $n = 8$ control, $n = 7$ DOI) and a downregulation of *Kcna3* (DOI vs control fold change: 0.79 ± 0.07; $p = 0.05$, unpaired t test with Welch correction; $n = 5$ control, $n = 7$ DOI). These results indicate that the changes in gene expression observed in ES animals at baseline contain a component that is found in common with 5-HT$_2$ receptor-evoked gene expression changes.

Together, our findings indicated that adult animals with a history of ES exhibit an altered prefrontal transcriptome, a component of which overlaps with gene expression changes that arise from 5-HT$_2$ stimulation. Furthermore, the ES-induced transcriptome showed a significant enrichment of genes associated with cellular development and signaling that may be associated with the enhanced 5-HT$_2$ responses we observe in ES animals.

Treatment with a 5-HT$_2$ antagonist reverses a component of the early stress-induced gene expression changes

Next, we examined whether chronic blockade of 5-HT$_2$ receptors in adulthood would normalize gene expression in the prefrontal cortex of ES animals. Controls and ES animals in adulthood were treated with the 5-HT$_2$ receptor antagonist ketanserin or vehicle ($n = 3$–5 animals per group) as described in Materials and Methods, and the expression of a subset of ES-regulated genes (*Nlgn1*, *Impa1*, *Adrb3*, *Kcnh2*, *CamK1g, Gnb1*, *Tnfrsf1b*) was analyzed. We observed that chronic treatment with ketanserin reversed the ES-induced changes in the expression of *Nlgn1*, *Impa1*, *Adrb3*, and *Kcnh2*. Two-way ANOVA analysis revealed a significant interaction between ES × ketanserin for the following: *Nlgn1* (fold change: control, 1.0 ± 0.05; ES, 1.71 ± 0.06; control plus ketanserin, 1.63 ± 0.82; ES plus ketanserin, 0.72 ± 0.06; $F_{(1,10)} = 9.72$, $p = 0.01$); *Impa1* (fold change: control, 1.00 ± 0.24; ES, 1.19 ± 0.19; control plus ketanserin, 1.32 ± 0.36; ES plus ketanserin, 0.34 ± 0.11; $F_{(1,12)} = 15.40$, p

= 0.002); *Adrb3* (fold change: control, 1.0 ± 0.12; ES, 0.63 ± 0.07; control plus ketanserin, 0.52 ± 0.16; ES plus ketanserin, 0.84 ± 0.16; $F_{(1,11)} = 6.54$, $p = 0.03$); and *Kcnh2* (fold change: control, 1.00 ± 0.17; ES, 1.91 ± 0.20; control plus ketanserin, 1.33 ± 0.29; ES plus ketanserin, 0.78 ± 0.09; $F_{(1,13)} = 13.18$, $p = 0.003$). In contrast, we found that ketanserin treatment did not specifically alter the ES-regulated changes in expression of *Camk1g, Tnfrsf1b*, and *Gnb1*. Two-way ANOVA revealed no significant ES × ketanserin interaction for *Camk1g* (fold change: control, 1.0 ± 0.21; ES, 2.14 ± 0.32; control plus ketanserin, 0.53 ± 0.10; ES plus ketanserin, 1.39 ± 0.36; $F_{(1,10)} = 0.25$, $p = 0.6$; main effect of ES, $p = 0.002$; main effect of ketanserin, $p = 0.003$); *Gnb1* (fold change: control, 1.0 ± 0.15; ES, 0.73 ± 0.09; control plus ketanserin, 0.71 ± 0.19; ES plus ketanserin, 0.35 ± 0.01; $F_{(1,11)} = 1.34$, $p = 0.2$; main effect of ES, $p = 0.01$; main effect of ketanserin, $p = 0.009$); and *Tnfrsf1b* (fold change: control, 1.0 ± 0.19; ES, 2.24 ± 0.41; control plus ketanserin, 0.94 ± 0.49; ES plus ketanserin, 1.37 ± 0.07; $F_{(1,11)} = 0.45$, $p = 0.5$; main effect of ES, $p = 0.06$). In both controls and ES animals, ketanserin treatment exerted significant effects on the expression of *Camk1g* and *Gnb1*. Our results suggest that 5-HT$_2$ receptor blockade in adulthood is capable of restoring some of the gene expression changes in the PFC of ES animals and that systemic 5-HT$_2$ receptor blockade may exert distinct effects on prefrontal gene transcription, depending on early life experience. Further experiments are necessary to ascertain whether the effects of systemic ketanserin treatment on the expression of specific PFC genes arise directly through the blockade of prefrontal 5-HT$_2$ receptors or involve possible indirect effects of ketanserin on raphe firing (Wright et al., 1990; Martín-Ruiz et al., 2001).

Discussion

A history of early life stress is linked to enhanced vulnerability for adult psychopathology. The major finding of our study is that adult animals with a history of ES show enhanced prefrontal 5-HT$_2$ receptor function. Evidence from multiple approaches supports this conclusion. First, 5-HT has atypical dominant excitatory effects, mediated via the 5-HT$_{2A}$ receptor, in a substantial portion of prefrontal layer V neurons of ES animals. Second, 5-HT$_2$-elicited excitatory inward currents in layer V neurons are enhanced in ES animals. Third, the frequency of network events generated in brain slices of PFC and the induction of immediate early gene expression *in vivo* following 5-HT$_2$ stimulation is significantly increased in ES animals compared with controls. Fourth, cortical 5-HT$_2$-mediated head shake behaviors are potentiated in ES animals. These changes occur without major alterations in the intrinsic excitability of the prefrontal cortex in ES animals. The changes also occur despite unaltered 5-HT$_{2A}$ and 5-HT$_{2C}$ receptor mRNA expression in PFC of ES animals and only a small increase in 5-HT$_2$ receptor binding. Our microarray results provide insight into the molecular changes that accompany the enhanced 5-HT$_2$ responses in ES animals. We show that several genes involved in cellular development, signal transduction, and G-protein signaling are regulated in the PFC following ES. Furthermore, we also find that a component of the gene expression changes in the PFC of ES animals can be reversed by chronic blockade of the 5-HT$_2$ receptor. Together, our results demonstrate enhanced 5-HT$_2$ function in animals with a vulnerability for anxiety and depressive behaviors and motivate a focus on 5-HT$_2$ receptors in the treatment of prodromal psychiatric disorders.

ES animals display aspects of an immature phenotype in prefrontal cortex

Electrophysiological responses to 5-HT in the adult PFC are dominated by its inhibitory receptors under normal circumstances (Gartside et al., 2000; Béïque et al., 2004; Puig et al., 2005), and our data from control animals are consistent with these earlier studies. Strikingly, we find that 5-HT elicits predominant excitatory inward currents via the 5-HT_{2A} receptor in ~40% of layer V pyramidal neurons of adult ES rats. The exclusive serotonergic excitation of layer V pyramidal neurons through 5-HT_2 receptors is typically only seen in the first two postnatal weeks in rodents (Zhang, 2003; Béïque et al., 2004). Our results suggest the retention of an immature response to 5-HT in almost half of the layer V PFC neurons from adult ES animals. The normal developmental period when 5-HT excites layer V pyramidal neurons coincides with a period of extensive cortical maturation, with large increases in dendritic branching and synaptic density (Roth et al., 1991; Zhang, 2003, 2004). It is noteworthy that our ES transcriptome profiling also indicates the enrichment of genes involved in cellular developmental processes that normally exhibit a higher expression during early postnatal development, such as $CamKI\gamma$ (Sawamura et al., 1996), the catalytic and regulatory domains of calcineurin (Schwartz et al., 2009), and the NR2D NMDA receptor subunit (Monyer et al., 1994). Interestingly, we also observed enhanced expression of neuroligin 1, a postsynaptic cell adhesion molecule that interacts with NMDA receptors at "hot spots" for the establishment of new excitatory synapses (Gerrow et al., 2006; Barrow et al., 2009). Together, our electrophysiological and transcriptome data indicate that early life stress results in an enriched expression of developmental growth pathways and an immature, excitatory 5-HT phenotype in adult PFC.

ES animals exhibit enhanced prefrontal 5-HT_2 receptor responses

A history of ES enhances adult excitatory inward currents, network activity, and Arc activation elicited by stimulation with the 5-HT_2 agonist DOI. Interestingly, in the absence of 5-HT stimulation there appeared to be little difference in the intrinsic excitability of prefrontal cortex. The changes in the effects of 5-HT_2 receptor stimulation occur without a change in 5-HT_{2A} mRNA expression in the PFC of adult animals with a history of ES and with a small increase in 5-HT_2 receptor binding. Though such an increase in binding could left shift a concentration response curve under some circumstances (Bourne and von Zastrow, 2007), we found that the 5-HT_{2A}-elicited inward currents in layer V neurons had similar sensitivity to 5-HT in control and ES animals. The induction of calcineurin, which directly participates in the phospholipase $C\beta$/inositol triphosphate intracellular cascade of 5-HT_2 receptors (Day et al., 2002), could contribute to enhanced signaling via 5-HT_2 receptors in ES animals. Consistent with the idea that 5-HT_2 receptor signaling is changed in ES animals, evidence from previous studies has demonstrated increased levels of $G\alpha_q$ mRNA following ES (Bhansali et al., 2007). We found that a $G\alpha_q$-mediated (Garcia et al., 2007) head shake behavior induced by DOI is significantly enhanced in ES animals. While the desensitization and G-protein uncoupling of 5-HT_{2A} receptors have been the subject of considerable study (Gray and Roth, 2001), the cellular mechanisms responsible for enhancing the function of 5-HT_{2A} receptors are still not well understood. Recent work indicates that stimulation of corticotrophin-releasing factor (CRF) receptor 1 can sensitize responses to 5-HT_2 receptor stimulation and influence receptor recycling (Magalhaes et al.,

2010). One can speculate that perturbed CRF levels in ES animals (Plotsky et al., 2005) may influence 5-HT$_2$ receptor responses. Yet, ES animals have reduced CRF receptor 1 expression and binding in prefrontal cortex (Plotsky et al., 2005). Additional studies will be required to understand how the enhanced prefrontal 5-HT$_2$ function arises in ES animals.

It is interesting to envisage whether the enhanced 5-HT$_{2A}$ responses in ES animals occur in parallel with altered prefrontal 5-HT release. Two scenarios can be envisioned. In the first, waking (Rueter and Jacobs, 1996) and stress-evoked (Kawahara et al., 1993) 5-HT release could result in abnormally high PFC activity by stimulation of 5-HT$_{2A}$ receptors on layer V neurons. Alternatively, if 5-HT$_{2A}$ receptor function was upregulated as homeostatic compensation for low baseline 5-HT levels (Cahir et al., 2007), this change would make the brain particularly vulnerable to abnormal function under conditions of stress when prefrontal 5-HT release is elevated (Kawahara et al., 1993; Yoshioka et al., 1995; Hashimoto et al., 1999; Daniels et al., 2004). However, studies of ES animals have not found consistent differences in baseline 5-HT levels in the PFC (Gartside et al., 2000; Matthews et al., 2001; Daniels et al., 2004; Jezierski et al., 2006). Our finding of increased 5-HT$_{2A}$-stimulated PFC excitability in ES animals is consistent with clinical evidence showing increased prefrontal activity in patients with affective disorders (Drevets, 2000). Of note, patients suffering from major depression have also been reported to show damage within the PFC (Rajkowska et al., 1999). It is tempting to speculate that early adverse experience may predispose the adult PFC to increased 5-HT$_{2A}$-mediated hyperexcitability under stressful conditions.

Some changes in gene expression may be compensatory. The normally expressed *Kcnh7* potassium channel (Kv 11.3; Erg3) (Saganich et al., 2001) was downregulated in ES animals, and this was accompanied by an upregulation of the relatively rare *Kcnh2* potassium channel (Kv11.1; Erg1) (Saganich et al., 2001). This change may decrease one potential source of G$_q$-mediated excitation, since the upregulated *Kcnh2* channel is less sensitive to G$_q$ inhibition than the downregulated *Kcnh7* channel (Schledermann et al., 2001). However, such compensation appears somewhat exceptional, since the PFC of adult ES animals exhibits heightened 5HT$_2$-induced inward currents, network activity, immediate early gene expression, and head shake behavior.

Clinical implications and conclusions

In rodents and primates, early life stress increases adult anxiety and depressive behaviors (Sánchez et al., 2001; Newport et al., 2002; Kaffman and Meaney, 2007) and thus serves as a useful model to identify the factors that contribute to the vulnerability of an individual for adult psychopathology. Our results suggest that the enhanced prefrontal 5-HT$_{2A}$ receptor function in adult ES animals may contribute to the vulnerability for anxiety and depressive-like behaviors observed in these animals. Interestingly, postmortem work in humans has shown enhanced 5-HT$_2$ receptor function in affective disorders, as measured by stimulated GTPγS binding in the PFC (Friedman and Wang, 1996). Our microarray analysis in ES animals not only identifies molecular pathways associated with cellular development and signal transduction that accompany the enhanced 5-HT$_2$ function, but also shows changes in genes such as *Adrb3* (Stemmelin et al., 2008) and *Impa1* (Parthasarathy et al., 2003), which have been implicated in the treatment of mood disorders. Remarkably, these transcriptional

alterations are observed >6 weeks following ES, invoking possible epigenetic mechanisms in the long-term maintenance of an altered gene expression pattern (Holmes et al., 2005). Such a persistent expression of an altered phenotype may underlie a life-long vulnerability for emotional dysfunction. Intriguingly, systemic treatment with a 5-HT$_2$ receptor antagonist, a class of molecules that exhibits antidepressant properties (Celada et al., 2004), reverses specific gene expression changes observed in ES animals, including those in *Adrb3*, *Impa1*, *Kcnh2*, and *Nlgn1*. Previous studies have shown that the regulation by adult stress of gene transcripts in the hippocampus can be blocked by 5-HT$_2$ receptor antagonist treatment (Olsson et al., 1997; Vaidya et al., 1997). Our results indicate that certain transcriptional differences observed many weeks after early life stress can also be reversed with chronic 5-HT$_2$ receptor blockade. This suggests that a component of the transcriptional changes observed in the PFC of ES animals may arise as a consequence of increased 5-HT$_2$ receptor function.

In conclusion, we demonstrate that a history of early adverse experience enhances adult 5-HT$_2$ responses and excitability in the PFC and results in a lasting alteration in the gene expression of molecular pathways that modulate cellular development, signal transduction, and stress responses. Our studies motivate a focus on the contribution of prefrontal 5-HT$_{2A}$ receptors to the vulnerability for anxiety and depressive disorders.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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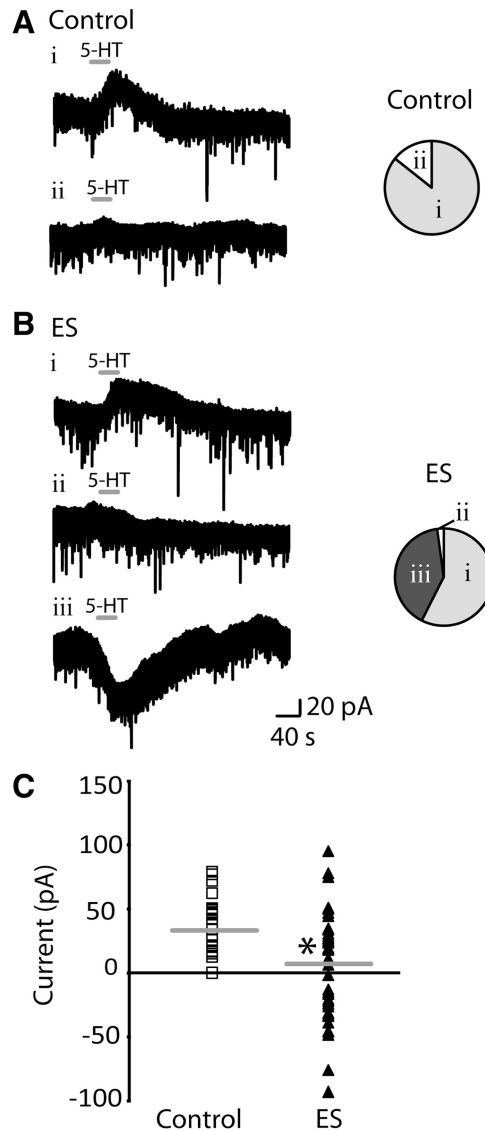
A Control

i 5-HT

Control

ii 5-HT

B ES

i 5-HT

ii 5-HT

ES

iii 5-HT

⌐20 pA
40 s

C

Figure 1. Serotonin elicits a different pattern of adult responses in layer V pyramidal neurons after early stress.
A, Examples of voltage-clamp traces from control animals show that bath application of 5-HT (10 μ_M; 30 s) elicited either a dominant outward current (**Ai**) or no net response (**Aii**) in layer V pyramidal neurons in the prefrontal cortex. The horizontal line denotes application of 5-HT. The pie chart shows that a large proportion of layer V pyramidal neurons in control animals displayed a 5-HT-induced outward current (**Ai**; $n = 30/35$), and a small subset of neurons showed no response (**Aii**; $n = 5/35$). **B**, Examples of voltage-clamp traces from ES animals show that the same application of 5-HT elicited one of three responses: a dominant outward current (**Bi**), no net response (**Bii**), or a dominant inward current (**Biii**) in layer V pyramidal neurons. The dominant inward current was only observed in brain slices from ES animals. The pie chart represents the proportion of layer V pyramidal neurons that displayed outward currents (**Bi**; $n = 27/47$), no response (**Bii**; $n = 1/47$), or inward currents in response to 5-HT (**Biii**; $n = 19/47$) in ES animals. **C**, Scattergram shows the range of responses in

layer V pyramidal neurons to 5-HT in control ($n = 35$) and ES animals ($n = 47$). Horizontal line denotes the mean response in layer V pyramidal neurons from control (34.2 ± 3.7 pA) and ES animals (5.9 ± 5.6 pA; *$p = 0.0002$, unpaired t test).
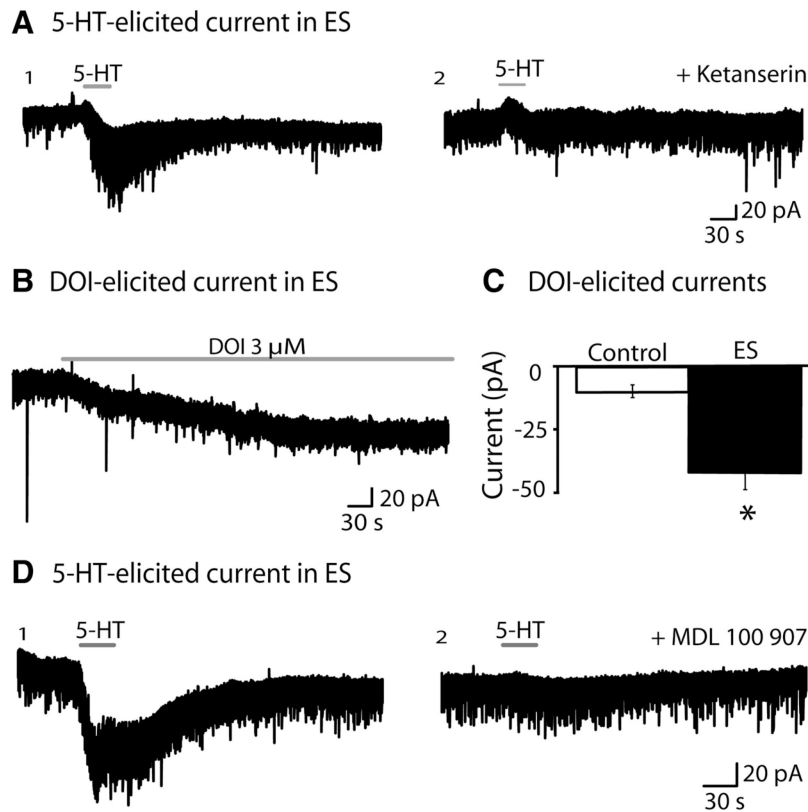
Figure 2. Serotonin-elicited inward currents in ES animals are mediated by 5-HT$_{2A}$ receptors.
A, A voltage-clamp trace from an ES animal shows that the dominant 5-HT-elicited inward current (*A1*) is completely blocked by the 5-HT$_2$ antagonist, ketanserin (*A2*) (2 μM; 10 min). *B*, A voltage-clamp trace shows the 5-HT$_2$ partial agonist DOI (3 μM; 15 min) eliciting an inward current in an ES layer V pyramidal neuron. *C*, The bar chart shows that the mean peak amplitude of the inward current elicited by bath application of DOI was significantly larger in ES animals (*n* = 9) relative to control animals (*n* = 11; *p = 0.0003, paired *t* test). *D*, A voltage-clamp trace from an ES animal shows that the 5-HT-elicited inward current (*D1*) is completely blocked by the selective 5-HT$_{2A}$ antagonist MDL 100907 (*D2*) (30 n$_M$, 10 min).
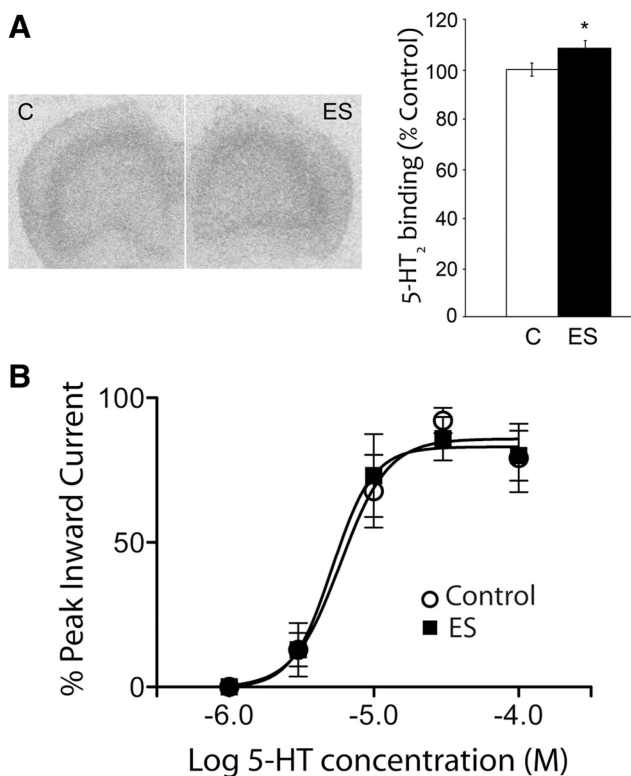
Figure 3. Influence of early stress on 5-HT$_2$ receptor binding and the sensitivity of the 5-HT$_{2A}$-elicited inward current in layer V pyramidal neurons.

A, ES caused a small increase in [^{3}H]ketanserin binding in the PFC in adulthood. Shown are representative autoradiograms and graphical representation of the 5HT$_2$ receptor binding density from the PFC of control (C) and ES animals. Data are percentage of control and are mean ± SEM (*p = 0.02, unpaired *t* test, n = 4 control, n = 7 ES). *B*, Concentration response curves for the 5-HT$_{2A}$-elicited inward current in layer V pyramidal neurons from control and ES animals. These experiments assessed the inward current mediated directly by 5-HT$_{2A}$ receptors on layer V pyramidal cells by recording after the application of WAY 100635 (30 n$_M$, 10 min) to block 5-HT$_{1A}$ receptors and in the presence of antagonists to block glutamate and GABA synaptic transmission (CNQX, 20 μ_M; APV, 50 μ_M; bicuculline, 10 μ_M). The EC$_{50}$ values are not significantly different between groups: (control, 5.6 ± 1.9 μ_M, n = 5; ES, 5.1 ± 1.9 μ_M, n = 5; unpaired *t* test, p = 0.8).

A Current-Clamp

i Control

ii ES
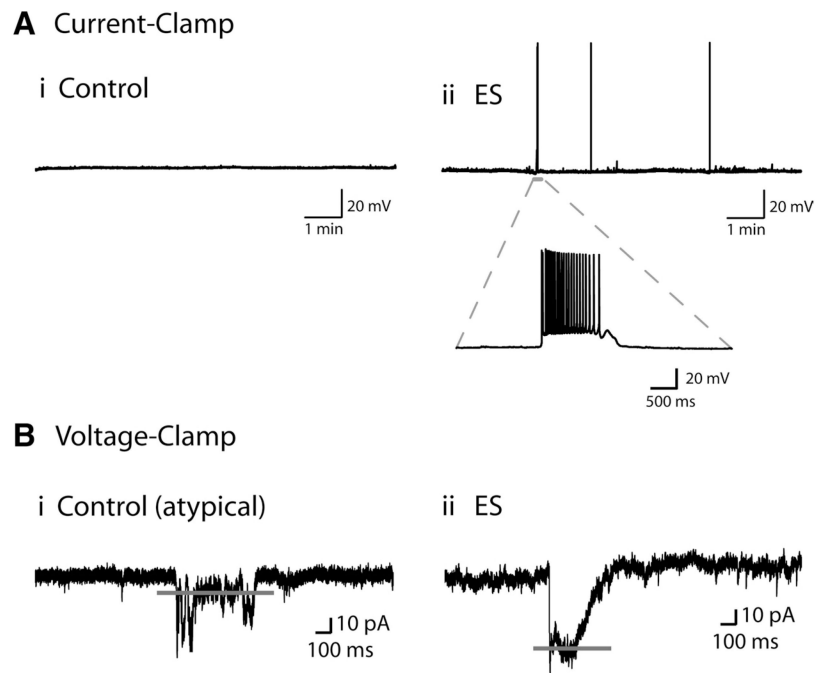

B Voltage-Clamp

i Control (atypical)

ii ES

Figure 4. Early stress increases the prevalence of spontaneous network activity (up states) in the presence of DOI.

A, Examples from control (*Ai*) and ES (*Aii*) recordings in current-clamp showing the lack (control) and the occurrence (ES) of episodes of spontaneous network activity detected by layer V pyramidal neurons after application of the 5-HT$_2$ partial agonist, DOI (3 μM; 15 min), to the brain slice. One episode is enlarged to show detail of the spontaneously occurring up state observed. These phenomena were identified based on previous description of network activity in cortical brain slices (see Materials and Methods). *B*, To quantify the amplitude of the network activity, voltage-clamp recording were performed. Examples of control (rare) (*Bi*) and ES (*Bii*) network activity are shown in voltage-clamp. The horizontal gray line denotes the measured amplitude of the up state.
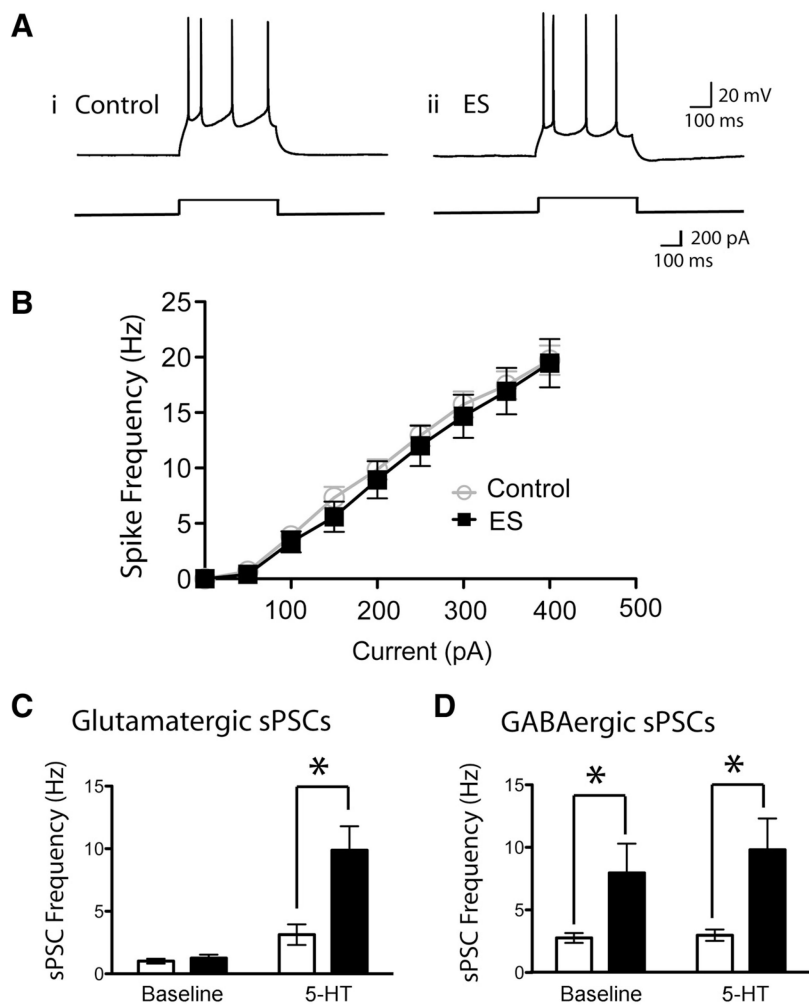
Figure 5. Early stress does not appear to enhance the intrinsic excitability of the prefrontal cortical slice as measured by input–output experiments and baseline levels of glutamatergic and GABAergic synaptic activity.

A, The intrinsic excitability of layer V pyramidal neurons was assessed by examining the frequency of action potentials elicited by injections of depolarizing current. Representative examples are shown from control (*Ai*) and ES (*Aii*) animals. *B*, A graph of the input–output relationships shows that layer V pyramidal cells from both groups have similar excitability when assessed from their resting potentials. *C*, The frequency of glutamatergic sPSCs measured in layer V pyramidal neurons at baseline and in response to 5-HT (10 μ_M, 30 s) in controls and ES. Both groups have a similar frequency of glutamatergic sPSCs at baseline. In the presence of 5-HT, the frequency of glutamatergic sPSCs is significantly enhanced in both controls and ES animals, and two-way ANOVA revealed an interaction where by the 5-HT-elicited increase in glutamatergic sPSCs is significantly enhanced in ES neurons (*$p = $ 0.0006). *D*, The frequency of GABAergic sPSCs measured (with high Cl^- patch solution in the presence of CNQX, 20 μ_M) in layer V pyramidal neurons at baseline and in response to 5-HT (10 μ_M, 30 s) in controls and ES. Interestingly, ES layer V neurons display a significantly higher frequency of GABAergic sPSCs at baseline (two-way ANOVA reveals a significant main effect of postnatal group, *$p = $ 0.001). Bath application of 5-HT (10 μ_M,

30 s) did not significantly alter the frequency of GABAergic sPSCs in either the control or ES animals. A higher concentration of 5-HT may be required to increase the frequency of GABAergic sPSCs overall (Zhou and Hablitz, 1999).
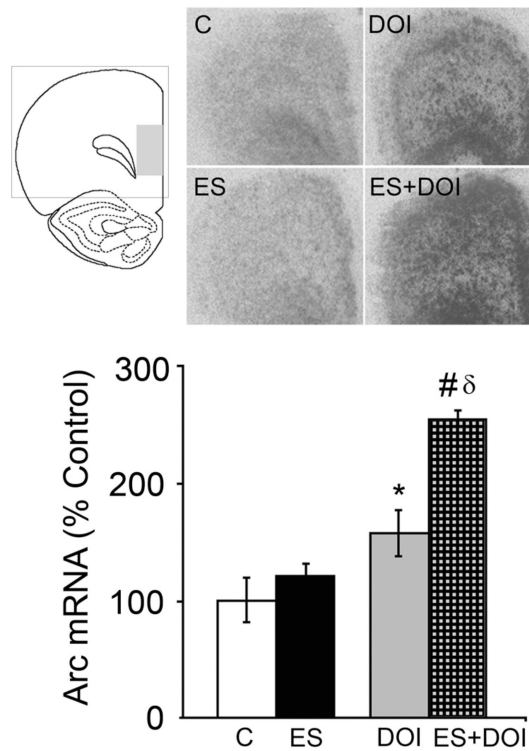
Figure 6. Early stress potentiates the DOI-induced Arc expression in the prefrontal cortex.
A history of ES potentiated the DOI-induced increase in Arc mRNA levels within the PFC.
Shown are representative autoradiograms of PFC sections and graphical representation of
the densitometric analysis from control (C), ES, DOI, and ES plus DOI groups. Results are
expressed as a percentage of control and are the mean ± SEM. Two-way ANOVA revealed a
significant ES × DOI interaction ($F_{(1,15)}$ = 11.39, p = 0.004). *p < 0.05, significantly
different from control; #p < 0.05, significantly different from ES; δp < 0.05, significantly
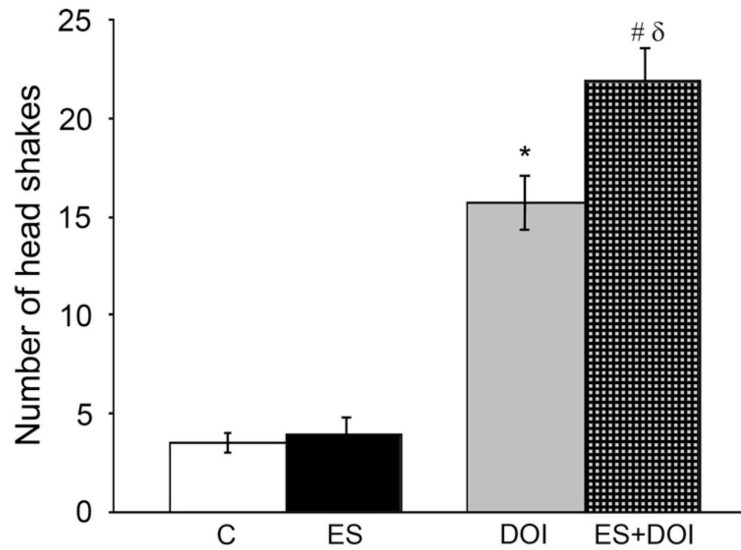different from DOI (ANOVA and Bonferroni *post hoc* test); n = 4–5 per group.

Figure 7. A history of early stress-enhanced DOI-induced head shake behavior.
The induction of head shakes by the 5-HT$_2$ partial agonist DOI was potentiated in ES animals. Results are expressed as the mean ± SEM. Two-way ANOVA analysis indicated a significant ES × DOI interaction effect ($F_{(1,14)} = 7.01$, $p = 0.02$). *$p < 0.05$, significantly different from control; #$p < 0.05$, significantly different from ES; δ$p < 0.05$, significantly different from DOI (ANOVA and Bonferroni *post hoc* test); $n = 4$–5 per group.
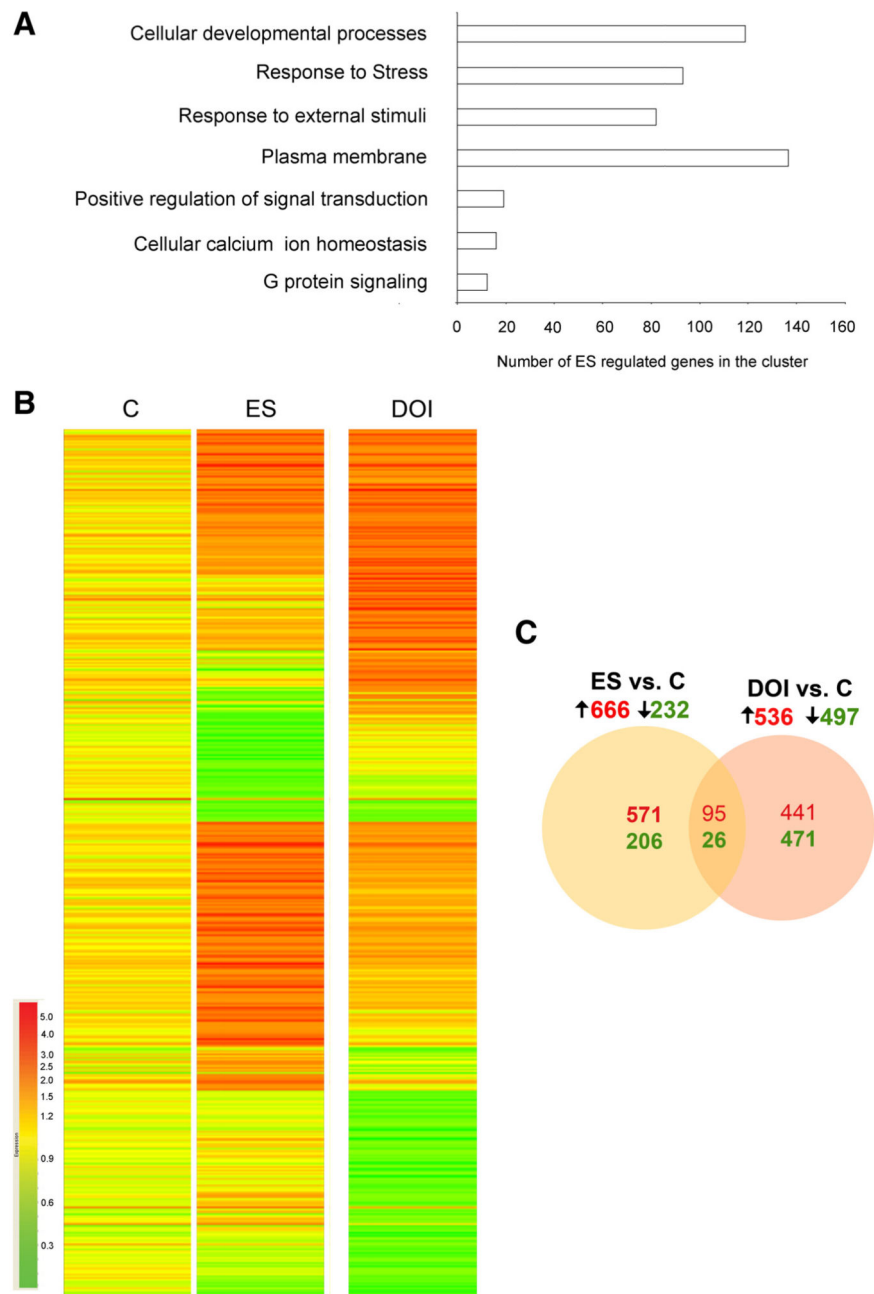
Figure 8. Exposure to early stress alters the adult prefrontal transcriptome.
A, Functional analysis of ES-regulated genes reveals enrichment of cellular development processes, stress-response pathways, transmembrane components, transduction of extracellular signal, G protein signaling, and ion homeostasis pathways in the PFC. *B*, Hierarchical cluster of significantly regulated genes ($p < 0.05$, *t* test corrected for multiple comparisons; upregulated genes are shown in red, downregulated genes are shown in green) across the control (C) and ES groups ($n = 3$ control; $n = 4$ ES). Each row represents a single gene and each column represents the average fold change for each group. The data are log-transformed, normalized intensity values. *C*, The numbers of upregulated (red) and

downregulated (green) genes in the ES and DOI groups (fold change cutoff: 1.5 for upregulated and 0.66 for downregulated genes, $p < 0.05$ compared with control; t test corrected for multiple comparisons) are represented using a Venn diagram. As represented in the Venn diagram, ~13% of the genes regulated by ES were similarly regulated in the DOI group.

Table 1

Early stress increases the prevalence of spontaneous network activity in the presence of the 5-HT$_2$ receptor agonist DOI

	Control	Early stress	*p* value
Number of cells displaying network activity/total number of cells	2/13 (15%)	12/13 (92%)	0.0002
Amplitude (pA)	−17.4±9.5	−67.9±8.3	0.06
Duration (ms)	450±50	651±205	0.7

Voltage-clamp recordings (10 min per neuron) were made from control and ES neurons following application of DOI (3 μM; 15 min) to the brain slice. Under these conditions, activity patterns consistent with network activity (Sanchez-Vives and McCormick, 2000) were observed in very few neurons from control animals and in almost all neurons from ES animals (significance assessed with Fisher's exact test). The amplitude and duration of the measured network events in each group are shown as mean ± SEM (controls: 2 events; ES: 17 events) and were compared with Student's unpaired *t* test.

Table 2

Layer V pyramidal neurons from ES animals are significantly more depolarized, but display no change in spike amplitude or input resistance

	Control (*n*=74)	Early stress (*n*=87)	*p* value
Resting membrane potential (mV)	−82.4±0.6	−80.5±0.6	0.03
Spike amplitude (mV)	86.4±0.7	85.8±0.8	0.5
Input resistance (MΩ)	102.2±6.3	99.9±5.1	0.7

Values are shown as mean±SEM and were compared with Student's unpaired *t* test.

Table 3

Validation of microarray results using quantitative PCR

Gene	ES versus C fold change	*p* value
Upregulated		
Art5	2.88±0.78	0.03
Camk1g	1.65±0.31	0.04
Grin2d	1.23±0.03	0.003
Impa1	1.21±0.04	0.006
Kcnh2	1.45±0.16	0.004
Nlgn1	1.58±0.23	0.03
Ppp3ca	1.29±0.01	0.006
Ppp3r2	2.86±0.64	0.03
Tnfrsf1b	1.22±0.01	0.01
Downregulated		
Adrb3	0.66±0.11	0.02
Gnb1	0.82±0.09	0.06
Kcna3	0.75±0.09	0.09
Kcnh7	0.70±0.04	0.006

Genes of particular interest implicated in cellular signaling, excitability, and neuronal plasticity were chosen for qPCR to validate the microarray results. The validation of the microarray by qPCR was carried out in tissue samples from an independent cohort of experimental animals. All genes were normalized to an average of four housekeeping genes (*18s rRNA, Gapdh, Hprt,β-actin*), which were not regulated in the microarray. Genes up-regulated in the early life stress group are shown in alphabetical order at the top of the table and down-regulated genes are shown in alphabetical order at the bottom. Data are expressed as fold change (mean±SEM). The p values were obtained using an unpaired *t* test, (*Nlgn1, Ppp3ca, Ppp3r2, Tnfrsf1b, Adrb3, Gnb1, Kcna3, Kcnh7, n*=4–7 per group; *Art5, Grin2d, Kcnh2, n*=6 –10 per group; *Camk1g, Impa1, n*=13–16 per group).