

Amphetamine in Adolescence Disrupts the Development of Medial Prefrontal Cortex Dopamine Connectivity in a *dcc*-Dependent Manner

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Initiation of drug use during adolescence is a strong predictor of both the incidence and severity of addiction throughout the lifetime. Intriguingly, adolescence is a period of dynamic refinement in the organization of neuronal connectivity, in particular medial prefrontal cortex (mPFC) dopamine circuitry. The guidance cue receptor, DCC (deleted in colorectal cancer), is highly expressed by dopamine neurons and orchestrates their innervation to the mPFC during adolescence. Furthermore, we have shown that amphetamine in adolescence regulates DCC expression in dopamine neurons. Drugs in adolescence may therefore induce their enduring behavioral effects *via* DCC-mediated disruption in mPFC dopamine development. In this study, we investigated the impact of repeated exposure to amphetamine during adolescence on both the development of mPFC dopamine connectivity and on salience attribution to drug context in adulthood. We compare these effects to those induced by adult exposure to an identical amphetamine regimen. Finally, we determine whether DCC signaling within dopamine neurons is necessary for these events. Exposure to amphetamine in adolescence, but not in adulthood, leads to an increase in the span of dopamine innervation to the mPFC, but a reduction of presynaptic sites present on these axons. Amphetamine treatment in adolescence, but not in adulthood, also produces an increase in salience attribution to a previously drug-paired context in adulthood. Remarkably, DCC signaling within dopamine neurons is required for both of these effects. Drugs of abuse in adolescence may therefore induce their detrimental behavioral consequences by disrupting mesocortical dopamine development through alterations in the DCC signaling cascade.

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INTRODUCTION

Initiation of drug use during adolescence is a strong predictor of both the incidence and severity of lifetime drug abuse and addiction (Robins and Przybeck, 1985; Anthony and Petronis, 1995; Grant and Dawson, 2003). This vulnerability is especially troubling considering that the majority of individuals who begin to experiment with drugs of abuse are under the age of 18 years old (Madras, 2010; Currie and Wild, 2012; Leatherdale and Burkhalter, 2012; SAMHSA, 2012; Swendsen *et al*, 2012). The risk of lifetime addiction appears to be greatest the earlier drug use commences (Grant and Dawson, 2003; McCabe *et al*, 2007), suggesting that the detrimental effects of drug exposure in adolescence are intimately linked to ongoing neurodevelopmental processes. Adolescence is a period of

dynamic refinement in the organization of neuronal circuitry. In particular, prefrontal cortex connectivity continues to develop until early adulthood, with this brain region being the last to achieve full maturity (Giedd *et al*, 1999; Sowell *et al*, 1999, 2003; Gogtay *et al*, 2004; Shaw *et al*, 2008). The protracted development of the prefrontal cortex confers an extended period of plasticity and vulnerability for disruption in its wiring patterns.

One of the most dramatic changes occurring in the prefrontal cortex during adolescence is the establishment of dopamine connectivity. In rodents, the dopamine innervation to the medial prefrontal cortex (mPFC) increases progressively beyond the weaning stage until early adulthood, with substantial modifications in fiber density, shape, and organization happening during adolescence (Kalsbeek *et al*, 1988; Benes *et al*, 2000; Manitt *et al*, 2011; Naneix *et al*, 2012). In parallel to these changes in dopamine circuitry, local mPFC neurons shift towards a mature state of responsiveness to the modulatory effects of dopamine (Spear, 2000; Tseng and O'Donnell, 2007; O'Donnell, 2011; Cass *et al*, 2013). The delayed maturation of the dopamine inputs to the mPFC is unique within the monoamine systems (Levitt and Moore, 1979; Lidov *et al*, 1980; Benes *et al*, 2000)

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and also occurs in primates, possibly including humans (Rosenberg and Lewis, 1995). Drugs of abuse during adolescence may therefore produce their enduring detrimental effects on behavior by disrupting the developmental trajectory of the dopamine innervation to the mPFC.

We recently identified *dcc* as the first gene shown to orchestrate the development of dopamine connectivity to the mPFC specifically in adolescence (Manitt *et al*, 2011, 2013). DCC (deleted in colorectal cancer) is a receptor for the guidance cue netrin-1 and is highly and conspicuously expressed by dopamine neurons in rodents and humans throughout the lifetime. DCC expression in dopamine neurons is high during early development and adolescence, in line with the maturational timeline of dopamine pathways. Interestingly, DCC expression is substantially lower during adulthood, suggesting that DCC signaling may play a different, more local, role in the adult brain (Osborne *et al*, 2005; Grant *et al*, 2007; Manitt *et al*, 2010; Reyes *et al*, 2013; Yetnikoff *et al*, 2007, 2010, 2011, 2013a). Using rodents, we showed that DCC signaling within the ventral tegmental area (VTA) dopamine neurons determines the extent of their innervation to the mPFC specifically during adolescence and, in turn, organizes the structure and function of mPFC local circuitries (Manitt *et al*, 2013). Variations in DCC expression in dopamine neurons during adolescence lead to dramatic changes in mPFC-dependent behaviors in adulthood, including changes in sensitivity to behavioral effects of drugs of abuse (Manitt *et al*, 2013). Remarkably, we have also demonstrated that repeated exposure to an abused dose of amphetamine during early adolescence downregulates DCC expression in the rodent VTA (Yetnikoff *et al*, 2011). Together, these findings raise the intriguing possibility that drugs of abuse during adolescence disrupt the development of dopamine connectivity by regulating DCC signaling within dopamine neurons.

Here, we sought to determine whether repeated exposure to an abused dose of amphetamine in early adolescent C57BL/6 mice disrupts the development of mPFC dopamine connectivity. To confirm that the effects of amphetamine exposure in adolescence result from perturbations in dopamine development, we also examine the effects of adult drug exposure on mPFC dopamine circuitry organization. We used conditional transgenic mouse models to assess whether DCC signaling within dopamine neurons mediates amphetamine-induced disruption in adolescent dopamine development. Finally, we examined behavioral correlates of altered mPFC dopamine development in adulthood.

MATERIALS AND METHODS

Animals

All experiments and procedures were performed in accordance with the guidelines of the Canadian Council of Animal Care and the McGill University/Douglas Mental Health University Institute Animal Care Committee. Mice were bred in the Douglas Mental Health University Institute Neurophenotyping center, maintained on a 12-h light–dark cycle (light on at 0700 h) and given ad libitum access to food and water. Pups were weaned at postnatal day (PND) 21 ± 1 and housed with same-sex littermates.

dcc conditional mice. We have generated a *dcc* loss-of-function mutation exclusively in dopamine neurons by crossing mice with loxP-insertions flanking exon 23 of the *dcc* gene (*dcc^{lox/lox}*) with a line in which iCre expression is regulated by the dopamine transporter (DAT; BAC-DATiCre mice). We have shown that *dcc^{lox/lox}DAT^{Cre}* mice lack DCC expression exclusively in dopamine neurons and that the absence of DCC expression does not affect DA neuron survival; for a complete description of the characterization of these mice, please refer to Manitt *et al* (2013). The *dcc^{lox/lox}DAT^{Cre}* mice survive to adulthood and do not exhibit any overt phenotypic differences from control *dcc*-floxed littermates. Floxed, cre-negative littermates (*dcc^{lox/lox}*) are indistinguishable from wild-type mice, and were utilized in the experiments examining adolescent or adult mice. Different cohorts of floxed controls and *dcc^{lox/lox}DAT^{Cre}* mice were used for each experiment. Each experiment had cohorts from a minimum of 5 litters.

Drugs and Dose

d-Amphetamine sulfate (AMPH; Sigma-Aldrich, Dorset, United Kingdom) was dissolved in 0.9% saline. All AMPH injections were administered i.p. at a volume of 0.1 ml/10 g. Although there is no clear formula for converting human doses to mice, comparable plasma concentrations of a drug in the two species would be expected to lead to comparable brain concentrations (Kuczenski and Segal, 2005). A dose of 4 mg/kg of AMPH achieves plasma concentrations > 250 ng/ml in mice (Riffée *et al*, 1978; Van Swearingen *et al*, 2013), which is within the range of plasma levels achieved by oral doses of AMPH that have been reported to be abused by adolescents (Kramer *et al*, 1967; Anggård *et al*, 1970; Gustavsen *et al*, 2006). Importantly, we have shown previously that this dose of AMPH, administered in the same regimen as the present experiments, alters the expression of DCC in the VTA (Yetnikoff *et al*, 2010, 2011).

Neuroanatomical Analysis

Perfusion. Mice received an overdose of sodium pentobarbital (> 75 mg/kg, i.p.) and were perfused intracardially with 50 ml of 0.9% saline followed by 75 ml of chilled fixative solution (4% paraformaldehyde in phosphate-buffered saline). Brains were dissected and placed in the fixative solution overnight at 4 °C and were then transferred to phosphate-buffered saline and stored for a maximum of 2 days. Brains were sectioned using a vibratome (35- μ m-thick slices).

Immunofluorescence. Every second section was processed for immunofluorescence (1:2 series) as before (Manitt *et al*, 2010, 2011, 2013). A rabbit polyclonal anti-TH antibody (1:500 dilution, catalog #AB152; Millipore Bioscience Research Reagents) and an Alexa Fluor 555-conjugated secondary antibody raised in goat (1:500 dilution, 1 h incubation, Invitrogen) were used.

Diaminobenzidine immunohistochemistry. Every second section was processed for immunohistochemistry (1:2 series) as before (Manitt *et al*, 2010, 2011, 2013), using the same rabbit polyclonal anti-TH antibody, a goat anti-rabbit biotinylated secondary antibody (1:250 dilution, catalog

BA1000, Vector Labs), and the Vectastain Elite ABC reagent. Staining was visualized by placing sections in nickel-intensified diaminobenzidine for 1 min.

Stereology. TH-positive varicosities were used as the counting unit to obtain a measure of dopamine presynaptic density, as nearly every dopamine varicosity in the mPFC forms a synapse with a dendritic spine or shaft (Séguéla *et al*, 1988). Varicosities represent sites where neurotransmitter synthesis, packaging, release, and reuptake most often occur (Benes *et al*, 1996). Furthermore, we and others have shown that the TH antibody used in these studies labels dopamine axons but rarely labels norepinephrine axons within the regions of interest (Miner *et al*, 2003; Manitt *et al*, 2011, 2013). As in all our previous neuroanatomical studies, we obtained counts only from the right hemisphere because of the lateralization of dopamine systems.

To assess differences in dopamine connectivity following early adolescent AMPH exposure, we performed stereological quantification of the span and density of TH-positive varicosities in the cingulate, prelimbic, and infralimbic subregions of the pregenual mPFC. We performed identical analyses in the nucleus accumbens (NAcc). We also evaluated the number of TH-positive neurons and their size in the VTA and substantia nigra pars compacta (SNc) using stereology in diaminobenzidine-stained tissue. To assess the total volume of TH-positive fiber innervation (in cubic micrometers) we used the Cavalieri method in StereoInvestigator® (MicroBrightField). To determine the density of TH-positive varicosities, we used the optical fractionator probe of the StereoInvestigator software (Manitt *et al*, 2011, 2013). The coefficient of error was below 0.1 for all regions of interest in all sampled brains. Counts were performed blind.

mPFC analysis. The mPFC subregions were delineated according to plates spanning 14–18 of the mouse brain atlas (Figures 1–3b; (Paxinos and Franklin, 2008)). Contours of the dense TH-positive innervation of the subregions were traced at $\times 5$ magnification using a Leica DM400B microscope (Figure 1c). An unbiased counting frame ($25 \times 25 \mu\text{m}$) was superimposed on each contour and counts were made at regular predetermined intervals ($x = 175 \mu\text{m}$, $y = 175 \mu\text{m}$) from a random start point. Counting of varicosities was performed at $\times 100$ magnification on 6 of the 12 sections contained within the rostrocaudal borders of our region of interest (Plates 14–18; 1:2 series). A guard zone of $5 \mu\text{m}$ was used and the optical disector height was set to $10 \mu\text{m}$.

NAcc analysis. An unbiased counting frame ($10 \times 10 \mu\text{m}$) was superimposed on the contour of the NAcc and counts were made at regular predetermined intervals ($x = 200 \mu\text{m}$, $y = 200 \mu\text{m}$) from a random start point. Counting was performed at $\times 100$ magnification on four of the eight sections contained within the rostrocaudal borders of our region of interest (Plates 15–18, 1:2 series; Figure 4a and b; (Paxinos and Franklin, 2008)). A guard zone of $4 \mu\text{m}$ was used and the optical disector height was set to $5 \mu\text{m}$ (Williams *et al*, 2003).

Number of DA neurons and cell size. The total number of dopamine neurons in the VTA and SNc was assessed using

the optical fractionator probe of StereoInvestigator as previously (Grant *et al*, 2007; Manitt *et al*, 2013). The counting scheme used a $60 \times 60 \mu\text{m}$ counting frame ($x = 150 \mu\text{m}$, $y = 150 \mu\text{m}$ intervals) with a random start point. Counting was performed at $\times 40$ magnification in a 1:4 series. A $3 \mu\text{m}$ guard zone and a probe depth of $10 \mu\text{m}$ were used. The average cell size of TH-positive neurons in the VTA and SNc were estimated using the nucleator function of StereoInvestigator. Measurements were performed with a $60 \times 60 \mu\text{m}$ counting frame ($x = 200 \mu\text{m}$, $y = 200 \mu\text{m}$ intervals) from a random start point. Cell body measurements were performed at $\times 40$ in a 1:8 series (four sections per animal).

Behavioral Evaluation

Apparatus. Locomotor activity boxes containing infrared sensors (AccuScan Instruments, Columbus, OH, USA) were adapted for use with mice (Yetnikoff *et al*, 2010, 2013b) by placing inserts that divide the space into four equal parts ($20 \times 20 \times 29.5 \text{ cm}$; Accuscan Instruments). Locomotor activity was defined as distance traveled (centimeters) in a given period of time.

Conditioned locomotor activity. Mice were placed in the previously drug- or saline-paired locomotor chamber and their locomotor activity was recorded for 15 min in a drug-free state.

Experimental Procedures and Timeline

Figures 1–3a show the drug treatment regimen used in all the experiments. We have shown that the exact amphetamine regimen, administered in early adolescence, decreases DCC receptor expression in the VTA (Yetnikoff *et al*, 2011). During a pretreatment phase, mice received one injection of AMPH (4 mg/kg) or vehicle (saline), once every other day for a total of 5 days. This pretreatment regimen was given during early adolescence (from PND 22 ± 1 to PND 31 ± 1) or during adulthood (from PND 75 ± 15 to 84 ± 15). As in our previous work, in this study, we define ‘early adolescence’ in C57BL/6 mice as the period between the day of weaning and PND 32. These definitions seem now to be a consensus in the mouse literature (eg, (Spear, 2000; Laviola *et al*, 2003; Schneider, 2013; Tirelli *et al*, 2003)). This is not an absolute margin, but rather an age range during which mice exhibit distinct neurobehavioral characteristics. Locomotor activity was measured 15 min prior and 90 min after each saline or AMPH injection. Following this pretreatment regimen, mice were left undisturbed for ~ 6 weeks to allow adolescent mice to reach adulthood (Figures 1a and 3a). Adult-treated mice were matched to this same interval (Figure 2a). At the end of this interval, mice were brought back to the activity room to assess conditioned locomotor activity. Mice were then perfused and neuroanatomical experiments were performed.

Quantitative Real-Time PCR

Early adolescent mice received amphetamine from PND 22 ± 1 to PND 31 ± 1 (Figure 1f). One week after completing this treatment regimen, mice were rapidly decapitated and their brains were flash-frozen in 2-methylbutane (Fisher

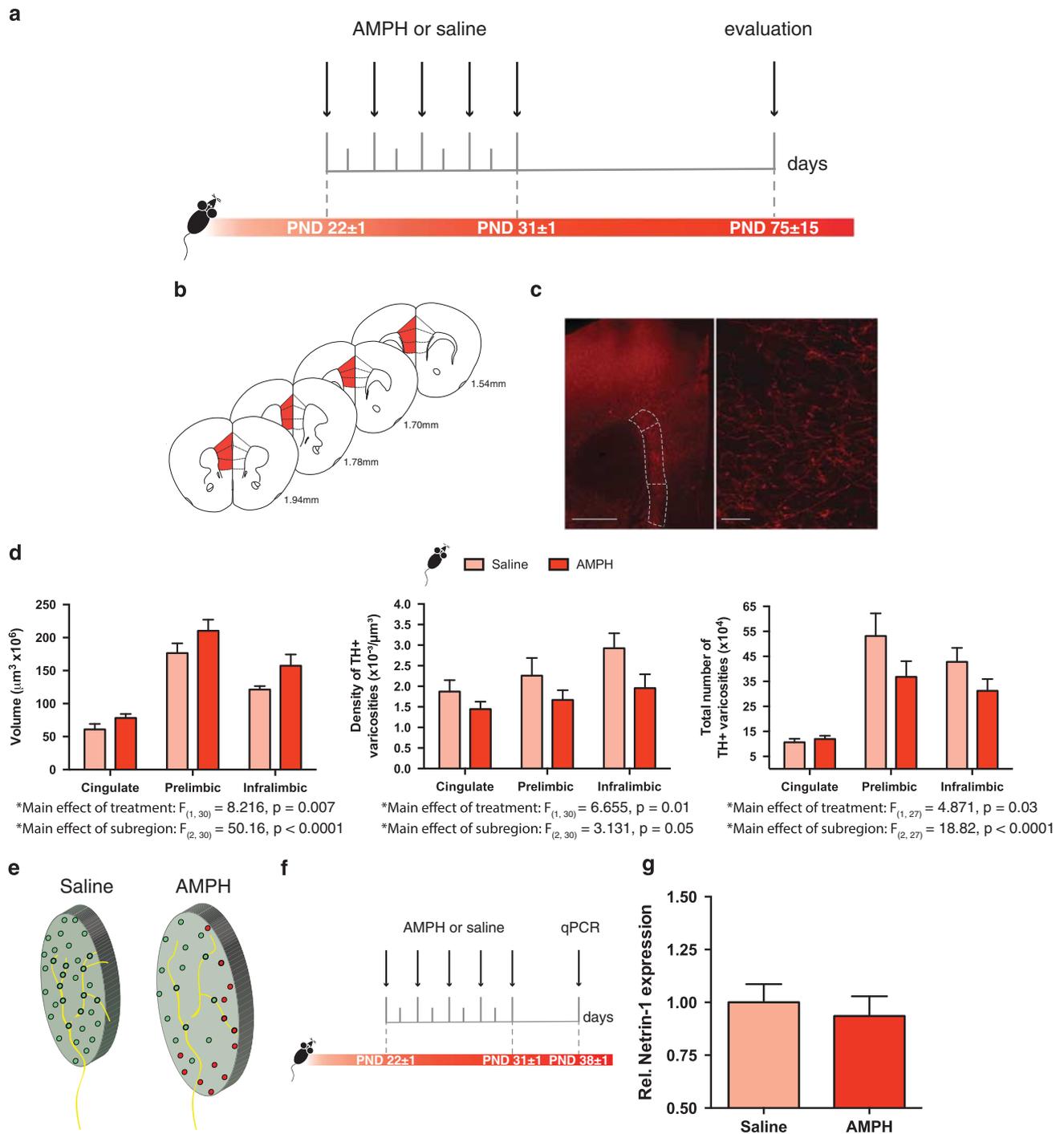


Figure 1 Exposure to amphetamine in adolescence results in altered dopamine connectivity in the adult medial prefrontal cortex. (a) Timeline of treatment in adolescence and experimental procedures. $n = 5-6$ per group. (b) Regions of interest in the medial prefrontal cortex (mPFC) were outlined according to the Mouse Brain Atlas (Paxinos and Franklin, 2008) and are highlighted in red. The cingulate, prelimbic, and infralimbic subregions of the mPFC were analyzed. (c) *Left panel*: micrograph of a coronal section of the pregenual mPFC at a low magnification ($\times 5$) showing an overlay of the contours traced to delineate subregions of interest. Scale bar = $500 \mu\text{m}$. *Right panel*: micrograph of a coronal section of the pregenual mPFC at a high magnification ($\times 100$) showing the tyrosine hydroxylase (TH)-positive varicosities. Scale bar = $10 \mu\text{m}$. (d) *Left panel*: volume of the TH-positive fiber innervation (mean \pm SEM) to the inner layers of the mPFC. Mice treated with AMPH in adolescence show increased fiber volume relative to their saline counterparts (significant main effect of treatment, $p = 0.0075$). *Middle panel*: density of TH-positive varicosities to the inner layers (mean \pm SEM) of the mPFC. Mice treated with AMPH in adolescence show decreased varicosity density relative to their saline counterparts (significant main effect of treatment, $p = 0.01$). *Right panel*: total number of TH-positive varicosities to the inner layers of the mPFC (mean \pm SEM). Mice treated with AMPH in adolescence show decreased total number of varicosities relative to their saline counterparts (significant main effect of treatment, $p = 0.03$). (e) Schematic representation of fiber expansion and varicosity density in the mPFC of adult mice exposed to AMPH or saline in adolescence. A putative axon is superimposed over both schematics. (f) Timeline of treatment in adolescence and analysis of netrin-1 expression by qPCR. $n = 5-6$ per group. (g) Netrin-1 mRNA expression normalized to GAPDH in the mPFC. There were no differences in relative Netrin-1 mRNA expression in the mPFC between mice that received amphetamine (AMPH) or saline in adolescence (from PND22 \pm 1—PND31 \pm 1) when examined 1 week later (PND38 \pm 1).

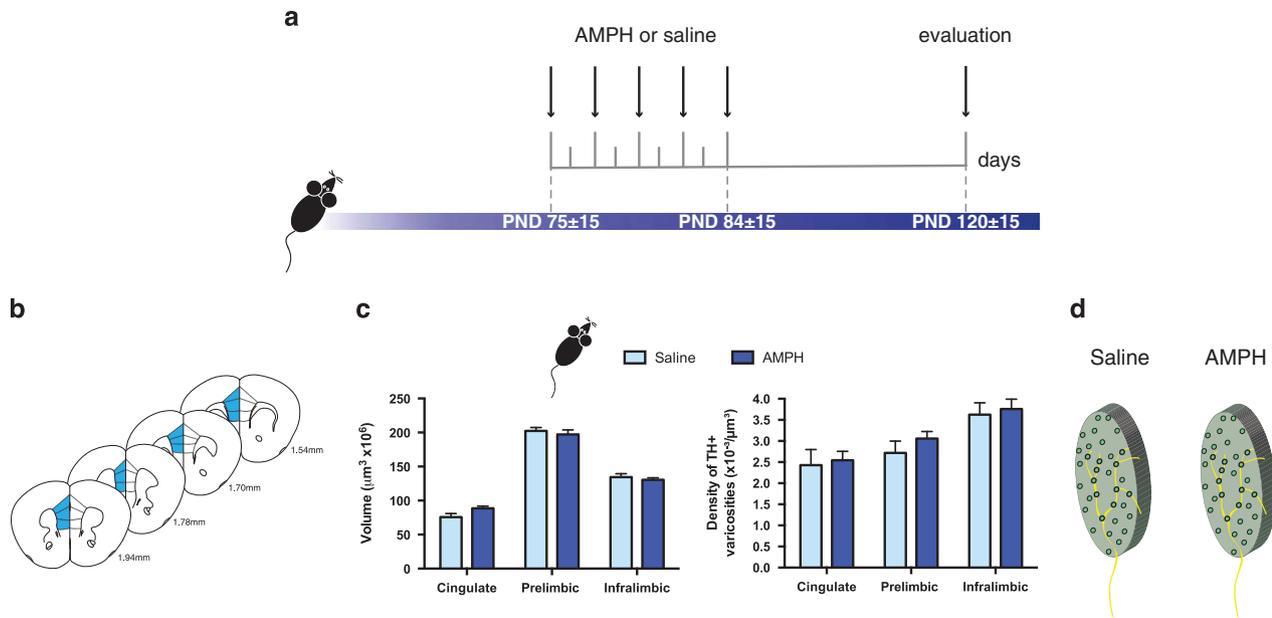


Figure 2 Exposure to amphetamine during adulthood does not alter dopamine connectivity in the medial prefrontal cortex. (a) Timeline of treatment in adulthood and experimental procedures, $n = 8$ per group. (b) Regions of interest in the medial prefrontal cortex (mPFC) were outlined according to the Mouse Brain Atlas (Paxinos and Franklin, 2008) and are highlighted in blue. The cingulate, prelimbic, and infralimbic subregions of the mPFC were analyzed. (c) *Left panel*: volume of the TH-positive fiber innervation (mean \pm SEM) to the inner layers of the mPFC. There were no differences in the volume of TH-positive fiber innervation to the mPFC inner layers between mice that received AMPH or saline in adulthood. *Right panel*: density of TH-positive varicosities to the inner layers (mean \pm SEM) of the mPFC. The density of TH-positive varicosities to the mPFC inner layers was not different between mice that received AMPH or saline in adulthood. (d) Schematic representation of fiber expansion and varicosity density in the mPFC of adult mice exposed to AMPH or saline in adulthood. A putative axon is superimposed over both schematics.

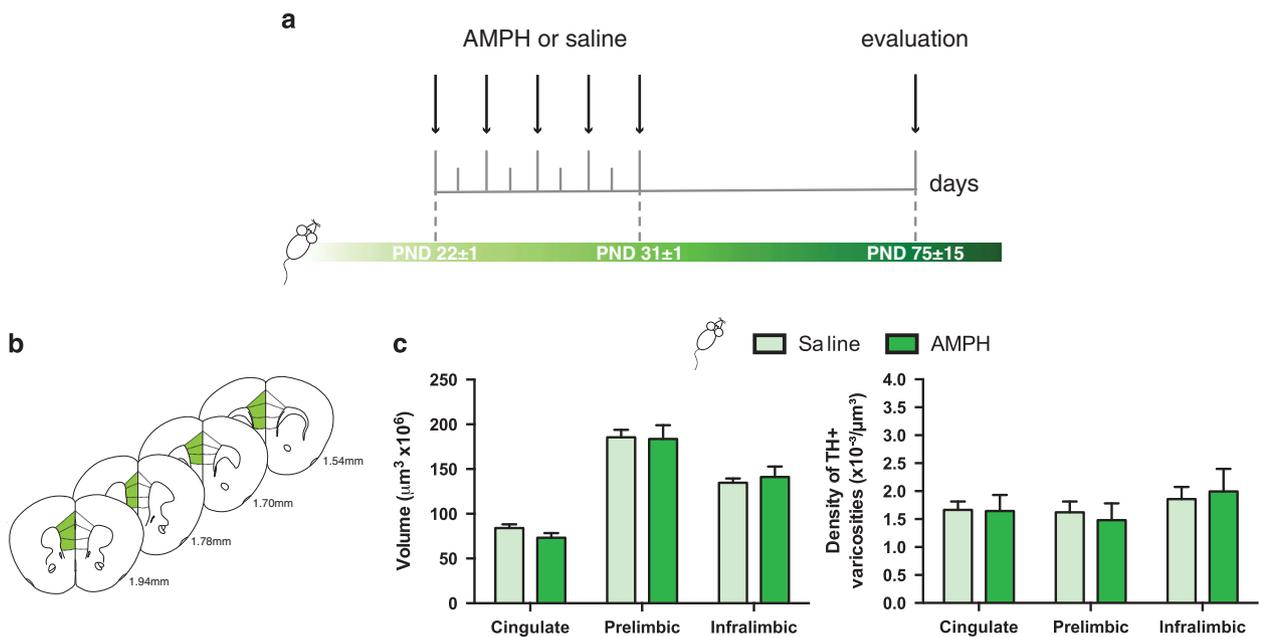


Figure 3 DCC signaling within dopamine neurons is necessary for the effects of amphetamine in adolescence on dopamine connectivity in the medial prefrontal cortex. (a) Timeline of treatment in adolescence and experimental procedures for $dcc^{lox/lox}DAT^{Cre}$ mice, $n = 5-9$ per group. (b) Regions of interest in the medial prefrontal cortex (mPFC) were outlined according to the Mouse Brain Atlas (Paxinos and Franklin, 2008) and are highlighted in green. The cingulate, prelimbic, and infralimbic subregions of the mPFC were analyzed. (c) *Left panel*: volume of the TH-positive fiber innervation (mean \pm SEM) to the inner layers of the mPFC. There were no differences in the volume of TH-positive fiber innervation to the mPFC inner layers between $dcc^{lox/lox}DAT^{Cre}$ mice that received AMPH or saline in adolescence. *Right panel*: density of TH-positive varicosities to the inner layers (mean \pm SEM) of the mPFC. The density of TH-positive varicosities to the mPFC inner layers was not different between $dcc^{lox/lox}DAT^{Cre}$ mice that received AMPH or saline during adolescence.

Scientific, Hampton, NH, USA) chilled with dry ice. Bilateral punches of mPFC, including the cingulate (Cg1), prelimbic (PrL), and infralimbic (IL) subregions, were excised from

1-mm-thick coronal sections corresponding to Plates 15–18 (Paxinos and Franklin, 2008). RNA was extracted using Trizol (Life Technologies) according to the manufacturer's

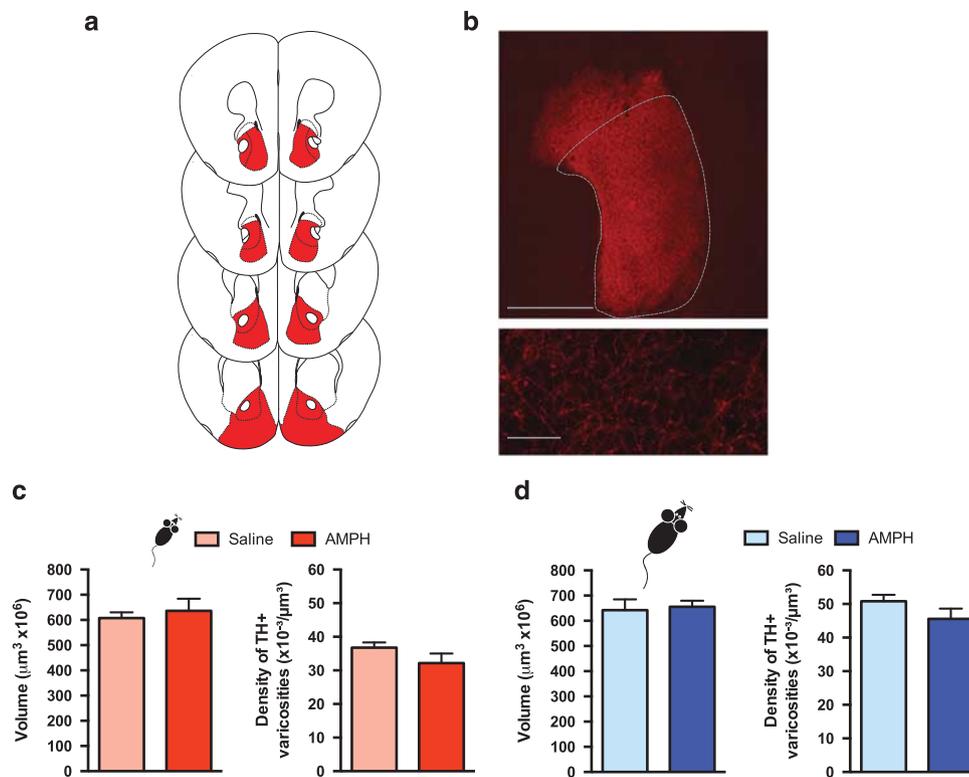


Figure 4 Exposure to amphetamine in adolescence does not result in altered dopamine connectivity in the nucleus accumbens in adulthood. (a) The nucleus accumbens (NAcc) was outlined according to the Mouse Brain Atlas (Paxinos and Franklin, 2008) and highlighted in red. (b) *Top panel*: micrograph of a coronal section of the pregenual NAcc at a low magnification ($\times 5$) showing an overlay of the contour traced to delineate this area. Scale bar = 500 μm . *Bottom panel*: micrograph of a coronal section of the pregenual NAcc at a high magnification ($\times 100$) showing the tyrosine hydroxylase (TH)-positive varicosities. Scale bar = 10 μm . (c) *Left panel*: volume of the TH-positive fiber innervation (mean \pm SEM) to the NAcc. There were no differences in the volume of TH-positive fiber innervation to the NAcc between mice that received amphetamine (AMPH) or saline in adolescence (from PND22 \pm 1—PND31 \pm 1) when examined in adulthood (PND75 \pm 15). *Right panel*: density of TH-positive varicosities to the NAcc (mean \pm SEM). The density of TH-positive varicosities to the NAcc was not different between mice that received AMPH or saline in adolescence. (d) *Left panel*: volume of the TH-positive fiber innervation (mean \pm SEM) to the NAcc. There were no differences in the volume of TH-positive fiber innervation to the NAcc between mice that received AMPH or saline in adulthood (from PND75 \pm 15—PND84 \pm 15) when examined after an approximately 6-week interval following treatment termination (PND120 \pm 15). *Right panel*: density of TH-positive varicosities to the NAcc (mean \pm SEM). The density of TH-positive varicosities to the NAcc was not different between mice that received AMPH or saline in adulthood. $n = 4-5$ for all groups.

protocol. The concentration was determined with an ND-1000 NanoDrop spectrophotometer. cDNA was synthesized using the MultiScribe reverse-transcription kit (Life Technologies) according to the manufacturer's instructions. In all mouse tissues, mRNA expression was assessed with quantitative PCR using TaqMan probes (Life Technologies) as outlined by the manufacturer. Netrin-1 gene expression was assessed with probe set Ntn1 (Mm00500896_m1). GAPDH (glyceraldehyde-3-phosphate; Mm99999915_g1) was used as standard and single-well amplification efficiency estimates and relative quantification of expression levels were performed as described (Pfaffl, 2001).

Statistical Analysis

The goal of this study was to determine the long-term effects of AMPH exposure on dopamine connectivity. Thus, we planned comparisons between data obtained from AMPH and saline treatment groups *within* each exposure age only (either adolescence or adulthood).

Neuroanatomy. Two-way ANOVAs with subregion \times treatment as between-subjects variables were used to analyze the

volume of TH-positive fibers and density of TH-positive varicosities in the mPFC for each exposure age group. The effects of *dcc* on the development of dopamine connectivity in the mPFC vary along the dorso-ventral axis (Manitt *et al*, 2011, 2013); thus here, as in our previous studies, we include subregion as one of the main factors in the analysis. To analyze TH-positive fiber volume and density of TH-positive varicosities in the NAcc, we used Student's *t*-tests of planned comparisons within each exposure age group. Two-way ANOVAs with region and treatment as between-subjects variables were used to analyze the number and cell size of DA neurons in the VTA and SNc for each exposure age group.

Conditioned locomotor activity. Student's *t*-tests of planned comparisons within each exposure age group were conducted.

Quantitative real-time PCR. Netrin-1 mRNA expression was compared between adolescent saline- or amphetamine-treated mice by Mann-Whitney rank sum test even if

normality tests and equal variance tests were positive, reflecting the small sample sizes (5–6).

RESULTS

AMPH in Adolescence, but not in Adulthood, Alters Dopamine Innervation in the mPFC

The span of TH-positive fiber innervation (in cubic micrometers) in the mPFC of adult mice that received AMPH during adolescence was significantly increased in comparison with mice that received saline during adolescence. Although this effect was distributed across the three subregions, the difference appeared to be more pronounced ventrally (Figure 1d, left panel: two-way ANOVA, main effect of treatment, $F_{(1, 30)} = 8.216$, $p = 0.0075$; no significant treatment \times subregion interaction, $F_{(2, 30)} = 0.33$, $p = 0.71$; main effect of subregion, $F_{(2, 30)} = 50.16$, $p < 0.0001$). Importantly, adolescent exposure to AMPH resulted in a concomitant *reduction* in the density of TH-positive varicosities in the mPFC. Again, this difference was more pronounced in ventral subregions (Figure 1d, middle panel: two-way ANOVA, main effect of treatment, $F_{(1, 30)} = 6.655$, $p = 0.01$; no significant treatment \times mPFC region interaction, $F_{(2, 30)} = 0.3923$, $p = 0.67$; main effect of mPFC region, $F_{(2, 30)} = 3.131$, $p = 0.05$). Correspondingly, mice that received AMPH during adolescence had a significant *decrease* in the total number of TH-positive varicosities; this effect was more evident in the prelimbic and infralimbic subregions (Figure 1d, right panel: two-way ANOVA main effect of treatment, $F_{(1, 27)} = 4.871$, $p = 0.036$; no significant treatment \times mPFC region interaction, $F_{(2, 27)} = 1.441$, $p = 0.25$; main effect of mPFC region, $F_{(2, 27)} = 18.82$, $p < 0.0001$). The vast majority of dopamine varicosities in the mPFC form synapses (Séguéla *et al*, 1988). Thus, the increase in the expanse of dopamine innervation coupled with reduced density and total number of dopamine varicosities indicates that AMPH in adolescence alters the development of mPFC dopamine axons and decreases their number of presynaptic sites (Figure 1e).

In contrast, exposure to AMPH during adulthood did not alter dopamine connectivity in the mPFC. There were no differences in the volume of TH-positive fibers between saline- and AMPH-treated littermates in any of the mPFC subregions analyzed (Figure 2c, left panel: two-way ANOVA, main effect of treatment, $F_{(1, 42)} = 0.1705$, $p = 0.68$; no significant treatment \times mPFC region interaction, $F_{(2, 42)} = 1.5$, $p = 0.23$; main effect of mPFC region, $F_{(2, 42)} = 282.5$, $p < 0.0001$). Moreover, there were no differences in the density of TH-positive varicosities in these cortical subregions between saline- and AMPH groups (Figure 2c, right panel: two-way ANOVA, main effect of treatment, $F_{(1, 42)} = 0.4883$, $p = 0.48$; no significant treatment \times mPFC region interaction, $F_{(2, 42)} = 0.0943$, $p = 0.91$; main effect of mPFC region, $F_{(2, 42)} = 11.23$, $p = 0.0001$). These data indicate that the same AMPH regimen that induces profound changes in adult mPFC DA synaptic connectivity when administered during early adolescence does not alter dopamine connectivity within this region when administered during adulthood (Figure 2d). We have previously shown, that in contrast to early adolescence (Yetnikoff *et al*, 2011), exposure to the exact same regimen

of amphetamine upregulates DCC expression in the VTA when given to adult mice and rats (Yetnikoff *et al*, 2007, 2010). This age-dependent effect is consistent with the idea that DCC signaling within dopamine neurons mediates different events at different developmental times (Flores, 2011).

Role of *dcc* in AMPH-Induced Disruption of mPFC Dopamine Wiring

To determine whether DCC signaling in dopamine neurons plays a role in the AMPH-induced disruption of mPFC dopamine wiring we observe in adult mice after adolescent exposure, we examined the effects of an identical treatment regimen in early adolescent *dcc^{lox/lox}DAT^{Cre}* mice. We found that AMPH exposure during adolescence fails to alter the development of dopamine synaptic connectivity in the mPFC in mice lacking *dcc* within dopamine neurons specifically. There were no differences in the volume of TH-positive fibers or density of TH-positive varicosities in the mPFC between saline- and AMPH-treated *dcc^{lox/lox}DAT^{Cre}* mice (*volume*: Figure 3c, left panel: two-way ANOVA, main effect of treatment, $F_{(1, 48)} = 0.07614$, $p = 0.78$, main effect of region, $F_{(2, 48)} = 66.61$, $p < 0.0001$; *density*: Figure 3c, right panel: two-way ANOVA, main effect of treatment, $F_{(1, 27)} = 0.0014$, $p = 0.96$, main effect of region, $F_{(2, 27)} = 1.105$, $p = 0.34$). These findings indicate that DCC signaling within dopamine neurons is required for the effects of AMPH on mPFC DA development (Figure 3d).

Netrin-1 mRNA Expression in the mPFC is not Altered by Adolescent Exposure to AMPH

Netrin-1, the ligand for the DCC receptor, is highly expressed in the mPFC (Manitt *et al*, 2011). We used real-time quantitative PCR to determine whether mice exposed to amphetamine in adolescence have altered netrin-1 mRNA expression in the mPFC, which may contribute to the observed disruption of dopamine connectivity. We found that 1 week after the last amphetamine dose, during the period where DCC-mediated netrin-1 signaling is necessary to organize mPFC dopamine circuitry, netrin-1 mRNA expression did not differ between saline and amphetamine groups (Figure 1g; $U = 14$, $p = 0.9$). This indicates that adolescent exposure to amphetamine does not alter netrin-1 expression in the mPFC 1 week following drug exposure.

AMPH in Adolescence does not Alter Dopamine Wiring in the NAcc

Adult mice that received AMPH during early adolescence showed no alterations in the span of TH-positive fibers or in the density of TH-positive varicosities in the NAcc in comparison with their saline counterparts (*volume*: Figure 4c; left panel: $t_{(6)} = 0.54$, $p = 0.61$; *density*: Figure 4c; right panel: $t_{(6)} = 1.423$, $p = 0.20$). Similarly, there were no differences in these two parameters between adult mice that received AMPH during adulthood and their saline counterparts (*volume*: Figure 4d; left panel: $t_{(8)} = 0.2658$, $p = 0.80$; *density*: Figure 4d; right panel: $t_{(8)} = 1.480$, $p = 0.18$).

Adolescent Exposure to AMPH does not Alter Soma Size or Number of Dopamine Neurons

To examine the effects of AMPH exposure on dopamine soma size and total numbers, we conducted stereological counts of TH-positive neurons in the VTA and SNc of adult mice treated with AMPH or saline in adolescence. There were no changes in these measurements between drug and saline groups (*Dopamine neuron number*: VTA, Mean \pm SEM: Saline = 8545 \pm 2046, AMPH = 8832 \pm 1224; SNc, Mean \pm SEM: Saline = 7519 \pm 1558, AMPH = 6689 \pm 1027; Main effect of treatment, $F_{(1, 8)} = 0.03222$, $p = 0.86$. *Dopamine neuron size*: VTA, Mean \pm SEM: Saline = 159.28 \pm 12.21 μm , AMPH = 152.97 \pm 17.65 μm ; SNc, Mean \pm SEM: Saline = 177.43 \pm 16.59 μm , AMPH = 166.21 \pm 21.037 μm ; Main effect of treatment, $F_{(1, 18)} = 0.2711$, $p = 0.61$; data not shown).

Augmented Salience Attribution Following Adolescent, but not Adult Exposure to Amphetamine

We next began to determine whether the effects of AMPH exposure in early adolescence on mPFC dopamine development have consequences on behavioral responses to drug-associated cues in adulthood. Mice were tested, in the absence of drug, for conditioned locomotor activity to their previously drug-paired environment, a behavior which depends on mPFC function (Franklin and Druhan, 2000). Adult mice that were exposed to AMPH during adolescence show significantly greater locomotor activity (95%) than their saline-treated littermates when re-exposed to the pretreatment environment (Figure 5a: $t_{(8)} = 3.71$, $p = 0.006$). In contrast, adult mice that received AMPH or saline during adulthood did not show differential locomotor activity when re-exposed to the pretreatment environment (Figure 5b: $t_{(13)} = 1.806$, $p = 0.094$). Furthermore, adult *dcc^{lox/lox}DAT^{Cre}* mice treated with AMPH or saline during adolescence did not show differences in locomotor activity when re-exposed to the pretreatment environment (Figure 5c: $t_{(15)} = 0.83$, $p = 0.42$). These findings suggest that the DCC-mediated disruption in mPFC dopamine development induced by

AMPH in adolescence leads to exaggerated contextual salience of drug-related cues in adulthood.

DISCUSSION

Here, we report that exposure to amphetamine in early adolescence disrupts mPFC dopamine development by altering DCC signaling within dopamine neurons, leading to augmented salience attribution in adulthood. First, we show that mice that receive amphetamine in adolescence, but not in adulthood, have aberrant mPFC dopamine innervation and a stark reduction in dopamine synapses in adulthood. Second, we find that AMPH decreases dopamine presynaptic sites selectively in the mPFC. Third, we show that these developmental effects of amphetamine require DCC signaling within dopamine neurons. Finally, we demonstrate that mice exposed to amphetamine in adolescence, but not in adulthood, show exaggerated contextual salience to previously drug-paired environments; an effect that also depends on DCC signaling within dopamine neurons. These findings support the idea that drugs of abuse in adolescence induce detrimental behavioral consequences by disrupting mesocortical dopamine development. Furthermore, we identify the DCC signaling cascade as a critical mechanistic component of these effects.

DCC-mediated Effects of Amphetamine on the Sculpting of mPFC Dopamine Axons

Presynaptic sites. In this study, we find that amphetamine administration during adolescence reduces the density of presynaptic sites from dopamine axons specifically in the mPFC. This effect requires DCC signaling within dopamine neurons. DCC-mediated synaptic decrease is in line with the fact that, in addition to their role in axonal guidance, DCC receptors continue to organize neuronal connectivity beyond axonal pathfinding (Dickson, 2002; Chao *et al*, 2009; Poon *et al*, 2013). DCC receptors function in a short-range capacity to dictate axonal target recognition, axon and dendrite arborization, and synaptogenesis (Deiner *et al*,

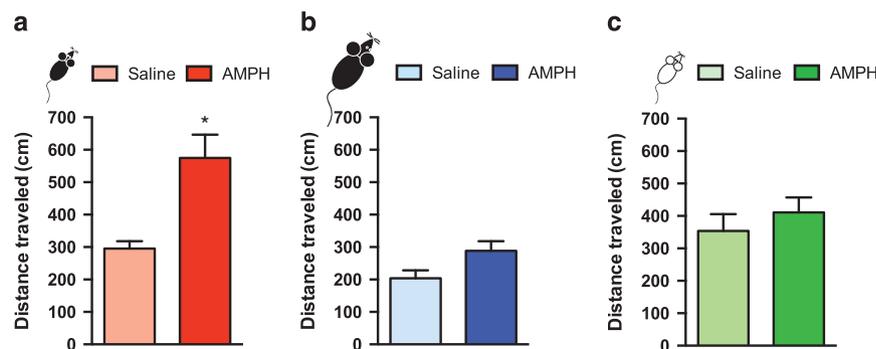


Figure 5 Amphetamine exposure in adolescence, but not in adulthood, leads to augmented salience attribution and requires *dcc* in dopamine neurons. Salience attribution was quantified by calculating the total locomotor activity across a 15-min test of conditioned locomotor activity (mean \pm SEM). (a) Mice exposed to amphetamine (AMPH) during adolescence (from PND 22 \pm 1 to PND 31 \pm 1) display augmented salience attribution when re-exposed to the previously drug-paired chamber in adulthood (PND 75 \pm 15), in comparison with their saline controls ($n = 5$ per group; * indicates $p = 0.006$). (b) Mice exposed to AMPH ($n = 7$) or saline ($n = 9$) during adulthood (from PND 75 \pm 15 to PND 845 \pm 1) show no differences in salience attribution when re-exposed to a previously drug-paired chamber approximately 6 weeks (\sim PND 120 \pm 15) after treatment termination. (c) *dcc^{lox/lox}DAT^{Cre}* mice ($n = 8$ per group) exposed to AMPH or saline during adolescence (from PND 22 \pm 1 to PND 31 \pm 1) show no differences in salience attribution when re-exposed to a previously drug-paired chamber in adulthood (PND 75 \pm 15).

1997; Winberg *et al*, 1998; Dent *et al*, 2004; Colón-Ramos *et al*, 2007; Manitt *et al*, 2009). Compromised DCC signaling induced by amphetamine during adolescence, a period of intense mPFC synaptic refinement, may therefore lead to the decrease of dopamine presynaptic sites by disrupting these post-axonal pathfinding events. Blocking DCC function has been shown to dramatically reduce presynaptic sites from retinal ganglion cell axons in a netrin-1-dependent manner (Manitt *et al*, 2009). Netrin-1 is highly expressed by mPFC neurons in layers that are densely innervated by dopamine fibers (Manitt *et al*, 2011). Therefore, amphetamine in adolescence may 'denude' dopamine axons by limiting the DCC/netrin-1 interaction. Indeed DCC-mediated netrin-1 signaling promotes synaptogenesis *in vitro* by increasing the probability of axon–dendrite adhesion and, in turn, triggering the formation of presynaptic sites (Goldman *et al*, 2013). Here, we show that netrin-1 mRNA expression in the mPFC during adolescent synaptic organization is not altered by exposure to amphetamine. Future studies will address whether increasing netrin-1 function in the mPFC would rescue the effects of amphetamine in adolescence on mPFC dopaminergic presynaptic density.

Aberrant innervation. Coupled with the reduction of presynaptic sites, exposure to amphetamine during adolescence induces an increase in the expanse of dopamine fibers to the mPFC. We propose that amphetamine administration, by reducing DCC expression in dopamine neurons (Yetnikoff *et al*, 2011), leads to the failure of dopamine axons to undergo target recognition processes in non-cortical regions, and that these axons instead ectopically innervate the mPFC. Evidence from our group suggests that in adolescence, DCC receptors promote target recognition events by dopamine axons within brain regions that express *low* levels of netrin-1. When DCC signaling is compromised during this particular developmental period, dopamine axons fail to undergo target recognition events within these regions. Instead, they continue to grow towards the inner layers of the mPFC, where netrin-1 is *highly* expressed (Manitt *et al*, 2011). Another possibility is that the increase in the expanse of dopamine innervation results from sprouting of fibers that have already reached the mPFC by adolescence (Kalsbeek *et al*, 1988; Van den Heuvel and Pasterkamp, 2008). However, blockade of DCC signaling has been shown to *reduce* axonal branching and differentiation of growing retinal ganglion cells (Manitt *et al*, 2009).

Because dopamine axons travel through the striatum on their way to cortical targets (Kalsbeek *et al*, 1988), we hypothesized that amphetamine induces the rerouting of dopamine axons that were destined to innervate the NAcc and/or the dorsal striatum. Dopamine synaptic connectivity undergoes intense dynamic changes in striatal regions during adolescence (Spear, 2000; Antonopoulos *et al*, 2002; McCutcheon *et al*, 2012; Naneix *et al*, 2012). In this experiment, we were not able to identify the differences in dopamine connectivity in the NAcc between adult mice treated with amphetamine or saline during adolescence. However, this apparent lack of effect should be interpreted cautiously. The increase in fiber expanse in the mPFC would correspond to a very small change in dopamine fiber

density in striatal regions that may be undetectable by our current anatomical analysis (Manitt *et al*, 2011). Future studies will explore this idea using conditional viral labeling techniques to investigate detailed dopamine axonal morphology in NAcc and striatal regions.

Behavioral Consequences of Amphetamine-Induced Disruption in mPFC Dopamine Development

Dopamine axons innervate the mPFC sparsely, but their synaptic connectivity appears to be organized in a very strategic manner (Séguéla *et al*, 1988). We have shown that small alterations to mPFC dopamine connectivity lead to sizeable changes in the pyramidal neuron morphology and excitability and, consequently, to changes in cognitive processing and responses to drugs of abuse (Grant *et al*, 2007; Manitt *et al*, 2011, 2013; Pokinko *et al*, 2014). Similarly, dopamine function in the human prefrontal cortex has been shown to play a critical role in higher-order cognitive processing, including behavioral inhibition and salience attribution, as well as in the regulation of subcortical reward circuitry (Robbins *et al*, 1994; Rogers *et al*, 1999; Tomasi *et al*, 2007; Goldstein and Volkow, 2011). Thus, by disrupting prefrontal cortex dopamine maturation, drug use in adolescence likely induces deficits in these processes, increasing vulnerability to develop addiction. Indeed, compelling evidence shows prefrontal cortex dysfunction and associated cognitive deficits in stimulant drug abusers (Verdejo-García *et al*, 2006; Tomasi *et al*, 2007; Woicik *et al*, 2011; Ersche *et al*, 2011; Goldstein and Volkow, 2011; Kohno *et al*, 2014;). Furthermore, prefrontal cortex activation in adolescent subjects appears to predict levels of future substance use and dependence symptoms, particularly in individuals who are already high-frequency drug users (Mahmood *et al*, 2013).

Here, we find that mice exposed to amphetamine in adolescence show exaggerated conditioned locomotor activation when re-exposed to the previously drug-paired environment in adulthood. Conditioned locomotor activity is a measure of contextual salience attribution and depends on mPFC function (Stewart *et al*, 1984; Cervo and Samanin, 1996; Di Ciano *et al*, 1998; Franklin and Druhan, 2000; Hall *et al*, 2008; Robinson and Berridge, 1993, 2008). Whereas the amphetamine regimen used in this experiment produced exaggerated salience attribution when administered in adolescence, it failed to induce salience attribution when administered in adulthood. These findings are the first evidence that drug exposure in adolescence, in comparison with adulthood, renders mice more sensitive to drug-paired cues in adulthood. Remarkably, DCC signaling mediates the effects of amphetamine in adolescence on both dopamine synaptic connectivity and salience attribution, suggesting strongly that these events are causally linked.

The changes in the development of dopamine connectivity induced by amphetamine are more pronounced in the infralimbic cortex subregion. Dopamine input to the mPFC dictates the functional maturation of local circuitry (Manitt *et al*, 2011, 2013). Thus, our results suggest that aberrant dopamine innervation leads to modifications in infralimbic cortex function. The infralimbic cortex controls cue-induced reinstatement of drug-seeking behavior following extinction (Peters *et al*, 2008; LaLumiere *et al*, 2012). In fact,

this region has been proposed as a key component of the 'stop circuit' substrate mediating the transition from regulated to compulsive drug use (Kalivas, 2008; Peters et al, 2009). We are currently investigating the consequences of disrupted development of dopamine connectivity on the structure and function of infralimbic pyramidal neurons and on behaviors that are linked to infralimbic cortical function.

CONCLUSION

In this study, we show that a regimen of exposure to an abused dose of amphetamine during adolescence, but not during adulthood, reduces mPFC dopamine connectivity and increases salience attribution of the drug-paired context in adulthood. DCC receptor signaling in dopamine neurons is necessary for both of these effects. Adolescent drug use may therefore influence individual vulnerability to drug addiction through DCC-mediated disruption in the development of mPFC dopamine circuitry, and ultimately, on cognitive function. We have previously shown that variations in DCC, which occur in humans (Srouf et al, 2010; Méneret et al, 2014), are associated with psychiatric disorders, including depression and schizophrenia (Grant et al, 2012; Manitt et al, 2013). We propose that factors that confer susceptibility or resilience to psychopathology, including addiction, regulate DCC in adolescence.

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The authors declare no conflict of interest.

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