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Early emergence of altered 5-HT_{2A} receptor-evoked behavior, neural activation and gene expression following maternal separation

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ABSTRACT

The early stress of Maternal Separation (MS) contributes to the establishment of adult psychopathology. The serotonergic (5-HT) system is implicated during this temporal window in mediating the development of mood-related behaviors. MS is reported to evoke altered 5-HT_{2A} receptor function in adulthood. However, the ontogeny of altered 5-HT_{2A} receptor responsivity following MS remains unknown. Here, we examined 5-HT_{2A} receptor agonist, DOI (1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane) (2 mg/kg) evoked responses, namely stereotypical head-twitch behaviors in control and MS Sprague-Dawley rat pups at postnatal day 21 (P21). MS involved a separation of pups from the dam for 3 h daily from postnatal day 2–14. MS pups at P21 exhibited significantly enhanced head-twitch behaviors compared to controls. Using c-Fos cell counting we examined neural activation in control and MS pups following DOI treatment. MS pups exhibited altered DOI-evoked c-Fos expression within all mPFC subdivisions, but not in the hippocampus, lateral septum and hypothalamus, suggesting differential prefrontal neural activation upon 5-HT_{2A} receptor stimulation following early stress. Gene profiling of 5-HT_{2A} receptor-regulated immediate early genes (IEGs) indicated a decline in the expression of *Fos*, *Fra1* and *Egr1* mRNA under baseline conditions in the mPFC of MS pups. MS pups also showed an altered pattern in the regulation of several 5-HT_{2A} receptor-regulated IEGs (*Fos*, *Fra1*, *Bdnf*, *Egr1*, *Egr3*) following DOI treatment. Collectively, these results highlight an early emergence of altered 5-HT_{2A} receptor-evoked behavioral responses and neural activation patterns in multiple brain regions in animals with a history of MS.

1. Introduction

Early life stress is a risk factor for the development of psychopathology (Carr et al., 2013; Lyons et al., 2011; Pechtel and Pizzagalli, 2012). Both preclinical and clinical studies have established the existence of critical periods during neurodevelopment wherein the developing brain is particularly sensitive to environmental perturbations (Ansorge et al., 2007; Carr et al., 2013; Gross and Hen, 2004). Early adverse experience is known to program enhanced anxiety and depressive-like behavior in adulthood, and to evoke perturbed neuroendocrine and behavioral responses to stress (Lyons et al., 2011). Amongst the critical substrates for early stress is the serotonergic neurocircuitry (Lesch and Waider, 2012; Ohta et al., 2014). Serotonin (5-HT) plays an important role during development and maturation of neural circuits, influencing progenitor turnover, differentiation, migration, synaptogenesis and dendritic remodeling (Lesch and Waider, 2012). Perturbations of serotonergic neurotransmission during critical windows of postnatal development either via pharmacological methods such as treatment with selective serotonin reuptake inhibitors (SSRIs) (Ansorge et al., 2007) or through genetic approaches evoke altered anxiety and depressive-like behavior (Ansorge et al., 2007; Gross and Hen, 2004). While several previous reports have implicated the 5-HT_{1A} receptor in the development of anxiety and depressive-like behavior (Gross et al., 2002; Richardson-Jones et al., 2011), more recently studies have also suggested an important role for the 5-HT_{2A} receptor in contributing to the behavioral consequences of early adversity (Benekareddy et al., 2011; Holloway et al., 2013; Wischhof et al., 2015). Recent reports indicate that diverse animal models of early life perturbations, including the classical model of maternal separation (MS) involving a 3 h daily separation of pups from the dam from postnatal day 2–14 (Kalinichev et al., 2002), postnatal exposure to SSRIs, as well as gestational models such as maternal immune activation (MIA), exhibit perturbed cortical 5-HT_{2A} receptor expression/function (Benekareddy et al., 2010; Holloway et al., 2013; Sarkar et al., 2014). Further, pharmacological blockade of 5-HT_{2A} receptors during the period of early life perturbations is reported to prevent the emergence of anxiety and depressive-like behaviors in some of these animal models, suggesting a key role for the 5-HT_{2A} receptor (Benekareddy

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et al., 2011; Sarkar et al., 2014).

Adult animals with a history of the early stress of MS, exhibit both enhanced cortical 5-HT_{2A} receptor-mediated electrophysiological responses, as well as 5-HT_{2A} receptor-evoked head-twitch behaviors (Benekareddy et al., 2010), which are known to be mediated via cortical 5-HT_{2A} receptors (Canal and Morgan, 2012). MS animals also exhibit an alteration in the 5-HT_{2A} receptor-regulated transcriptome in the medial prefrontal cortex (mPFC), in particular pertaining to genes associated with signal transduction, neuronal plasticity and cellular development (Benekareddy et al., 2010). Further, enhanced cortical 5-HT_{2A} receptor expression accompanied by increased head-twitch behavior has been observed in adult animals with a history of MIA (Holloway et al., 2013; Malkova et al., 2014). Interestingly, animal models of schizophrenia and psychosis, based on treatment with psychotomimetics in adulthood also exhibit enhanced responses to DOI, namely increased head-twitch behavior (Chiu et al., 2014; Santini et al., 2013) and neuroinflammatory insults that are linked to psychosis-like behavior also result in enhanced neocortical 5-HT_{2A} receptor expression (Holloway et al., 2013; Savignac et al., 2016). While these previous reports have focused on studies in adulthood, we sought to examine the regulation of 5-HT_{2A} receptor-mediated behaviors, neuronal circuit activation using the marker c-Fos and immediate early gene (IEG) expression within the mPFC at a postnatal time point, soon after peak expression of cortical 5-HT_{2A} receptors. 5-HT_{2A} receptor expression shows a dynamic regulation during the first few weeks of postnatal life. 5-HT_{2A} receptor expression appears to first acquire adult-like levels by the end of the first postnatal week (P7-P8), followed by a transient increase reaching peak receptor levels between P10 and P17, after which it declines and is maintained at adult levels commencing around P18- P20 (Basura et al., 2008; Morilak and Ciaranello, 1993). The ontogeny of 5-HT_{2A} receptors indicates highest expression in cortical brain regions during postnatal development overlapping with critical periods in which early stress can program life-long behavioral consequences (Basura et al., 2008; Morilak and Ciaranello, 1993).

Our results indicate that MS animals exhibit enhanced 5-HT_{2A} receptor-evoked behavioral responses at postnatal day 21 soon after the cessation of the MS paradigm. This enhanced 5-HT_{2A} receptor responsivity is accompanied by an altered pattern of neural activation, based on c-Fos cell counting analysis in response to 5-HT_{2A} receptor stimulation in postnatal MS animals. This is also noted in the altered expression of multiple IEGs within the mPFC of MS animals under baseline conditions and in response to 5-HT_{2A} receptor stimulation. Our results indicate that early stress evokes rapid changes in 5-HT_{2A} receptor-evoked responses, including altered transcriptional responses, patterns of neural activation and 5-HT_{2A} receptor-induced behaviors that emerge during postnatal life.

2. Material and methods

2.1. Animals

Sprague-Dawley rat pups maintained on a 12 h light/dark cycle (lights on at 7:00 a.m.) with *ad libitum* access to food and water were used for all experiments. All experimental procedures were conducted as per the national guidelines of the Committee for Supervision and Care of Experimental Animals (CPCSEA) and were approved by the TIFR Institutional Animal Ethics committee.

2.2. Maternal separation paradigm

The early stress paradigm of maternal separation (MS) was performed as described previously (Benekareddy et al., 2010). Dams and their litters were assigned at random to either control or MS groups on postnatal day (P1). All litter sizes ranged between 10 and 12 pups per litter. Pups in the MS group were separated from the dam for a duration of three hours daily from P2- P14. During the period of separation the entire litter was placed in a beaker lined with bedding material and kept on a heating pad in a different room. The dam was placed in a fresh cage throughout the separation period. At the end of the separation, pups were returned to the home cage first followed by the dam. Control litters were left undisturbed apart from standard animal facility maintenance cleaning every three days, which involved minimal disturbance. For all experiments (behavioral and molecular), rat pups were sacrificed at P21. There were three separate experimental cohorts of animals with the following experimental groups, namely Control, MS, Control + DOI and MS + DOI. Within each experimental cohort, the individual experimental groups had animals derived from a minimum of three litters to avoid any litter-specific effects, and included both male and female pups. Experimental cohort 1 was used for the measurement of head-twitch behavior at P21 following DOI treatment ($n = 5 - 7$/group). Experimental cohort 2 was perfused at two hours post DOI treatment for c-Fos immunohistochemistry ($n = 4 - 5$/group). Experimental cohort 3 was sacrificed two hours post DOI treatment and was used for qPCR analysis ($n = 7 - 9$/group).

2.3. Drug treatment and behavioral analysis

Control and MS rat pups (P21) were intraperitoneally administered the 5-HT_{2A} receptor agonist DOI ((±)-1-(2, 5-Dimethoxy-4-iodophenyl)-2-aminopropane hydrochloride) (2 mg/kg) (Sigma-Aldrich, USA) or vehicle (0.9% saline) [Treatment groups: Control + Vehicle, Control + DOI, MS + Vehicle and MS + DOI, ($n = 5 - 7$ per group)]. DOI evokes stereotypical head-twitch behaviors defined by a rapid radial movement of the head. During behavioral analysis rat pups were individually housed in a separate cage. Head twitch behavior was recorded in rat pups administered vehicle or DOI commencing twenty minutes after drug treatment for a thirty minute duration. The number of head-twitch events was manually scored by an experimenter blind to the experimental treatment groups.

2.4. Immunohistochemistry and cell counting

All animals (P21) were sacrificed two hours post vehicle or DOI treatment by first anesthetizing them with an overdose of sodium thiopentone followed by transcardial perfusion with 4% paraformaldehyde in 0.1 M phosphate buffer pH 7.4 (0.1 M PB). Free-floating 40um thick coronal sections of the brain were obtained using a vibrating blade microtome (Leica, Germany). Sections were incubated with the blocking solution (0.3% Triton X-100, 10% horse serum, 0.1 M PB) for 2 h at room temperature followed by overnight incubation with the primary antibody (1:1000, rabbit anti c-Fos, Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). Sections were washed in 0.1 M PB and incubated with the biotinylated secondary antibody (1:250, biotinylated donkey anti rabbit, Vector Labs, USA) for 3 h. Sections were then washed with 0.1 M PB and incubated with avidin-biotin complex (Vectastain Elite ABC HRP kit, Vector Labs, USA) for 2 h at room temperature. Following ABC treatment, sections were treated with diaminobenzidine (SigmaFast DAB, Sigma Aldrich, USA) to visualize the signal.

The number of c-Fos positive cells was counted using a Zeiss Axioskop (Carl Zeiss, Germany) with a 20X objective by an experimenter blind to the experimental treatment groups. c-Fos immunoreactive cells were counted in the following brain regions bilaterally: mPFC (6 sections per animal; 4–6 animals per group), hippocampal subfields (4 sections per animal; 4–6 animals per group), lateral septum (LS; 4 sections per animal; 4–6 animals per group) and the paraventricular nucleus of the hypothalamus (PVN; 3 sections per animal; 4–6 animals per group). Different brain regions were identified using the postnatal rat brain atlas (Khazipov et al., 2015). Only intensely stained cells were considered to be c-Fos positive. The cell counts were expressed as number of c-Fos positive cells per section and results are represented as the mean ± S.E.M.

2.5. Quantitative PCR

Control and MS rat pups (P21) were rapidly decapitated 2 h following vehicle or DOI treatment following CO_2 inhalation anesthesia (n = 7-9 animals per group). The medial prefrontal cortex (mPFC) was rapidly dissected, snap frozen in liquid nitrogen and stored at −80 °C. RNA was extracted using Trizol (TRI reagent, Sigma-Aldrich, USA), quantified on a Nanodrop (Thermo scientific, USA) and reverse transcribed to generate cDNA (PrimeScript RT Reagent Kit, Takara, Clonetech, Japan). cDNA was then subjected to qPCR (CF96X Real Time System, BioRad, USA) using specific primers against the genes of interest (Supplementary Table 1). qPCR data was analyzed using the ΔΔCt method as described previously (Bookout and Mangelsdorf, 2003). Gene expression levels were normalized to the endogenous housekeeping gene *Hprt,* (hypoxanthine guanine phosphoribosyl transferase) which was unchanged across experimental treatment groups. Results are expressed as fold change ± S.E.M of the control group. Heat maps for mean fold change values were constructed using MATLAB.

2.6. Statistical analysis

Statistical data analysis was performed using the software Prism (Graphpad Software Inc., USA). All results are expressed as mean ± S.E.M. Experiments were subjected to a two-way ANOVA followed by a Bonferroni *post hoc* test with significance set at $p < 0.05$.

3. Results

3.1. Maternally separated animals exhibit enhanced 5-HT$_{2A}$ receptor-mediated head-twitch behavior on postnatal day 21

DOI, a 5-HT$_{2A}$ receptor agonist, elicits a unique head-twitch response in rats, characterized by rapid, radial movements of the head. Here, we sought to address whether 5-HT$_{2A}$ receptor-evoked head-twitch behavior is perturbed at an early postnatal time point of P21, soon after the cessation of MS at P14. Control and MS pups received vehicle or DOI treatment and were analyzed for head-twitch behavior (Fig. 1A). Two-way ANOVA analysis revealed significant main effects of both DOI treatment ($F_{(1.18)} = 10.545$, $p = 0.0045$) and MS ($F_{(1.18)} = 117.22$, $p < 0.00010$). Further, we also noted a significant MS x DOI interaction effect ($F_{(1.18)} = 15.14$, $p = 0.001$). Bonferroni *post hoc* comparisons indicated that the head-twitch behavior was significantly potentiated in DOI treated P21 MS animals as compared to DOI treated control groups. Baseline head-twitch events in vehicle treated controls and MS animals did not differ. These results indicate that enhanced 5-HT$_{2A}$ receptor-mediated behavioral responses observed in MS animals are noted as early as P21 soon after termination of MS.

3.2. Maternally separated pups exhibit altered DOI-evoked c-Fos immunoreactivity within multiple brain regions

Previous studies indicate that treatment with the 5-HT$_{2A}$ receptor agonist, DOI evokes robust and widespread increases in c-Fos expression in several cortical brain regions within the adult rat brain. Here we sought to address whether DOI treatment on postnatal day 21 alters the numbers of c-Fos positive cells within cortical brain regions, and further whether the c-Fos positive cell numbers were distinct in 21 day old pups with a history of MS.

The DOI-evoked c-Fos immunoreactivity within all subdivisions of the mPFC (Fig. 2A-D) differed significantly between control and MS pups. Two-way ANOVA analysis indicated significant MS x DOI interaction effects for c-Fos positive cell numbers in the Cg ($F_{(1.14)} = 6.71$, $p = 0.021$) and a significant main effect of MS ($F_{(1.14)} = 9.43$, $p = 0.008$) (Fig. 2B). In the PrL and the IL subdivisons of the mPFC, two-way ANOVA analysis revealed only significant MS x DOI

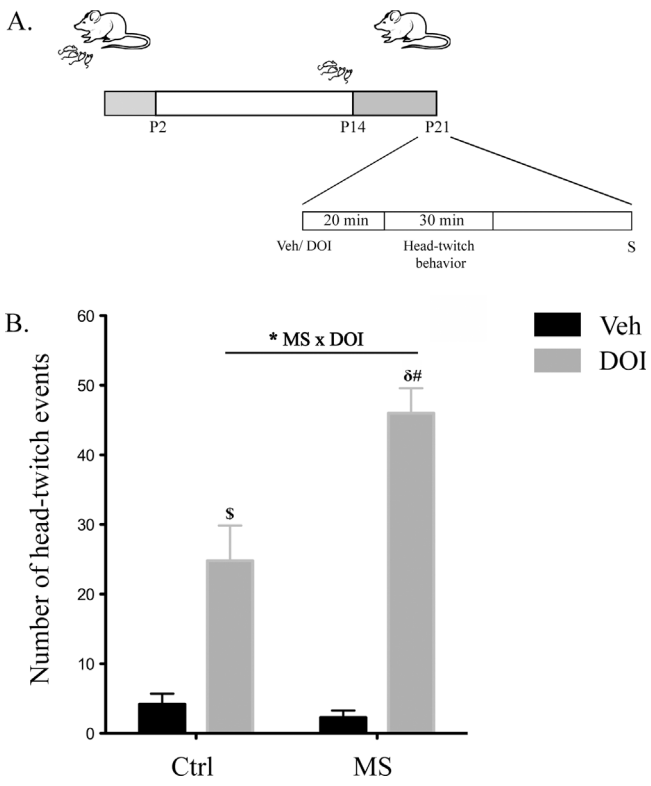

Fig. 1. Animals with a history of maternal separation show enhanced 5-HT$_{2A}$ receptor-mediated head-twitch behavior at postnatal day 21.
(A) Shown is a schematic of the experimental paradigm for maternal separation (MS) followed by treatment with vehicle or 5-HT$_{2A}$ receptor agonist, DOI (2 mg/kg). Head-twitch behavior was recorded and analyzed on postnatal day 21 (P21) as described in Methods. (B) MS pups at P21 exhibited a significant increase in 5-HT$_{2A}$ receptor-evoked head-twitch behavior. Results are expressed as the mean ± SEM. *$p < 0.05$ MS x DOI interaction effect, Two-way ANOVA analysis. Significance in Bonferroni *post hoc* comparisons ($p < 0.05$) as compared to vehicle treated controls ($), as compared to vehicle treated MS (#), and as compared to DOI-treated controls (δ), n = 5-7 animals per group.

interaction effects (PrL: $F_{(1.14)} = 6.14$, $p = 0.026$; Il: $F_{(1.12)} = 7.11$, $p = 0.021$) but no significant main effects. Bonferroni *post hoc* comparisons indicated DOI treatment to naive rat pups with no stress history did not significantly alter the number of c-Fos positive cells within all subdivisions of the mPFC (Cg: Fig. 2B, PrL: Fig. 2C, IL: Fig. 2D) as compared to the vehicle-treated control pups. In contrast, MS rat pups revealed a robust and significant increase in c-Fos positive cell numbers within the Cg (Fig. 2B) following DOI treatment as compared to the vehicle-treated MS group. While Bonferroni *post hoc* comparisons did not indicate a significant increase in c-Fos positive cell numbers in the PrL and IL of DOI treated MS pups as compared to the vehicle-treated MS group, the directionality of change and pattern is similar across all subdivisions of the mPFC. Within the hippocampal subfields (Fig. 2A), c-Fos positive cell numbers did not differ across control and MS animals at P21. Two-way ANOVA analysis indicated no significant MS x DOI interaction effects for all of the hippocampal subfields examined (Fig. 2E-H), however we did observe a significant main effect of MS within the CA1 ($F_{(1,16)} = 19.57$, p = 0.0004) (Fig. 2E) and hilar subfield ($F_{(1,16)} = 224.48$, p < 0.0001) (Fig. 2H).

We next addressed whether DOI influenced the number of c-Fos positive cells within the lateral septum and PVN in control and MS pups at P21. Two-way ANOVA analysis indicated no significant MS x DOI interaction effects for either the lateral septum (Fig. 3A, 3B) or the PVN (Fig. 3A, 3C). We observed a significant main effect of DOI within the LS ($F_{(1.15)} = 18.43$, p = 0.0006) and a significant main effect of MS in the PVN ($F_{(1,15)} = 22.66$, p = 0.0003). Taken together, these results indicate that the DOI-evoked c-Fos immunoreactivity within the mPFC, but not the hippocampus, lateral septum and PVN, differs significantly

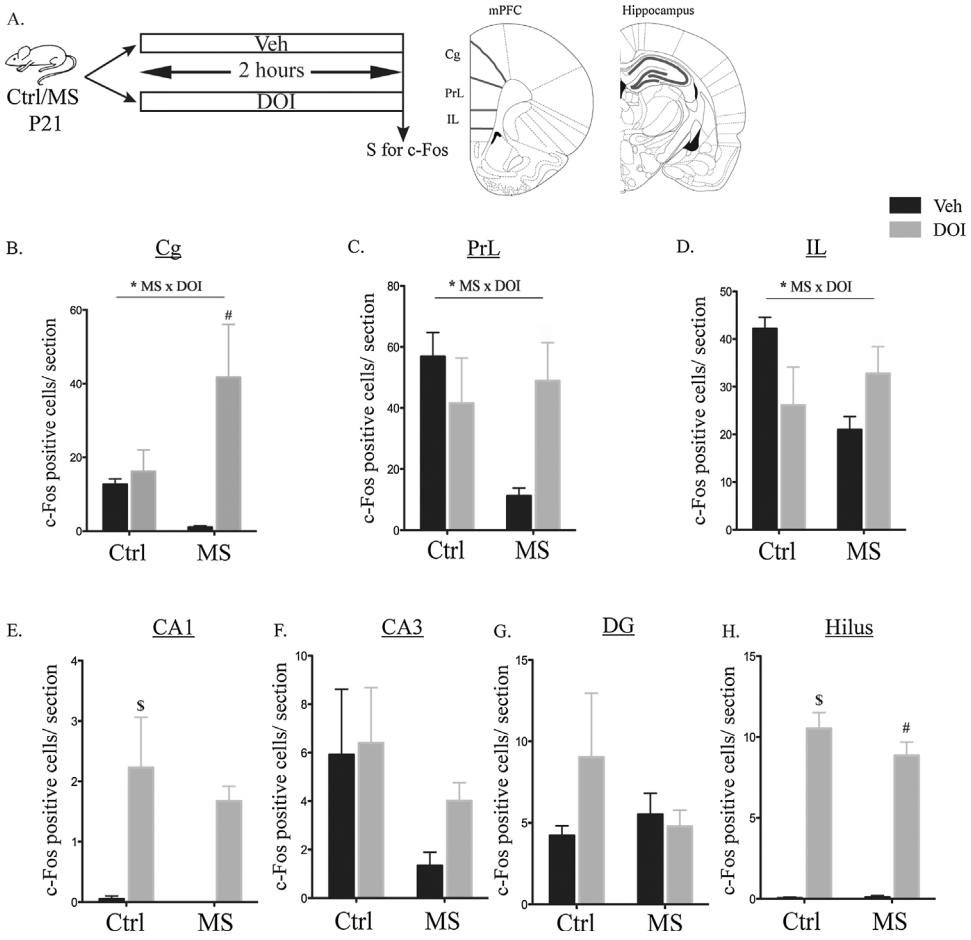

Fig. 2. Pattern of neural activation following DOI is altered in the mPFC of MS animals.
(A) Shown is a schematic of the experimental paradigm for treatment of control (Ctrl) or MS P21 pups with vehicle or 5-HT_{2A} receptor agonist, DOI (2 mg/kg) for c-Fos immunohistochemistry and schematics of coronal sections of the medial prefrontal cortex (mPFC) and the hippocampus. (B-D) Two-way ANOVA analysis revealed significant MS x DOI interaction effects within all subdivisions of the mPFC namely the cingulate (Cg), prelimbic (PrL) and infralimbic (IL) cortices ($*p < 0.05$, Two-way ANOVA). DOI administration enhanced c-Fos positive cell numbers within all mPFC subdivisions in DOI-treated MS animals, but not in DOI-treated controls with no early stress history. Analysis of c-Fos positive cell numbers within the different hippocampal subfields of the CA1 (E), CA3 (F), dentate gyrus (DG) (G) and hilus (H) indicated no significant MS x DOI interaction in two-way ANOVA analysis. DOI treatment enhanced c-Fos positive cell numbers in the CA1 (E) and hilar (H) hippocampal subfields in both control and MS pups. DOI treatment did not influence c-Fos positive cell number in the CA3 (F) and DG (G) hippocampal subfields. Results are expressed as the mean ± SEM. $*p < 0.05$ MS x DOI interaction, Two-way ANOVA analysis. Significance in Bonferroni *post hoc* comparisons ($p < 0.05$) as compared to vehicle treated controls ($) and as compared to vehicle treated MS (#), n = 4-6 animals per group.

between naive rat pups and rat pups with a recent history of MS.

3.3. Maternally separated pups exhibit an altered DOI-evoked immediate-early gene expression pattern in the mPFC

Given that we noted starkly differing c-Fos positive cell numbers following DOI treatment in MS animals in the mPFC, we next sought to

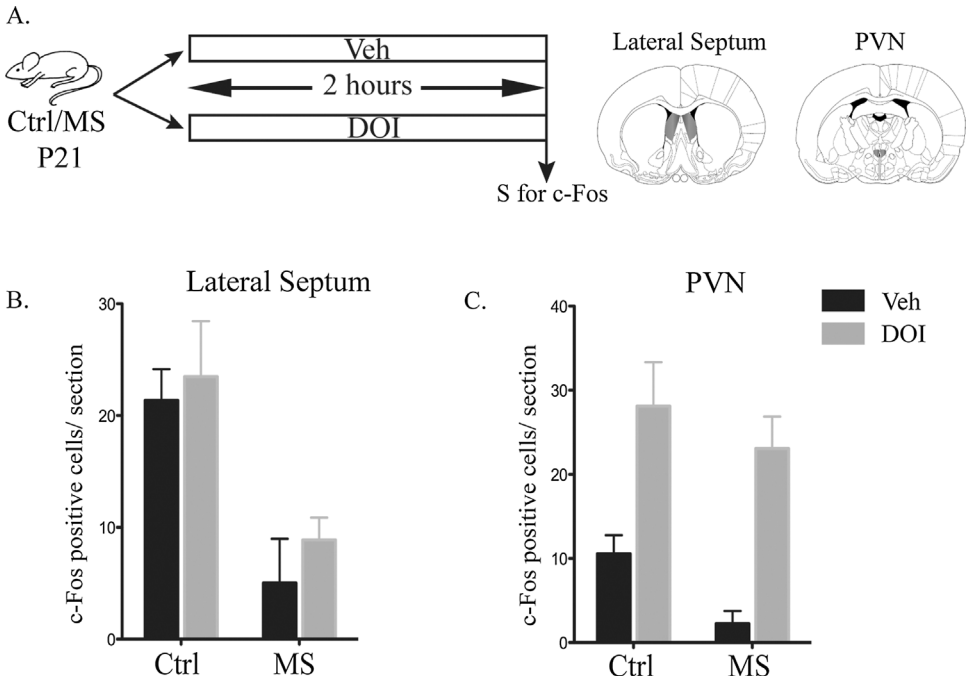

Fig. 3. Pattern of neural activation following DOI in the lateral septum and hypothalamic paraventricular nucleus in MS animals.
(A) Shown is a schematic of the experimental paradigm for treatment of control (Ctrl) or MS P21 pups with vehicle or 5-HT_{2A} receptor agonist, DOI (2 mg/kg) for c-Fos immunohistochemistry and schematics of coronal sections at the level of the lateral septum (LS) and paraventricular nucleus (PVN) of the hypothalamus. We noted no significant MS x DOI interaction effects in either the LS (B) or the PVN (C), n = 5-6 animals per group.

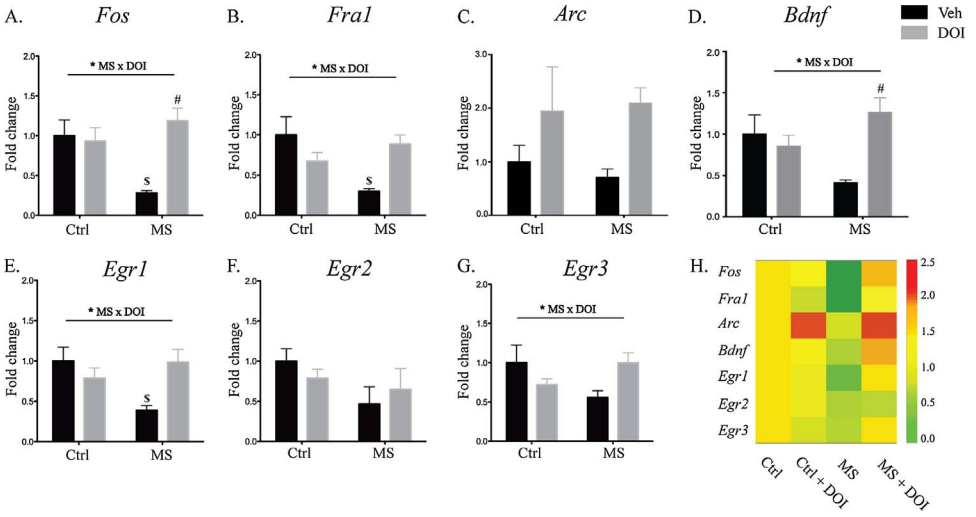

Fig. 4. A history of early stress alters the DOI-evoked pattern of immediate early gene expression in the medial prefrontal cortex.
Shown are qPCR results to determine the expression levels of mRNA for multiple immediate early genes (IEGs) expressed as fold change of the vehicle treated control group. Two-way ANOVA analysis revealed significant MS x DOI interaction effects for the regulation of *Fos* (A), *Fra1* (B), *Bdnf* (D), *Egr1* (E), and *Egr3* (G) mRNA expression, but not for *Arc* (C) and *Egr2* (F) mRNA levels. Results are expressed as the mean ± SEM. * $p < 0.05$, MS x DOI interaction, Two-way ANOVA analysis. Significance in Bonferroni *post hoc* comparisons ($p < 0.05$) as compared to vehicle treated controls ($) and as compared to vehicle treated MS (#). (H) Shown is a representative heat map indicating the magnitude of up (red) and down (green) regulation of IEG expression in the mPFC of Control, Control + DOI, MS and MS + DOI treatment groups, n = 7-9 animals per group.

examine whether activity-dependent IEG expression was also altered within the mPFC of MS pups, both under baseline conditions and in response to DOI administration. We focused on those IEGs (*Fos*, *Fra1*, *Arc*, *Bdnf, Egr1-3*,) that have previously been described to be regulated within the neocortex following 5-HT_{2A} receptor stimulation, and are considered to be components of the 5-HT_{2A} receptor-stimulated "transcriptome fingerprint".

Two-way ANOVA analysis revealed that the pattern of IEG regulation evoked by DOI within the mPFC of MS pups differed significantly from that observed in DOI-treated pups with no stress history. We observed significant MS x DOI interaction effects for the regulation of *Fos* ($F_{(1.27)} = 8.99$, $p = 0.006$) (Fig. 4A), *Fra1* ($F_{(1.27)} = 8.76$, $p = 0.0063$) (Fig. 4B), *Bdnf* ($F_{(1.27)} = 8.27$, $p = 0.0078$) (Fig. 4D), *Egr1* ($F_{(1.27)} = 8.15$, $p = 0.0082$) (Fig. 4E) and *Egr3* ($F_{(1.27)} = 5.53$, $p = 0.026$) (Fig. 4G) within the mPFC, with a significant main effect of DOI observed for *Fos* ($F_{(1,27)} = 6.69$, $p = 0.0154$) and *Arc* ($F_{(1,27)} = 5.67$, $p = 0.024$). Bonferroni *post hoc* comparisons revealed a significant baseline reduction in expression of *Fos* (Fig. 4A), *Fra1* (Fig. 4B), and *Egr1* (Fig. 4E) in the mPFC of MS animals as compared to controls. Interestingly, *post hoc* comparisons indicated that while DOI treatment to pups with no stress history did not alter the expression of any of the IEGs profiled, MS rat pups showed a significant DOI-evoked induction in *Fos* (Fig. 4A) and *Bdnf* (Fig. 4D) mRNA levels within the mPFC as compared to the vehicle-treated MS group. In summary, our results indicate that MS evokes a dysregulation in the expression of specific IEGs within the mPFC both under basal conditions and following 5-HT_{2A} receptor stimulation.

We also assessed the expression of the 5-HT_{2A} receptor within the mPFC of MS animals on postnatal day 21. qPCR analysis for *Htr2a* mRNA levels revealed no change in 5-HT_{2A} receptor expression within the mPFC of P21 MS animals as compared to age-matched controls (*Htr2a* mRNA levels: Control = 1 ± 0.18; MS = 0.78 ± 0.12, n = 7–9 animals per group; $p > 0.05$, Student's *t* test).

4. Discussion

Our results indicate that soon after early stress, MS animals at P21 exhibit enhanced behavioral head-twitch responses following 5-HT_{2A} receptor stimulation. Under baseline conditions MS animals exhibit reduced expression of c-Fos positive cell numbers within the mPFC, and show a significantly altered DOI-evoked c-Fos expression pattern as compared to control pups at P21. This suggests that a history of MS results in a distinct pattern of neural activation, as determined by c-Fos positive cell counting, within the mPFC following 5-HT_{2A} receptor stimulation. In addition, our results demonstrate that MS pups also show a decline in the mRNA expression of specific IEGs (*Fos*, *Fra1*, *Egr1*) in the mPFC under baseline conditions, and exhibit an altered pattern of DOI-evoked IEG expression. These results indicate that soon after the cessation of MS, P21 MS pups exhibit altered 5-HT_{2A} receptor responsivity, with potentiated 5-HT_{2A} receptor-evoked behavioral responses, and altered c-Fos positive cell numbers and IEG expression within the mPFC.

Previous studies indicate that adult animals with a history of early stress exhibit enhanced cortical 5-HT_{2A} receptor function, including enhanced 5-HT_{2A} receptor-evoked head-twitch behavior (Benekareddy et al., 2010; Holloway et al., 2013). Our results provide novel evidence indicating that such enhanced 5-HT_{2A} receptor-evoked responses, namely enhanced head-twitch behavior, exhibit an early onset following MS, emerging during postnatal life (P21) soon after the termination of stress. The 5-HT_{2A} receptor-evoked head-twitch behavior is a stereotypical, rhythmic rotational movement of the head observed in both rats and mice, and mediated via cortical 5-HT_{2A} receptors (Canal and Morgan, 2012). Prior studies examining the normal ontogeny of 5-HT_{2A} receptor-evoked head-twitch responses indicate that head-twitches are first noted at P18, and not observed at earlier ages of P7 and P14 in mouse models (Darmani et al., 1996). This suggests that although the cortical excitation evoked by 5-HT_{2A} receptor stimulation appears to peak at P10 (Zhang, 2003), and cortical 5-HT_{2A} receptor mRNA and receptor binding show maximal expression by P12-17 (Basura et al., 2008; Morilak and Ciaranello, 1993), the functional maturation of 5-HT_{2A} receptor-mediated head-twitch behavior emerges only following the second week of life (Darmani et al., 1996). Our findings indicate that this behavioral response to 5-HT_{2A} receptor activation is robustly enhanced in P21 pups with a history of MS.

In our experiments, we used c-Fos cell counting analysis in key limbic brain regions as a molecular marker to assess neural activation (Sheng and Greenberg, 1990) in control and MS pups at P21 following 5-HT_{2A} receptor stimulation. Further, our IEG profiling analysis also provides a measure of degree of neuronal activation within the mPFC of MS animals both baseline and following DOI treatment. Our gene profiling analysis indicates a decline in neuronal activity within the mPFC of MS animals under baseline conditions, and although the c-Fos cell counts within mPFC subdivisions did not indicate statistically significant decreases in activity the pattern is suggestive of reduced neuronal activation in the mPFC of P21 MS pups. Interestingly, prior reports have indicated reduced neural activity within the mPFC during postnatal life in both a model of neonatal maltreatment (Rincón-Cortés and Sullivan, 2016), as well as in a social isolation stress model (Ieraci et al., 2016). These observations bear clinical relevance as mPFC hypoactivity has been previously observed in studies of patients with a history of early adversity (van Harmelen et al., 2014). The mPFC plays a key role in top-down regulation of several limbic neurocircuits,

modulating anxiety and depressive-like behaviors, stress response and fear pathways (Arnsten, 2015; Davidson, 2002; Giustino and Maren, 2015; McKlveen et al., 2015). We did note a significant baseline decrease in neuronal activity within the LS of MS pups. The LS plays a key role in the modulation of anxiety-like behavior and regulation of neuroendocrine stress responses (Anthony et al., 2014; Herman et al., 2002; Parfitt et al., 2017). A prior report suggests that MS blunts septal activation in response to vasopressin in adulthood thus impairing social recognition (Lukas et al., 2011). Collectively our findings of possible blunted neural activity within the mPFC and LS and PVN of P21 MS pups suggest that such alterations in a network that regulates stress response pathways may play a key role in the perturbed stress responses that arise following early adversity (van Bodegom et al., 2017).

DOI is a hallucinogenic ligand of the 5-$HT_{2A/2C}$ receptor, and acute administration of DOI results in increased head-twitch behavior and a robust increase in c-Fos positive cell numbers across multiple brain regions (Canal and Morgan, 2012). This is also accompanied by a signature transcriptome in the neocortex following acute DOI-mediated 5-HT_{2A} receptor stimulation (González-Maeso et al., 2007, 2003). Previous reports suggest that the behavioral and transcriptional effects of DOI are predominantly mediated via the 5-HT_{2A} receptor. DOI-induced head-twitch behavior and neocortical regulation of c-Fos and Arc mRNA expression is inhibited by pretreatment with a selective 5-HT_{2A}, but not 5-HT_{2C}, receptor antagonist (Willins and Meltzer, 1997; Pei et al., 2004; Santini et al., 2011; Scruggs et al., 2000). Further, local infusion of DOI in the rat medial prefrontal cortex leads to an increase in the levels of 5-HT, an effect that is blocked by a selective 5-HT_{2A}, but not 5-HT_{2C}, receptor antagonist (Martín-Ruiz et al., 2001). Given the evidence that the effects of the 5-HT_2 receptor agonist DOI on head-twitch and neocortical IEG expression predominantly involve a role for the 5-HT_{2A} receptor, it is likely that the effects observed in our study also involve altered function of the 5-HT_{2A} receptor in MS animals at P21. However, the studies done on the contribution of the 5-HT_{2A} receptor to the behavioral and gene expression effects of DOI were carried out in adult animals, and it is important to take this in consideration prior to ruling out a contribution of the $5HT_{2C}$ receptor to the effects we observed of enhanced DOI-evoked head-twitch behaviors and perturbed neocortical IEG expression in MS animals at P21, a developmental time point at which both $5HT_{2A}$ and $5HT_{2C}$ receptors are expressed in the rat neocortex (Li et al., 2004). The ontogeny of emergence of such an enhanced c-Fos expression and the 5-$HT_{2A/C}$ receptor-evoked "transcriptome fingerprint" remains unknown at present. Cortical $5HT_{2A}$ receptor expression reaches adult-like levels around P7-8 and is followed by a rapid increase to peak expression levels around P10-12. This increase is followed by a decline and maintenance at adult-like levels commencing around P18 and stabilized by P21 (Basura et al., 2008; Morilak and Ciaranello, 1993). Our results indicate that DOI treatment to control P21 pups with no stress history does not alter c-Fos positive cell number or IEG expression patterns within the mPFC. This suggests that while electrophysiological responses peak at P10 (Zhang, 2003) and functional (head-twitch) responses are already observed at P21 (Darmani et al., 1996) and receptor expression levels are similar to adult brain, the coupling of 5-HT_{2A} receptor activation to enhanced neocortical c-Fos immunoreactivity and IEG expression may not be in place at P21. Currently, the ontogeny of the DOI-evoked pattern of cortical IEG expression remains unknown. It is unlikely that DOI treatment at this age does not evoke neural activation, given both electrophysiological and biochemical studies clearly indicate that the 5-HT_{2A} receptor is functionally coupled to down-stream signaling (Ike et al., 1995; Zhang, 2003). This raises the possibility that "activity-transcription" coupling downstream of 5-HT_{2A} receptor activation resulting in enhanced IEG expression may not have fully matured by P21 in the neocortex, in particular in the PFC which is known to show substantial maturation of local microcircuits and connectivity during adolescence (McEwen and Morrison, 2013; Spear, 2000). However, what is striking is that MS pups at P21 exhibit both enhanced c-Fos positive cell numbers in all subdivisions of the mPFC, as well as increased expression of *Fos, Fra1, Bdnf, Egr1* and *Egr3* in the mPFC, but show no significant difference in 5-HT_{2A} receptor mRNA levels as compared to control pups. It is of interest to note that Egr3 has been implicated in the transcriptional regulation of 5-HT_{2A} receptor expression in response to environmental stimuli (Maple et al., 2016). In our study we have checked for IEG expression two hours after DOI administration, whereas some previous studies have examined DOI-mediated IEG expression one hour following treatment (González-Maeso et al., 2003, 2007; Santini et al., 2011). It is possible that in our experimental design we might have missed the peak of gene expression for some of the IEGs following DOI administration. Our observations raise the speculative possibility that as a consequence of early adversity, MS pups exhibit altered 5-HT_{2A} receptor function/sensitivity/signaling supported not only by the observation of enhanced head-twitch responses, but also the distinct nature of cortical c-Fos and IEG expression in response to DOI stimulation. We also observed that the perturbed c-Fos immunoreactivity evoked by 5-HT_{2A} receptor activation in MS pups appears to be localized to the PFC, as the DOI-mediated increase in c-Fos expression within the hippocampus (CA1 and hilus) and PVN did not differ between control and MS pups at P21. This also indicates that while the normal ontogeny of 5-HT_{2A} receptor activation–transcription coupling may not have developed in the PFC by P21, within the hippocampus and PVN 5-HT_{2A} receptor activation at P21 already evokes enhanced c-Fos positive cell numbers.

Our results provide novel evidence that soon after the cessation of early stress, MS pups at P21 already exhibit enhanced 5-HT_{2A} receptor-evoked behavioral and IEG responses. The underlying mechanisms for the altered 5-HT_{2A} receptor responses remain unclear. Prior evidence does indicate altered expression of several genes involved in Gαq- and Ca^{2+}-mediated signaling at P21 in the mPFC of MS pups (Proulx et al., 2014). Further studies are required to examine whether a history of MS alters 5-HT_{2A} receptor-Gαq coupling and/or stabilizes differential signaling states. Given that the metabotropic glutamate receptor 2 (mGlu2) is reported to form heteromers with the 5-HT_{2A} receptor, and is required for the behavioral effects of hallucinogenic 5-HT_{2A} receptor ligands (Moreno et al., 2011), this raises the speculative possibility of altered heterocomplex formation based on early adversity. Indeed, along with evidence from other models of early adversity such as MIA and postnatal SSRI exposure, there is building evidence that early adversity evokes persistent changes in 5-HT_{2A} receptor function (Holloway et al., 2013; Malkova et al., 2014; Sarkar et al., 2014). Here, we show that such changes emerge early and are already in place in peri-weaning periods. Given that serotonergic neurotransmission and the 5-HT_{2A} receptor plays an important role in contributing to the emergence of anxiety and depressive-like behavior following early adversity and in the heightened hallucinogen responsivity noted in these models of early trauma, our results motivate future investigation to determine the underlying mechanisms for the early stress induced altered 5-HT_{2A} receptor responses.

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Conflict of interest

The authors have no conflict of interest to declare.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ijdevneu.2017.10.005.

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