Non-Contingent Exposure to Amphetamine in Adolescence Recruits miR-218 to Regulate *Dcc* Expression in the VTA

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The development of the dopamine input to the medial prefrontal cortex occurs during adolescence and is a process that is vulnerable to disruption by stimulant drugs such as amphetamine. We have previously linked the amphetamine-induced disruption of dopamine connectivity and prefrontal cortex maturation during adolescence to the downregulation of the Netrin-1 receptor, DCC, in dopamine neurons. However, how DCC expression in dopamine neurons is itself regulated is completely unknown. MicroRNA (miRNA) regulation of mRNA translation and stability is a prominent mechanism linking environmental events to changes in protein expression. Here, using male mice, we show that miR-218 is expressed in dopamine neurons and is a repressor of DCC. Whereas *Dcc* mRNA levels increase from early adolescence to adulthood, miR-218 exhibits the exact opposite switch, most likely maintaining postnatal *Dcc* expression. This dynamic regulation appears to be selective to *Dcc* since the expression of *Robo*1, the other guidance cue receptor target of miR-218, does not vary with age. Amphetamine in adolescence, but not in adulthood, increases miR-218 in the VTA and this event is required for drug-induced downregulation of *Dcc* mRNA and protein expression. This effect seems to be specific to *Dcc* because amphetamine does not alter *Robo*1. Furthermore, the upregulation of miR-218 by amphetamine requires dopamine D2 receptor activation. These findings identify miR-218 as regulator of DCC in the VTA both in normal development and after drug exposure in adolescence.
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INTRODUCTION

Exposure to drugs, including amphetamine, have different long-term effects on the brain and on behavior depending on the age at the first exposure. Epidemiological studies have shown, for example, that drug use in adolescence is associated with an elevated risk for lifetime abuse and dependence (Grant and Dawson, 1998; McCabe *et al*, 2007; Swendsen *et al*, 2012). Although it is easy to imagine that stimulant drugs disrupt the patterns of neural networks that are still developing during adolescence, it is only recently that we have begun to understand the molecular mechanisms by which they interact with ongoing developmental processes in adolescence to induce enduring changes in brain connectivity and function.

Reorganization of dopamine input connectivity of the medial prefrontal cortex is one of the most notable developmental events observed in the brain in adolescence (Manitt *et al*, 2013, 2011; Money and Stanwood, 2013; Reynolds *et al*, 2015) and, as such, is an obvious target of

disruption by stimulant drugs. Our work in rodents has identified the Netrin-1 receptor, Deleted in Colorectal Cancer (DCC), as a key mediator for medial prefrontal cortex dopamine connectivity in adolescence and its disruption by drugs (Flores, 2011; Manitt *et al*, 2013, 2011; Reynolds *et al*, 2017). We have shown that DCC receptors within mesolimbic dopamine axons promote target recognition events in the nucleus accumbens, thereby preventing them to grow ectopically to the medial prefrontal cortex. By limiting the extent of the dopaminergic input to the medial prefrontal cortex, DCC receptors influence the organization and function of local synaptic circuitry and cognitive processing (Manitt *et al*, 2013; Reynolds *et al*, 2017). What is more, DCC expression in dopamine neurons is altered after drug exposure: amphetamine treatment in early adolescence downregulates DCC protein expression in ventral tegmental (VTA) dopamine neurons (Yetnikoff *et al*, 2011). As a result, amphetamine in adolescence leads to (a) altered dopamine innervation to the medial prefrontal cortex, (b) reorganization of medial prefrontal cortex local circuitry, and (c) impaired cognitive function in adulthood (Reynolds *et al*, 2015). These results indicate that variations in DCC receptor expression in dopamine neurons and specifically in adolescence influence the developmental trajectory of dopamine neurons and, therefore, adult medial prefrontal cortex function. However, how DCC expression is

regulated during the normal course of adolescent development and whether this process is disrupted by stimulant drugs is not known.

MicroRNA regulation of mRNA translation and stability is a prominent mechanism linking environmental events to changes in protein expression (Codocedo and Inestrosa, 2016; Hollins and Cairns, 2016). Recently, we demonstrated that the microRNA-218 (miR-218) is a potent repressor of DCC expression in human and mouse medial prefrontal cortex pyramidal neurons (Torres-Berrío *et al*, 2017). In fact, we showed that downregulation of miR-218 mediates stress-induced upregulation of *Dcc* mRNA and protein expression in the adult medial prefrontal cortex (Torres-Berrío *et al*, 2017). Because microRNAs are emerging as master regulators of many cellular processes, including those involved in brain development (Hollander *et al*, 2010; Hollins and Cairns, 2016; Im *et al*, 2010; Issler and Chen, 2015; Rao and Pak, 2016; Sun *et al*, 2013; Torres-Berrío *et al*, 2017), we hypothesize that miR-218 is the key regulator of DCC expression in dopamine neurons and the molecule that serves as a sensor of environmental perturbations, including exposure to amphetamine in adolescence. First, we determined if miR-218 is expressed by VTA dopamine neurons and whether its expression varies across postnatal life according to changes in DCC levels. We then assessed the possibility that amphetamine in adolescence, in comparison to adulthood, recruits miR-218 in dopamine neurons to repress DCC. Finally, we assessed whether dopamine D2 or D1 receptor activation is required for miR-218 signaling.

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. Male C57BL/6 wild-type mice were obtained from Charles River Canada and maintained in the colony room of the Douglas Mental Health University Institute Neurophenotyping center on a 12-h light–dark cycle (light on at 0800 h) with food and water available *ad libitum*. All the experiments were conducted during the light part of the cycle.

In Situ Hybridization

Adult male C57BL/6 mice were anesthetized using an overdose of intraperitoneal ketamine 50 mg/kg, xylazine 5 mg/kg, and acepromazine 1 mg/kg, and perfused intracardially with 50 ml of 0.9% saline followed by 75 ml of chilled fixative solution (2% paraformaldehyde in phosphate-buffered solution). After removal, brains were rapidly frozen in dry ice-cooled 2-methylbutane (Fisher Scientific, Hampton, NH, USA). Coronal sections of the VTA at 12 μm were obtained using a cryostat, were mounted onto superfrost slides and endogenous peroxidase was inactivated with 0.3% H_2O_2. Slices were then permeabilized with Proteinase K solution and underwent acetylation with triethanolamine and acetic anhydride. Sense and antisense 5′ digoxigenin-labeled LNA probes against miR-218 (hsa-miR-218-5p: 5′-ACATGGTTAGATCAAGCACA-3′; Exiqon, Vedbaek,

Denmark) were then hybridized to the slices for 14 h at 60 °C. After hybridization, brain sections were stringently washed in saline-sodium citrate (SSC) and 50% formamide for 30 min at 60 °C, treated with RNAse A for 30 min at 37 °C and washed in decreasing concentrations of SSC. VTA tissue was incubated overnight at 4 °C with an anti-DIG antibody coupled to horseradish peroxidase (Roche, Mississauga, ON, Canada) and rabbit polyclonal anti-Tyrosine Hydroxylase (TH) antibody (1:500 dilution, catalog #AB152, RRID: AB_390204; Millipore Bioscience Research Reagents) or rabbit polyclonal anti-DCC No. 2473 (1:200) (Manitt *et al*, 2010). To reveal miRNA expression, sections were incubated with tyramide-coupled to Cy3 (Perkin Elmer, Montréal, QC, Canada) and Alexa 488-coupled secondary antibody raised in goat (Life Technologies, Toronto, ON, Canada) to reveal TH immunofluorescence. Sections were rinsed in PBS and coverslipped with DAPI containing media (Vector laboratories, Burlingame, CA, USA).

Drugs

D-Amphetamine sulfate (Sigma-Aldrich, Dorset, United Kingdom) was dissolved in 0.9% saline. All amphetamine injections were administered i.p. at a volume of 0.1 ml/10 g and at a dose of 4 mg/kg of amphetamine. Raclopride (Sigma-Aldrich, Dorset, United Kingdom) was dissolved in 0.9% saline and the pH was adjusted to 7.4. SCH 23390 (Tocris Bioscience, Bristol, United Kingdom) was dissolved in 0.9% saline. All raclopride and SCH 23390 injections were administered i.p. at a volume of 0.1 ml/10 g 30 min prior each amphetamine injection. The dose used for raclopride was 1 mg/kg and the dose used for SCH 23390, 0.1 mg/kg (Fan and Hess, 2007).

Amphetamine treatment protocol. Male C57BL/6 mice received one injection of amphetamine (4 mg/kg) or vehicle (saline), once every other day, for a total of 5 days. Mice were treated during early adolescence, from postnatal day (PND) 22 ± 1 to PND 31 ± 1, or in adulthood, from PND 75 ± 15 to PND 84 ± 15. This exact drug regimen, when administered in adolescence, but not in adulthood, decreases DCC receptor expression in the VTA 1 week after the last injection (Yetnikoff *et al*, 2011). Consistent with our previous work, we define early adolescence in mice as the period between the day of weaning and PND 32 (Manitt *et al*, 2013; Reynolds *et al*, 2015; Yetnikoff *et al*, 2011); this definition seems now to be a consensus in the rodent literature (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. Horizontal activity was measured 15 min prior and 90 min after each saline or AMPH injection.

RNA Extraction and Quantitative Real-Time PCR for Mouse Tissue

One week after either early adolescent or adult amphetamine treatment, bilateral punches of the VTA were taken from coronal sections obtained as described previously (Manitt *et al*, 2010). Briefly, after mice euthanasia, brains were then removed and rapidly frozen in dry ice-cooled

2-methylbutane (Fisher Scientific, Hampton, NH, USA). Bilateral punches of the VTA were excised from 1-mm-thick coronal slices starting from sections corresponding to Plate 55 of Paxinos and Franklin (2001). Total RNA and microRNA fraction were isolated from the mouse frozen tissue with the miRNeasy Micro Kit protocol (Qiagen, Toronto, ON, Canada) as previously (Torres-Berrío *et al*, 2017). All mouse RNA samples were determined to have 260/280 and 260/230 values ⩾ 1.8, using the Nanodrop 1000 system (Thermo Scientific, Toronto, ON, Canada). Furthermore, we assessed RNA integrity using the denaturing gel electrophoresis method proposed by Aranda *et al* (2012) and detected the three well-defined 28S, 18S, and 5.8S/5S ribosomal bands without any background. Reverse transcription for *Dcc, Roundabout homolog 1 (Robo1)*, and *Glyceraldehyde-3-phosphatedehydrogenase (GAPDH)* mRNA were performed using a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA) according to manufacturer's instructions. Real time PCR, using TaqMan assay (Applied Biosystems, Foster City, CA) was carried out with an Applied Biosystems 7900HT RT PCR system. Data for *Dcc* and *Robo1* mRNA expression were analyzed by using the absolute quantification (AQ) standard curve method and the levels of these transcripts were relativized to the expression of the reference gene *Gapdh*. Reverse transcription for miR-218 was performed using the TaqMan MicroRNA Reverse Transcription Kit together with the corresponding miRNA TaqMan probes (Applied Biosystems, Foster City, CA) and expression levels were calculated using AQ standard curve method. The small nucleolar RNA (snoRNA) RNU6B was used as endogenous control to normalize the expression of miR-218. In all cases, the real-time PCR was run in technical triplicates.

Protein Extraction and Western Blot Analysis

In total, 0.3 ml of 100% ethanol was added to the remaining interphase and phenol phase separated from the total RNA extraction, incubated at room temperature for 3 min and centrifugated at 2000 g at 4 °C for 2 min to sediment DNA. The phenol/ethanol supernatant was transferred to a new tube and mixed with 1.5 ml of isopropanol, incubated for 10 min and centrifugated at 12 000 g for 10 min at 4 °C to precipitate the protein. After removal of the supernatant, the pellet was washed three times with 2 ml of 100% ethanol solution, air dried and resuspended in TNE buffer (50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1% Nonidet P-40, 1% Triton X-100, 0.1% SDS and 1 mM EDTA) supplemented with phosphatase and protease. Protein extracts were heated to 95 °C for 5 min with Laemmli buffer as a reducing treatment and processed for western blot as before (Torres-Berrío *et al*, 2017). Antibodies: anti-DCC (1:1000, Cat#554223, RRID: AB_395314, BD Pharmingen, Mississauga, Canada), anti-ROBO1 (1:1500, Cat#ab85312, Abcam, Toronto, Canada), and anti-β-actin (1:15 000, Sigma-Aldrich, Oakville, ON, Canada).

Locked Nucleic Acid Oligonucleotides Preparation

In vivo LNA oligonucleotides targeting miRNA-218-5p (antimiR-218; MIMAT0000663; mmu-miR-218-5; sequence: 5′-UUG UGC UUG AUC UAA CCA UGU-3′) and scramble

(Scramble, sequence: 5′-GGT TAG ATC AAG CAC A-3′) were customized and synthetized by Exiqon (Vedbaek, Denmark). This technology was developed to inhibit a specific microRNA *in vivo* and induce its degradation (Hollander *et al*, 2010; Im *et al*, 2010; Krutzfeldt *et al*, 2007). Both LNA oligonucleotides were dissolved in sterile PBS (pH 7.4).

Stereotaxic Surgery and LNA Oligonucleotides Administration

One day before the starting of the amphetamine regimen, at PND21 ± 1, mice were anesthetized with isoflurane (5% for induction and 2% for maintenance) and placed in a stereotaxic apparatus. Simultaneous bilateral microinfusions of LNA oligonucleotides containing the antimiR-218 or scramble sequences (Exiqon, Vedbaek, Denmark) into the VTA were performed stereotaxically using Hamilton syringes. *VTA coordinates*: − 2.56 mm (anterior/posterior), +0.9 mm (lateral), and − 4.1 mm (dorsal/ventral) relative to Bregma, at a 10° angle as previously (Manitt *et al*, 2013). A total of 0.5 μl of LNA oligonucleotides solution was delivered on each side over an 8-min period followed by a pause of 6 min.

Experimental Design

The experimental design and procedures employed in this study are indicated with diagrams on each figure.

Statistical Analysis

All values are represented as means ± SEM. A significance threshold of $\alpha = 0.05$ was used in all the experiments. Statistical differences between two groups were analyzed with Student's *t*-tests. Correlations were calculated using the Pearson correlation coefficient with two-tailed analysis on the fold-change data. All data are normally distributed and the variance is similar between groups. Statistical differences between more than two groups were analyzed with one-way, two-way, or two-way repeated-measures ANOVAs, followed by Bonferroni multiple comparison *post hoc* tests. The sample size in all the experiment varied from 4 to 8 animals per group.

RESULTS

Expression of miR-218 in Dopamine Neurons in the VTA

Combined *in situ* hybridization and immunofluorescence experiments show that miR-218 is highly expressed by dopamine neurons in the adult VTA (Figure 1a). Careful qualitative inspection revealed that miR-218 expression is confined to TH-positive neurons (Figure 1b). The expression of miR-218 to a specific subpopulation of neurons within one region is consistent with previous reports showing that in the hippocampus miR-218 is specifically enriched in pyramidal neurons compared to neuropeptide somatostatin expressing GABAergic interneurons or to microglia, astrocytes and oligodendrocytes (Butovsky *et al*, 2014; He *et al*, 2012). We find DCC and miR-218 coexpression in the adult VTA (Figure 1c).
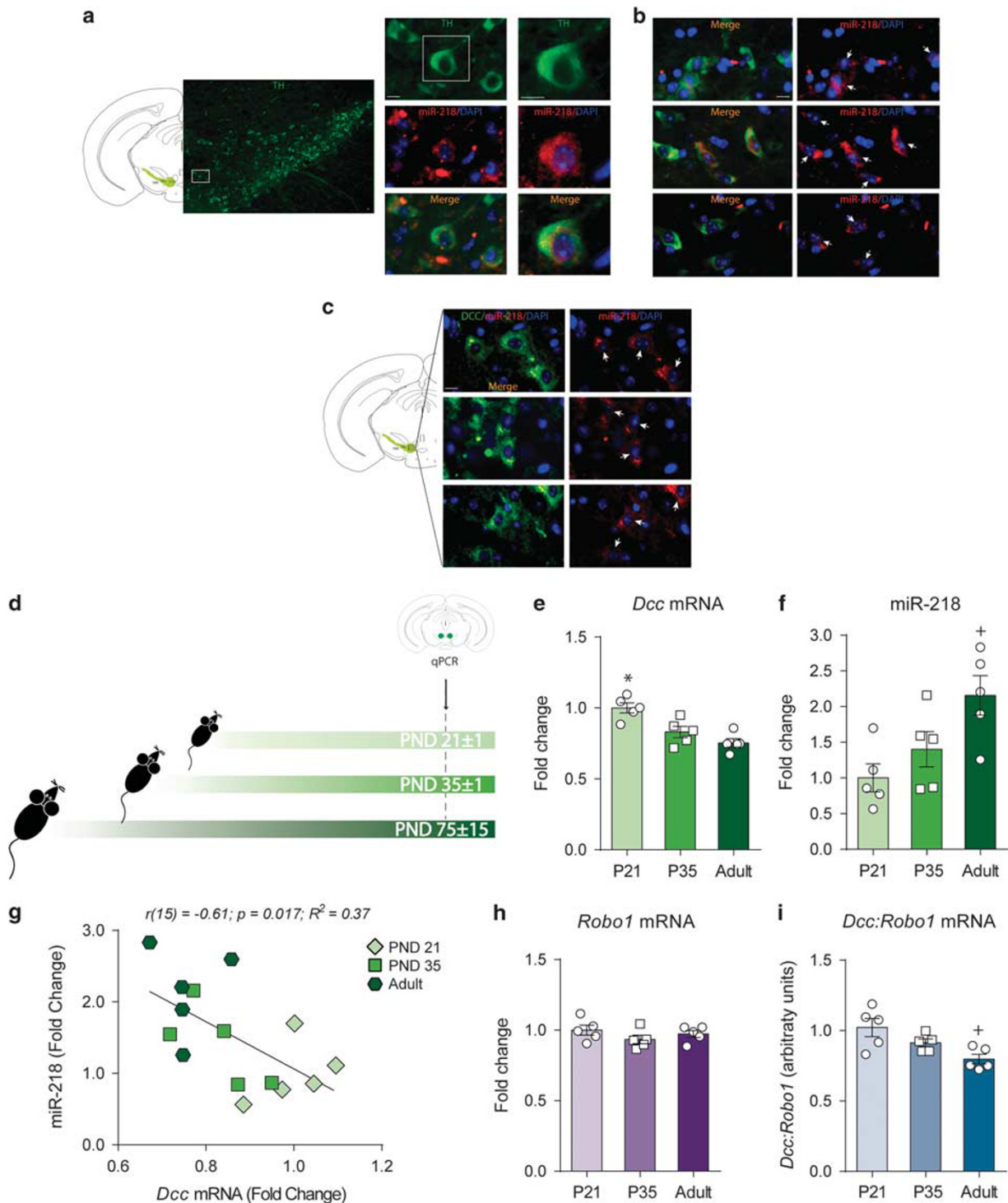
Figure 1 Dopamine neurons expressed miR-218, with levels varying across postnatal development. (a) Representative low- and high-magnification micrographs of coronal sections from an adult mouse showing miR-218 (red) expression in tyrosine hydroxylase (TH) positive neurons (green) in the VTA. Scale bar 10 μm. (b) MiR-218 is confined to TH-positive neurons (white arrows). Scale bar 10 μm. (c) Co-expression of DCC (green) and miR-218 (red) in the adult VTA (white arrows). Scale bar 10 μm. (d) Timeline and experimental procedures. (e) Levels of *Dcc* mRNA in the VTA of mice at three different postnatal ages. (f) Levels of miR-218 in mice at three different postnatal ages. Note the opposite dynamic expression pattern of *Dcc* and miR-218 and the switch in levels of expression from early adolescence to adulthood (*significantly different from the other ages, $p < 0.05$. [+]significantly different from adulthood, $p < 0.05$). (g) There is a negative correlation between *Dcc* and miR-218 expression in the VTA during postnatal development. (h) The levels of expression of another miR-218 target, *Robo1*, do not change in the VTA from early adolescence to adulthood. (i) There is an adolescent switch in the *Dcc:Robo1* mRNA expression ratio in the VTA with predominance of *Robo1* mRNA expression in adulthood *vs* adolescence ([+]significantly different from adulthood, $p < 0.05$). All data are shown as mean ± SEM. DAPI, 4′,6-diamidino-2-phenylindole; SNC, substantia nigra pars compacta; SNR, substantia nigra pars reticulata.

miR-218 and *Dcc* Levels are Opposite in the Postnatal VTA with Expression Switching in Adolescence

We have shown that DCC receptors are conspicuously expressed by VTA dopamine neurons throughout life, but that levels decrease from adolescence onwards (Manitt *et al*, 2010). Thus, we hypothesized that if miR-218 controls *Dcc* gene expression, its levels should be opposite to those of *Dcc* and should also shift from adolescence into adulthood. To address this issue, we measured miR-218 and *Dcc* mRNA expression in tissue punches excised from the VTA of PND21 ± 1, PND35 ± 1, and PND75 ± 15 mice (Figure 1d). We find that, similar to DCC protein expression, *Dcc* mRNA levels are high in early adolescence, but begin to significantly decrease afterwards (Figure 1e; one-way ANOVA: $F_{(2, 12)} = 12.70$, $p = 0.0011$; values at PND21 are significantly higher than at PND35 and PND75; *post hoc* Bonferroni, $p < 0.05$). Importantly, miR-218 expression is opposite to *Dcc* mRNA expression at all ages examined, it changes from low to high from adolescence to adulthood (Figure 1f; one-way ANOVA: $F_{(2,12)} = 5.89$, $p = 0.0017$; values at PND21 are

significantly lower than at PND75; *post hoc* Bonferroni, $p < 0.05$). There is a significant negative correlation between postnatal *Dcc* mRNA and miR-218 expression, suggesting that miR-218 controls *Dcc* mRNA expression in the VTA across the lifespan ($r_{(15)} = -0.61$; $p = 0.02$; $R^2 = 0.37$) (Figure 1g).

In addition to *Dcc*, miR-218 has been shown to repress the expression of another guidance cue receptor gene, *Robo1* (Small *et al*, 2010; Thiebes *et al*, 2015; Torres-Berrío *et al*, 2017), which is also expressed by VTA dopamine neurons in rodents (Marillat *et al*, 2002). Therefore, we determined VTA *Robo1* mRNA expression at the three different postnatal ages. Unlike *Dcc* mRNA levels, *Robo1* expression remains constant across postnatal life (Figure 1h; one-way ANOVA: $F_{(2,12)} = 1.17$). Thus, whereas the developmental expression of *Dcc* in the VTA is controlled by opposite variations in miR-218 levels, *Robo1* expression is not sensitive to these changes. This result is in line with the fact that miRNA control of mRNA expression has spatiotemporal specificity and is dependent on the rate of transcription of a particular gene (Mukherji *et al*, 2011; Sambandan *et al*, 2017). Whether
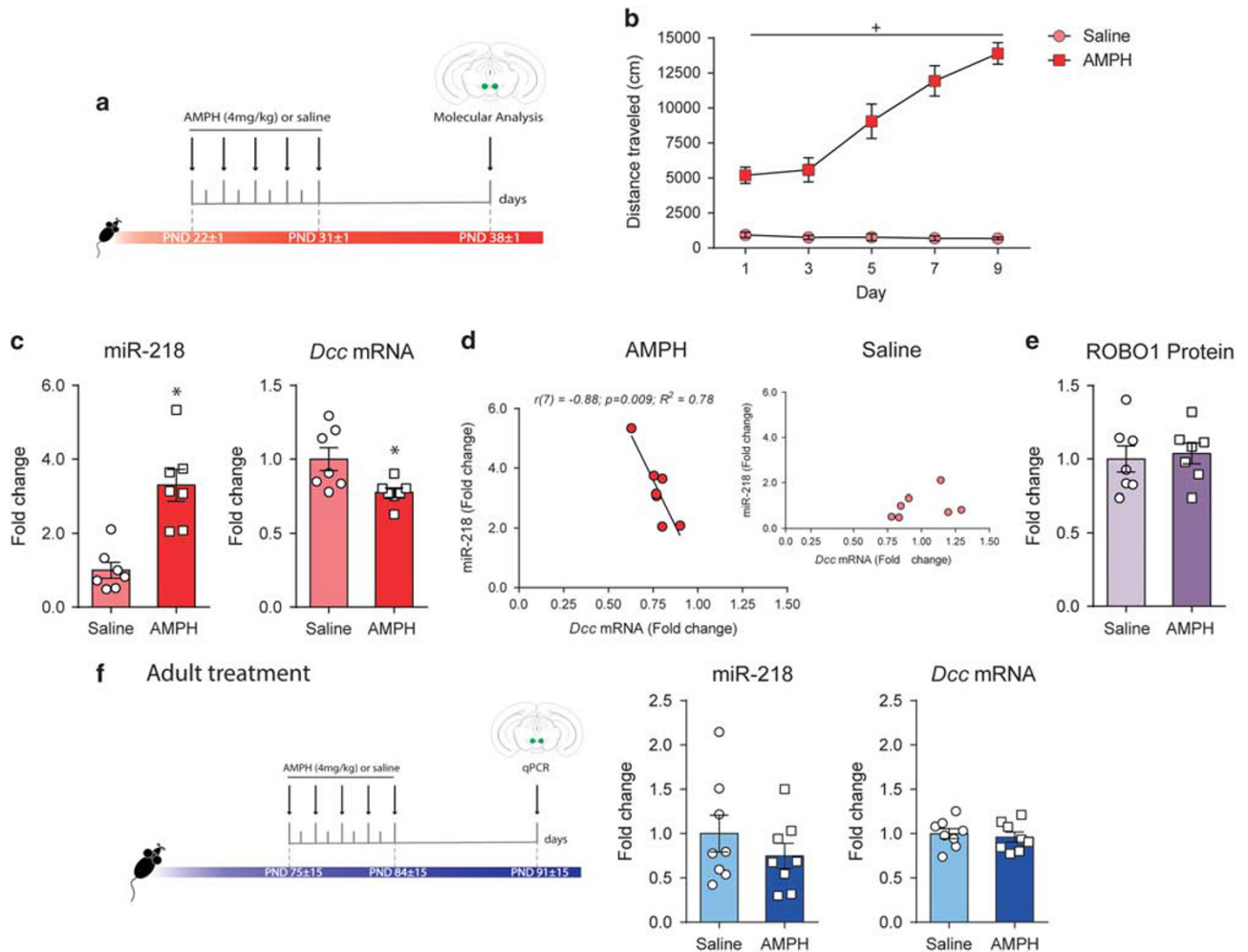

Figure 2 Amphetamine in adolescence upregulates miR-218, but downregulates *Dcc* mRNA expression in the VTA. (a) Timeline of treatment and experimental procedures. (b) Locomotor activity during the 90 min test performed after each treatment injection; [+]significantly different from saline group, $p < 0.05$. (c) MiR-218 expression in the VTA is significantly increased in the VTA one week after amphetamine (AMPH) treatment in adolescence; *significantly different from saline group, $p < 0.05$. (d) There is a negative correlation between miR-218 and *Dcc* mRNA expression in the VTA in the amphetamine-treated group, but not in saline controls. (e) AMPH exposure during adolescence does not change ROBO1 protein expression. (f) The exact same amphetamine regimen given during adulthood fails to alter miR-218 or *Dcc* mRNA expression in the VTA 1 week later. All data are shown as mean ± SEM.

Robo1 is expressed in VTA neurons other than dopamine cells and/or its transcription rate is outside the range of miR-218 regulation needs to be determined.

In motor neurons, ROBO1 receptor activation has been shown to switch off DCC-mediated Netrin-1 attraction (Dudanova and Klein, 2013). Then, we measured the relative levels of *Dcc* and *Robo1* mRNA in the VTA within each subject (ie, *Dcc:Robo1* mRNA ratio). We find that a significant shift towards *Robo1* predominance occurs from PND21 to adulthood (Figure 1i, one-way ANOVA: $F_{(2,12)} = 6.19$, $p = 0.014$; values at PND21 are significantly higher than at PND75; *post hoc* Bonferroni, $p < 0.05$). This finding is intriguing considering the critical role that DCC-mediated Netrin-1 signaling plays on axon targeting in the nucleus accumbens in adolescence, controlling in turn dopamine input to the medial prefrontal cortex (Reynolds *et al*, 2017). Robo1 receptors may contribute to this process.

Amphetamine in Adolescence Upregulates miR-218, but Downregulates *Dcc* mRNA Expression in the VTA

Next, we measured miR-218 in the VTA following amphetamine exposure in adolescence (Figure 2a). Figure 2b shows the locomotor activity displayed by mice after each amphetamine or saline injection. As expected, amphetamine-treated animals show higher activity compared with saline controls during all the sessions (two-way ANOVA for repeated measures: main effect of treatment, $F_{(1,12)} = 193.3$, $p < 0.0001$; main effect of the day, $F_{(4,48)} = 21.09$; $p < 0.0001$; and significant treatment × day interaction, $F_{(4,48)} = 23.01$, $p < 0.0001$. *Post hoc* Bonferroni; significant differences between amphetamine and saline in distance traveled each day, $p < 0.001$). Amphetamine exposure in early adolescence, in comparison to saline, produces a large increase (~2.5 fold) in miR-218 expression in the VTA 1 week later (Figure 2c; $t_{(12)} = 4.82$, $p = 0.0004$). Amphetamine decreases *Dcc* mRNA expression in this region (Figure 2c; $t_{(12)} = 2.70$, $p = 0.02$). This result is in line with our previous reports of decreased DCC protein expression (Yetnikoff *et al*, 2011), suggesting that amphetamine in adolescence controls DCC receptor at the levels of transcription. Interestingly, there is a significant negative correlation between miR-218 and *Dcc* mRNA expression in the VTA in the amphetamine group, but not in saline-treated mice (Figure 2d; $r_{(7)} = -0.88$, $p = 0.009$, $R^2 = 0.78$). This finding suggests that amphetamine induces increases in miR-218 levels that are high enough to downregulate *Dcc* mRNA expression. The effects of amphetamine on miR-218 and *Dcc* expression are robust: we replicated them in a second cohort of mice.

To verify if the increase in miR-218 levels induced by amphetamine in the VTA affect the expression of the other receptor guidance cue, ROBO1, we extracted the protein fraction from the samples used in our original cohort. We found that ROBO1 expression is not altered by amphetamine treatment in the VTA (Figure 2e; $t_{(12)} = 0.33$, $p = 0.75$).

Adult Exposure to Amphetamine Does Not Alter miR-218 or *Dcc* mRNA Expression in the VTA

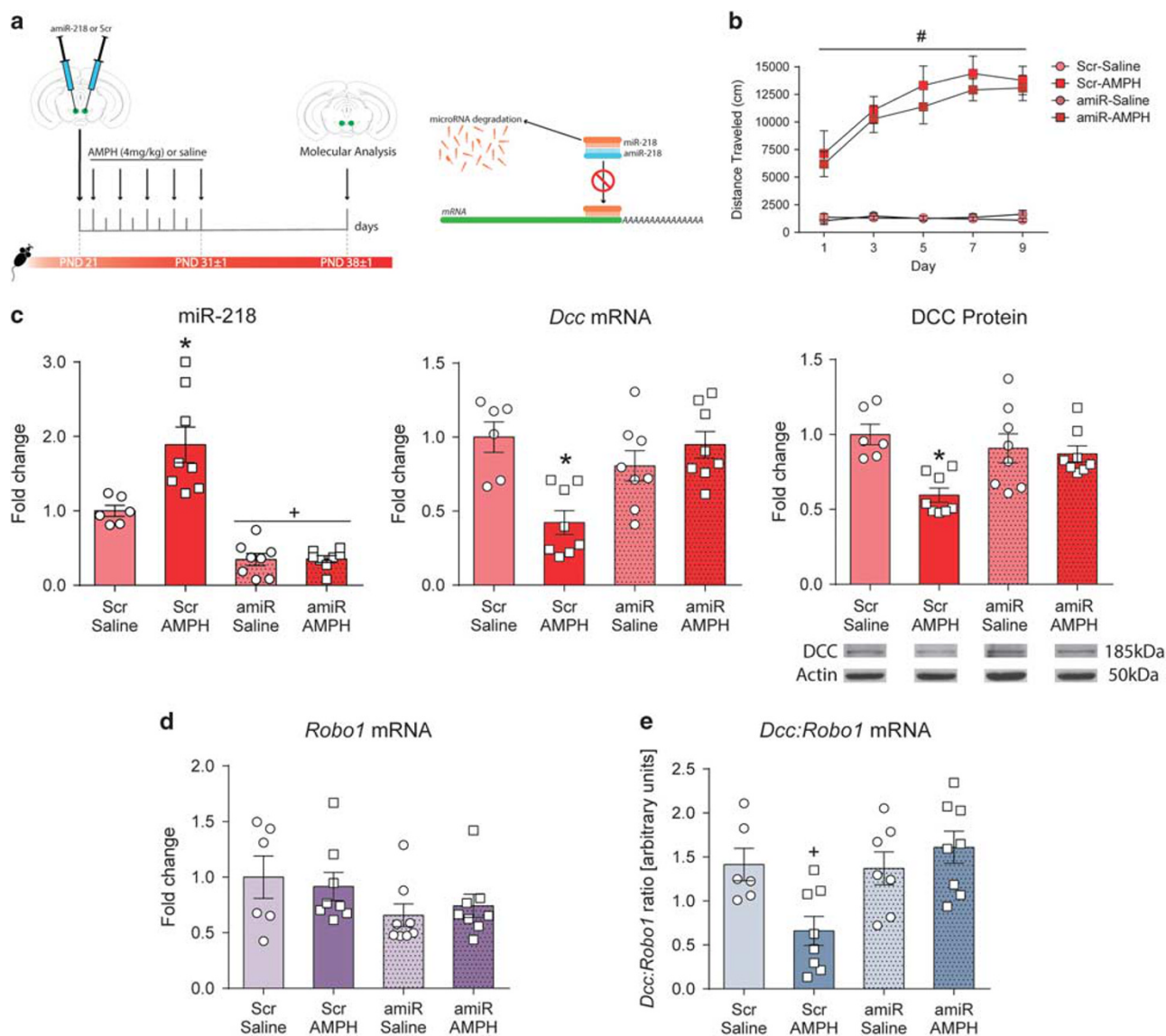To determine whether the effects of amphetamine on miR-218 and *Dcc* mRNA expression are restricted to the adolescent period, we conducted the exact same experiment, but in adult mice. Animals were treated from PND75 ± 15 to PND84 ± 15 and euthanized 1 week after (Figure 2f). In contrast to adolescent exposure, amphetamine in adulthood does not alter miR-218 or *Dcc* mRNA expression in the VTA (Figure 2f; miR-218: $t_{(14)} = 0.99$, $p = 0.34$; *Dcc* mRNA $t_{(14)} = 0.50$, $p = 0.62$).

miR-218 Is Required for Amphetamine-Induced Downregulation of *Dcc* in the VTA

To directly demonstrate that amphetamine downregulates *Dcc* mRNA expression in the VTA by upregulation of miR-218, we used LNA oligonucleotide technology ('antag-omiRs'). Based on previous reports (Hollander *et al*, 2010; Im *et al*, 2010; Krutzfeldt *et al*, 2007), we first tested two different concentrations of LNA oligonucleotides (0.3 mM and 0.6 mM) by microinfusing them bilaterally into the VTA and assessing miR-218 levels 10 days later. Since both doses were equally efficient in reducing VTA miR-218 expression, we selected the lowest dose (0.3 mM) to minimize undesirable effects.

One day before the start of the amphetamine or saline treatment, mice received bilateral VTA microinfusions of an antagomiR with scramble sequence (Scramble) or with antimiR-218 (Figure 3a). There were four groups: Scramble-saline, Scramble-amphetamine, antimiR-218-saline, and antimiR-218-amphetamine. The behavioral analysis revealed no effects of the antimiR-218 microinfusion on locomotor activity observed in mice treated with amphetamine or saline (Figure 3b; three-way ANOVA for repeated measures, main effect of treatment, $F_{(1,26)} = 112.20$, $p < 0.0001$, *post hoc* Bonferroni; significant differences Scramble-amphetamine and antimir-218-amphetamine *vs* Scramble-saline and antimir-saline in the distance traveled each day; $p < 0.05$). As expected, in mice with microinfusions of the Scramble sequence, amphetamine treatment significantly increases miR-218 expression in comparison to their saline counterparts. In contrast, in mice with antimiR-218 microinfusions, miR-218 is reduced (~70%) in comparison to mice with Scramble sequence, regardless of treatment. Furthermore, in the antimiR-218 group, there are no differences in miR-218 expression between amphetamine and saline groups. (Figure 3c; two-way ANOVA: main effect of microinfusion, $F_{(1,26)} = 59.66$, $p < 0.0001$; main effect of treatment, $F_{(1,26)} = 8.91$; $p = 0.0040$; and significant microinfusion × treatment interaction, $F_{(1,26)} = 8.67$, $p = 0.0045$, *post hoc* Bonferroni; significant differences Scramble-amphetamine *vs* all the other groups, antimiR-218-saline and amphetamine *vs* Scramble-saline; $p < 0.05$).

MiR-218 degradation blocked the downregulation of *Dcc* mRNA expression induced by amphetamine in the VTA. As anticipated, amphetamine induced a downregulation of *Dcc* mRNA expression in mice with scramble sequence micro-infusions. This reduction, however, was abolished by the antimiR-218 microinfusion (Figure 3c; two-way ANOVA: main effect of treatment, $F_{(1,26)} = 10.65$, $p = 0.031$, significant microinfusion × treatment interaction, $F_{(1,26)} = 14.53$, $p = 0.0008$; *post hoc* Bonferroni, significant differences between scramble-amphetamine and the rest of the groups, $p < 0.05$). It is interesting to note that microinfusion of antimiR-218 alone does not increase *Dcc* mRNA expression beyond control levels. This finding is consistent with our previous results showing that

Figure 3 Amphetamine in adolescence recruits miR-218 to downregulate *Dcc* mRNA and protein expression in the VTA. (a) Left panel: timeline of antimiR-218 (amiR-218) or scramble (Scr) sequence microinfusions, amphetamine (AMPH) or saline treatment, and experimental procedures. Right panel: schematic representation of the mechanism of action of antagomiRs. (b) Behavioral activity during the 90 min test performed after each treatment injection, #significantly different from saline-treated mice, $p < 0.05$. (c) AMPH upregulates miR-218 in Scr-treated mice, but not in those treated with antimiR-218. MiR-218 levels are significantly reduced following antimir-218 infusion, regardless of drug exposure *significantly different from the other groups, $p < 0.01$; +significantly different from Scr-Saline, $p < 0.05$). AntimiR-218 infusion abolishes AMPH-induced downregulation of *Dcc* mRNA and DCC protein expression in the VTA; *significantly different from the rest of the groups, $p < 0.05$. (d) *Robo1* expression in the VTA is not altered by AMPH treatment or by antimiR-218 microinfusion. (e) AMPH in adolescence induces a significant shift in the *Dcc:Robo1* mRNA expression ratio in the VTA with a predominance of *Robo1* mRNA in amphetamine *vs* saline groups. This effect is abolished when miR-218 degradation is induced; +significantly different from Scramble-Saline, $p < 0.05$. All data are shown as mean ± SEM.

blockade of miR-218–*DCC* binding in a human cell line does not increase *DCC* mRNA levels beyond those see in control conditions (Torres-Berrío *et al*, 2017).

To verify that anitmiR-218 also abolishes the effects of amphetamine on DCC protein levels, we conducted western blot analysis in the same VTA samples. Indeed, amphetamine exposure in adolescence reduces DCC protein expression in mice with Scramble sequence microinfusions, but not in mice with antimiR-218 microinfusions (Figure 3c; two-way ANOVA: main effect of treatment, $F_{(1,26)} = 9.84$, $p = 0.0042$, significant microinfusion × treatment interaction,

$F_{(1,26)} = 6.80$, $p = 0.015$; *post hoc* Bonferroni, significant differences between scramble-amphetamine *vs* all the other groups, $p < 0.05$). These results indicate that the repression of *Dcc* mRNA by miR-218 in the VTA impacts on protein translation. These results show, for the first time, that microRNAs can function as molecular mediators of the effects of stimulant drugs on gene in expression in reward-relevant circuitry in adolescence.

Unlike *Dcc* and, as expected, *Robo1* mRNA expression is not altered by amphetamine treatment regardless of microinfusion (Figure 3d; two-way ANOVA: microinfusion: $F_{(1,26)} = 4.01$;

treatment: $F_{(1,26)} = 8e - 6$; microinfusion × treatment: $F_{(1,26)} = 0.43$), demonstrating, again, that amphetamine-induced regulation of miR-218 targets *Dcc* selectively. However, in mice with scramble sequence microinfusion, amphetamine treatment led to a significant reduction in the relative *Dcc:Robo1* mRNA expression ratio. This change was not observed in samples derived from mice with antimiR-218. (Figure 3e; two-way ANOVA: main effect of microinfusion: $F_{(1,25)} = 6.28$, $p = 0.019$, infusion × treatment interaction: $F_{(1,25)} = 7.56$, $p = 0.011$, *post hoc* Bonferroni, $p < 0.05$).

Amphetamine in Adolescence Increases miR-218 in the VTA via Activation of D2 Receptors

Activation of D2 receptors in VTA dopamine neurons is known to initiate a series of cellular and molecular events that lead to long-term neuronal and behavioral adaptations induced by amphetamine (Kalivas and Duffy, 1991; Vezina, 2004). Thus, we tested whether D2 receptor activation is required for the effects of amphetamine on miR-218 and *Dcc* mRNA expression (Figure 4a). Figure 4b shows that raclopride pretreatment reduces amphetamine-induced locomotor activity (three-way ANOVA for repeated measures, main effect of pretreatment, $F_{(1,23)} = 79.79$, $p < 0.0001$; of treatment $F_{(1,23)} = 210.66$, $p < 0.0001$, and of pretreatment × treatment interaction, $F_{(1,23)} = 62.35$, $p < 0.0001$, *post hoc* Bonferroni; significant differences saline-amphetamine *vs* all the other groups in the distance traveled each day and in raclopride-amphetamine *vs* saline-amphetamine and raclopride-saline during the two last days of the treatment; $p < 0.05$).

Importantly, blockade of D2 receptors prevents amphetamine-induced increase in miR-218 (Figure 4c; two-way ANOVA, main effect of pretreatment $F_{(1,23)} = 5.26$, $p = 0.0316$ and significant pretreatment × treatment interaction, $F_{(1,23)} = 5.35$, $p = 0.030$; *post hoc* Bonferroni, $p < 0.05$) and the reduction on *Dcc* mRNA expression in the VTA (Figure 4c; two-way ANOVA, main effect of pretreatment, $F_{(1,23)} = 8.91$, $p = 0.006$, treatment $F_{(1,23)} = 10.93$, $p = 0.003$ and significant pretreatment × treatment interaction, $F_{(1,23)} = 4.67$, $p = 0.041$; *post hoc* Bonferroni, $p < 0.05$).

Mesolimbic but not mesocortical dopamine neurons, express high levels of DCC receptors across postnatal life (Manitt *et al*, 2013). Furthermore, we demonstrated that DCC receptors in mesolimbic dopamine neurons promote target recognition in the nucleus accumbens in adolescence, preventing them from ectopically innervating the medial prefrontal cortex (Reynolds *et al*, 2017). Thus, the fact that D2 receptors are necessary for the effects of amphetamine on VTA miR-218 and *Dcc* mRNA expression is particularly important because mesolimbic, but not mesocortical dopamine neurons express somatodendritic D2-like receptors (Lammel *et al*, 2008).

Blockade of D1 Receptors Does Not Alter the Effects of Amphetamine in Adolescence on miR-218 and *Dcc* mRNA Expression in the VTA

To further elucidate the mechanism by which amphetamine exposure in adolescence modulates the expression of miR-218 in the VTA we also assessed the effects of systemic administration of a D1 receptor antagonist. As shown in

Figure 4f, pretreatment with the D1 antagonist SCH 23390 before each amphetamine injection blocks drug-induced increase in locomotor activity (Three-way ANOVA for repeated measures, main effect of pretreatment, $F_{(1,20)} = 245.40$, $p < 0.0001$; of treatment $F_{(1,20)} = 328.25$, $p < 0.0001$, of pretreatment × treatment interaction, $F_{(1,20)} = 210.64$, $p < 0.0001$. *Post hoc* Bonferroni analysis revealed significant differences between saline-amphetamine *vs* all the other groups, between SCH 23390-amphetamine *vs* saline–saline and between SCH 23390-amphetamine *vs* SCH 23390-saline on the first day of treatment; $p < 0.05$).

The molecular analysis indicates that blocking the D1 receptors does not affect amphetamine-induced increase in miR-218 (Figure 4g; two-way ANOVA, main effect of treatment, $F_{(1,20)} = 25.73$, $p < 0.0001$. *Post hoc* Bonferroni analysis revealed significant differences between saline-amphetamine *vs* saline–saline, saline-amphetamine *vs* SCH 23390-saline, SCH 23390-amphetamine *vs* saline–saline and between SCH 23390-amphetamine *vs* SCH 23390-saline, $p < 0.05$) and reduction on *Dcc* mRNA expression in the VTA (Figure 4g; two-way ANOVA, main effect of treatment, $F_{(1,20)} = 27.92$, $p < 0.0001$. *Post hoc* Bonferroni analysis revealed significant differences between saline-amphetamine *vs* saline–saline, saline-amphetamine *vs* SCH 23390-saline, SCH 23390-amphetamine *vs* saline–saline and between SCH 23390-amphetamine *vs* SCH 23390-saline, $p < 0.05$). Moreover, *Robo1* mRNA is similar among the different treatments (Figure 4h; two-way ANOVA, pretreatment: $F_{(1,20)} = 0.58$; treatment: $F_{(1,20)} = 0.010$; pretreatment × treatment: $F_{(1,20)} = 0.38$).

DISCUSSION

DCC signaling in dopamine neurons determines the development and function of the prefrontal cortex by controlling the extent of dopamine axons that continue to grow to this region during adolescence (Manitt *et al*, 2013, 2011; Reynolds *et al*, 2015, 2017). However, the precise mechanism that maintains and regulates the levels of DCC itself in dopamine neurons has remained unknown. Here we show that miR-218, a potent repressor of DCC (Torres-Berrío *et al*, 2017), is expressed in VTA dopamine neurons and might be controlling their *Dcc* mRNA expression across postnatal life. Furthermore, we show that repeated exposure to amphetamine during early adolescence, but not in adulthood, suppresses *Dcc* mRNA and protein expression in VTA neurons through upregulating miR-218. This upregulation of miR-218 by amphetamine targets specifically *Dcc* and requires dopamine D2 receptor activation. Thus, miR-218 in the VTA, most likely in dopamine neurons, appears to be a molecular link between stimulant drugs in adolescence and changes in the expression of a key gene involved in prefrontal cortex and cognitive maturation (Reynolds *et al*, 2015, 2017). We propose that miR-218 expression in dopamine neurons is a marker of vulnerability to environmental risk factors in adolescence as well as a mediator of their enduring effects.

The Dynamic Expression of miR-218 and *Dcc* in the VTA

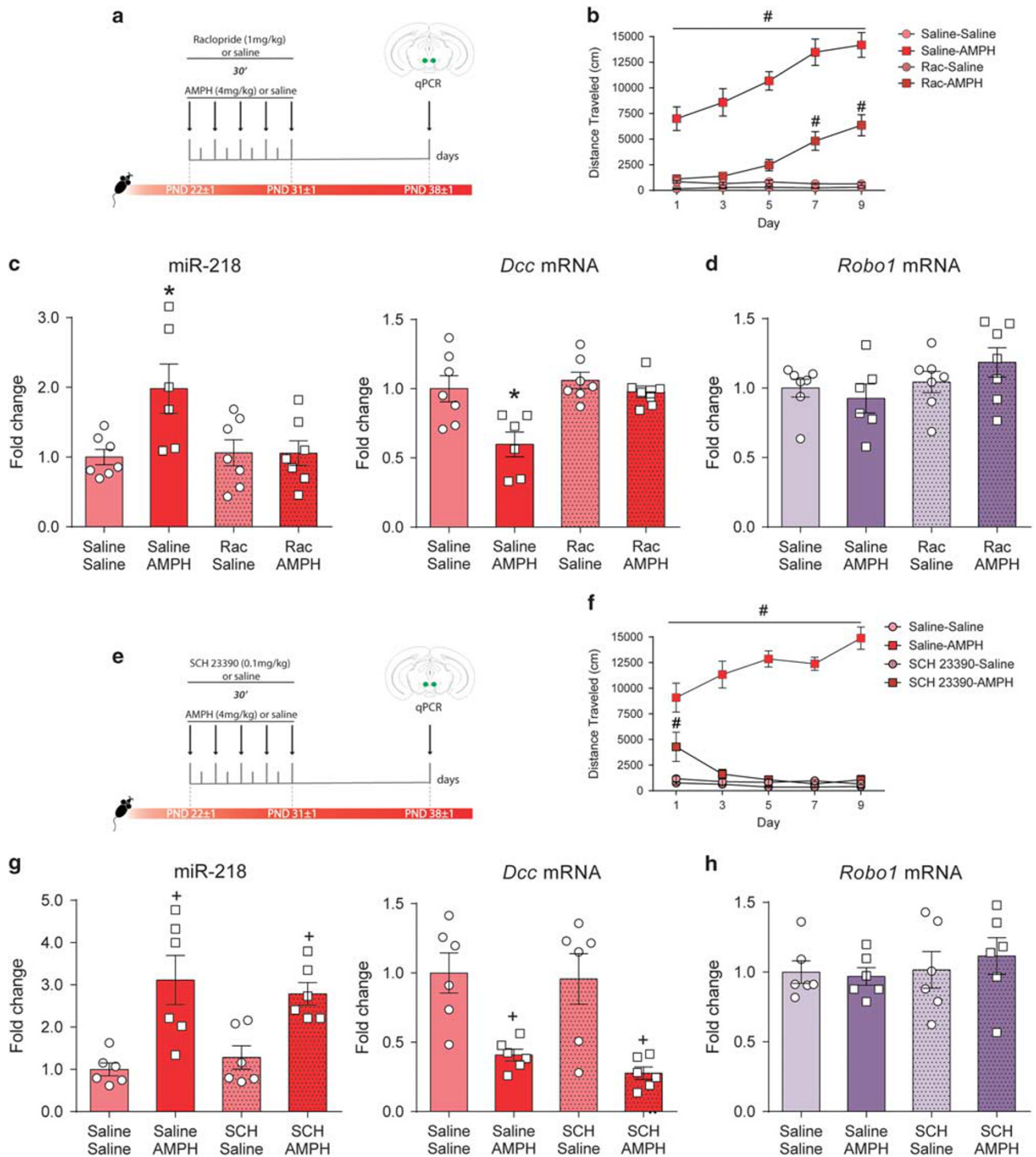We have previously shown the pattern of DCC protein expression in VTA dopamine neurons changes during

Figure 4 Amphetamine in adolescence requires D2 receptors activation to upregulate miR-218 and downregulate *Dcc* mRNA in the VTA. (a) Timeline of treatment and experimental procedures. (b) Locomotor activity during the 90 min test performed after each treatment injection; #significantly different from the other groups, $p < 0.05$. (c) Amphetamine (AMPH)-induced upregulation of miR-218 and, in turn, downregulation of *Dcc* in the VTA requires D2 receptors activation; *significantly different from the other groups, $p < 0.05$. (d) *Robo1 mRNA* expression in the VTA is not altered by AMPH treatment or by raclopride (Rac) pretreatment. (e) Timeline of treatment and experimental procedures. (f) Locomotor activity during the 90 min test performed after each treatment injection; #significantly different from the other groups, $p < 0.05$. (g) Blockade of D1 receptors does not alter amphetamine-induced regulation of miR-218 or *Dcc* mRNA; +significantly different from saline-treated mice, $p < 0.05$. (h) *Robo1 mRNA* expression in the VTA is not altered by AMPH treatment or SCH 23390 pretreatment. All data are shown as mean ± SEM.

postnatal life, with high levels occurring in early adolescence but low levels in adulthood (Manitt *et al*, 2010). Our present data suggest that this dynamic expression is controlled at the transcription level (ie, *Dcc* mRNA expression) and, importantly, is maintained by miR-218. We have shown that miR-218 controls *DCC* mRNA and protein expression in prefrontal cortex pyramidal neurons in both humans and rodents (Torres-Berrío *et al*, 2017), and in fact DCC protein

expression in this region also switches in adolescence (Goldman *et al*, 2013). Thus, we speculate that miR-218 may control the spatiotemporal expression of DCC and may be implicated in the adolescent maturation of reward-relevant circuits. Future quantitative and subcellular fractionation studies will be conducted to further characterize the expression of miR-218 in the VTA.

Although the mechanisms controlling the fluctuation of VTA miR-218 levels over development remain to be determined, endogenous fluctuations in gonadal hormones are likely to play a role. Gonadal hormones may affect miR-218 by altering activity-dependent transcription of pri-miR-218, cleavage and translocation of pre-miR-218, and/or stability of the mature transcript (Joilin *et al*, 2014; Sambandan *et al*, 2017). Indeed, the expression of other microRNAs in the hippocampus has been shown to vary across postnatal life and to be sensitive to alterations in circulating levels of gonadal steroids (Kucherenko *et al*, 2012; Prins *et al*, 2014; Rao *et al*, 2013b, 2015). Future experiments are needed to determine whether gonadal hormones influence miR-218 expression on dopamine neurons during adolescence.

miR-218 as a Potential Mediator of Amphetamine-Induced Regulation of DCC Receptors in the VTA

There is an increasing number of studies showing that exposure to drugs, including ethanol, cocaine, and heroin, leads to changes in miRNA expression in reward-relevant brain regions of adult rodents (Darcq *et al*, 2015; Eipper-Mains *et al*, 2011; Hollander *et al*, 2010; Im *et al*, 2010; Yan *et al*, 2017), and in fact, these would include changes in miR-218 expression in the striatum after repeated exposure to heroin and cocaine during adulthood in rats (Eipper-Mains *et al*, 2011; Yan *et al*, 2017). Our study, however, goes a step further to show that the upregulation of miR-218 induced by amphetamine exposure during adolescence targets *Dcc* mRNA expression. Furthermore, this effect appears to be selective to *Dcc* because the expression of *Robo1*, which is the other guidance cue receptor target of miR-218, is unaltered by amphetamine. We also demonstrate how amphetamine regulates miR-218 and show that this regulation is restricted to adolescence.

In the VTA, dopamine neurons projecting to the nucleus accumbens, but not to the medial prefrontal cortex, express D2 receptors (Lammel *et al*, 2008). The activation of these autoreceptors initiates a series of cellular and molecular processes that lead to long-term neuronal and behavioral adaptations (Kalivas and Duffy, 1991; Vezina, 2004). In this study, we show that D2, but not the D1, receptor activation is required for the upregulation of miR-218 expression by amphetamine in adolescence. This is consistent with previous reports showing that changes in D2 receptors expression in cultured renal cells is associated with changes in the expression of several microRNAs (Han *et al*, 2015). While the mechanisms mediating these effects are unknown, changes in neuronal activity may be at play (Sim *et al*, 2014). In fact, we suggest that systemic raclopride administration abolishes the effect of amphetamine on miR-218 by blocking D2 receptors specifically in VTA mesolimbic dopamine neurons. Indeed, amphetamine induces somatodendritic dopamine release in the VTA and we have shown that

mesolimbic, but not mesocortical dopamine neurons, express DCC receptors. Furthermore, the lack of effect of the D1 antagonist on miR-218 upregulation by amphetamine supports the idea that the effects of raclopride are mediated by D2 receptors localized to dopamine neurons because dopamine cells do not express D1 receptors (Mansour *et al*, 1992). Nonetheless, at this point we cannot rule out the involvement of postsynaptic D2 receptors, indirectly affecting miR-218 expression in dopamine neurons.

A major strength of our study is that we used antagomir technology *in vivo* to demonstrate that miR-218 functions as molecular mediator of the effects of amphetamine on DCC expression. Although this strategy is effective when assessing short-term changes in miRNA expression, it has important limitations when assessing long-term effects. MiR-218 seems to regulate *Dcc* mRNA expression across postnatal life and it has been shown that a single intracerebroventricular infusion of antagomir can induce the degradation of microRNAs for at least 30 days (Nolan *et al*, 2014). Such a prolonged reduction of miR-218 would continue to influence DCC expression well into adulthood, making it difficult to assess the role of miR-218 in the enduring effects of drug exposure selectively in adolescence.

Age Dependent Effects of Amphetamine on DCC Protein Expression in the VTA

In previous studies we showed that amphetamine exposure in adulthood induces an increase in VTA DCC protein expression and that this increase is necessary for the development of drug-induced behavioral sensitization (Yetnikoff *et al*, 2010). In this study, however, we show that amphetamine exposure in adulthood does not alter *Dcc* mRNA expression. In contrast, amphetamine in adolescence downregulates both *Dcc* mRNA and DCC protein expression in the VTA. Thus, the present data suggest that not only the direction of the effects of amphetamine on DCC protein expression in the VTA are opposite between early adolescence and adulthood, but that also different mechanisms may be recruited. The fact that amphetamine in adolescence, but not in adulthood, upregulates miR-218 in the VTA may be an important mechanism accounting for age effects of the drug on DCC protein expression.

CONCLUSION

Considerable efforts are being made to identify biomarkers of vulnerability, particularly during adolescence. MicroRNAs are emerging as promising targets since they are highly stable in peripheral fluids (Chakraborty and Das, 2016), with patters of expression in these fluids matching those observed in the tissue of origin, including brain (Issler *et al*, 2014; Jin *et al*, 2013; Rao *et al*, 2013a). Thus, miR-218 has translational potential as both a mediator and a predictor biomarker, especially since it can be readily detected in blood, urine, and saliva samples in humans (Weber *et al*, 2010).

FUNDING AND DISCLOSURE

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AUTHOR CONTRIBUTIONS

SC and CF conceived the experiments. SC, AA, and CF designed the experiments. SC and JMR-L performed the developmental neuroanatomical and molecular characterization, drug, and antagomir experiments. SS performed all the stereotaxic surgeries. DN contributed to the in situ hybridization experiments. AT-B and LMR provided technical training necessary to perform part of the experiments. SC, JMR-L, AA and CF analyzed the results. SC, AA and CF wrote the paper.

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