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Striatal modulation of BDNF expression using microRNA124a-expressing lentiviral vectors impairs ethanol-induced conditioned-place preference and voluntary alcohol consumption

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Abstract

Alcohol abuse is a major health, economic and social concern in modern societies, but the exact molecular mechanisms underlying ethanol addiction remain elusive. Recent findings show that small non-coding microRNA (miRNA) signaling contributes to complex behavioral disorders including drug addiction. However, the role of miRNAs in ethanol-induced conditioned-place preference (CPP) and voluntary alcohol consumption has not yet been directly addressed. Here, we assessed the expression profile of miR124a in the dorsal striatum of rats upon ethanol intake. The results show that miR124a was downregulated in the dorso-lateral striatum (DLS) following alcohol drinking. Then, we identified brain-derived neurotrophic factor (BDNF) as a direct target of miR124a. In fact, BDNF mRNA was upregulated following ethanol drinking. We used lentiviral vector (LV) gene transfer technology to further address the role of miR124a and its direct target BDNF in ethanol-induced CPP and alcohol consumption. Results reveal that stereotaxic injection of LV-miR124a in the DLS enhances ethanol-induced CPP as well as voluntary alcohol consumption in a two-bottle choice drinking paradigm. Moreover, miR124a-silencer (LV-siR124a) as well as LV-BDNF infusion in the DLS attenuates ethanol-induced CPP as well as voluntary alcohol consumption. Importantly, LV-miR124a, LV-siR124a and LV-BDNF have no effect on saccharin and quinine intake. Our findings indicate that striatal miR124a and BDNF signaling have crucial roles in alcohol consumption and ethanol conditioned reward.

Introduction

Ethanol abuse is a major health economic and social concern in modern societies. Substance abuse, including alcohol, is characterised by compulsive drug taking and seeking, and the consumption of substances of abuse converges on a shared pathway within the limbic system that mediates motivated behaviors (Nestler & Carlezon, 2006; Stuber *et al.*, 2010). The dorso-lateral striatum (DLS) is involved in the development of habits, including maladaptive persistent habits (Yin *et al.*, 2004, 2006; Yin & Knowlton, 2006; Tricomi *et al.*, 2009), and is involved in advanced stages of addiction, when drug use progresses towards a compulsive, habitual pathology and behaviors directed mostly at drug seeking (Gerdeman *et al.*, 2003). The transition from voluntary drug use to more compulsive and habitual modes of drug-seeking behavior represents a transition from prefrontal cortical to striatal control over-responding, and from ventral to more dorsal striatal subregions (Everitt & Robbins, 2005; Everitt *et al.*, 2008; Everitt & Robbins, 2013). An increase in dopaminergic transmission is observed to occur either by direct action of drugs on dopaminergic neurons (cocaine, nicotine) or indirectly by inhibition of γ-aminobutyric acid-ergic interneurons in the ventral tegmental area (alcohol, opiates; Nestler & Carlezon, 2006). As for most substances of abuse, ethanol abuse is typically a multigenetic brain disorder, implying combined changes of expression of several hundred genes, producing its rewarding effects through an interaction with a large array of brain pathways, and leading to persistent alterations (neuroplastic, structural and functional) in related brain centers. Neuroadaptations underlying behavioral sensitisation result in long-lasting functional changes in these brain circuits, increasing compulsive patterns of ethanol seeking and craving (Simerly, 2006). Epigenetic modifications and neuroadaptations imply gene expression changes of a large array of genes whose control is not yet established, as the exact molecular mechanisms underlying ethanol addiction are poorly understood.

Recently, however, the roles of some microRNAs (miRNAs) in mammalian midbrain dopaminergic neurons have been identified. miRNAs are small non-protein-coding RNA transcripts that can regulate the expression of messenger RNAs that code for proteins. Because of their highly pleiotropic nature, each miRNA has the potential to regulate hundreds or even thousands of protein-coding RNA transcripts (Thomson *et al.*, 2006; Filipowicz *et al.*, 2008;

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Jonkman & Kenny, 2013). miRNAs therefore are important epigenetic regulators, and control important signaling patterns (Cao *et al.*, 2006; Franke *et al.*, 2012), contributing to various disease processes (Kuss & Chen, 2008; Silber *et al.*, 2009; Sato *et al.*, 2011). miRNAs act as key spatiotemporal regulators in post-transcriptional control of gene expression during dendritic morphogenesis and synapse development, controlling the expression of hundreds of genes involved in neuroplasticity and in the function of synapses (Silber *et al.*, 2009; Dreyer, 2010; Sato *et al.*, 2011; Sun *et al.*, 2012). Moreover, miRNAs have been found to relate to addictive behaviors (Kim *et al.*, 2007; Dreyer, 2010; Impey *et al.*, 2010). miRNAs in the dorsal striatum control the escalation of drug intake in rats (Jonkman & Kenny, 2013), highlighting the central role for miRNAs in drug-induced neuroplasticity in brain reward systems that drive the emergence of compulsive-like drug use in animals. For example, expression changes of miR124a in the brain reward pathway strongly affect behavior towards drugs of abuse, for example cocaine (Chandrasekar & Dreyer, 2011), and miR124a regulates cocaine-induced expression plasticity (Chandrasekar & Dreyer, 2009). miR124a is strongly expressed in the brain and regulates adult neurogenesis (Cao *et al.*, 2007; Cheng *et al.*, 2009; Kawahara *et al.*, 2012), and promotes neuronal differentiation by triggering brain-specific alternative pre-mRNA splicing (Makeyev *et al.*, 2007). In addition, it is involved in several brain pathologies, including aging and Alzheimer's disease (Fang *et al.*, 2012; Qi *et al.*, 2012). miR124a targets and regulates the expression of both the mRNA of the anti-neural function protein SCP1 (small C-terminal domain phosphatase 1; Visvanathan *et al.*, 2007) and of the Sry-related box Sox9 transcription factor whose overexpression abolishes neuronal differentiation (Cheng *et al.*, 2009). Besides these important functions, miR124 also plays putative roles in hepatocellular carcinoma (Zheng *et al.*, 2012), gastric cancer (Xia *et al.*, 2012b), and in brain tumor differentiation and progression (Silber *et al.*, 2008; Xia *et al.*, 2012a). Finally, miR124a in the dopaminergic midbrain pathway also strongly affects the expression of several neurotransmitters, mainly brain-derived neurotrophic factor (BDNF; Chandrasekar & Dreyer, 2009, 2011), which in turn is involved in the survival and function of midbrain dopaminergic neurons and also in drug reward and relapse (Corominas *et al.*, 2007; Briand & Blendy, 2010; Ghitza *et al.*, 2010). Furthermore, alcohol-induced expression changes of some miRNAs induce changes at protein levels, disrupt tight junctions, and increase cell permeability and drastically affect cell signaling (Kim *et al.*, 2007; Dreyer, 2010; Hollander *et al.*, 2010), thus providing evidence of the control of mRNA expression by miRNAs in alcohol abuse.

Our previous studies have shown a crucial function of BDNF, through its receptor tropomyosin-related kinase B (TrkB), in the enhancement of locomotor activity, behavioral sensitisation, cocaine-induced conditioned-place preference (CPP) acquisition as well as extinguished cocaine-induced CPP reinstatement following cocaine priming (Bahi *et al.*, 2008). Human BDNF expression is controlled by complex mechanisms, and its transcription is regulated by multiple promoters driving the expression of different coding transcripts (Pruunsild *et al.*, 2007; Caputo *et al.*, 2011). Genes bearing multiple binding sites for transcription factors show higher probabilities to be targeted by miRNAs and to harbor more miRNA-binding sites on average (Cui *et al.*, 2007; Caputo *et al.*, 2011). Indeed, BDNF expression is regulated by a group of miRNAs, and to date some of these sites have been experimentally validated [miR-1/206 (Lewis *et al.*, 2003); miR-30a, miR-30a-5p and miR-195 (Mellios *et al.*, 2008); miR-124 and let-7d (Chandrasekar & Dreyer, 2009); miR-15a (Friedman *et al.*, 2009); and miR-210 (Fasanaro *et al.*, 2009)].

In the present study we further evaluate the role of BDNF and miR124a in alcohol abuse, using viral manipulation of local gene expression of either miR124a or BDNF as described in our previous studies (Karpova *et al.*, 2011; Bahi & Dreyer, 2012), and we show that striatal modulation of both miR124a and BDNF expression impairs ethanol-induced CPP and voluntary alcohol intake in rats.

Materials and methods

Animals

Adult male Wistar rats were group-housed (five per cage) at room temperature (approximately 22 °C) with a 12 : 12 h light : dark cycle (light on at 06:00 h), and allowed to adapt to this environment for a period of 7 days before the experiments began. Bedding was produced locally and autoclaved before use, and rats had free access to tap water and standard rodent chow diet obtained from the National Feed and Flour Production and Marketing Company LLC (Abu Dhabi, UAE). All animal care and use were in accordance with the National Institutes of Health 'Guide for the Care and Use of Laboratory Animals'. The procedures were approved by the institutional Animal Research Ethics Committee (Approval No. A17-12).

BDNF and miR124a quantification after voluntary ethanol intake

Rats were singly housed 10 days prior to the initiation of ethanol access. Fluid solutions were provided in cylindrical bottles equipped with regular steel drinking spouts. Rats were given two bottles – one containing tap water, and the other containing either water ($n = 8$) or a solution of 5% ethanol in tap water (v/v; $n = 8$) for 15 days. The placement order of the bottles was inverted daily to ensure that consumption levels were not due to a side preference.

Rats were killed 24 h after the final ethanol access by decapitation, and brain regions were dissected out and frozen in TRizol according to the manufacturer's instructions. The micro-dissection procedure was performed according to rat stereotaxic coordinates (Paxinos & Watson, 1998), yielding lateral and medial subdivisions of the dorsal striatum [DLS and dorso-medial striatum (DMS), respectively]. The tissue was isolated via punch of a coronal slice 2.5 mm wide in the anterior–posterior direction, beginning immediately posterior to the prefrontal cortex (Supporting Information Fig. S1).

Total RNA was extracted from animal tissues using TRizol, and tested for purity ($A_{260/280}$ ratios). Reverse transcription and real-time polymerase chain reaction (PCR) amplification was performed using Oligo-dT standard primers. Real-time PCR was performed with SYBR-Green. Reactions were performed in a 20-μL final volume using 2 μL of cDNA. The PCR protocol used consisted of a 45-s denaturation at 94 °C, followed by 45-s annealing and extension at 64 °C for 40 cycles. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as reference gene. The primers used were: *miR124a*: 5′-TCC GTG TTC ACA GCG.GAC-3′ and 5′-CAT TCA CCG CGT GCC TTA-3′ (Chandrasekar & Dreyer, 2009); *BDNF*: 5′-GGT TCG AGA GGT CTG ACG AC-3′ and 5′-CAA AGG CAC TTG ACT GCT GA-3′; *GAPDH*: 5′-ATG ACT CTA CCC ACG GCA AG-3′ and 5′-CAT ACT CAG CAC CAG CAT CAC-3′ (Bahi *et al.*, 2008).

Lentiviral constructions and production

The construction of the lentiviral vector (LV)-BDNF-expressing vector was performed as described previously (Bahi *et al.*, 2008). Briefly, rat BDNF cDNA was amplified by PCR and cloned into pTK431 using *BamHI* and *XhoI* restriction sites. The construction of LV-miR124a- and LV-siR124a-expressing vectors was described in a previous study (Chandrasekar & Dreyer, 2011). All constructions were confirmed by sequencing. For lentiviral production a triple transfection approach was used, as described previously (Bahi *et al.*, 2005; Bahi & Dreyer, 2008, 2012; Chandrasekar & Dreyer, 2011). Briefly, the pTK431-expressing vectors were co-transfected into HEK293T cells together with the packaging pΔNRF and the envelope pMDG-VSV-G plasmids. The viral particles were purified and concentrated using ultracentrifugation.

Stereotaxic injection of LV-miR124a, LV-siR124a and LV-BDNF in the dorsal striatum

For stereotaxic surgery, rats were anesthetised with a ketamine/xylazine mixture [100 mg/kg and 10 mg/kg, respectively, intraperitoneally (i.p.)] and installed in a stereotaxic frame. Using a precision Hamilton micro-syringe with a 26G needle, rats were bilaterally infused with 2 μL viral solution using the following coordinates: DLS (1.2 mm anterior to Bregma, ± 3.7 mm lateral to the medial suture, 4.5 mm ventral to the skull surface); medio-lateral striatum (MLS; 1.2 mm anterior to Bregma, ± 1.5 mm lateral to the medial suture, 4.5 mm ventral to the skull surface). All viral injections were performed according to the rat stereotaxic coordinates (Paxinos & Watson, 1998). After surgery, animals were left in recovery for 7 days.

Ethanol-induced CPP

The place conditioning apparatus was a rectangular chamber that consisted of two conditioning compartments (30 × 30 × 30 cm) separated by a removable door (10 cm). One of the conditioning chambers had white walls and a stainless-steel large grid floor; the other had black walls and a narrow grid floor. Rats could access the entire apparatus when the guillotine door was removed (Bahi, 2012; Bahi & Dreyer, 2012). The place conditioning procedure consisted of three phases: pre-conditioning, conditioning and post-conditioning tests, as previously described (Bahi & Dreyer, 2012).

Pre-conditioning

The rats were injected with saline and placed between the two compartments with the guillotine doors removed, allowing free access to the two conditioning compartments for a 15-min period. The time spent in each conditioning compartment was manually recorded. Most rats spent approximately 50% of the time in each chamber ($P > 0.05$). Therefore, conditioning was performed using an unbiased, balanced protocol.

After baseline assessment, the rats were stereotaxically injected with viral vectors as described above, and given a 7-day recovery period.

Conditioning

During conditioning, rats were injected i.p. with 0.5 g/kg of ethanol (prepared from 20% ethanol solution dissolved in 0.9% isotonic saline v/v) once, and immediately confined to one conditioning compartment for 30 min. On alternate schedules, rats received saline injections (volumes were calculated according to body weight) and were confined to the other compartment for 30 min. The conditioning phase with ethanol and saline on alternative schedules (morning and afternoon) lasted for 5 days.

Post-conditioning test

Animals were injected with saline and immediately placed between the two compartments, with free access to both conditioning compartments for 15 min. The time spent in each box was manually recorded. The preference for the ethanol-paired place was expressed as the mean difference between the duration spent in it during the pre- and post-conditioning tests.

Two-bottle choice voluntary ethanol consumption and preference

This procedure has been described previously (Bahi & Dreyer, 2012). Briefly, two drinking bottles with 5% (v/v) alcohol or drinking tap water were available to the animals *ad libitum* during a 5-day period. In order to avoid a possible side preference, the positions of the bottles were changed daily. The drinking bottles (g) and the body weight (g) were weighed daily. The amount of consumed ethanol (g) was calculated after every measurement and expressed per 1000 g of body weight (1 mL = 0.789 g). The total fluid intake was worked out by calculating the volumes of water and ethanol consumed and adjusting to 1000 g of body weight.

Saccharin and quinine consumption and preference were measured in the same rats 7 days after measurement of ethanol drinking. Methods were as described for ethanol voluntary intake, and two concentrations of each tastant were evaluated (saccharin: 0.035 and 0.07%; quinine: 0.015 mM and 0.03 mM) in ascending order, consistent with our previous similar studies (Bahi & Dreyer, 2012).

BDNF and miR124a quantification following viral injection

After completion of the quinine voluntary intake, rats were given access to two bottles – one containing tap water, and the other containing either water or a solution of 5% ethanol in tap water for 15 days. The placement order of the bottles was inverted daily to ensure that consumption levels were not due to a side preference. Rats were killed 24 h later by decapitation and tissue was processed as described previously.

Determination of blood ethanol concentration (BEC)

In brief, rats were given an i.p. injection with a 20% (v/v) solution of ethanol (3 g/kg of body weight) in isotonic sterile saline. Tail blood samples were collected in heparinised capillary tubes at 30, 60, 120 and 240 min following injection. The determinations of BEC expressed as g/dL were made using the nicotinamide adenine dinucleotide (NAD)-NADH enzymatic assay, and concentrations were calculated using an ethanol standard curve.

Statistical analysis

For statistical comparisons, the software package SPSS (version 19.0) was used. All data were expressed as means ± SEM. BDNF and miR124a mRNA expression were analysed using a two-way analysis of variance (ANOVA), with treatment and region as the between-subject factors. Ethanol-induced CPP behavior was analy-

sed using a one-way ANOVA. Voluntary ethanol or tastant consumption and total volume intake were analysed using a two-way ANOVA with repeated measures (virus group × ethanol/tastant concentration). BDNF and miR124a mRNA expression following viral injection were analysed using one-way ANOVAs. BEC data were analysed using one-way ANOVAs with repeated measures (virus groups were the between-subject factor). *Post hoc* individual mean comparisons were performed with the Bonferroni's test when *F*-values were significant. The level of statistical significance was set at $P < 0.05$ at all times.

Results

Expression of miR124a and BDNF in the dorsal striatum of rats following voluntary alcohol intake

Figure 1 shows that miR124a and BDNF levels were affected following 15 days of voluntary alcohol intake in rats. In fact, and as revealed by a two-way ANOVA, miR124a expression was decreased about threefold in the DLS but not changed in the DMS (main effect of treatment $F_{1,28} = 10.597$, $P = 0.003$; main effect of region $F_{1,28} = 5.921$, $P = 0.022$). More importantly the interaction between the treatment and the region was significant ($F_{1,28} = 9.001$, $P = 0.006$; Fig. 1A). Together these data indicate that chronic ethanol drinking results in the reduction of miR124a expression in the dorsal striatum, and that this effect is specific to the dorso-lateral region. To determine the potential role of miR124a in ethanol addiction, we studied the effect of miR124a on the expression of BDNF, a common signal molecule involved in basically all drugs of abuse, including alcohol (for review, see for example Corominas *et al.*, 2007; Davis, 2008; McGinty *et al.*, 2010; Autry & Monteggia, 2012), in the rat dorsal striatum. As expected, 15-day alcohol intake significantly increased about twofold the expression of BDNF, as depicted in Fig. 1B; a two-way ANOVA analysis revealed that, compared with the water-drinking group, BDNF expression was increased in the DLS of ethanol-exposed animals as indicated by a main effect of treatment ($F_{1,28} = 5.492$, $P = 0.026$) and a main effect of region ($F_{1,28} = 30.121$, $P < 0.001$). More interestingly, the interaction between the two factors was significant ($F_{1,28} = 7.464$, $P = 0.011$). These findings demonstrate that 2 weeks of ethanol intake upregulates dorso-lateral striatal BDNF.

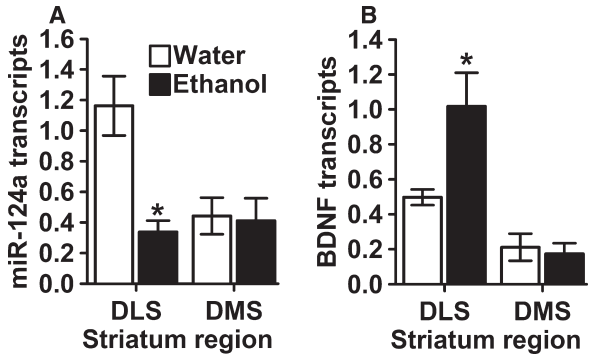

FIG. 1. Voluntary ethanol consumption induced changes in the miRNA124a and BDNF mRNA levels in the rat striatum. (A) miR124a and (B) BDNF mRNA levels were normalised against GAPDH in the corresponding samples. Histograms represent mean ± SEM, $n = 8$, $^{*}P < 0.05$ (two-way ANOVA, Bonferroni's *post hoc* test).

The effects of DLS LV-miR124a and LV-BDNF expression on ethanol-induced CPP

To test the effects of miR124a on ethanol-induced CPP, both gain-of-function and loss-of-function approaches were used. In this experiment, LV-siR124a ($n = 9$) was used to knock down its expression, and a lentivirus-expressing miR124a (LV-miR124a; $n = 8$) was used to upregulate its expression in the DLS. LV-Mock- ($n = 14$) and LV-BDNF- ($n = 12$) expressing vectors were also used. During habituation to the CPP apparatus, no side preference was present in any of the groups, and there was no difference between the four groups ($F_{3,39} = 0.528$, $P = 0.666$; data not shown). The effects of DLS lentiviral injections on ethanol-induced (0.5 g/kg) CPP are shown in Fig. 2. One-way ANOVA analysis has shown that the difference in time spent in the ethanol-paired box varied as a function of virus ($F_{3,39} = 14.338$, $P < 0.001$). *Post hoc* evaluations revealed that rats injected with LV-BDNF or LV-siR124a showed about a threefold reduced CPP score compared with control rats (LV-Mock; $P = 0.007$ and $P = 0.009$, respectively). In addition, there was a slight difference between LV-Mock and LV-miR124a, but it did not reach significance ($P = 0.075$). More importantly, miR124a overexpression (LV-miR124a) enhanced about fivefold ethamol-induced CPP compared with LV-BDNF ($P < 0.001$) and LV-siR124a ($P < 0.001$). These findings suggested that miR124a is a critical regulator for BDNF in rat DLS. We hypothesise that miR124a might participate in the formation of alcohol conditioned reward by regulating the gene expression of BDNF.

LV-siR124a in the DLS, but not LV-miR124a, attenuated voluntary alcohol intake in rats

To determine levels of voluntary ethanol intake and preference following DLS viral injection of LV-Mock ($n = 7$), LV-miR124a ($n = 8$) and its silencer LV-siR124a ($n = 9$), we conducted a 5-day continuous-access two-bottle choice drinking test where animals could drink either water or a 5% ethanol solution. As depicted in Fig. 3A, one-way ANOVA repeated measure with virus as the between-subject factor showed no significant effect of time ($F_{4,84} = 1.693$, $P = 0.159$). In contrast, there was a main significant effect of viral injection in the DLS on ethanol consumption

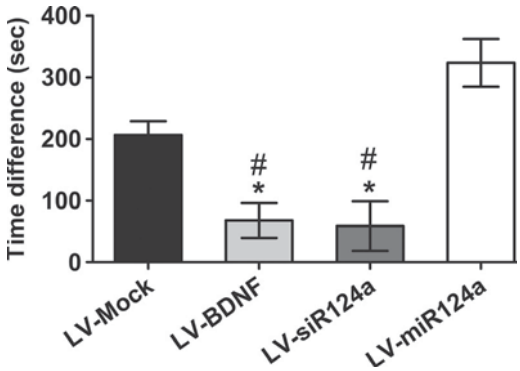

FIG. 2. Ethanol-induced CPP following viral injection in the DLS. Histograms show the mean ± SEM of the time difference between post-conditioning and pre-conditioning tests that each group of rats spent in the ethanol-paired compartment. After baseline testing, animals were stereotaxically injected with LV-Mock, LV-BDNF, LV-siR124a or LV-miR124a. After recovery, animals were conditioned with either saline or ethanol (five conditioning sessions each), and then tested for their preference on day 11. $n = 8$–14, $^{*}P < 0.05$, compared with LV-Mock; $^{\#}P < 0.01$ compared with LV-miR124a (one-way ANOVA, Bonferroni's *post hoc* test).

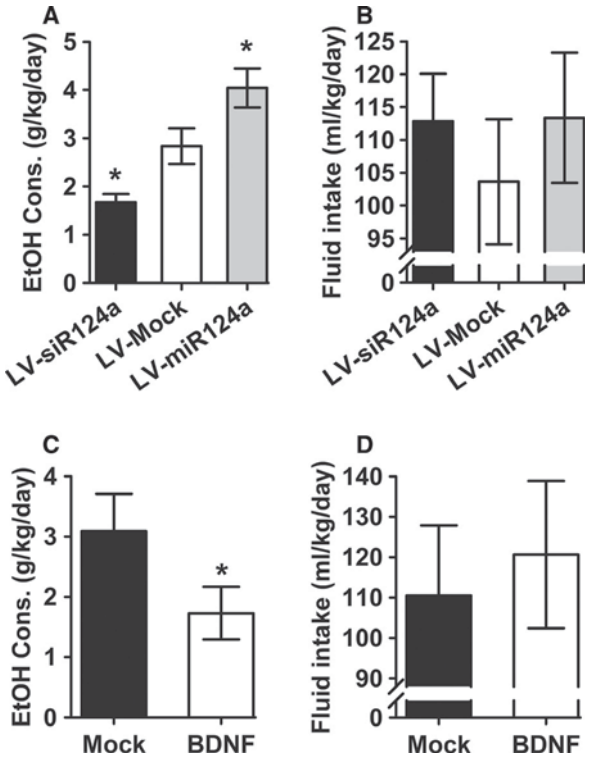

FIG. 3. Voluntary ethanol consumption and total fluid intake following viral injection in the DLS. Groups of Wistar rats were stereotaxically injected with LV-siR124a, LV-miR124a, LV-BDNF or LV-Mock, and given access to 5% ethanol and water in a two-bottle choice drinking paradigm for 5 days. (A and C) Daily g/kg ethanol consumption across the experiment. (B and D) The total fluid intake was the sum of the volume of ethanol solution and water consumed per kg of body weight per day. The data represent mean ± SEM. The number of animals per group was $n = 7$–9. *$P < 0.05$ vs. LV-Mock (two-way ANOVA, Bonferroni's *post hoc* test).

($F_{2,21} = 18.479$, $P < 0.001$). *Post hoc* evaluations revealed that, compared with LV-Mock, LV-siR124a decreased (about 30%, $P = 0.027$) but LV-miR124a increased (about 25%, $P = 0.025$) voluntary ethanol intake. Interestingly, and as shown in Fig. 3B, daily total fluid intake did not differ between the three groups during the continuous-access two-bottle procedures as the virus main effect was not significant ($F_{2,21} = 0.408$, $P = 0.670$). These findings suggest that the changed ethanol consumption by LV-siR124a- and LV-miR124a-injected rats was not caused by an overall change in total amount of fluid consumed.

LV-BDNF in the DLS also attenuated voluntary alcohol intake in rats, similar to LV-siR124a

The aim of this experiment was to determine whether the changes of expression of BDNF in the DLS can affect the voluntary alcohol drinking behaviors using the standard procedure in which the position of the bottles was alternated every day and drinking averaged across days. For this purpose, male rats were stereotaxically injected into the DLS with either LV-Mock- ($n = 7$) or LV-BDNF- ($n = 12$) expressing vectors, then given access to either 5% ethanol or tap water for five consecutive days. A significant difference in ethanol consumption was observed between LV-Mock- and LV-BDNF-injected rats ($F_{1,17} = 16.671$, $P = 0.001$), with LV-BDNF rats displaying a 1.7-fold decrease in ethanol intake when compared with controls (Fig. 3C). There was no significant effect of days of exposure to ethanol on alcohol intake ($F_{4,68} = 1.422$, $P = 0.236$). As depicted in Fig. 3D, the one-way ANOVA with repeated measure indicated a non-significant DLS viral injection effect on total fluid intake ($F_{1,17} = 0.892$, $P = 0.358$). Thus, LV-Mock and LV-BDNF showed similar liquid consumption.

LV-siR124a, LV-miR124a and LV-BDNF in the DLS did not affect taste discrimination

To determine whether differences in ethanol voluntary intake might reflect changes in taste preferences caused by LV-siR124a and LV-miR124a injection, drinking studies with saccharin and quinine were performed. As shown in Fig. 4A, there was a significant difference in saccharin consumption across the two concentrations groups [($F_{1,42} = 91.167$, $P < 0.001$) main effect of saccharin concentration]. This pattern was not altered by viral injection in the DLS ($F_{2,42} = 0.209$, $P = 0.812$). More importantly, the interaction between DLS viral injection and saccharin concentration was not significant ($F_{2,42} = 0.127$, $P = 0.881$; two-way ANOVA with repeated measures, factors were: virus and saccharin concentration). Also, and as depicted in Fig. 4B, there were no differences between LV-siR124a-, LV-Mock- and LV-miR124a-injected rats in total fluid intake groups [($F_{1,42} = 6.872$, $P = 0.012$) main effect of saccharin concentration; ($F_{2,42} = 0.315$, $P = 0.732$) main effect of virus; ($F_{2,42} = 0.020$, $P = 0.980$) main effect of saccharin concentration × virus interaction]. After 7 days of the saccharin intake study, the same rats were given access to quinine solution vs. water, the results are depicted in Fig. 4C. The consumption of quinine between the three viral-injected groups was not different ($F_{2,42} = 0.225$, $P = 0.800$), but rats consumed more quinine for the higher concentration (0.03 mM) compared with the lower concentration (0.015 mM), as revealed by a main effect of quinine concentration ($F_{1,42} = 19.845$, $P < 0.001$). No significant interaction between quinine concentration and viral injection in the DLS was found ($F_{2,42} = 0.142$, $P = 0.868$). As for saccharin, the difference in total fluid intake in between the three groups was not significant [($F_{1,42} = 0.301$, $P = 0.586$) main effect of quinine concentration; ($F_{2,42} = 0.226$, $P = 0.799$) main effect of virus; ($F_{2,42} = 0.521$, $P = 0.598$) quinine concentration × virus interaction; Fig. 4D]. We compared the body weights of rats injected with LV-Mock, LV-siR124 and LV-miR124 at the end of the experiment, and the results have shown no main effect of virus ($F_{2,21} = 2.309$, $P = 0.124$; data not shown).

To test the possibility that the differences in ethanol voluntary intake and preference for ethanol in LV-BDNF rats was due to differences in taste sensations rather than rewarding effects, we exposed rats to sweet solutions of saccharin and bitter solutions of quinine. As depicted in Fig. 4E, a two-way ANOVA with repeated measure revealed that there was no difference in the consumption for saccharin between LV-Mock and LV-BDNF ($F_{1,34} = 0.263$, $P = 0.612$). In contrast, there was a significant main effect of saccharin concentration ($F_{1,34} = 72.023$, $P < 0.001$). More importantly, the interaction between viral injection in the DLS and saccharin concentration was not significant ($F_{1,34} = 0.315$, $P = 0.578$). In addition, Fig. 4F showed that there were no differences between LV-Mock- and LV-BDNF-injected rats in total fluid intake groups [($F_{1,34} = 6.480$, $P = 0.016$) main effect of saccharin concentration; ($F_{1,34} = 0.103$, $P = 0.750$) main effect of virus; ($F_{1,34} = 0.179$, $P = 0.675$) saccharin concentration × virus interaction].

After 7 days of the saccharin intake study, the same rats were given access to quinine solution vs. water, the results are depicted

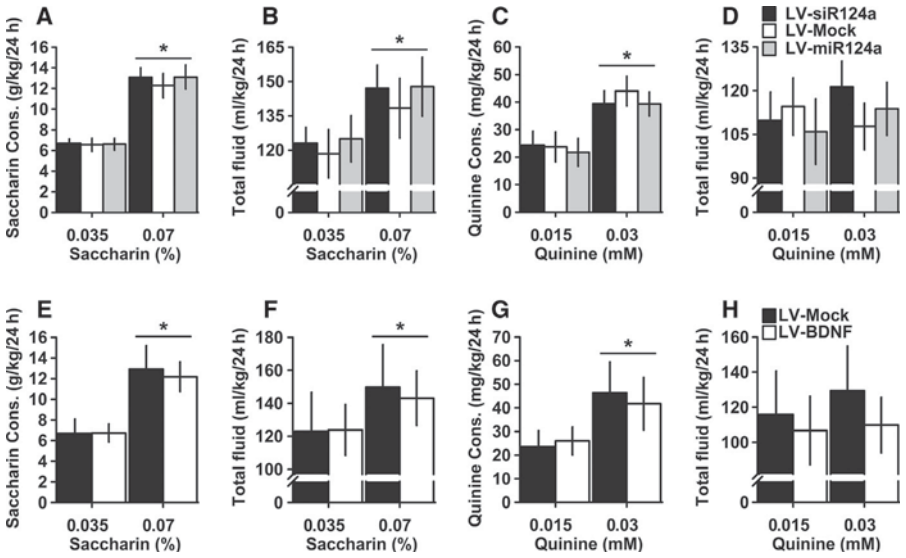

FIG. 4. Voluntary saccharin and quinine consumption, and total fluid intake following viral injection in the DLS. (A and E) Saccharin consumption; and (B and F) total fluid intake displayed by LV-siR124a, LV-miR124a, LV-BDNF and LV-Mock rats are indicated. Saccharin consumption was expressed as a gram of saccharin solution consumed per kg of body weight per day. The total fluid intake was the sum of the volume of the saccharin solution and water consumed per kg of body weight per day. (C and G) Quinine consumption; and (D and H) total fluid intake displayed by LV-siR124a, LV-miR124a, LV-BDNF and LV-Mock rats are shown. Quinine consumption was expressed as a mg of quinine solution consumed per kg of body weight per day. The total fluid intake was the sum of the volume of quinine solution and water consumed per kg of body weight per day. Data are presented as mean ± SEM. $^{*}P < 0.05$, the difference between the indicated group (two-way ANOVA, Bonferroni's *post hoc* test).

in Fig. 4G. There was no difference ($F_{1,34} = 1.136$, $P = 0.294$) between the two groups (LV-Mock and LV-BDNF) in terms of quinine consumption (mg/kg/24 h). The ANOVA revealed a significant concentration effect ($F_{1,34} = 20.507$, $P < 0.001$), which reflected a general tendency towards higher intake of more concentrated quinine solutions. More importantly, the interaction between DLS viral injection and the quinine concentration was not significant ($F_{1,34} = 0.061$, $P = 0.807$). However, the total fluid intake when the quinine solution was presented vs. water was similar between LV-Mock- and LV-BDNF-injected rats and was significant [($F_{1,34} = 0.838$, $P = 0.366$) main effect of quinine concentration; ($F_{1,34} = 2.520$, $P = 0.122$) main effect of virus; ($F_{1,34} = 0.322$, $P = 0.574$) interaction between quinine concentration and viral injection in the DLS; Fig. 4H]. Also, BDNF overexpression did not affect body weight ($F_{1,17} = 1.743$, $P = 0.204$; data not shown).

Expression of miR124a and BDNF in the dorsal striatum of rats following viral injections

This experiment was designed to assess whether ethanol-induced upregulation of BDNF mRNA reported in Fig. 2 can be prevented by upregulation of miR124a. To test this hypothesis, LV-siR124a and LV-miR124a were infused into the DLS, and rats were given access to 5% ethanol for 15 days. As shown in Fig. 5 and as expected, one-way ANOVA revealed that LV-miR124a enhanced miR124a expression ($F_{2,13} = 22.484$, $P < 0.001$). *Post hoc* evaluation indicated that, compared with LV-Mock, miRNA transcripts were upregulated with LV-miR124a ($P = 0.002$) and downregulated with LV-siR124a ($P = 0.031$). More importantly, BDNF mRNA was also regulated following miR124a modulation ($F_{2,13} = 25.78$, $P < 0.001$). In fact, and compared with LV-Mock, BDNF transcripts decreased following LV-miR124a injection (about threefold decrease, $P = 0.001$) and increased upon LV-siR124a injection ($P = 0.024$).

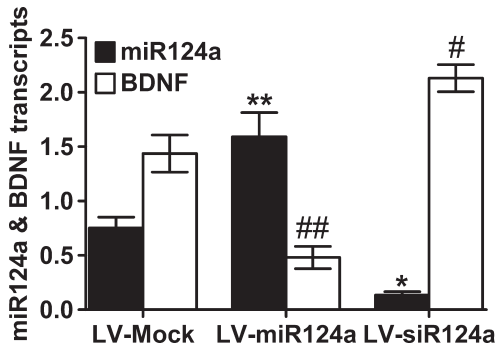

FIG. 5. Brain-derived neurotrophic factor (BDNF) and miR124a mRNA quantification following viral injection in the DLS and free access to 5% ethanol for 15 days. miR124a and BDNF quantification in LV-Mock-, LV-siR124a- and LV-miR124a-injected rats. Transcript levels were normalised against GAPDH ($n = 7, 4, 5$). $^{*}P < 0.05$, $^{**}P < 0.005$, $^{\#}P < 0.05$, $^{\#\#}P < 0.005$ (one-way ANOVA, Bonferroni's *post hoc* test).

In order to provide more convincing evidence for the direct regulation of the BDNF level by miR124A, rats were stereotaxically injected with LV-Mock, LV-miR124a or LV-siR124a in the DLS and, after recovery, BDNF mRNA was quantified after systemic ethanol injection (2 g/kg, i.p.). As depicted in Fig. 6, there was a main effect of ethanol on BDNF mRNA expression ($F_{1,24} = 22.128$, $P < 0.001$). In fact, and compared with saline, LV-Mock-expressing rats displayed a 2.3-fold increase in BDNF mRNA upon ethanol administration. However, pre-miR124a injection abolished ethanol-induced BDNF mRNA increase as reflected by a main effect of virus injection ($F_{2,24} = 9.536$, $P = 0.001$) and a significant interaction between the two factors ($F_{2,24} = 10.534$, $P = 0.001$). More importantly, siR124a-injected rats displayed comparable BDNF mRNA expression as LV-Mock-expressing rats. *Post hoc* evaluations revealed a significant difference between Mock and miR124a

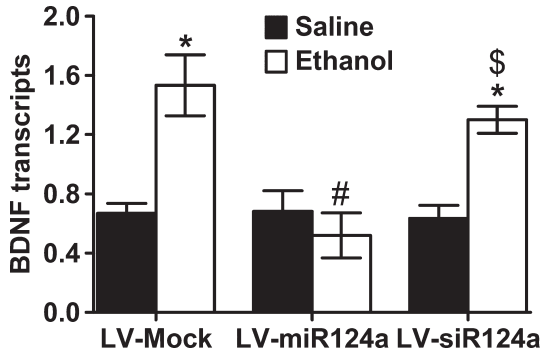

FIG. 6. Brain-derived neurotrophic factor (BDNF) mRNA quantification following viral infusion and passive i.p. injection of ethanol (2 g/kg). BDNF quantification in LV-Mock-, LV-siR124a- and LV-miR124a-injected rats. Transcript levels were normalised against GAPDH ($n = 5$). $^{*}P < 0.01$ for ethanol vs. saline, $^{\#}P < 0.01$ for LV-miR124a vs. LV-Mock, $^{\$}P < 0.01$ for LV-siR124a vs. LV-miR124a (two-way ANOVA, Bonferroni's *post hoc* test).

($P = 0.001$), but no difference between Mock and siR124a ($P = 0.820$). Taken together, these findings indicated that overexpression of miR124a indeed prevented alcohol-induced upregulation of BDNF.

BEC following viral injections of LV-miR124a, LV-siR124a and LV-BDNF

Finally, the blood concentration of alcohol in LV-Mock-, LV-miR124a-, LV-siR124a- and LV-BDNF-injected rats was assessed. Results have shown that the viral vectors utilised in this study did not elicit their effects by altering ethanol pharmacodynamics (Supporting Information Fig. S2).

Discussion

In this study, we investigated ethanol-induced alteration of miR124a and BDNF mRNA expression in the dorsal striatum following voluntary ethanol intake. We found that ethanol consumption during 15 days resulted in a decrease in miR124a and an increase in BDNF expression in the DLS but not in the MLS. More importantly, we also demonstrated that lentiviral-mediated overexpression of miR124a in the DLS enhanced ethanol-induced CPP and voluntary alcohol intake in rats. In addition, knocking-down miR124a expression in the same brain region reduced ethanol-induced CPP and alcohol consumption. These last ethanol-induced behaviors were also observed with BDNF overexpression in the DLS. A control experiment revealed that tastants (saccharin and quinine) consumption was not affected following miR124a and BDNF modulation. In fact, both gain-of-function and loss-of-function approaches have demonstrated that miR124a was a strong regulator for the expression of BDNF in rat DLS. Moreover, the ethanol-mediated upregulation of BDNF was significantly blocked by overexpression of miR124a. Thus, miR124a indeed participated in alcohol-induced gene regulation in the rat brain. Based on these results, we hypothesise that continuous exposure to alcohol results in the induction of BDNF mRNA expression via miR124a downregulation.

miRNAs are small non-coding RNAs involved in the regulation of gene expression and protein translation. Along this line, we speculate that miRNAs may be involved in the regulation of drug-related genes in general (Hollander *et al.*, 2010; Nudelman *et al.*, 2010; Eipper-Mains *et al.*, 2011) and ethanol in particular (Pietrzykowski *et al.*, 2008; Miranda *et al.*, 2010; Guo *et al.*, 2012). To afford a direct support for ethanol-induced changes in miR124a expression, we assessed the expression pattern of miR124a in rat dorsal striatum after 15 days of exposure to voluntary alcohol intake. Our results clearly revealed that, compared with water, miR124a expression was downregulated in the DLS of alcohol-exposed rats. This result is in agreement with a previous study that reported that miR124a expression in the dorsal striatum of the rat brain was downregulated following cocaine exposure (Chandrasekar & Dreyer, 2009). This downregulation was accompanied by a profound increase in mRNA levels of the miR124a direct target gene, BDNF (Chandrasekar & Dreyer, 2009). In the same study, *in silico* computational analysis predicted that BDNF is a direct target gene of miR124a. Our results revealed that in rat DLS *in vivo*, alcohol-mediated increase of BDNF mRNA was attenuated by viral-mediated overexpression of miR124a.

Ethanol-induced modifications of gene expression in reward-associated brain regions are considered to contribute to long-lasting neuroadaptations and behavioral abnormalities (Lewohl *et al.*, 2000; Kerns & Miles, 2008; Piechota *et al.*, 2010). In addition, extensive research has implicated BDNF neurotransmission in the regulation of responses to drug-related behavioral consequences in general (for review, see for example Corominas *et al.*, 2007; Thomas *et al.*, 2008; Dietz *et al.*, 2009; Ghitza *et al.*, 2010) and ethanol in particular (McGough *et al.*, 2004; Davis, 2008; Bosse & Mathews, 2011). How BDNF may contribute to the rewarding properties of alcohol is not yet known, but it has been shown that exposure to ethanol alters BDNF expression in several brain regions. In fact, a significant increase in BDNF mRNA was reported in the dorsal striatum upon voluntary ethanol consumption (McGough *et al.*, 2004; Jeanblanc *et al.*, 2009; Logrip *et al.*, 2009).

The role of BDNF in alcohol addiction was assessed in rats using ethanol-induced CPP and voluntary access to two-bottle choice drinking paradigm. Our findings revealed that lentiviral-mediated overexpression of BDNF in rat DLS inhibited the development of ethanol-induced CPP and reduced voluntary alcohol consumption and preference. The current findings are in agreement with a previous report where McGough and co-workers have shown that BDNF ± mice exhibit greater ethanol-induced place preference than control mice (McGough *et al.*, 2004). In fact, genetic depletion of the levels of BDNF by 50% results in a significantly higher preference for the chamber associated to ethanol injection than that observed in wild-type control mice.

The importance of this BDNF mRNA regulatory mechanism is supported by several reports demonstrating a significant correlation between BDNF levels and alcohol intake. Thus, reduction in BDNF increases animal sensitivity for ethanol. In fact, it has been shown that after a deprivation period, both BDNF heterozygote mice consumed more ethanol than wild-type control male (McGough *et al.*, 2004) and female mice (Hensler *et al.*, 2003). Also, chronic heavy alcohol ingestion lowered BDNF levels in rats (Jung *et al.*, 2011). More importantly and in humans, peripheral BDNF levels measured in alcohol-dependent patients and control subjects using an ELISA assay revealed that the BDNF level was lower in the alcohol-dependence group 'alcoholics' than in the normal controls (Joe *et al.*, 2007; Huang *et al.*, 2011). We hypothesise that ethanol exposure decreased the expression of miR124a and consequently increases the expression of BDNF in the dorsal striatum. Secreted BDNF, and through its receptor TrkB (Lamballe *et al.*, 1991; Soppet *et al.*, 1991), modulates the activity of neurotransmitter systems, especially dopamine and serotonin. Thus, BDNF signaling pathway activation was shown to induce expression of the dopamine D3 receptor (Jean-

blanc *et al.*, 2006), and increase serotonin synthesis and release (Mamounas *et al.*, 2000). More importantly, it has been reported that activation of the dopamine D3 receptor negatively regulates ethanol intake (Thanos *et al.*, 2005). Also, decreased serotonergic function was associated with increased alcohol preference and consumption in rodents (for review, see LeMarquand *et al.*, 1994a, b). Together, these data support the theory that BDNF functions as a positive modulator of ethanol intake in both laboratory animals and humans, most probably through the dopamine D3 receptor and the serotoninergic system by blocking the progression to addiction.

In addition, we found that striatal miR124a overexpression exacerbated the stimulatory effect of ethanol. Moreover, inhibition of striatal miR124a signaling using a lentiviral-mediated silencer expression 'rescued' the decreased alcohol intake and ethanol-induced CPP intake seen in BDNF-overexpressing rats. Therefore, we hypothesise that miR124a controls alcohol intake, and may influence vulnerability to ethanol addiction, by regulating the stimulatory effects of the drug on striatal BDNF expression. Whatever the underlying mechanisms, our data demonstrate that siR124a and miR124a exert opposite effects on striatal BDNF expression levels, and suggest that the balance between these two factors may play a crucial role in determining vulnerability to ethanol-rewarding properties and addiction.

In summary, the expression profile of miR124a in rat dorsal striatum after ethanol exposure was investigated. The results suggested that miR124a was downregulated following alcohol. Consequently, we hypothesise that miR124a is an important regulator of alcohol addiction via its direct target gene BDNF. The current findings focus attention on the fact that miR124a enables fine-tuning of BDNF-induced transcriptional neuroplastic responses to drugs of abuse. Indeed, the highly coordinated action of miR124a on BDNF, and perhaps on many other addiction-related genes, suggests that miR124a may be a key focal point in controlling ethanol-induced striatal neuroplasticity and vulnerability to alcohol addiction.

Supporting Information

Additional supporting information can be found in the online version of this article:

Fig. S1. Histological representation of dissection placements in the dorso-lateral "DLS" (A) and medio-lateral "MLS" striatum (B).

Fig. S2. Mean blood ethanol concentrations following viral injection. (A) Mean BEC of LV-siR124a, LV-Mock and LV-miR124a –injected rats (n = 4, 3, 4). (B) Mean BEC of LV-Mock and LV-BDNF –injected rats (n = 3 & 6). For all groups, viral vectors were injected into the DLS and BEC was assessed following a single injection with ethanol (3 g/kg body weight, i.p.). Blood was collected from the tail vein at various times after injection and analysed to determine the ethanol concentration. Values represent the mean ± SEM. *$P < 0.000$, the difference between 0.5- and 4-h (repeated measures one-way ANOVA, Bonferroni's *post hoc* test).

Disclosure/Conflict of Interest

The authors report no conflicts of interest.

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Abbreviations

BDNF, brain-derived neurotrophic factor; BEC, blood ethanol concentration; CPP, conditioned-place preference; DLS, dorso-lateral striatum; DMS, dorso-medial striatum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; i.p., intraperitoneally; LV, lentiviral vector; miRNA, microRNA; MLS, medio-lateral striatum; PCR, polymerase chain reaction; TrkB, tropomyosin-related kinase B.

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