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ORIGINAL ARTICLE

Dorsolateral striatal miR-134 modulates excessive methamphetamine intake in self-administering rats

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Abstract

Increasing evidence indicates that excessive drug consumption is sufficient for the transition from recreational and controlled drug use to uncontrolled use and addiction. However, the underlying mechanisms are debated. Some neurobehavioral and neuroimaging evidence indicates that dorsolateral striatum (dlStr)-dependent habit learning plays a key role in excessive drug intake and the transition to addiction, but little is known about the molecular events. The present study investigated whether dlStr miR-134, an important regulator of synaptic transmission and plasticity, is involved in excessive methamphetamine intake. We established excessive and uncontrolled methamphetamine self-administration model in rats by permitting animals extended access to drug (6 h/session/d, LgA group), whereas animals that were limited to access to drug (2 h/session/d, ShA group) exhibited low and controlled self-administration. miR-134 expression in dlStr was significantly increased and its target LIMK1 expression was decreased in the LgA group, but not in the ShA group, compared with the saline control group. However, passive methamphetamine exposure did not alter miR-134 and LIMK1 levels in dlStr. We also found that down-regulation of miR-134 in dlStr through local microinjection of a lentivirus carrying miR-134 sponge (LV-miR-134-Sil) significantly reduced methamphetamine infusions and excessive consumption in LgA group, rather than ShA group. These results indicated that dlStr miR-134, perhaps via its target LIMK1, contributed to excessive and uncontrolled methamphetamine intake, supporting the hypothesis that stimulus-response habit formation is an important mechanism underlying the transition from controlled drug use to uncontrolled drug use and addiction.

Keywords Methamphetamine addiction · Excessive drug intake · miR-134 · Dorsolateral striatum · Stimulus-response habit learning

Introduction

Methamphetamine is one of the most widely used psychoactive drugs worldwide. Initial methamphetamine use is motivated by its hedonic effect, while long-term use becomes escalation of the frequency and amount of methamphetamine consumption, resulting in addiction (Kawa et al. 2016). However, there is no approved pharmacological intervention for treating methamphetamine addiction. Understanding the neurobiological mechanisms in depth is essential for the successful development of medications to treat methamphetamine addiction.

Although initial drug use is associated with positive reinforcement and impulsive actions, drug addiction is characterized by a loss of flexible control over drug use despite negative consequences (Koob et al. 1998). Behavioral studies in rodents have shown that this shift from casual and regular drug use to compulsive drug use may be related to the development of habitual drug seeking and taking from goal-directed behaviors (Everitt and Robbins 2005; Smith and Laiks 2018), and excessive drug consumption drives and accelerates the transition (Leong et al. 2016), but the underlying mechanism remains unclear. Recently, clinical studies have

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provided behavioral and neuroimaging evidence for overreliance on habit learning in alcohol addicts (Lopez et al. 2016). Therefore, excessive drug intake is sufficient for the development of addictive behaviors and is thought to be a hallmark of persistent addiction liability (Edwards and Koob 2013).

The change from recreational drug use to addiction is represented by a transition from prefrontal cortical control to subcortical striatal control and by a shift in control from ventral domain to dorsal domain within the striatum (Everitt and Robbins 2013). The striatum can be divided into three functional domains on the basis of corticostriatal connectivity patterns: the ventral striatum (also known as the nucleus accumbens, NAc), which controls motivation; the dorsomedial striatum (dmStr, or the caudate nucleus in nonhuman primates), which contributes to goal-directed actions; and the dorsolateral striatum (dlStr, or the putamen in nonhuman primates), which drives stimulus-response (S-R) habit learning (Balleine and O'Doherty 2010). In either self-administering rats and monkeys or human addicts, initial or limited exposure to addictive substances, such as cocaine, methamphetamine and alcohol, evoked functional alterations in the ventral striatum. After prolonged and excessive exposures, these alterations spread to the dorsal domains (Gozzi et al. 2011; Meyer et al. 2016; Porrino et al. 2004). In particular, a study using positron emission tomography (PET) imaging of ^{11}C-raclopride binding in human cocaine addicts showed that cocaine cue-elicited dopamine response in the putamen was correlated significantly with addiction severity (Volkow et al. 2006). In contrast, inactivation of or functional intervention in the dlStr, but not the dmStr, selectively disrupted habitual and compulsive drug-seeking and taking behaviors (Jonkman et al. 2012a). Although increasing evidence suggests that the dlStr plays a key role in the transition to addiction, little is known about the associated molecular events.

MicroRNAs (miRNAs) are a class of noncoding short-chain RNAs that regulate gene expression by inducing messenger RNA (mRNA) degradation and suppressing mRNA translation into protein (Bartel 2004). In the brain, miRNAs are involved in various functions such as neuronal differentiation and maturation, synapse formation and plasticity, and miRNA dysfunction contributes to numerous psychiatric disorders (Im and Kenny 2012; Saba and Schratt 2010). Many studies have revealed linkages between several miRNAs and actions of cocaine, opioids and alcohol, including addiction, but few reports have focused on methamphetamine exposure (Bali and Kenny 2013; Smith and Kenny 2018). miR-134, a brain-specific miRNA molecule, is enriched in the dendrites of neurons and regulates dendritic morphology and function. Recent studies have demonstrated that miR-134 contributes to cognitive deficits, status epilepticus, ischemic stroke and depression by acting on LIM kinase 1 (LIMK1) (Fan et al. 2018); however, whether miR-134 participates in drug addiction remains unknown. In the present study, therefore, the role of dlStr miR-134 in excessive methamphetamine intake was investigated. Because extended (6 h/session/d) access to addictive substances leads to excessive and escalating drug intake in self-administering rats (Kitamura et al. 2006), we used this model to investigate methamphetamine exposure-induced alterations in the levels of miR-134 and its target LIMK1 and to observe the effects of dlStr miR-134 downregulation on excessive drug-taking behavior.

Materials and methods

Animals

Male Sprague-Dawley (SD) rats (SPF [Beijing] Laboratory Animal Technology Co., Ltd., Beijing, China) weighing 320–350 g at the beginning of the experiment were individually housed under controlled environmental conditions of 22 ± 2 °C and 40–60% humidity with a 12:12 h light: dark cycle (light: 8:00–20:00). The animals received food and water ad libitum, except during the self-administration experimental sessions. All animal procedures were approved by the Institutional Animal Care and Use Committee and were conducted in strict accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals.

Intravenous methamphetamine-induced self-administration in rats

After 5–7 days of habituation, rats were implanted with a catheter into the jugular vein according to the method in our previous publication (Cao et al. 2016). Briefly, each rat was anesthetized with 50 mg/kg sodium pentobarbital (intraperitoneal (ip); Serva, Shanghai, China), and the right jugular vein was surgically separated. Then, an intravenous catheter constructed of Micro-Renathane (Braintree Scientific Inc., Braintree, MA, USA) was inserted into the jugular vein and secured using suture thread. The catheter was subcutaneously passed to the top of the skull with an exit into a modified 22-gauge cannula (Anilab Software & Instruments Co., Ltd., China).

After 5–7 days of recovery from surgery, the rats received methamphetamine self-administration training. Training was conducted in operant test chambers (30 × 30 × 30 cm^3) linked to a computerized data collection program (Anilab SuperState Version 4.0). The procedure was in accordance with previous reports with some modifications (Du et al. 2016), which involved an acquisition phase and an escalation phase. First, the rats were trained to acquire methamphetamine self-administration behavior for 5 consecutive days with 2 h of access to d-methamphetamine hydrochloride (provided by the Narcotics Control Bureau of the National Ministry of

Public Security, Beijing, China; 0.05 mg/kg/infusion) under a fixed ratio 1 (FR1) schedule of reinforcement. Then, the rats were divided into two groups balanced for methamphetamine consumption within 5 days. During the escalation phase, the drug access time of long-access (LgA) group was increased to 6 h daily, while that of the short-access (ShA) group was maintained at 2 h. The escalation training phase lasted for 13–15 days. A control group was trained in the same way as the LgA group but with 0.9% saline infusion. The control, ShA and LgA groups contained 16, 17 and 17 rats, respectively. Samples from 7 rats in each group were used for Western Blot analysis, while samples from the other rats were used for reverse transcription quantitative PCR (RT-qPCR) assays. At 24 h after the last self-administration, the animals were sacrificed by decapitation, and the brain was removed rapidly. Three subregions of the striatum, including the NAc, dmStr and dlStr, were isolated. The samples for RT-qPCR assays were immersed in RNase reagent and stored at −80 °C, while those for Western Blot analysis were immediately cryopreserved in liquid nitrogen and then stored at −80 °C.

Passive methamphetamine treatment by experimenter-mediated injection

In this experimental procedure, rats were injected subcutaneously (sc) with methamphetamine by the experimenter. The low-dose group received 1 mg/kg methamphetamine injection once daily, while the high-dose group received 5 mg/kg methamphetamine injection. Each group was further divided into three subgroups, each of which was administered methamphetamine for 7, 14 or 28 consecutive days. The control group received 0.9% saline injection for 28 days. The animals were sacrificed 24 h after the last injection by decapitation, and the brain was removed rapidly. Then, the NAc, dmStr and dlStr were isolated. The samples were immersed in RNase reagent and stored at −80 °C for RNA extraction and RT-qPCR assays.

Stereotaxic injection of lentiviral vectors into the dlStr and methamphetamine self-administration training in rats

A lentiviral vector containing miR-134 sponge (LV-miR-134-Sil; viral supernatant concentration: 1×10^8 TU/ml) and negative control vector containing a scrambled sequence (LV-NC) were obtained from Gene Pharma Co. Ltd. (Suzhou, China). The lentiviral vectors also contained enhanced green fluorescent protein (eGFP) as a reporter of infection. For stereotaxic injection, rats were anesthetized with 50 mg/kg sodium pentobarbital and installed in a stereotaxic frame (RWD Life Science Co. Ltd., China). Using a precision micro-syringe with a 35 G blunt needle (WPI, USA), the rats were bilaterally injected with lentiviral vectors (LV-miR-134-Sil or LV-NC) into the dlStr. The stereotaxic injection site coordinates were as follows: anteroposterior (AP), 1.00 mm from the Bregma; mediolateral (ML), ± 3.50 mm from the midline; and dorsoventral (DV), 4.25 and 5.50 mm below the skull surface. We infused 1 μl of virus stock per site at a rate of 0.2 μl/min. To allow diffusion of the solution into the brain tissue, the needle was left in place for an additional 5 min after each infusion. Two weeks after surgery, the position of the stereotaxic injection was verified by detecting eGFP fluorescence, and fluorescence images were acquired using a VS120 virtual slide microscope (Olympus, Japan).

To investigate the effect of the miR-134 sponge on methamphetamine self-administration behavior, rats were subjected to jugular vein catheterization as described above and then immediately received stereotaxic virus injection. After 5–7 days of recovery, the rats were trained for methamphetamine self-administration. The procedure was same as that described above. The animals were sacrificed 24 h after the last self-administration training session. The brain was removed and the dlStr region was isolated. Each sample was divided into two parts: one was immersed in RNase reagent and stored at −80 °C for RT-qPCR assay, and the other was cryopreserved in liquid nitrogen and then stored at −80 °C for Western Blot analysis.

Luciferase reporter assay

A luciferase reporter assay was used to test the effectiveness of the miR-134 sponge in vitro. We generated a recombinant luciferase reporter vector that contained miR-134 binding sites. An 842 bp fragment containing the 3′-untranslated region (3′-UTR) of LIMK1 was cloned from rat striatum and then subcloned into the Xho I/Not I site of the psi-CHECK-2 luciferase expression plasmid (Promega, USA). The recombinant psi-CHECK-2 reporter plasmid and the lentiviral vector expression plasmid containing the miR-134 sponge or a negative control sequence were co-transfected into HEK293T cells in 96-well plates by using Lipofectamine 2000 (Invitrogen, USA). After 48 h of culture, luciferase activity was measured with a dual luciferase reporter assay kit (Cat. E1910, Promega, USA). Firefly luciferase activity was normalized to Renilla luciferase activity.

Total RNA isolation and quantification by real-time RT-qPCR

Total RNA from the tissue samples was isolated using a miRNeasy Mini Kit (Qiagen, Germany) according to the manufacturer's instruction. The expression levels of miR-134 and LIMK1 mRNA were verified by RT-qPCR. Complementary DNA (cDNA) was synthesized using a reverse transcriptase reaction (Qiagen) in accordance with the manufacturer's instruction. We used specific stem-loop RT primers for miR-134

analysis and random primers for LIMK1 mRNA analysis. Real-time RT-qPCR was performed using a QuantiTect SYBR Green PCR Kit (Qiagen) with an ABI 7500 system (Applied Biosystems, USA). Each sample was run in triplicate. Small nuclear RNA U6 (U6 snRNA) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were used as endogenous control to normalize miR-134 and LIMK1 mRNA expression, respectively. Relative expression was calculated with the 2^{-ddCT} method. The sequences of RT primers and qPCR primers are shown in Table 1.

Protein extraction and Western blot analysis

Total protein was extracted from tissue samples with T-PER Tissue Protein Extraction Reagent (Thermo Scientific, USA) containing a protease inhibitor cocktail (Roche, Suisse). The protein concentrations were measured with bicinchoninic acid (BCA) method (Biomed, China). Total protein (40 μg) was separated by 10% SDS polyacrylamide gel electrophoresis and transferred to a polyvinylidene fluoride membrane (Millipore, MA). Subsequently, the membrane was blocked with 5% skim milk in TBST (10 mM Tris, 150 mM NaCl and 0.1% Tween 20, pH 7.4) for 2 h at room temperature and then incubated with primary antibody overnight at 4 °C. After washing 3 times in TBST, the membrane was incubated with horseradish peroxidase (HRP)-labeled secondary antibody to bind the primary antibody for 1–2 h at room temperature. Finally, the specific bands were detected using enhanced chemiluminescence detection kit (Millipore, USA) and quantified with AlphaView System (Alpha Technologies, USA). The relative density values were calculated by using β-actin as an internal control. The following antibodies were used: anti-LIMK1 rabbit monoclonal antibody (Cat. 3842, Cell Signaling Technology, USA), anti-β-actin mouse monoclonal antibody (Cat. CW0096M, CWBIO, China), HRP-conjugated goat anti-rabbit IgG (Cat. ZB5301, ZSGB-bio, China) and HRP-conjugated goat anti-mouse IgG (Cat. ZB5305, ZSGB-bio, China).

Immunofluorescence staining

After two weeks, the rats that had been stereotactically injected with lentiviral vectors were anesthetized with 50 mg/kg sodium pentobarbital (ip), and perfused transcardially with cold 4% paraformaldehyde (PFA) in PBS and fixed in 4% PFA at 4 °C overnight. Then, each brain was transferred into a solution of 30% sucrose in PBS and left at 4 °C for 2–3 days until it sank. Subsequently, the brain was frozen in optimal cutting temperature (OCT) compound at −20 °C and cut coronally at a thickness of 30 μm with a freezing microtome (Leica, Germany). Then, the sections were transferred to slides. After incubation with 10% bovine serum albumin containing 0.3% Triton X-100 for 30 min at room temperature, the sections incubated with mouse anti-GFAP (Cat. AB5541, Millipore, USA) or mouse anti-NeuN (Cat. ABN78, Millipore, USA) at 4 °C overnight, and then incubated with Alexa 488-conjugated secondary antibody (Cat. ZF0512, ZSGB-bio, China). Finally, the fluorescence quenching agent containing DAPI dye was used to seal the slices, and staining was visualized by using a VS120 fluorescence microscope (Olympus, Japan).

Statistical analysis

All data are presented as the mean ± SEM. The data for methamphetamine self-administration in the escalation phase were tested using two-way analysis of variance (ANOVA) with repeated measures followed by Bonferroni test, and the data for methamphetamine consumption and luciferase reporter assays were tested with Student's t test or two-way ANOVA followed by Bonferroni test. In addition, statistical analysis of miR-134 and LIMK1 expression was performed with one-way or two-way ANOVA followed by Bonferroni test. The level of statistical significance was defined as $P < 0.05$.

Results

Methamphetamine self-administration with limited and extended drug access

The time course of methamphetamine self-administration following extended or limited drug availability was shown in Fig. 1a. For the escalation phase, two-way ANOVA with repeated measures revealed the significances in a main effect of treatment ($F_{(2,47)} = 265.97$, $P < 0.001$), a main effect of time ($F_{(14,658)} = 11.57$, $P < 0.001$) and a treatment × time interaction ($F_{(28,749)} = 9.87$, $P < 0.001$). During the escalation phase, the number of methamphetamine infusions progressively increased in LgA rats, and Bonferroni test revealed that escalation occurred on days 9–20 compared with day 6. In contrast, the number of drug infusions in ShA rats remained low and stable. As a result, the total methamphetamine consumption in the LgA group was significantly higher than that in the ShA group (Fig. 1a). Furthermore, we analyzed the number of methamphetamine infusions and the drug consumption during the first two hours of extended and limited self-administration of the escalation phase. Similar to the data shown in Fig. 1a, an escalation in the number of methamphetamine infusions was observed in the first 2 h of each session in the LgA group, and the number in the LgA group was higher than that in the ShA group ($F_{\text{treatment}\ (1,32)} = 26.11$, $P < 0.001$, $F_{\text{time}\ (14,448)} = 15.68$, $P < 0.001$, $F_{\text{interaction}\ (14,509)} = 5.63$, $P < 0.001$, two-way ANOVA with repeated measures; Fig. 1b). Accordingly, during the escalation phase, LgA self-administering rats cumulatively consumed more methamphetamine in the first 2 h

Table 1 Sequences of the RT and qPCR primers

	Primer	Sequence
miR-134	RT	5′-GTCGTATCCAGTGCGGCCGCTGTTCT CAT
		CTGTCTCGCACTGGATACGACCCCCTC-3′
	Upstream	5′-GCCGCTGTTCTCATCTGTCTC-3′
	Downstream	5′-CGGGCTGTGACTGGTTGAC-3′
U6 snRNA	RT	5′-AACGCTTCACGAATTTGCGT-3′
	Upstream	5′-CTCGCTTC GGCAGCACA-3′
	Downstream	5′-AACGCTTCACGAATTTGCGT-3′
LIMK1	Upstream	5′-GCCTTCGCTCTTGCTTCGT-3′
	Downstream	5′-TTCCCTCTGCCTAGCCTCTGT-3′
GAPDH	Upstream	5′-GTATTGGGCGCCTGGTCACC-3′
	Downstream	5′-CGCTCCTGGAAGATGGTGATGG-3′

of each session than ShA rats (Fig. 1b). Thus, the rats with extended access to methamphetamine displayed excessive and uncontrolled drug consumption, whereas those with limited access showed moderate and controlled drug consumption.

Extended methamphetamine self-administration, but not passive exposure, altered the levels of miR-134 and its target LIMK1 in the dlStr of rats

In methamphetamine self-administered rats, the expression of miR-134 in the dlStr was robustly increased in the LgA group, but not in the ShA group, compared with the saline control group (Fig. 2a, dlStr: $F(2,26) = 23.71$, $P < 0.001$; by one-way ANOVA). In addition, the miR-134 level in the LgA group was significantly higher than that in the ShA group (Fig. 2a). However, miR-134 levels in the dmStr were not significantly different among the three groups, although the levels in the NAc were slightly higher in the LgA group than in the control group (Fig. 2a, dmStr: $F(2,26) = 2.26$, $P > 0.05$; and NAc: $F(2,26) = 4.62$, $P < 0.05$; by one-way ANOVA). LIMK1 has been demonstrated be an important target of miR-134, and its expression is negatively regulated by miR-134. Accordingly, both mRNA and protein levels of LIMK1 in the dlStr were significantly lower in the LgA group than in the control group, while this difference was not observed in the ShA group (Fig. 2b, dlStr: $F(2,26) = 5.29$, $P < 0.05$; and Fig. 2c, dlStr: $F(2,18) = 4.21$, $P < 0.05$; by one-way ANOVA). In addition, no differences in LIMK1 mRNA or protein levels were detected among the control, ShA and LgA groups in either the dmStr or the NAc (Fig. 2b, dmStr: $F(2,26) = 0.05$, $P > 0.05$; NAc: $F(2,26) = 0.19$, $P > 0.05$; and Fig. 2c, dmStr: $F(2,18) = 2.77$, $P > 0.05$; NAc: $F(2,18) = 0.49$, $P > 0.05$; by one-way ANOVA). These results indicated that extended, rather than limited, methamphetamine self-administration resulted in notable increases in the expression of miR-134 and decreases in the expression of its target LIMK1 in the dlStr of rats.

The effects of passive methamphetamine exposure on miR-134 and LIMK1 levels were tested as well. Compared with saline injection, neither low-dose (1 mg/kg, sc) nor high-dose (5 mg/kg, sc) methamphetamine injection by the experimenter for 7, 14 or 28 days altered the levels of miR-134 in the dlStr, dmStr or NAc of rats (Fig. 3a, dlStr: $F_{(6,69)} = 0.97$, $P > 0.05$; dmStr: $F_{(6,98)} = 1.80$, $P > 0.05$; and NAc: $F_{(6,76)} = 0.40$, $P > 0.05$; by one-way ANOVA). Additionally, the same results were observed for LIMK1 mRNA levels (Fig. 3a, dlStr: $F_{(6,91)} = 0.04$, $P > 0.05$; dmStr: $F_{(6,98)} = 1.80$, $P > 0.05$; and NAc: $F_{(6,70)} = 0.55$, $P > 0.05$; by one-way ANOVA). These results suggested that excessive methamphetamine-induced alterations in dlStr miR-134 and LIMK1 levels relied on the instrumental learning of rats.

LV-miR-134-Sil down-regulated functional miR-134

We used lentivirus-mediated miR-134 sponge expression to silence endogenous miR-134. The sponge contained four recognition elements of miR-134 to absorb the miRNA molecule (Fig. 4a) and induce miR-134 loss-of-function. Luciferase activity was significantly higher in HEK293T cells cotransfected with the plasmid expressing the miR-134 sponge and the recombinant luciferase reporter with the 3'-UTR of LIMK1 than in cells cotransfected with the scrambled negative control plasmid and the recombinant luciferase reporter (Fig. 4b), suggesting that the sponge could down-regulate functional miR-134 in vitro. Next, we used a lentiviral vector to express the miR-134 sponge (LV-miR-134-Sil) in the dlStr. Two weeks after stereotaxic microinjection of LV-miR-134-Sil into the dlStr, the green fluorescence had diffused into the region of the dlStr (Fig. 4c). Immunostaining with the neuron-specific marker NeuN and the astrocyte-specific marker GFAP showed that LV-miR-134-Sil was mainly located at neurons, rather than at astrocytes, in the dlStr region of rats (Fig. 4c).

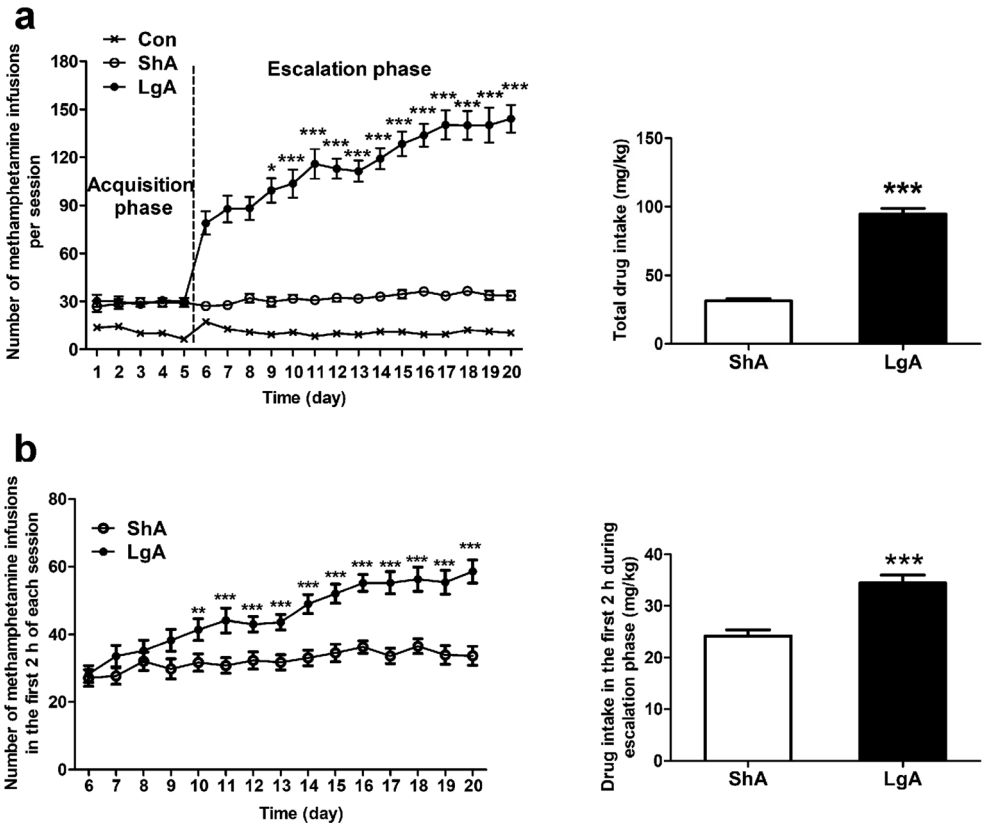


Fig. 1 Methamphetamine self-administration in rats with limited and extended access. **a** Number of methamphetamine infusions and total drug consumption during the whole self-administration training period. **b** Number of methamphetamine infusions and drug consumption in the first 2 h of each session during the escalation phase. $n = 16–17$. Number of drug infusions: $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ vs day 6 (the first day of the escalation phase); two-way ANOVA with repeated measures followed by Bonferroni test. Drug consumption: $^{***}P < 0.001$ for LgA vs ShA; Student's t test. Con: control group, ShA: short-access group, LgA: long-access group

Down-regulation of miR-134 in the dlStr reduced methamphetamine self-administration and excessive intake in rats with extended access

During the acquisition phase of methamphetamine self-administration, LV-miR-134-Sil rats and rats treated with scrambled negative control lentivirus (LV-NC) learned to respond to methamphetamine reinforcement at similar rates (Fig. 5a), demonstrating that LV-miR-134-Sil bilateral microinjection into the dlStr did not alter operant performance. Under the extended access condition, LV-miR-134-Sil rats displayed less self-administration behavior and methamphetamine consumption than LV-NC rats (Fig. 5a; number of methamphetamine infusions: $F_{\text{treatment }(1,16)} = 6.02$, $P < 0.05$, $F_{\text{time }(12,192)} = 13.51$, $P < 0.001$, $F_{\text{interaction }(12,233)} = 1.34$, $P > 0.05$, two-way ANOVA with repeated measures; drug consumption: $P < 0.05$, Student's t test). Conversely, methamphetamine self-administration behavior and drug consumption did not differ between LV-miR-134-Sil rats and LV-NC rats with limited access (Fig. 5b; number of methamphetamine infusions: $F_{\text{treatment }(1,12)} = 0.10$, $P > 0.05$, $F_{\text{time }(12,120)} = 1.53$, $P > 0.05$, $F_{\text{interaction }(12,155)} = 0.43$, $P > 0.05$, two-way ANOVA with repeated measures; drug consumption: $P > 0.05$, Student's t test). Furthermore, the data derived from the first two hours of each self-administration session confirmed these results. In LV-NC rats during the escalation phase, the cumulative methamphetamine intake in the first 2 h was significantly higher in the LV-NC-LgA group than in the LV-NC-ShA group (Fig. 5c; drug consumption: $F(1,26) = 4.47$, $P < 0.05$, two-way ANOVA followed by Bonferroni test), which was in consistent with the results shown in Fig. 1b. Under the 6 h access condition, two-way ANOVA followed by Bonferroni test revealed that methamphetamine consumption in the first 2 h was significantly lower in LV-miR-134-Sil rats (LV-miR-134-Sil-LgA) than in negative control rats (LV-NC-LgA), having decreased to the levels in the LV-miR-134-Sil-ShA and LV-NC-ShA rats (Fig. 5c). The results regarding the number of methamphetamine infusions in the first 2 h on each day were similar (Fig. 5c; self-administration behavior: $F_{\text{treatment }(3,26)} = 11.29$, $P < 0.05$, $F_{\text{time }(12,312)} = 13.34$, $P < 0.001$, $F_{\text{interaction }(36,389)} = 5.23$, $P > 0.05$, two-way ANOVA with repeated measures). These results demonstrated that LV-miR-134-Sil microinjection into the dlStr reduced excessive methamphetamine consumption under the extended, but not the limited, drug access condition.

After the behavioral test, we examined the changes in miR-134 and LIMK1 levels in the dlStr. With regard to miR-134 expression, two-way ANOVA revealed significant main effects of lentivirus treatment ($F_{(1,26)} = 19.13$, $P < 0.001$) and drug access pattern ($F_{(1,26)} = 5.29$, $P < 0.05$). Similarly, there were significant differences in LIMK1 protein levels due to main effects of lentivirus treatment ($F_{(1,26)} = 36.49$, $P < 0.001$) and drug access pattern (LIMK1 protein: $F_{(1,26)} = 8.05$, $P < 0.01$). Compared with LV-NC

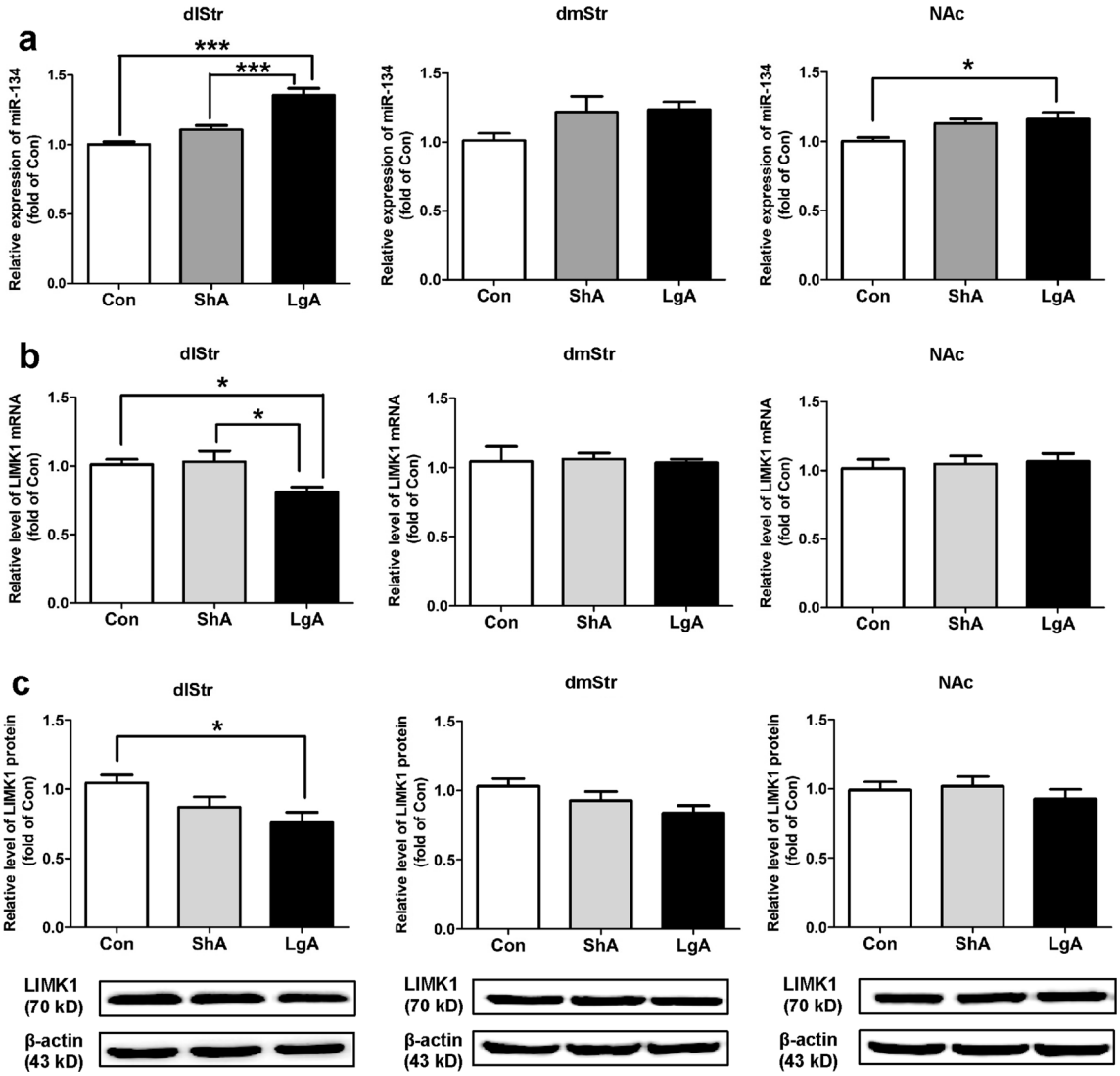


Fig. 2 Methamphetamine self-administration altered the levels of miR-134 and its target LIMK1 in rats. **a** miR-134 expression, **b** LIMK1 mRNA levels, and **c** LIMK1 protein levels. (**a**) and (**b**), $n = 9–10$; (c), $n = 7$. $^{*}P < 0.05$ and $^{***}P < 0.001$, one-way ANOVA followed by Bonferroni test. Con, control; ShA, short-access; LgA, long-access. dlStr, dorsolateral striatum; dmStr, dorsomedial striatum; NAc, nucleus accumbens

microinjection, LV-miR-134-Sil microinjection decreased miR-134 levels by approximately 30% and increased LIMK1 protein levels by approximately 50% in the dlStr in both the ShA and LgA groups (Fig. 5d), suggesting that miR-134 sponge expressed in the dlStr could down-regulate miR-134 levels and function in vivo. Furthermore, among LV-NC rats, the LgA group exhibited a higher miR-134 level and a lower LIMK1 level than the ShA group (Fig. 5d), which was in consistent with our above result. Therefore, lentivirus-mediated miR-134 sponge expression in the dlStr resulted in down-regulation of miR-134 in vivo and a subsequent reduction in LIMK1 suppression, which contributed to the decrease in excessive methamphetamine intake under the extended access condition.

Discussion

Increasing evidence indicates that excessive drug consumption is sufficient for the emergence of addictive behaviors such as compulsive drug seeking and taking, increased motivation and high risk of relapse (Jonkman et al. 2012b). Therefore, disruption of excessive drug intake may be beneficial for impeding the development of drug addiction. The underlying mechanisms responsible for excessive intake and the transition to addiction are debated. A number of neurobehavioral mechanisms have been suggested, including reward allostasis, tolerance, incentive sensitization and habit formation (Ahmed and Koob 2005; Berridge 2007; Everitt and Robbins 2005; Oleson and Roberts 2009). The results of the present study indicated that dlStr miR-134, a brain-specific miRNA

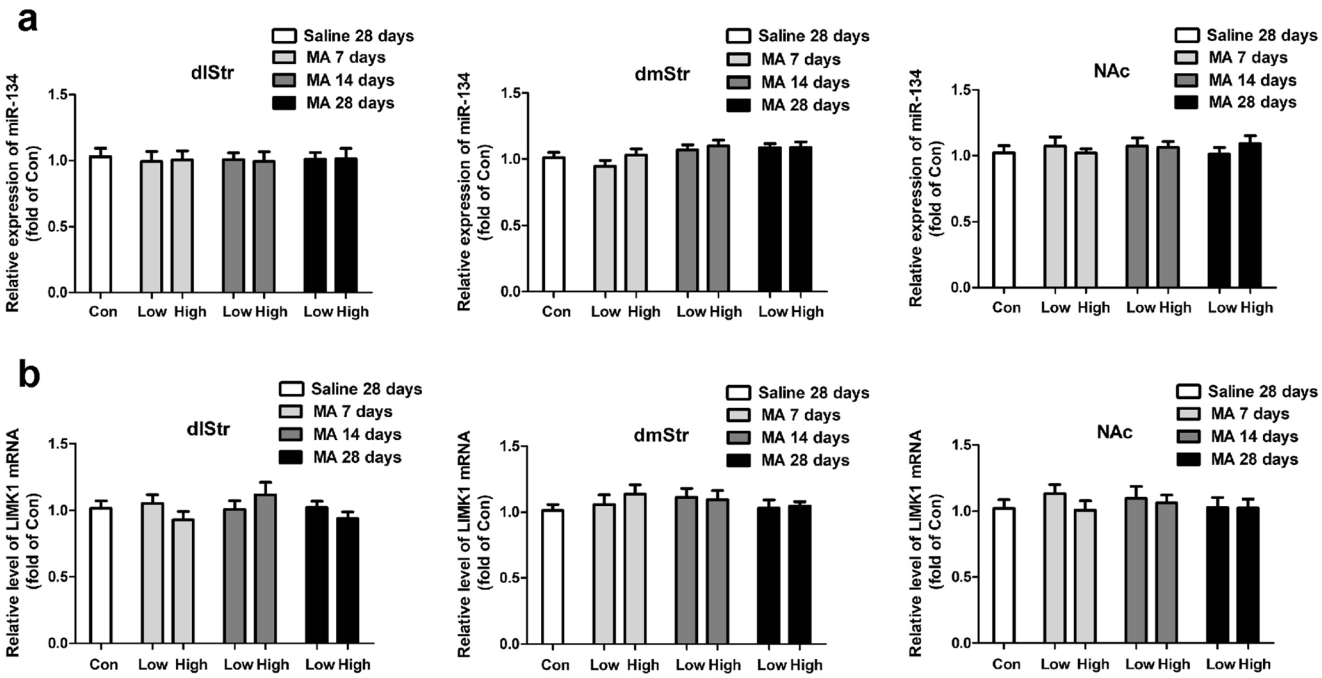


Fig. 3 Passive methamphetamine injection did not alter miR-134 and LIMK1 levels in rats. **a** miR-134 expression and **b** LIMK1 mRNA levels. $n = 10–15$. One-way ANOVA followed by Bonferroni test. Con, control; Low, methamphetamine 1 mg/kg; High, methamphetamine 5 mg/kg; dlStr, dorsolateral striatum; dmStr, dorsomedial striatum; NAc, nucleus accumbens. The control group received saline injections for 28 days

regulating synaptic transmission and plasticity, contributed to excessive and uncontrolled methamphetamine self-administration under an extended access condition, but not to moderate drug consumption under a limited access condition, in rats, supporting the hypothesis of S-R habit formation.

Drug addiction is characterized by a compulsion to take drugs and a loss of control in limiting intake. An animal model that represents the switch from recreational drug use to addictive-like use was first developed in cocaine self-administering rats by permitting the animals extended access to the drug (Ahmed and Koob 1998). Typically, rats given cocaine access for only 1 or 2 h daily exhibited regular and stable drug consumption that did not change over months of repeated access. In contrast, following extended drug access (6 h or more), a transition to increased drug intake was observed in the rats, usually within 7–14 days. In addition, excessive drug consumption was shown in the extended-access rats. This pattern arguably resembles the transition from controlled (limited) to uncontrolled ("binge and run") drug use typical for human addicts. This model has since been replicated with other abused drugs including heroin, methamphetamine, amphetamine, methylphenidate and phencyclidine (Ahmed et al. 2000; Carroll et al. 2005; Gipson and Bardo 2009; Kitamura et al. 2006; Marusich et al. 2010), indicating that excessive and uncontrolled intake with extended access is a general phenomenon that occurs across multiple drug classes. Importantly, cocaine self-administration with extended access in rats has been proven to produce neuroimaging alterations that closely mimic hallmark imaging findings in human addicts (Gozzi et al. 2011). Therefore, the limited-to-extended daily access self-administration model is suitable for examining the neurobiological mechanisms underlying the transition from regular drug use to drug addiction. In this study, we replicated the same animal model. An escalation of methamphetamine intake was observed in rats switched to extended access (6 h/session/d) but not in rats maintained on limited access (2 h/session/d), consistent with our and others' previous reports (Du et al. 2016; Kitamura et al. 2006; Rogers et al. 2008). As a result, we observed robust methamphetamine consumption approaching 100 mg/kg during the approximately 3-week training period in LgA rats. Thus, our self-administering rats displayed excessive and uncontrolled methamphetamine intake under the extended access condition that resembled the drug use pattern prevalent in methamphetamine addicts (Li et al. 2010).

Using the rat model of extended access self-administration, some neurobehavioral and neuroimaging evidence has suggested that excessive drug exposure causes functional and structural alterations in the dlStr that critically contribute to the switch to addiction (Corbit et al. 2012). However, little has been reported on the intracellular molecular events. miRNAs are considered to be master regulators of gene expression in mammals, and they spatiotemporally control the morphology and function, as well as the plasticity, of the central nervous system. Recently, a class of miRNAs (including miR-124, miR-132, miR-134

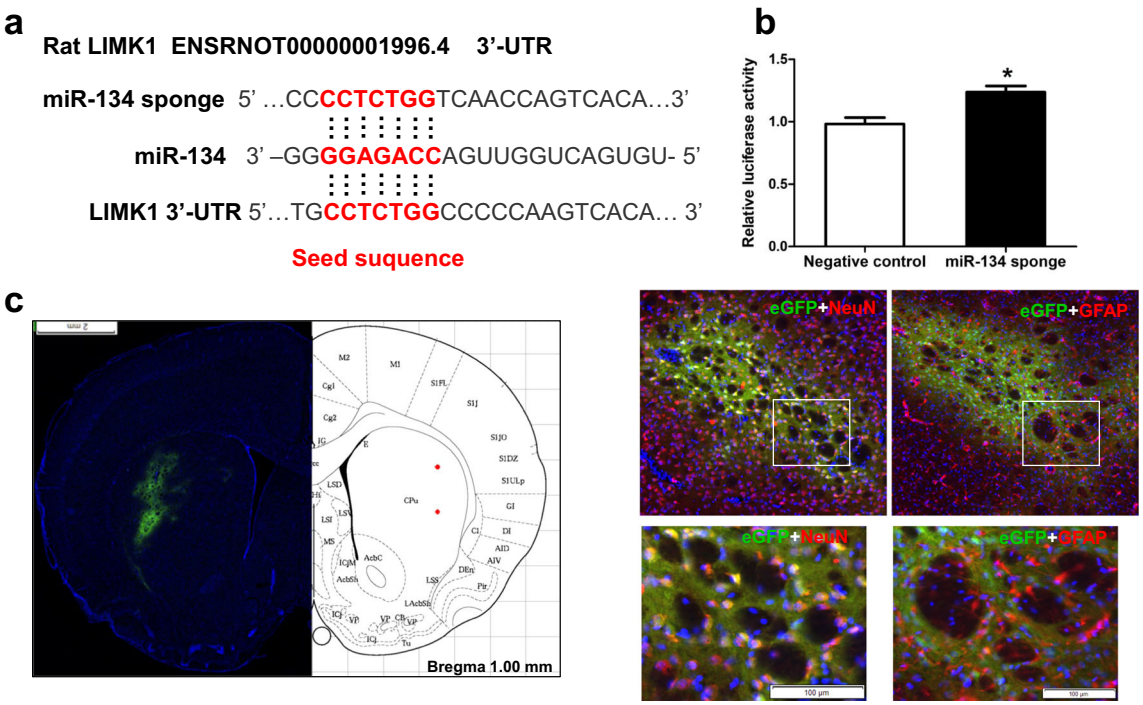
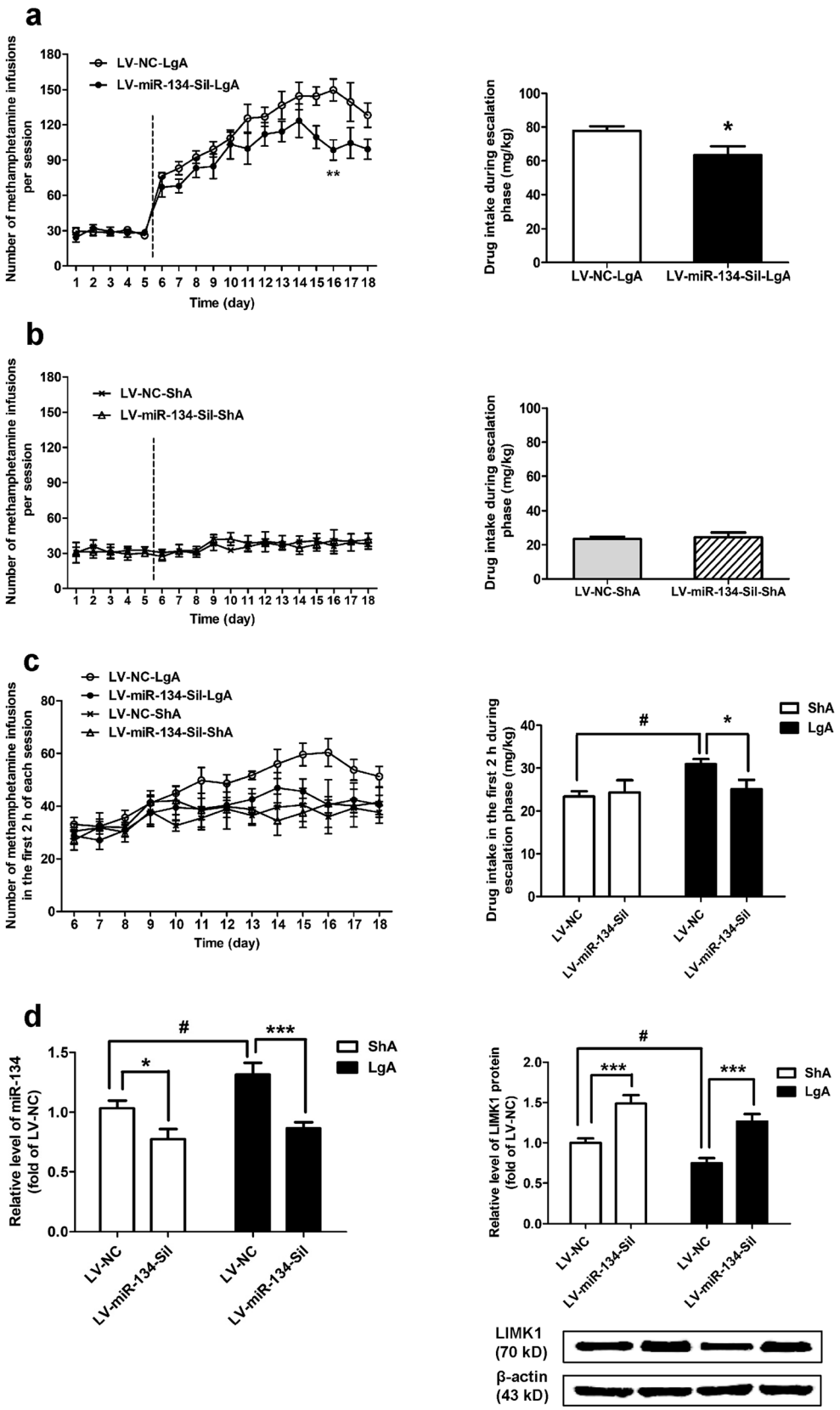


Fig. 4 The miR-134 sponge down-regulated functional miR-134. **a** Recognition element sequence of the miR-134 sponge. **b** Results of the in vitro luciferase reporter assay. $n = 4$, $^*P < 0.05$ vs negative control, Student's t test. **c** Rats were stereotactically infused with the miR-134 sponge LV-miR-134-Sil. The left panel showed the rat brain in stereotaxic coordinates and the locations in the dlStr targeted by the virus infusions (The coronal section was based on Paxinos and Watson. The Rat Brain in Stereotaxic Coordinates, 5th ed. Elsevier Academic Press, 2005), and the right panel showed representative immunostaining labeled with GFAP or NeuN (20× magnification). Green staining indicated eGFP from the virus, while red staining indicated NeuN or GFAP immunofluorescence. The cell nuclei were stained with DAPI (blue). 3'-UTR, 3′-untranslated region; dlStr, dorsolateral striatum; GFP, green fluorescent protein; GFAP, glial fibrillary acidic protein; NeuN, neuronal nuclei; Sil, silence

and miR-212) that primarily localize to the synaptodendritic compartment have been found to be especially important for neuronal function and synaptic plasticity (Hollander et al. 2010; Sadakierska-Chudy et al. 2017). Dorsal striatal miR-132 and miR-212 have been reported to be involved in the transition to escalated cocaine intake in rats, but the studies did not distinguish between the dorsolateral and dorsomedial subregions (Hollander et al. 2010, Sadakierska-Chudy et al. 2017). In this study, we found that miR-134 in the dlStr contributed to excessive voluntary methamphetamine consumption in rats. Compared with saline self-administration, extended, but not limited, methamphetamine self-administration led to a robust increase in miR-134 expression in the dlStr of rats and a decrease in LIMK1, a known target of miR-134. In addition, in the dlStr of LgA rats, the decrease in miR-134 was accompanied by increases in both mRNA and protein levels of LIMK1, indicating that miR-134 negatively regulates LIMK1 expression by targeting both mRNA degradation and translational repression. In experimenter-administered rats, however, passive exposure to methamphetamine (1 and 5 mg/kg for 7, 14 and 28 days, sc) did not alter miR-134 and LIMK1 levels in the dlStr. We first considered whether or not the total drug amount was sufficient. The cumulative methamphetamine exposure in the high-dose group (5 mg/kg injection for 28 days, sc) was as high as 140 mg/kg; therefore, the failure of the drug to alter miR-134 and LIMK1 expression was likely not due to insufficiency of drug exposure. In fact, considering the bioavailability of methamphetamine delivered by sc injection (Gentry et al. 2004), the plasma concentration of methamphetamine exposure to the rats in the high-dose group was not lower than that in the LgA group. Notably, the main difference between experimenter-administration and self-administration is that self-administered rats experience instrumental learning, and extended-session self-administering rats may also experience S-R habit learning. A previous study revealed that miR-134 expression was synapse activity-dependent (Valluy et al. 2015); thus, we presumed that the miR-134 up-regulation in the dlStr of LgA self-administering rats not only resulted from excessive methamphetamine exposure but also relied on habit learning. In fact, elevated expression of synapse-related miR-132 and miR-212 in the dorsal striatum has also been observed in cocaine self-administered rats but not in animals that passively receive the same amount of cocaine (Sadakierska-Chudy et al. 2017).



In general, loss-of-function or gain-of-function experiments are necessary to elucidate the relationship between a certain molecule and behavioral alterations. As a brain-specific miRNA, miR-134 has been reported to predominantly

◀ **Fig. 5** Down-regulation of miR-134 in the dlStr reduced methamphetamine self-administration and drug consumption under the extended access condition. **a** LV-miR-134-Sil bilateral microinjection into the dlStr reduced the number of methamphetamine infusions and drug intake under the extended (6 h) access condition. $n = 9$. Drug infusion: $^{**}P < 0.01$, compared with the LV-NC-LgA group, two-way ANOVA with repeated measures followed by Bonferroni test; drug intake: $^{*}P < 0.05$, Student's t test. **b** LV-miR-134-Sil failed to alter the number of methamphetamine infusion or drug intake under the limited (2 h) access condition. $n = 6$. **c** The number of methamphetamine infusions and drug intake in the first 2 h of each session. n = 6–9. Drug infusion: two-way ANOVA with repeated measures followed by Bonferroni test; drug intake: $^{*}P < 0.05$, $^{\#}P < 0.05$, two-way ANOVA followed by Bonferroni test. **d** LV-miR-134-Sil bilateral microinjection into the dlStr decreased miR-134 levels and increased LIMK1 protein levels in vivo. n = 6–9. $^{*}P < 0.05$, $^{***}P < 0.001$, $^{\#}P < 0.05$, two-way ANOVA followed by Bonferroni test. NC: negative control; Sil: silence; ShA: short-access; LgA: long-access

localize to neuronal dendrites, and it negatively regulates dendritic spine morphology by targeting LIMK1 (Schratt et al. 2006). The previous studies showed that inactivation or intervention of dlStr (rather than dmstr) in rodents can selectively destroy cocaine habitual and compulsive drug use and drug-seeking behavior (Jonkman et al. 2012a). Animal studies have shown that after long-term cocaine self-administration, the release of dopamine in dlStr (not NAc) increased significantly when the drug cues were exposed, and microinjection of dopamine receptor blockers or glutamate AMPA receptor blockers in dlStr could weaken the drug seeking behavior maintained by the cues in rats (Ito et al. 2002; Vanderschuren et al. 2005; Willuhn et al. 2012). All these studies have shown that dlStr, perhaps not dmStr or NAc, played an important role in excessive and compulsive drug intaking and seeking behavior. Considering that excessive and uncontrolled methamphetamine intake significantly increased miR-134 expression in dlStr, the following study focused on the effect of down-regulation of dlStr miR-134 on long-term methamphetamine self-administration. In the present study, local microinjection of a lentivirus carrying miR-134 sponge (LV-miR-134-Sil) was used to silence endogenous miR-134 in vivo. We found that down-regulation of miR-134 in the dlStr clearly reduced the number of methamphetamine infusions and excessive consumption in self-administering rats under the extended, but not the limited, access condition. The dlStr is known to be a critical site for S-R habit learning; therefore, our current finding not only demonstrates the contribution of dlStr miR-134 to excessive and uncontrolled methamphetamine intake but also supports the hypothesis that habit formation is an important mechanism underlying the transition from recreational and controlled drug use to uncontrolled drug use and addiction. In contrast, drug self-administration with limited access is considered to be a goal-directed behavior and is controlled by the dmStr, which may explain why miR-134 down-regulation in the dlStr did not alter methamphetamine intake in ShA rats. Undoubtly, to exclude the effects of NAc or dmStr miR-134 on excessive methamphetamine consumption, further study on the changes of self-administration behaviors and drug intake after down-regulation of NAc or dmStr miR-134 in rats should be conducted.

Drug addiction is a classic disease of dysfunctional neuroplasticity. Many studies have focused on synaptic plasticity in the ventral tegmental area (VTA), NAc, amygdala and prefrontal cortex (PFC) that is associated with reward and motivation learning and executive control related to addiction (Jones and Bonci 2005; Luscher and Malenka 2011). However, less attention has been paid to dorsal striatal plasticity. In term of functional plasticity, chronic nicotine exposure induced long-term potentiation (LTP) and increased the number of silent synapses in medium-spiny neurons (MSNs) in the dlStr in mice (Xia et al. 2017), while chronic ethanol intake attenuated ERK phosphorylation and long-term depression (LTD) induction in the dlStr of rats (Cui et al. 2011). With regard to structural plasticity, chronic nicotine produced a lasting expansion in the dendritic complexity of MSNs in the dlStr, but not the dmStr (Ehlinger et al. 2017). As to methamphetamine in particular, Jedynak et al. reported that passive exposure to methamphetamine for 1 month and withdrawal for 3 months produced a significant increase in mushroom and thin spines on MSNs in the dlStr but a significant decrease in mushroom spines in the dmStr (Jedynak et al. 2007). These findings not only reveal that exposure to addictive drugs induces persistent and selective changes in the circuit connectivity of the dlStr that may facilitate the development of S-R habits, thereby promoting and sustaining addiction-related behavior, but also indicate that the elaborate patterns of plasticity depend on drug type. Some studies on excitatory synapses in the hippocampus and PFC have revealed that miR-134, by affecting LIMK1-cofilin signaling, negatively regulated dendritic spine size and thereby participated in recognition deficits, status epilepticus like-activity, and depression-like behaviors (Fan et al. 2018; Schratt et al. 2006). In addition to affecting dendritic structure, LIMK1 knockout has also been reported to enhance LTP (Meng et al. 2002), suggesting that miR-134 and LIMK1 regulate synaptic transmission and plasticity through complex mechanisms. In this study, we found that extended methamphetamine self-administration resulted in both miR-134 up-regulation and LIMK1 down-regulation, consistent with a negative effect of miR-134 on LIMK1 expression. In addition, along with increasing LIMK1 expression, down-regulation of dlStr miR-134 reduced excessive methamphetamine intake and extended self-administration. These results indicated that LIMK1 and its downstream signals may be key links in the pathway by which dlStr miR-134 modulates excessive methamphetamine consumption. Unfortunately, until now, there has been a lack of detailed studies on structural and functional changes in striatal synaptic plasticity during the transition to methamphetamine addiction.

Moreover, there have been no reports on the role of miR-134-LIMK1 signaling in synaptic transmission and plasticity in striatal MSNs. These topics should be addressed in future research.

In conclusion, in experiments using a methamphetamine self-administration model with extended access, we found that compared with moderate and controlled drug use, excessive and uncontrolled methamphetamine intake specifically led to up regulation of dlStr miR-134 in rats. Meanwhile, the mRNA and protein levels of dlStr LIMK1, a target of miR-134 that contributes to dendritic spine reorganization, were decreased. Furthermore, down-regulation of dlStr miR-134 reduced methamphetamine self-administration behavior and excessive drug intake under conditions of extended, but not limited, access. These findings indicated that dlStr miR-134, perhaps via its target LIMK1, contributed to excessive and uncontrolled methamphetamine consumption, supporting the hypothesis that S-R habit formation is an important mechanism underlying the transition from controlled drug use to uncontrolled drug use and addiction.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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