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Developmental exposure to psychostimulant primes activity-dependent gene induction in frontal cortex

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Abstract

Perinatal neurodevelopment involves extensive formation of neural connections and onset of activity-dependent gene expression for synaptic function and plasticity. Exposure to psychostimulants at this stage imposes significant risks for developing cognitive and affective disorders later in life. However, how developmental exposure to psychostimulants may induce long lasting molecular changes relevant to neural circuit function remains incompletely understood. In this study, we investigated the impacts of psychostimulant amphetamine on the activity-dependent induction of synaptic adaptor molecule *Arc* in the frontal cortex of neonatal mice. We found that transient exposure to amphetamine not only amplifies activity-dependent *Arc* mRNA expression immediately, but also potentiates subsequent induction of *Arc* mRNA in the absence of amphetamine. This priming effect is associated with a rapid and persistent increase of histone mono-methylation (H3K4me1), a marker for transcriptionally permissive chromatin, at the *Arc* locus, but not any long-lasting change in the phosphorylation of upstream transcription factor CREB. Furthermore, the increase of H3K4me1 at the *Arc* locus requires dopamine receptor signaling, and the priming of *Arc* expression correlates with the dopaminergic innervation pattern in the frontal cortex. Together, our results demonstrate that developmental exposure to psychostimulant amphetamine induces long-lasting chromatin changes and primes activity-dependent *Arc* gene induction. These findings reveal the molecular targets of psychostimulant during perinatal development that may contribute to long-term psychiatric risks.

Keywords

Psychostimulant; development; activity-dependent gene; amphetamine; *Arc*; Histone; H3K4me1

Introduction

The perinatal period is a critical stage in the development of the brain during which extensive growth in neural connections and rapid changes in neuronal gene expression occur (Sur and Rubenstein, 2005; Workman et al., 2013). These cellular and molecular changes lay down the foundations of brain circuits in preparation for the environmental and social challenges that individuals will face later in life. It is also during this perinatal period that

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the brain is particularly vulnerable to the insult of psychostimulants that overly stimulate the developing neuromodulator or neurotransmitter systems (Thompson et al., 2009).

Prenatal exposure to psychostimulants occurs in a significant human population. For example, 5% of pregnant women in the United States reported seeking psychoactive and addictive drugs such as amphetamine, methamphetamine, and cocaine (Substance Abuse and Mental Health Services Administration, 2012). Such developmental drug exposure places the fetus at significant risk for developing psychiatric symptoms later in life (Smith et al., 2015). Pre-clinical animal studies of developmental drug exposure typically use mice as a model, an altricial species whose brain development in the first two postnatal weeks (P0~P14) is comparable to the third trimester in human (Clancy et al., 2007; Workman et al., 2013). These studies have found that exposure to psychostimulants in neonatal mice causes significant cognitive and emotional behavioral symptoms in adulthood (Archer and Kostrzewa, 2016; Jablonski et al., 2016). However, the downstream targets and mechanisms that contribute to the developmental vulnerability to psychostimulants remain incompletely understood.

A common effect of psychostimulants is to increase the concentration of neuromodulator dopamine in the brain (Sulzer, 2011). Dopamine is known to play important roles in regulating cognitive, motivational, and motor functions through distinct anatomical projection pathways (Bjorklund and Dunnett, 2007). The mesofrontal dopamine projection innervates the frontal cortex and is crucially involved in cognitive control functions (Robbins and Arnsten, 2009; Weinstein et al., 2017). During development, this projection already reaches the frontal cortex at the perinatal stage and continues to elaborate until adulthood (Berger et al., 1991; Kalsbeek et al., 1988; Mastwal et al., 2014; Naneix et al., 2012). This developmental profile raises the possibility that excessive dopaminergic stimulation during perinatal development may cause long-lasting changes in frontal cortical neurons.

Our recent studies have revealed a role of dopamine in amplifying neural activity-dependent gene expression in the developing frontal cortex in mice (Ye et al., 2017). Neural activity-dependent genes are defined by their rapid and robust transcriptional response to increase in neural activity (Flavell and Greenberg, 2008). These genes include both transcription factors such as cFos and synaptic adaptor molecules such as *Arc*. The function of *Arc* has been particularly well studied as a master regulator of synaptic plasticity, including long-term potentiation (LTP), long-term depression (LTD), homeostatic scaling and persistent firing activity (Bramham et al., 2008; Marballi and Gallitano, 2018; Mastwal et al., 2016; Ren et al., 2014; Shepherd and Bear, 2011). Both loss of function and overexpression of *Arc* can disrupt synaptic plasticity and lead to cognitive dysfunctions and psychiatric phenotypes (Jenks et al., 2017; Manago et al., 2016; Plath et al., 2006; Wall et al., 2018). In addition, recent human genetic studies have implicated a list of proteins that directly interact with *Arc* in neurodevelopmental psychiatric disorders such as schizophrenia and intellectual disability disorders (Fromer et al., 2014; Kirov et al., 2012; Purcell et al., 2014).

Interestingly, the inducibility of *Arc* gene by neural activity is tightly regulated during development. Despite the presence of neural network activity in early postnatal mouse frontal cortex, *Arc* gene expression is barely detectable at this stage even with direct

electrical stimulation of the cortex (Ye et al., 2017). Robust induction of *Arc* gene occurs with the maturation of frontal dopaminergic innervation after two weeks of postnatal development and requires dopaminergic signaling through D1-type receptors (D1R) (Ye et al., 2017). In addition, simultaneous application of amphetamine and electrical stimulation to early postnatal cortex is sufficient to induce *Arc* expression acutely (Ye et al., 2017). However, it is unknown whether such neonatal exposure to psychostimulant amphetamine would lead to long-lasting changes in *Arc* expression in the frontal cortex.

In this study, we used biochemical and histological methods to evaluate the consequence of neonatal amphetamine exposure on the expression level and inducibility of *Arc* mRNA in the mouse frontal cortex, and the associated biochemical and epigenetic changes. Our findings indicate that developmental exposure to amphetamine induces long-lasting changes in histone methylation and primes the *Arc* gene for activity-dependent induction. This excessive induction of a master regulator of synaptic plasticity may distort the normal trajectory of frontal circuit maturation and contribute to behavior deficits later in life.

Materials and Methods

Animals

Wild-type and heterozygous *Arc-GFP* mice (Wang et al., 2006) in C57BL/6 strain were group housed (2–4 animals per cage) in a temperature and humidity controlled animal facility, maintained on a 12 hour light/dark cycle, and fed *ad libitum*. Birth was defined as postnatal day zero (P0). Both male and female mice were included in experiments and at least three litters of mice were used for each experiment. Experimental procedures were approved by the NIMH Animal Care and Use Committee.

RT-PCR

Frontal cortex tissue samples were dissected from mice, either from the home cage, 0.5 hour, 2 hours or 24 hours after amphetamine treatment. Samples were then homogenized in TRIzol with a Polytron homogenizer. Total RNA was then isolated using the RNeasy kit (Qiagen). To analyze mRNA expression level, cDNA was produced using reverse transcriptase (Life technologies) with random hexamer primers, and then measured with real-time quantitative PCR using TaqMan probes in the 7900HT Real-time PCR system (Applied Biosystems). Primers and Taqman probes were designed using PrimerExpress (Applied Biosystems). Sequences for primers and probes are included in Table 1. The *Arc* mRNA expression level was determined by the *Arc* cDNA concentration normalized to the *Actin* cDNA concentration.

Immunohistochemistry

Mouse brains were fixed in 4% paraformaldehyde and imbedded in 1.5~3% agarose. 50 μ m brain sections were cut with vibratome. Those sections were permeabilized by 0.3% Triton X-100 in PBS (PBST) for 20 min and blocked with 10% goat serum in the PBST for 1 hour. The sections were then incubated in antibodies against tyrosine hydroxylase (1:10,000, EMD Millipore Cat# AB152) overnight at 4°C. After washing with PBS for 30 min at room temperature, sections were incubated for 2 hours with Alexa 488 goat anti-rabbit IgG (1:100,

Molecular Probes (Invitrogen) Cat# A11008) at room temperature. Frontal brain sections were labeled immunofluorescently with TH-specific antibodies then imaged with confocal microscopy (FV1000, Olympus). Acquired images were analyzed automatically using ImageJ, blind with regard to experimental conditions. After applying line filters based on Hessian matrix (FeatureJ plugin) with a smoothing scale of 2 pixels, the images were thresholded at 3 standard deviations above background. Line-like structures that were larger than 30 pixels and had a circularity index less than 0.5 were automatically selected. The total number of selected pixels in each image was calculated to indicate the extent of tyrosine hydroxylase staining.

Drugs

Amphetamine sulphate, 5 mg/kg and SCH23390 hydrochloride, 0.2 mg/kg, were dissolved in saline and administered intraperitoneally.

Electrical Stimulation (ES)

Mice were gently restrained by scruffing. After wetting the skin around the ear with saline, electrical conductive gel was applied, and an electrical stimulus (2s, 100Hz, 10mA) was delivered across skull. The animal was visually monitored until recovery from muscle contraction, normally within 5 min. After this recovery period the animal was placed back in its home cage. For combined amphetamine and ES treatment, mouse received ES 15 min after amphetamine administration. In order to test for the potential priming effect of Amph/ES pretreatment, another ES stimulus (duration shortened to 1 s) was delivered a day after the pretreatment.

Chromatin Immunoprecipitation

Frontal cortex tissue was collected from mice in their home cage, 2 hr or 24 hr after treatment. Immunoprecipitation was performed following a published protocol (Wang et al., 2008) with minor modification. Fresh brain tissue was cross-linked in 1% formaldehyde for 10 min at room temperature, and the cross-linking reaction was quenched by adding glycine to 0.125 M. The brain tissue was then homogenized in lysis buffer (50 mM Hepes-KOH, pH 7.5, 140 mM NaCl, 1 mM EDTA, pH 8.0, 10% Glycerol, 0.5% NP-40, 0.25% Triton X-100, protease inhibitors (Roche)), and incubated in protein extraction buffer (200 mM NaCl, 1 mM EDTA, pH 8.0, 0.5 mM EGTA, pH 8.0, 10 mM Tris-HCl, pH 8.0, protease inhibitors) for 10 min at 4 °C. Isolated nuclei were sonicated in chromatin extraction buffer (1 mM EDTA, pH 8.0, 0.5 mM EGTA, pH 8.0, 10 mM Tris-HCl, pH 8.0, protease inhibitors) for 5 cycles (5 min of bursts with 30 s intervals) with Bioruptor (Diagenode). Insoluble debris was removed by centrifuging at 12,000 rpm for 10 min at 4 °C, then the sonicated chromatin fragments were adjusted into RIPA buffer (10 mM Tris, pH 7.6, 1 mM EDTA, 0.1% SDS, 0.1% Na-Deoxycholate, 1% Triton X-100). Forty µL of Protein A Dynabeads (Invitrogen) were used for each immunoprecipitation. Beads were pre-blocked with 5% BSA and 100 µg salmon sperm DNA for 1.5 h at 4 °C and then incubated with 3 µg of anti-H3K4me1 antibody (Abcam Cat# ab8895 RRID:AB_306847) for 2h at 4 °C. Chromatin fragments of 50 µl were saved as input DNA, and those of 1 ml were used for immunoprecipitation with H3K4me1-antibody-conjugated Dynabeads overnight at 4 °C. The immunoprecipitates were washed with the following combination of wash buffers: once with low salt RIPA buffer (20

mM Tris-HCl pH 8.0, 150 mM NaCl, 2 mM EDTA, 1% Triton X-100, 0.1% SDS), once with high salt RIPA buffer (20 mM Tris-HCl pH 8.0, 500 mM NaCl, 1 mM EDTA, 1% TritonX-100, 0.1% SDS), once with LiCl wash buffer (10 mM Tris-HCl pH 8.0, 250 mM LiCl, 1 mM EDTA, 1% NP40, and 1% deoxycholic acid (sodium salt)), and twice with TE buffer (10 mM Tris-HCl pH 8.0 and 1 mM EDTA). Bound chromatin and input DNA were reverse cross-linked overnight at 65 °C in elution buffer (10 mM Tris-HCl, pH 8.0, 5 mM EDTA, 300 mM NaCl, and 0.5% SDS). Immunoprecipitated DNA and input DNA were treated with RNase A (Sigma-Aldrich) and proteinase K (Roche), and purified with a QIAquick PCR purification kit (Qiagen). Immunoprecipitated DNA was analyzed by quantitative PCR (Applied Biosystems). The amount of target sequence in the *Arc* gene in the immunoprecipitated DNA was determined by comparison to that in the input DNA. These results were then normalized to that of a control sequence in the *Actin* gene.

Arc-GFP Expression

Brains from heterozygous *Arc-GFP* were fixed with 4% paraformaldehyde, imbedded in ~2% agarose, and sectioned with a vibratome into 50 µm sections. Mounted sections were imaged with a confocal fluorescent microscope (Olympus FV1000). Custom-written computer scripts (using Matlab Image Processing Toolbox) blind to experimental conditions were used to detect Arc-GFP expressing neurons in the confocal images (Cao et al., 2015; Wang et al., 2006). Briefly, all images were smoothed with a Gaussian filter of one-cell diameter to reduce noise. Local maxima were identified as candidate GFP+ cell centers, and corresponding cell body regions were defined as within one-cell radius (5 µm) from the centers. A background surround region was defined outside the cell body as a donut-shaped region with outer diameter of 3 cell radii, and an inner diameter of 2 cell radii. Cells were detected as GFP+ if the pixel intensity distributions of the cell body and the surround region were distinguishable at 95% confidence level (i.e., the difference of the median pixel intensity in the cell region versus the surround region was larger than 2 standard deviations of pixel noise). Two sections from each animal were used for quantification and the values were averaged per animal.

Western blotting.

Frontal cortex tissue samples were dissected from mice 2 hours after ES treatment, and homogenized in buffer containing 320 mM sucrose, 5 mM Hepes, 1% SDS, phosphatase inhibitor cocktails (Sigma) and protease inhibitors (Roche). Western blotting was done with antibodies against pCREB (1:1,000; EMD Millipore Cat# 06-519 RRID:AB_310153), CREB (1:1,000; Cell Signaling Technology Cat# 9197S RRID:AB_10695751), and tubulin (1:100,000; Sigma-Aldrich Cat# T6074 RRID:AB_477582). Images of the blots were quantified using ImageJ (NIH). The amount of protein blotted on each lane was normalized to levels of tubulin.

Statistical analysis

Statistical differences between two groups were determined with two-sided Student's *t*-test. Statistical differences among three groups or more were determined using one-way or two-way analysis of variance (ANOVA), followed by the Bonferroni multiple comparison tests. Data are displayed as mean ± SE.

Results

Neonatal amphetamine exposure primes the induction of *Arc* mRNA by electrical stimulation

Perinatal exposure to psychostimulant such as amphetamine occurs in a significant human population and such exposure is also associated with incidents of seizures in infants (Chasnoff et al., 1989; Kramer et al., 1990; Oro and Dixon, 1987). However, the downstream molecular targets and mechanisms that may contribute to the long-lasting effects of developmental psychostimulant exposure remain little understood. Our previous study has shown that the presentation of psychostimulant amphetamine (Amph) to neonatal mice significantly amplifies *Arc* mRNA level in the frontal cortex in response to a brief high-frequency electrical stimulus (ES) (Ye et al., 2017). To investigate whether there are any long-lasting molecular changes following this pathological exposure to psychostimulant and intensified neural network activity, we first examined the kinetics of Amph/ES-induced *Arc* mRNA levels.

Arc expression in the frontal cortex is very low and barely detectably by RT-PCR at postnatal day 7 (P7), but amplifies sharply from P11–13 during normal development (Ye et al., 2017). We therefore chose P9 as an intermediate time point to study how *Arc* expression might be regulated by Amph/ES. We collected frontal cortical tissue before and at various time points after administrating Amph/ES to P9 mice, and found that Amph/ES induced substantial increase in *Arc* mRNA levels within 30 minutes. *Arc* mRNA expression remained at a high level at 2 hours, but returned to baseline 24 hours after administration (Fig. 1A; one-way ANOVA, $F(3, 8) = 99$, $p < 0.0001$, $N = 3$ mice per group; post tests: Home Cage vs. 0.5hr after Amph/ES, $p < 0.0001$; Home Cage vs. 2hr after Amph/ES, $p < 0.0001$; Home Cage vs. 24 hr after Amph/ES, $p = 0.23$). Those results showed that *Arc* mRNA expression is transiently elevated after psychostimulant and electrical stimulation in neonates, and decays back to baseline within a day.

Next, we investigated whether this transient onset of *Arc* expression predicts the inducibility of *Arc* gene in response to subsequent electrical activation. On Day 1 of this experimental procedure, one group of P9 mice received Amph/ES pretreatment, while another comparable group remained in their home cage. On Day 2, half the mice in each group were given ES stimulation. Quantitative RT-PCR analysis showed that on Day 2, as expected, animals that did not receive ES stimulation had comparable levels of *Arc* mRNA, whether or not they had received pretreatment with Amph/ES (Fig. 1B). In contrast, when ES stimulation was administered on Day 2, it elicited significantly higher levels of *Arc* mRNA expression in animals pretreated with Amph/ES than in animals receiving no pretreatment (two-way ANOVA, pretreatment-by-stimulation interaction, $F(1,31) = 7.3$, $p = 0.011$, $N = 8\sim9$ mice per group; post-test, no ES without pretreatment vs. no ES with pretreatment, $p = 0.96$; ES without pretreatment vs. ES with pretreatment, $p = 0.0010$;). These results suggest that Amph/ES pretreatment primes *Arc* transcriptional response to subsequent electrical activation.

Neonatal amphetamine exposure does not lead to long-lasting changes in the phosphorylation of transcription factor CREB

The priming of *Arc* transcription could potentially reflect changes in the neural activity and upstream signal transduction pathways that lead to the transcriptional activation of *Arc*. Alternatively, it could also reflect changes in the chromatin modifications at the *Arc* locus that facilitates subsequent transcription.

To investigate those possibilities, we first examined the level of phosphorylated CREB (pCREB) under the Amph/ES pretreatment paradigm. CREB is a transcription factor whose phosphorylation is involved in activity-dependent *Arc* gene expression (Kawashima et al., 2009; West and Greenberg, 2011). Our Western blot analysis showed that as predicted, Day 2 ES stimulation significantly increased pCREB/CREB ratio over the baseline (Fig. 2A, B). However, there was no significant interaction between Day 1 Amph/ES pretreatment and Day 2 ES stimulation (two-way ANOVA, stimulation effect, $F(1,12) = 29.19$, $p = 0.0002$; pretreatment and stimulation interaction, $F(1,12) = 1.06$, $p = 0.32$; $N = 4$ mice for each group). Mice that had received Amph/ES pretreatment showed pCREB levels comparable to those that had received no pretreatment at the baseline level on Day 2 ($p = 0.92$), suggesting that there was no long-lasting change in pCREB after Amph/ES pretreatment. Amph/ES pretreatment also did not significantly affect the subsequent pCREB response to ES ($p = 0.21$). In addition, the total amount of CREB protein as normalized against the house-keeping protein tubulin remained the same across all the conditions (Fig 2C, two-way ANOVA, stimulation effect, $F(1,12) = 0.019$, $p = 0.89$; pretreatment effect, $F(1,12) = 0.23$, $p = 0.64$; interaction effect, $F(1,12) = 2.78$, $p = 0.12$). The lack of long-lasting change in upstream neuronal signaling events after pretreatment raises the possibility that the priming of *Arc* expression may be associated with persistent changes in other factors such as chromatin modifications.

Neonatal amphetamine exposure leads to long-lasting dopamine signaling dependent increase of H3K4me1 modification at the *Arc* locus

Previous studies have suggested that H3K4me1 modification keeps inducible genes in a transcriptionally responsive state (Calo and Wysocka, 2013; Herz et al., 2012; Ostuni et al., 2013; Whyte et al., 2012). The enhancer site responsible for activity-dependent *Arc* transcription has been characterized before (Kawashima et al., 2009; Kim et al., 2010; Pintchovski et al., 2009; Smith-Hicks et al., 2010), and H3K4me1 marks this enhancer, as well as the *Arc* coding region, in both resting and activated neurons in mature culture (Kim et al., 2010). However, whether the level of H3K4me1 at the *Arc* locus is dynamically regulated in neonatal cortex remains yet unknown. We therefore tested whether neonatal exposure to amphetamine may increase the H3K4me1 level at the *Arc* locus in P9 frontal cortex.

We collected frontal cortical tissue before and at various time points after administrating Amph/ES to P9 mice, and conducted chromatin immunoprecipitation (CHIP) assays with H3K4me1-specific antibody. The immunoprecipitated DNA was then analyzed with qPCR, using primers targeting four different sites (the activity-dependent enhancer, the promoter, exon 1 and exon 3) at the *Arc* locus (Fig. 3A). In contrast to the transient increase in *Arc*

mRNA or pCREB levels, H3K4me1 levels were induced by Amph/ES and remained high up to 24 hours after treatment (two-way ANOVA, treatment effect averaged across genomic sites, $F(2,83) = 80.33$, $p < 0.0001$; post tests, Home Cage vs. 2hr after Amph/ES, $p < 0.0001$; Home Cage vs. 24 hr after Amph/ES, $p < 0.0001$). These results indicate that Amph and electrical activation induce H3K4me1 modification at the *Arc* locus that endures far longer than the increase in *Arc* mRNA and CREB phosphorylation. Together with our earlier results showing primed *Arc* transcription after Amph/ES treatments (Fig. 1), these findings suggest that H3K4me1 levels at the *Arc* locus are stably maintained after initial induction and predict greater transcriptional response to subsequent electrical activation.

Since our previous study showed that the effects of Amph and ES on *Arc* mRNA expression is synergistic and single treatment has little effect (Ye et al., 2017), we investigated whether Amph and ES also have synergistic effect on H3K4me1 levels at the *Arc* locus. Compared with those from the untreated group, treatment with Amph/ES greatly enhanced the overall level of H3K4me1 at the *Arc* locus in P9 frontal cortex. Treatment with Amph alone, by contrast, enhanced the H3K4me1 level only slightly, and with ES alone, not at all (Fig. 3B; two-way ANOVA, treatment effect, $F(3, 78) = 14.45$, $p < 0.0001$, $N = 6$ mice per group; post tests: Amph/ES vs. Home Cage, $p < 0.0001$; Amph vs. Home Cage, $p = 0.039$; ES vs. Home Cage, $p = 0.99$). Consistent with our *Arc* mRNA data (Ye et al., 2017), we found that treatment with Amph and ES combined had a synergistic effect on H3K4me1 modification at the *Arc* enhancer (two-way ANOVA, Amph-by-ES interaction, $F(1, 19) = 22.29$, $p = 0.0001$).

To further test whether those changes in H3K4me1 levels depend on dopaminergic signaling through D1R, as what has been reported for *Arc* mRNA (Ye et al., 2017), we administered the D1R antagonist (ANT) SCH23390 before Amph/ES treatment. The comparison between “Saline+Amph/ES” and “D1R ANT+Amph/ES” experimental groups indicates that the effect of Amph/ES is significantly blocked by D1R ANT (Fig. 3C, two-way ANOVA, D1R ANT effect, $F(1, 32) = 17.37$, $p = 0.0002$; $N = 5\sim6$ mice per group). Further comparison between “Saline+Amph/ES” and “Amph/ES” groups shows no difference (Fig 3B and C, two-way ANOVA, saline effect, $F(1,35) = 0.62$, $p = 0.44$), nor does the comparison between “D1R ANT+Amph/ES” and “Home Cage” groups (Fig 3B and C, two-way ANOVA, treatment effect, $F(1,35) = 0.024$, $p = 0.88$). Together, these results indicate that the increased H3K4me1 modification normally induced by Amph/ES was completely blocked by D1R antagonist to the baseline level. Therefore, the induction of H3K4me1 modification at the *Arc* locus depends on dopaminergic signaling.

Amphetamine's priming effect correlates with the innervation pattern of dopaminergic axons in neonatal frontal cortex

To further investigate the correlation between dopaminergic inputs and the priming of *Arc* response to neural activity, we examined the spatial distribution of dopaminergic axons and *Arc* expression patterns in the frontal cortex. Using tyrosine hydroxylase (TH) immunostaining (Mastwal et al., 2014; Ye et al., 2017) to highlight axonal distribution in regularly housed P9 mice, we found that dopaminergic innervation was significantly denser

in the deep layers (L5/6) than in the superficial layers (L2/3) of the frontal cortex (Fig. 4A, B; *t*-test, $p = 0.0352$, $N = 5$ mice per group).

To examine the spatial distribution of *Arc* expression, we used our previously generated *Arc-GFP* knock-in mouse line in which the endogenous *Arc*-coding region is replaced by a destabilized *GFP*-coding region (Cao et al., 2015; Wang et al., 2006). Under the control of the endogenous *Arc* enhancer and promoter, *Arc*-expressing neurons in this heterozygous *Arc-GFP* mouse line also express GFP. We used confocal microscopy to image frontal cortical sections from a set of P9 *Arc-GFP* mice under the Amph/ES pretreatment paradigm. Our results showed that without Day 2 ES stimulation, few *Arc*-GFP expressing neurons occur in the deep layers (Fig. 4C, D). With Day 2 ES stimulation, however, the number of *Arc*-GFP expressing neurons was significantly greater in the groups pretreated with Amph/ES than in those receiving no pretreatment (two-way ANOVA, pretreatment-by-stimulation interaction, $F(1,18) = 7.002$, $p = 0.0164$, $N = 5\sim6$ mice per group; post test, ES without pretreatment vs. ES with pretreatment, $p = 0.0056$). These results are consistent with those from our qRT-PCR analysis of RNA extracted from the whole frontal cortex, including both superficial and deep layers (Fig. 1B). They further suggest that prior dopaminergic stimulation primes more neurons in the deep frontal cortex to express *Arc* following electrical activation.

Neurons in the superficial layers, by contrast, showed little *Arc*-GFP expression with or without Day 2 ES stimulation, pretreated with Amph/ES or not (Fig. 4E, F; two-way ANOVA, stimulation effect, $F(1,21) = 0.8217$, $p = 0.3750$; pretreatment effect, $F(1,21) = 1.321$, $p = 0.2633$). Increased *Arc*-GFP expression in deep but not in superficial layers following dopaminergic pretreatment is consistent with the spatial distribution of dopaminergic axons, suggesting that in deep layers, more neurons are capable of expressing *Arc* in response to electrical activation as they have received strong dopaminergic input.

Adult levels of H3K4me1 at *Arc* locus are constitutively high and not affected by amphetamine exposure

Since dopaminergic input to the frontal cortex is significantly higher in adults than in P9 mice (Ye et al., 2017), we investigated whether H3K4me1 modification at the *Arc* locus would follow the same trend. Our finding showed that the level of H3K4me1 modification was indeed significantly higher in adults than in P9 mice (Fig. 5A; two-way ANOVA, age effect, $F(1,49) = 138.5$, $p < 0.0001$; P9, $N = 9$ mice, Adult, $N = 6$ mice).

Recognizing that the level of H3K4me1 modification is already high in adult mice, we next sought to test whether combined dopaminergic and electrical stimulation would increase it still further. Comparing H3K4me1 levels between Amph/ES treated adult groups and undisturbed home-cage controls, we found that Amph/ES treatment did not enhance H3K4me1 level at the *Arc* locus (Fig 5B; two-way ANOVA, treatment effect, $F(1,29) = 0.17$, $p = 0.68$; Home cage, $N = 7$ mice, Amph/ES, $N = 4$ mice).

Our earlier findings showed that in neonates, potentiation of *Arc* mRNA expression was associated with increased H3K4me1 modification after dopaminergic activation (Fig. 3). Since Amph/ES did not affect the already high level of H3K4me1 in adults, we hypothesized

that Amph/ES pretreatment would not further increase *Arc* transcriptional response to subsequent electrical stimulation in adults. Indeed, our qRT-PCR analysis of samples from adult mice showed that their *Arc* mRNA expression levels remained approximately the same between Amph/ES pretreated and control groups (Fig 5C; two-way ANOVA, pretreatment effect, $F(1,8) = 0.83$, $p = 0.39$; $N = 3$ mice per group). These results suggest that in adult animals, H3K4me1 level was sufficiently high for dopamine to produce no additional priming effect on *Arc* expression. Therefore, dopaminergic priming of activity-dependent *Arc* gene expression is likely completed early during postnatal brain development.

Discussion

Our study reveals that during neonatal frontal cortical development, transient amphetamine exposure can synergize with electrical activity to induce long-lasting histone methylation changes and potentiate the subsequent induction of *Arc* gene expression by neural activity. *Arc* protein plays crucial roles in synaptic plasticity and its expression level needs to be tightly regulated for normal circuit functions (Jenks et al., 2017; Manago et al., 2016; Plath et al., 2006; Ren et al., 2014; Wall et al., 2018). The excessive induction of *Arc* as a consequence of neonatal exposure to amphetamine therefore may introduce a significant disruption to normal circuit maturation and contribute to the long-term behavioral deficits resulting from such exposures.

Histone methylation, which is involved in the maintenance of gene expression patterns during cell differentiation, has been considered more stable than acetylation and phosphorylation based on metabolic labeling data (Bhaumik et al., 2007; Klose and Zhang, 2007; Shi, 2007). Previous studies suggested that the level of H3K4me1 histone methylation can be used to classify enhancers into permissive vs. non-permissive states (Calo and Wysocka, 2013; Kim et al., 2010; Ostuni et al., 2013), and H3K4me1 modification plays an important role in transcription priming by modulating long-range chromatin interactions between enhancers and gene body regions (Local et al., 2018; Yan et al., 2018). It is traditionally thought that DNA binding proteins intrinsic to each cell type predetermine the level of H3K4me1 at specific genes (Kim et al., 2010; Natoli et al., 2011). According to this view, transient external stimuli would not change H3K4me1 level at inducible genes, but only activate genes that are already in a permissive state. Our study presents the surprising finding that during perinatal cortical development, transient extracellular stimulation with dopamine and electrical activity can rapidly induce long-lasting H3K4me1 modification at the *Arc* locus, which predicts greater induction of *Arc* mRNA in response to subsequent neural activation.

In contrast, our phospho-CREB analysis did not show evidence of long-lasting change in the upstream neuronal signaling events after dopaminergic pretreatment. Those results therefore suggest that the potentiation of *Arc* expression is more consistent with persistent H3K4me1 modifications. Interestingly, in cell culture studies, macrophage stimulation can induce H3K4me1 modification in specific genes lasting at least 48 hours (maximum duration unknown), whereas other covalent modifications decay more rapidly (Ostuni et al., 2013). The maximum duration of H3K4me1 modification induced by neonatal Amph/ES treatment is currently unknown and will need to be evaluated against the normal developmental

increase of this modification in future longitudinal studies. Since we found that *Arc* is a target of H3K4me1 modification and *Arc* expression level regulates long-term synaptic plasticity (Jenks et al., 2017; Manago et al., 2016; Plath et al., 2006; Ren et al., 2014; Wall et al., 2018), alteration of H3K4me1 at *Arc* locus in neonates is likely to affect activity-dependent developmental plasticity. Future research to establish techniques for locus-specific enzymatic modulation of H3K4me1 (Gallegos et al., 2018) in neonatal mouse brain may help to further dissect the specific roles of H3K4me1 in *Arc* expression and neural circuit development.

We show that the increase of H3K4me1 at the *Arc* locus is dependent on dopaminergic signaling in neonates. In adult, dopaminergic inputs to the frontal cortex are significantly higher than in neonates. Correspondingly, the baseline level of H3K4me1 at the *Arc* locus is already high, which appears to preclude further H3K4me1 modification in response to dopaminergic activation. As expected, therefore, electrical activity alone can induce *Arc* mRNA and further dopaminergic activation has no priming effect. Although dopamine activates multiple signaling pathways, some of which intersect with calcium-dependent pathways and can modulate activity-dependent gene expression induced by concurrent neural stimuli in adult brain (Berke and Hyman, 2000; Nestler, 2014), our results suggest that dopamine's priming effect on neural activation of *Arc* is completed early on in postnatal development.

In neonates, by contrast, the *Arc* locus in frontal cortical neurons has yet to be primed, and it takes both dopaminergic and electrical activity to do so. The critical role of dopaminergic signaling in initiating this priming process may help to explain earlier findings that pharmacologically-induced seizure activity and electrical stimulation fail to evoke immediate-early gene expression in the neonatal cortex (Jensen et al., 1993; Jungnitz et al., 2014; Schreiber et al., 1992; Vendrell et al., 1998).

Moreover, as the neonatal period in mice is comparable to the third trimester stage in human brain development (Workman et al., 2013), our findings highlight the potential impact of perinatal psychostimulant on neuronal chromatin and gene expression changes related to synaptic plasticity and cognitive functions. It has been reported that dopaminergic inputs are already present in human cerebral cortex before birth and continue to increase after birth (Berger et al., 1991). *Arc* mRNA expression in the human frontal cortex also increases significantly after birth (Colantuoni et al., 2011). As the *Arc* signaling complex has been implicated in neurodevelopmental psychiatric disorders such as schizophrenia and intellectual disability disorders (Fromer et al., 2014; Kirov et al., 2012; Purcell et al., 2014), perinatal amphetamine exposure may disrupt the normal maturation of *Arc* signaling and synaptic functions, thereby increasing the risk for psychiatric disorders later in life.

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A **B**

Figure 1. Neonatal amphetamine exposure primes the induction of *Arc* mRNA by electrical stimulation

(A) *Arc* mRNA expression levels increase in the frontal cortex at 30 minute after Amph/ES treatment in P9 mice, remain high after 2 hours, but decay back to the basal level after 24 hours (one-way ANOVA, $F(3,8) = 99$, $p < 0.0001$, $N = 3$ mice per group; Post tests: Home Cage vs. 0.5 hr after Amph/ES, $p < 0.0001$; Home Cage vs. 2 hr after Amph/ES, $p < 0.0001$; Home Cage vs. 24 hr after Amph/ES, $p = 0.23$).

(B) Diagram (left) of experimental design used to test the effects of Amph/ES pretreatments on the induction of *Arc* expression by electrical stimulation 24 hours later. qRT-PCR analysis (right) shows that ES stimulation induces *Arc* mRNA expression to a significantly higher degree following pretreatment with Amph/ES (two-way ANOVA, pretreatment-by-stimulation interaction, $F(1,31) = 7.3$, $p = 0.011$, $N = 8\sim 9$ mice per group; Post tests: ES without pretreatment vs. ES with pretreatment, $p = 0.0010$; no-ES without pretreatment vs. no-ES with pretreatment, $p = 0.96$). The expression level of *Arc* is normalized to that of *Actin*. All error bars indicate SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

Figure 2. Neonatal amphetamine exposure does not lead to long-lasting changes in the phosphorylation of transcription factor CREB

(A) Example Western blot showing the level of phosphorylated CREB, total CREB, and tubulin in frontal brain lysate under various experimental conditions.

(B) Western blot analysis of frontal cortical tissue lysates showed that Day 2 ES stimulation increased CREB phosphorylation as indicated by pCREB/CREB ratio (two-way ANOVA, stimulation effect, $F(1,12) = 29.19$, $p = 0.0002$; $N = 4$ mice for each group). However, mice that received Amph/ES pretreatment had pCREB levels comparable to those received no pretreatment at the baseline level on Day 2 ($p = 0.92$), and only an insignificant increasing trend after ES stimulation ($p = 0.21$).

(C) The total level of CREB protein (as normalized to tubulin) is not affected by treatments (two-way ANOVA, stimulation effect, $F(1,12) = 0.019$, $p = 0.89$; pretreatment effect, $F(1,12) = 0.23$, $p = 0.64$; pretreatment-by-stimulation interaction, $F(1,12) = 2.78$, $p = 0.12$). Ratio of

protein levels in each group is standardized to the No-Pretreatment/No-ES-stimulation group. All error bars indicate SEM.

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Figure 3. Neonatal amphetamine exposure leads to long-lasting dopamine-signaling dependent increase of H3K4me1 modification at the *Arc* locus

(A) CHIP-qPCR analysis shows that the levels of H3K4me1 modification at the *Arc* locus were induced by Amph/ES in P9 mice and remained high at both 2 and 24 hours after treatment (two-way ANOVA, treatment effect averaged across genomic sites, $F(2,83) = 80.33$, $p < 0.0001$; Post tests: Home Cage vs. 2 hr after Amph/ES, $p < 0.0001$; Home Cage vs. 24 hr after Amph/ES, $p < 0.0001$). H3K4me1 levels at different sites in the *Arc* gene were normalized to that of a reference site in the *Actin* gene. Diagram (below the bar graph)

shows the positions of PCR-targeted genomic sites (black lines) relative to the *Arc* coding region (black boxes) and transcription start site (grey arrow).

(B) The levels of H3K4me1 modification at the *Arc* locus increase significantly after Amph/ES treatment in P9 mice (two-way ANOVA, treatment effect, $F(3, 78) = 14.45$, $p < 0.0001$, $N = 6$ mice per group; Post tests: Amph/ES vs. Home Cage, $p < 0.0001$; Amph vs. Home Cage, $p = 0.039$; ES vs. Home Cage, $p = 0.99$), and the effect of Amph and ES on the *Arc* enhancer is synergistic (two-way ANOVA, Amph-by-ES interaction, $F(1, 19) = 22.29$, $p = 0.0001$).

(C) Injection of D1R antagonist SCH23390 blocks the effect of Amph/ES treatment on H3K4me1 levels at the *Arc* locus in P9 mice (two-way ANOVA, D1R ANT effect, $F(1, 32) = 17.37$, $p = 0.0002$; $N = 5\sim6$ mice per group). All error bars indicate SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

Figure 4. Amphetamine's priming effect correlates with the innervation pattern of dopaminergic axons in neonatal frontal cortex

(A) Confocal images of frontal cortex sections taken from a P9 mouse and immunostained with TH-specific antibody. Two regions of interest in the superficial (L2/3) and deep (L5/6) cortical layers were shown. Scale bar is 60 μ m.

(B) Dopaminergic innervation is significantly denser in deep than in superficial layers of the frontal cortex in P9 mice (*t*-test, $p = 0.0352$, $N = 5$ mice per group).

(C, E) Confocal images of the deep (C) and superficial (E) layers of the frontal cortex taken from P10 Arc-GFP mice that were tested using the Amph/ES pretreatment paradigm from P9 to P10. Scale bar is 60 μ m.

(D, F) After ES stimulation, significantly more neurons in the deep (D) frontal cortical layers expressed Arc-GFP in mice pretreated with Amph/ES than those receiving no pretreatment (two-way ANOVA, pretreatment-by-stimulation interaction, $F(1,18) = 7.002$, $p = 0.0164$, $N = 5\sim 6$ mice per group; Post tests: ES without pretreatment vs. ES with pretreatment, $p = 0.0056$). Few neurons expressed Arc-GFP in the superficial (F) layers of the frontal cortex. ES stimulation or Amph/ES pretreatment had no effect (two-way ANOVA, stimulation effect, $F(1,21) = 0.8217$, $p = 0.3750$; pretreatment effect, $F(1,21) = 1.321$, $p = 0.2633$). The total number of Arc-GFP positive neurons was measured in an area of $310 \times 310 \mu\text{m}$. All error bars indicate SEM. * $p < 0.05$, ** $p < 0.01$.

Figure 5. Adult levels of H3K4me1 at *Arc* locus are constitutively high and not affected by amphetamine exposure.

(A) H3K4me1 modification levels are significantly higher in adult (P60) than in P9 mouse frontal cortex (two-way ANOVA, age effect, $F(1,49) = 138.5$, $p < 0.0001$; P9, $N = 9$ mice, Adult, $N = 6$ mice).

(B) In adult mice, Amph/ES treatment does not further increase the H3K4me1 levels at the *Arc* locus (two-way ANOVA, treatment effect, $F(1, 29) = 0.17$, $p = 0.68$; Home cage, $N = 7$ mice, Amph/ES, $N = 4$ mice).

(C) In adult mice, Amph/ES pretreatment does not facilitate subsequent induction of *Arc* mRNA by ES (two-way ANOVA, pretreatment effect, $F(1,8) = 0.83$, $p = 0.39$; $N = 3$ mice per group). All error bars indicate SEM. ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

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Table 1.

PCR Primer and probe sequences

Actin chr5:142905326 – 142905394	5' ACCCCATTGAACATGGCATT 3' 5' TGTAGAAGGTGTGGTGCCAGAT 3'	6FAM TTACCAACTGGGACGACATGGAGAA TAMRA
Arc Enhancer chr15:74679267 – 74679326	5' AGCCTGCCTGCTTCCG 3' 5' GCGTCATGGCTCAGCTATTCT 3'	6FAM CAGCCTCTCTCCTTTTATG TAMRA
Arc Promoter chr15:74672480 – 74672541	5' GGAGGAGCTTAGCGAGTGT 3' 5' GCAGTCTCTGGGCCTCTCTA 3'	6FAM CTCGTCGCCGCTGAAG TAMRA
Arc Exon 1 chr15:74671300 – 74671382	5' CCGTCCTGCACTTCCATACC 3' 5' CCCAAACTCAAGCGCTTTCTG 3'	6FAM CTGCTCCAGGGTCTTG TAMRA
Arc Exon 3 chr15:74669892 – 74669969	5' CATGTAGGCAGCTTCAGGAGAA 3' 5' TGCTGCCATGTCTGGAAAGG 3'	6FAM ACCACACCACCAGCACC TAMRA