Lentiviral-mediated up-regulation of let-7d microRNA decreases alcohol intake through down-regulating the dopamine D3 receptor

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Abstract
Recent studies have shown that Lethal-7 (let-7) microRNA (miRNA) is involved in a wide range of psychiatric disorders such as anxiety, depression, schizophrenia, and cocaine addiction. However, the exact role of let-7d miRNA in regulating ethanol intake and preference remains to be elucidated. The aim of the present study was to clarify the role of accumbal let-7d in controlling ethanol-related behaviors in adult rats. For this purpose, stereotaxic injections of let-7d-overexpressing lentiviral vectors (LV) were administered bilaterally into the nucleus accumbens (Nacc) of Wistar rats. The ethanol-related behaviors were investigated using the two-bottle choice (TBC) access paradigm, in which the rats had access to 2.5, 5, and 10% ethanol solutions, the grid hanging test (GHT) and ethanol-induced loss-of-righting-reflex (LORR) test. The results showed that intra-accumbally administered let-7d-overexpressing LV significantly decreased ethanol intake and preference without having significant effects on body weight, consumption or preference for tastants (saccharin and quinine) or ethanol metabolism. Furthermore, accumbal let-7d increased resistance to ethanol-induced sedation in the GHT and LORR test. Most importantly, the data showed that the dopamine D3 receptor (D3R) was a can-

Abbreviations: BEC, blood ethanol concentration; D3R, dopamine D3 receptor; GHT, grid hanging test; let-7d, lethal-7d; LORR, loss-of-righting-reflex; LV, lentiviral vectors; miRNA, microRNA; Nacc, Nucleus accumbens; TBC, two-bottle choice.

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didate target of let-7d In fact, and using real time PCR, let-7d was found to directly target D3R mRNA to decrease its expression. Further analyses proved that D3R expression was negatively correlated with the levels of let-7d and ethanol-related behaviors parameters. Taken together, the data indicating that let-7d impaired ethanol-related behaviors by targeting D3R will open up new exciting possibilities and might provide potential therapeutic evidence for alcoholism.

1. Introduction

Alcohol use disorder (AUD), defined by DSM-5 criteria, is a devastating disease with a complex etiology affecting a significant proportion of the US population. Yearly and lifetime prevalence of AUD were, respectively, 13.9% and 29.1% (Grant et al., 2015). One of the main challenges in alcoholism research is to understand the neurological dysregulation, and by extension the molecular mechanisms involved in its neuropathophysiology. Substantial research has focused on the role that the dopaminergic mesolimbic system plays in craving and drug-seeking behavior, yet promising preclinical findings have not translated effectively into successful clinical applications.

It is well established that the reinforcing effects of drugs of abuse are, in large part, mediated by the mesolimbic dopamine (DA) system [for review see (Ikemoto and Bonci, 2014; Pierce and Kumaresan, 2006; Ritz and Kuhar, 1993)]. DA exerts its action through five receptor subtypes identified 30 years ago. After the cloning of the D1R (Zhou et al., 1990) and D2R (Bunzow et al., 1988), several extra DA receptors were identified including the D3R (Sokoloff et al., 1990) and D4R (Van Tol et al., 1991), which are homologous to the D2R, whereas the D5R (Sunahara et al., 1991), is homologous to the D1R. A large body of evidence suggests that the D3R subtype plays a crucial role in the modulation of mesolimbic dopaminergic neurotransmission involved in drug-seeking behavior, including for ethanol [For review see (Galaj et al., 2018; Heidbreder et al., 2004; Le Foll and Di Ciano, 2015)]. For example, the D2R/D3R agonist, quinpirole, dose-dependently decreased ethanol-reinforced responding (Hodge et al., 1999) and reduced hyperactivity induced by ethanol (Cohen et al., 1997). Also, the putative D3R agonist 7-OH-DPAT produced a decrease in ethanol intake in the male alcohol-preferring (P) and high alcohol drinking (HAD) rats (Russell et al., 1996), as well as in rats trained to self-administer ethanol (10%; *v*/*v*) orally in a free-choice two-lever operant task (Cohen et al., 1998). However the D3R antagonist, U-99194A, did not change oral ethanol self-administration in male C57BL/6J mice (Boyce and Risinger, 2002) but enhanced the acquisition of ethanol preference in male Swiss-Webster mice (Boyce and Risinger, 2000) in a place conditioning paradigm. Moreover, chronic co-administration of low-dose U99194A blocked the induction of ethanol sensitization, while acute U99194A had no effect in mice that were already sensitized (Harrison and Nobrega, 2009). The selective D3R antagonist SB-277011-A, induced a significant attenuation in ethanol preference, intake and lick responses in alcohol-preferring (P) rats (Thanos et al., 2005), and caused a dose-dependent reduction of relapse-like drinking in the alcohol deprivation effect model, as well as a decrease in cue-induced ethanol-seeking behavior (Vengeliene et al., 2006). These studies suggest a necessary role for the D3R in ethanol intake, sensitization, and conditioned place preference.

In recent years, miRNAs have been shown to regulate mRNA expression. miRNAs binding to their mRNA targets results in a significant downregulation of gene expression via translational repression and/or mRNA degradation [For review see (Pu et al., 2019; Saj and Lai, 2011; Ying et al., 2013)]. Despite the ever-growing important roles of miRNAs in the central nervous system, the possible molecular connections between miRNAs and DA receptors in general, and D3R in particular, are still, unexpectedly, not fully understood.

The lethal-7 (let-7) miRNA, one of the first identified and characterized miRNAs, is present in multiple copies in a genome and has diverse functions in animals. As an important regulator of gene expression, let-7 family miRNAs members are involved in multiple physiological processes, such as signal regulation (Zhao et al., 2011), early development (Ouchi et al., 2014), temporal regulation (Pasquinelli et al., 2000), lens regeneration (Nakamura et al., 2010), protein ubiquitylation (Rybak et al., 2009), sexual identity (Fagegaltier et al., 2014), and cell proliferation and differentiation (Ecsedi and Grosshans, 2013). More importantly, let-7 family miRNAs are also expressed throughout the brain (Akerblom et al., 2012; Coleman et al., 2017; Shinohara et al., 2011), where they are implicated in several neurophysiological functions such as mood and emotional behaviors (Hunsberger et al., 2015; Maffioletti et al., 2016). In recent years, evidence has emerged that shows that let-7 miRNAs are also involved with neurobiological responses to drugs of abuse (He and Wang, 2012; Lopez-Bellido et al., 2012). López-Bellido and colleagues reported that, in the zebrafish embryos, cocaine upregulated dre-let-7d and its precursors (dre-let-7d-1 and dre-let-7d-2), which in turn modulated the expression of opioid receptors (Lopez-Bellido et al., 2012). Previous studies from our laboratory, using qRT-PCR, in situ hybridization, Northern blots, FISH analysis and RNase protection assay, have shown that precursor and mature let-7d transcription levels were significantly downregulated, in several brain regions, following cocaine injection (Chandrasekar and Dreyer, 2009). Interestingly, D3R mRNA and protein expression decreased significantly upon let-7d overexpression (Chandrasekar and Dreyer, 2009). Follow-up studies revealed that lentiviral-mediated overexpression of let-7d in the Nacc attenuated cocaine-induced conditioned place preference (CPP) in Wistar rats (Chandrasekar and Dreyer, 2011). We have also reported

that, in adult C57BL/6J mice, hippocampal overexpression of let-7d induced an anxiolytic-like effect in the open field and elevated plus maze tests, as well as an anti-depressant-like effect in the tail suspension and forced swim tests. In addition, a significant negative correlation between D3R mRNA and let-7d has been found (Bahi and Dreyer, 2018).

Taking into account all of the above aspects, we hypothesized that accumbal let-7d overexpression might result in D3R downregulation which in turn will be associated with decreased ethanol-related behavior responses. Therefore, in the present report, the role of let-7d in the Nacc in various ethanol-related behaviors has been examined. Initially the effect of intra-accumbal let-7d overexpression on voluntary ethanol intake and preference using a standard TBC drinking procedure has been determined. Based on these data, the effects of intra-accumbal let-7d overexpression on *i)* ethanol-induced motor, and *ii)* sedation responses, as well as *iii)* ethanol clearance were further determined. This assessed the mRNA expression of D3R, a direct target of let-7d, and the correlations between let-7d and D3R expression levels with the different parameters of ethanol-related behaviors.

2. Experimental procedures

2.1. Animals

Male Wistar rats, obtained from the local breeding facility, weighing approximately 220 g were housed in standard observation cages. The rats were bred locally and were kept under environmentally controlled standard laboratory conditions [ambient temperature ~23 ± 2 °C; humidity ~60 ± 15%; 12 h light/12 h dark cycle (0600–1800 light on)]. The animal facility at our college typically used a woodchip bedding, which when touched feels somewhat prickly, even though the wood pieces are small. Woodchip bedding was produced locally and autoclaved before use. The 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). The experimental procedures were approved by the local Animal Research and Ethics Committee (Protocol No A17–12).

2.2. Construction of lentiviral vectors and stereotaxic injections

The let-7d overexpressing lentiviral vectors were prepared as described previously (Chandrasekar and Dreyer, 2011). In brief, let-7d miRNA, driven by an RNA Pol III mouse U6 promoter, was constructed by cloning an approximately 450 bp fragment containing the rat let-7d precursor hairpin loop into pTK431 transfer vector (obtained from Dr. Tal Kafri, Gene Therapy Center, UNC). Viral particles were produced as fully described in our previous studies (Bahi et al., 2004a, 2004b), (Bahi et al., 2016; Bahi and Dreyer, 2017).

For stereotaxic injections, the rats were bilaterally infused with a 0.5 μl viral solution at two stereotaxic coordinates: 1st injection (AP + 2.7, ML +/- 1.2, DV - 6.8); 2nd injection (AP +1.3, ML +/- 1, DV - 7.6) (Bahi et al., 2005; Bahi and Dreyer, 2014; Paxinos and Watson, 1998). Sham-operated controls were injected with an empty cloning vector (Mock). The behavioral tests started 7 days later as depicted in Fig. 1.

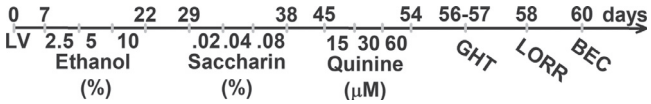

Fig. 1 Experimental timeline. The timelines show sequence and duration of experimental protocols.

2.3. The two-bottle choice (TBC) drinking paradigm

This test was performed to study the effects of let-7d overexpression on voluntary alcohol intake and preference in Wistar rats as described previously (Bahi and Dreyer, 2014, 2017). In brief, rats were given access to two bottles, one with tap water and the other with ethanol, starting at a concentration of 2.5% ethanol for five days, followed by 5%, and continuing with 10% ethanol for another five days. Amounts consumed from each drinking bottle were measured at the end of each drinking period with values being used to calculate consumption of ethanol (g/kg/day), preference for ethanol (ethanol intake/total volume fluid consumed), and total volume of fluid ingested (ml/kg/day). For each ethanol solution, the values for ethanol consumption, preference and the total fluid intake were averaged across the 5-days period. The general design of the study is displayed in Fig. 1.

2.4. Continuous access to saccharin and quinine in the TBC drinking paradigm

To assess whether the decreased ethanol consumption and preference observed in let-7d-overexpressing rats was not due to differences in taste discrimination, the same rats were tested in a standard TBC drinking procedure for saccharin and quinine as depicted in Fig. 1. In brief, the rats were given two bottles containing either tap water or increasing concentrations of saccharin (0.02, 0.04 and 0.08%). After completion of the saccharin drinking test and a respective 7-day washout period, rats were given access to increasing concentrations of quinine (15, 30, and 60 μM) and tap water. For both tastants, each concentration was offered for 3 consecutive days and with bottle position changed daily to control for side preference. For both tastants, the values for consumption, preference and the total fluid intake were averaged across the 3-days period. The general design of the study is displayed in Fig. 1.

2.5. The grid hanging test (GHT)

This test was used to assess motor strength and was performed as described previously for mice (Bahi et al., 2016). Briefly, each rat was injected with saline and placed on the center of a wire mesh platform. The experimenter supported the rat until it grabbed the grid, which was then inverted and placed 30 cm above a plastic cage filled with sawdust bedding, so the rat was hanging upside-down. The duration of time that the rat remained hanging to the grid was manually recorded. The following day, each rat was given an intraperitoneal injection of 1.2 g/kg ethanol (prepared as a 20% (*v*/*v*) solution in 0.9% saline), and the ability to hang from the wire, as measured by latency to fall, was manually recorded.

2.6. Loss-of-righting-reflex (LORR) test

As described previously (Al Mansouri et al., 2014; Bahi et al., 2016), this test was performed to assess the sedative effects of ethanol 24 h after completing the GHT. For this purpose, the rats were

given an intraperitoneal injection of 3 g/kg ethanol (prepared as a 20% (*v*/*v*) solution in 0.9% saline) and placed on their back in a U-shaped trough after losing the ability to right themselves. The onset and the duration of LORR (sedation time) were manually recorded until this reflex was regained. Sedation time is defined as the time needed to regain the ability to right onto three of four paws, three times within 30 s.

2.7. Blood ethanol concentration (BEC)

To assess whether let-7d overexpression affected ethanol metabolism pharmacokinetics and clearance, the two experimental groups of rats were given an intraperitoneal injection of 1.2 g/kg ethanol (prepared as a 20% (*v*/*v*) solution in 0.9% saline). 0.5, 1, 2, and 3 h later, the rats were gently restrained, and a 30 μl blood sample was drawn from the end of the nicked tail with a capillary tube for BEC determinations. The samples were analyzed using an alcohol dehydrogenase activity colorimetric assay from BioVision Research Products (CA, USA) as described previously (Bahi et al., 2013). The area under the blood ethanol concentration-time curve between 0 and 3 h was determined for each of the 2 experimental groups.

2.8. RNA isolation and real-time qRT-PCR quantification

On conclusion of behavioral experiments, rats were killed by rapid decapitation. Brains were removed and immediately placed on pre-chilled stainless matrix. Using a blade, 2-mm thick brain sections were prepared using the matrix and tissue punches of the NAc were procured rapidly, using a stainless-steel cannula with an inner diameter of 1.5 mm. Tissue samples were immediately frozen on dry ice and stored at -80 °C for further RNA analysis.

Total RNA was isolated using TRIzol reagent according to the manufacturer's guidelines. In brief, using a glass homogenizer, NAc tissue samples were homogenized in TRIzol and vortexed for 30 s before being incubated at room temperature for 5 min. Residual proteins were removed by addition of 0.2 mL of chloroform, for 30 s, incubation at room temperature for 3 min, and centrifugation for 15 min at 12,000 × *g* and 4 °C. The aqueous phase was precipitated with 0.5 mL isopropanol by mixing for 15 s, incubation at room temperature for 10 min, and centrifugation for 10 min at 12,000 × g and 4 °C. The resulting RNA pellet was washed with RNase-free 75% ethanol and centrifuged for 5 min at 7500 × g and 4 °C. The RNA pellet was resuspended in RNase-free water, incubated at 60 °C for 10 min. RNA was quantified by spectrophotometry, and its integrity was verified by agarose gel electrophoresis and visualized with ethidium bromide staining.

Expression profiling of let-7d miRNA was performed by reverse transcription using the TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystem) according to the manufacturer's instructions. To quantify D3R mRNA levels the cDNA was generated from total RNA and oligo(dT_{20}) primer with the SuperScript™ III RT according to the manufacturer's protocol. In brief, the following components were added to a nuclease-free microcentrifuge tube: 4 μg of total RNA, 1 μl of 50 μM oligo(dT_{20}), 1 μl 10 mM dNTP Mix (10 mM each dATP, dGTP, dCTP and dTTP at neutral pH), RNase-free water to 12 μl. The mixture was heated to 65 °C for 5 min and incubated on ice for 2 min. The contents of the tube were collected by brief centrifugation and 4 μl 5X First-Strand Buffer, 1 μl 0.1 M DTT, 1 μl RNase-OUT™ Recombinant RNase Inhibitor, and 2 μl of SuperScript™ III RT (200 units/μl) were added to the tube. The contentment was mixed by pipetting gently up and down. The tube was incubated at 50 °C for 60 min and the reaction was inactivated by heating at 70 °C for 15 min. To remove RNA complementary to the cDNA, 1 μl (2 units) of E. coli RNase H were added and the reaction was incubated at 37 °C for 20 min.

PCR was subsequently performed using the IQ SYBR Green Supermix in the iCycler System (Bio-Rad Laboratories, Inc.). The cycling parameters were 95 °C for 4 min, followed by 40 cycles of 95 °C for 45 s, and 64 °C for 45 s. Expression levels were determined using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001; Androvic et al., 2019) and normalized to the internal reference genes U6 snRNA or Cyclophilin. It should be emphasized that the stability of the selected reference mRNA (cyclophilin) and microRNA (U6 snRNA) was not evaluated. The primer pairs used were as follows: Cyclophilin forward, 5′-GTG AGA AGG GC TTT GGC TAC-3′ and reverse, 5′-TTC TCG TCA GGA AAG CGG-3′; D3R forward, 5′-GGG GTG ACT GTC CTG GTC TA-3′ and reverse, 5′-TGG CCC TTA TTG AAA ACT GC-3′; let-7d forward, 5′-AGG TTG CATAGT TTT AGG GCA-3′ and reverse, 5′-AAG GCA GGT CGT ATA GT-3′; U6 snRNA forward, 5′-CTC GCT TCG GCA CA-3′ and reverse, 5′-AAC GCTTCA CGA ATT TGC GT-3′ (Bahi et al., 2005; Bahi and Dreyer, 2014; Chandrasekar and Dreyer, 2009, 2011). The Cq values are included in the supplementary material.

2.9. Statistical analysis

Statistical analysis was performed using IBM SPSS Statistics 26. All data were expressed as the mean ± SEM. The data representing the effect of let-7d overexpression on ethanol and tastants consumption and preference were analyzed using a two-way ANOVA repeated measures with viral-injection as the between-subjects factor and concentration as the within-subjects factor. The data representing the effect of let-7d overexpression on hanging duration, LORR onset and duration, transcripts' levels and the AUC of blood alcohol metabolism were analyzed using one-way ANOVA. Simple linear regression (Pearson's) analysis was performed to examine the correlation between let-7d and D3R expression with ethanol-related behaviors. The data were checked for homogeneity of the variances with the Levene test. In case of a significant main effect, post hoc comparisons were performed with Bonferroni's test. The criterion for statistical significance was set at $p \leq 0.05$.

3. Results

3.1. Accumbal let-7d overexpression decreased ethanol consumption and preference

First voluntary ethanol consumption (ml/kg/day) has been examined in let-7d-overexpressing rats ($n = 12$) and their Mock controls ($n = 12$) in a standard TBC paradigm using ascending concentrations of ethanol solutions (2.5, 5, and 10%). The data were then submitted to analysis of variance with repeated measures using viral-injection as the between-subjects factor and concentration as the within-subjects factor. As depicted in Fig. 2A, the results indicated a main effect of viral-injection ($F_{(1,22)} = 30.315$, $p < 0.0001$) and ethanol concentration ($F_{(2,44)} = 85.771$, $p < 0.0001$), with a significant interaction between the two factors ($F_{(2,44)} = 12.120$, $p = 0.001$). Bonferroni's post-hoc tests showed that let-7d-overexpressing rats drank less ethanol from the 5 and 10% solutions (both $p < 0.0001$) but not from the 2.5% ($p = 0.944$). After this initial study voluntary ethanol intake (g/kg/day) was examined in the two experimental groups and results are displayed in Fig. 2B. The two-way ANOVA with repeated measures revealed significant effects of viral-injection ($F_{(1,22)} = 26.202$, $p < 0.0001$) and

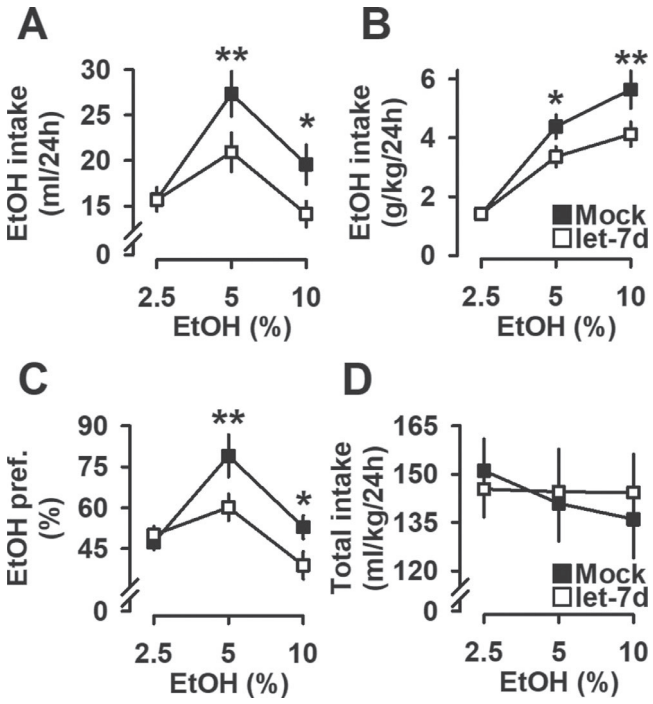

Fig. 2 Effect of Mock and let-7d in the Nacc on ethanol drinking behavior. The data are expressed as mean ± SEM for ethanol consumption calculated as A) milliliters and B) grams of alcohol consumed per kilogram of body weight in male Wistar rats, C) ethanol preference expressed as ethanol consumed versus total fluid consumed, and D) total fluid (water + ethanol) intake. $^{*}p < 0.05$, $^{**}p < 0.001$. For both groups $n = 12$.

ethanol concentration ($F_{(2,44)} = 260.393$, $p < 0.0001$). Interestingly, the viral-injection × concentration interaction was also found significant ($F_{(2,44)} = 12.354$, $p < 0.0001$). Bonferroni's post-hoc tests indicated that let-7d-overexpressing rats consumed less ethanol from the 5 and 10% solutions (both $p < 0.0001$) but not from the 2.5% ($p = 0.891$). Next, we examined whether let-7d overexpression could alter ethanol preference and results showed that both experimental groups displayed an inverted U-shaped ethanol concentration dependent curve. The two-way ANOVA statistical analysis revealed significant effects of viral-injection ($F_{(1,22)} = 29.730$, $p < 0.0001$) and ethanol concentration ($F_{(2,44)} = 95.477$, $p < 0.0001$), with significant interaction between the two factors ($F_{(2,44)} = 18.178$, $p < 0.0001$) (Fig. 2C). Pairwise comparisons revealed that, compared to Mock controls, let-7d overexpression produced a strong and persistent decrease in ethanol preference at the 5 and 10% solutions (both $p < 0.0001$) but not at the 2.5% ($p = 0.144$). Interestingly, and as shown in Fig. 2D, averaged daily total fluid intake did not differ between the two experimental groups at any concentration. In fact, the two-way ANOVA with repeated measures analysis showed no significant effect of viral-injection ($F_{(1,22)} = 0.165$, $p = 0.688$), or ethanol concentration ($F_{(2,44)} = 2.595$, $p = 0.086$). Also, the interaction between the two factors ($F_{(2,44)} = 1.975$, $p = 0.153$) was not found statistically significant. These findings indicate that *i)* accumbal let-7d overexpression reduces ethanol consumption and preference, and *ii)* suggest that let-7d-altered ethanol-related behaviors were not caused by an overall change in the total amount of fluid consumed, or body weight (data not shown).

3.2. Accumbal let-7d overexpression had no effect on taste sensitivity

Since differential taste reactivity may affect ethanol consumption and preference, a taste preference experiment has been performed, using a standard TBC drinking paradigm in which the rats were exposed to sweet solutions of saccharin and bitter solutions of quinine. For the saccharin solutions, voluntary saccharin consumption (ml/kg/day) in the two experimental groups was first examined. The two-way ANOVA with repeated measures revealed no significant effects of viral-injection ($F_{(1,22)} = 0.007$, $p = 0.936$) or saccharin concentration ($F_{(2,44)} = 1.298$, $p = 0.283$). Also, the viral-injection × concentration interaction was not found significant ($F_{(2,44)} = 0.749$, $p = 0.479$) (Fig. 3A). Similarly, and as displayed in Fig. 3B, saccharin preference tended to be affected by concentration ($F_{(2,44)} = 3.110$, $p = 0.055$). However, the main effect of viral-injection ($F_{(1,22)} = 0.008$, $p = 0.930$) and the interaction between the two factors ($F_{(2,44)} = 0.521$, $p = 0.597$) were not found significant. Finally, relative total fluid consumption was not affected following let-7d overexpression: there was no main effect of viral-injection ($F_{(1,22)} = 0.002$, $p = 0.962$) or concentration ($F_{(2,44)} = 0.442$, $p = 0.645$) on total fluid intake. Furthermore, the viral-injection × concentration interaction ($F_{(2,44)} = 0.793$, $p = 0.459$) was not found significant (Fig. 3C).

Fig. 3D-F shows quinine consumption (ml/kg/day) and preference for Mock- and let-7d-injected rats across three different quinine solutions. Seven days after completion of the saccharin intake experiment, the same rats could drink either tap water or an ascending series of quinine concentrations (20, 40, and 80 μM). The two-way ANOVA with repeated measures revealed no significant effects of viral-injection ($F_{(1,22)} = 0.114$, $p = 0.739$) or quinine concentration ($F_{(2,44)} = 1.018$, $p = 0.370$). Also, the interaction between the two factors was not found significant ($F_{(2,44)} = 0.415$, $p = 0.663$) (Fig. 3D). For quinine preference, the two-way repeated measures ANOVA yielded no significant main effect of viral-injection ($F_{(1,22)} = 0.057$, $p = 0.814$), concentration ($F_{(2,44)} = 1.618$, $p = 0.210$), or interaction ($F_{(2,44)} = 0.162$, $p = 0.851$). Overall, the rats showed low preference ratios for the three quinine solutions (mean preference ˜ 3.8 to 4.67%) (Fig. 3E). Finally, analysis of total fluid consumption (per kilogram of body weight) showed no significant main effects of viral-injection ($F_{(1,22)} = 0.020$, $p = 0.889$), or concentration ($F_{(2,44)} = 0.236$, $p = 0.791$). Also, the interaction between the two factors was not found significant ($F_{(2,44)} = 0.308$, $p = 0.737$) (Fig. 3F).

Taken together, these results indicated that let-7d-decreased ethanol consumption and preference was not caused by an overall change in taste perception or sensitivity.

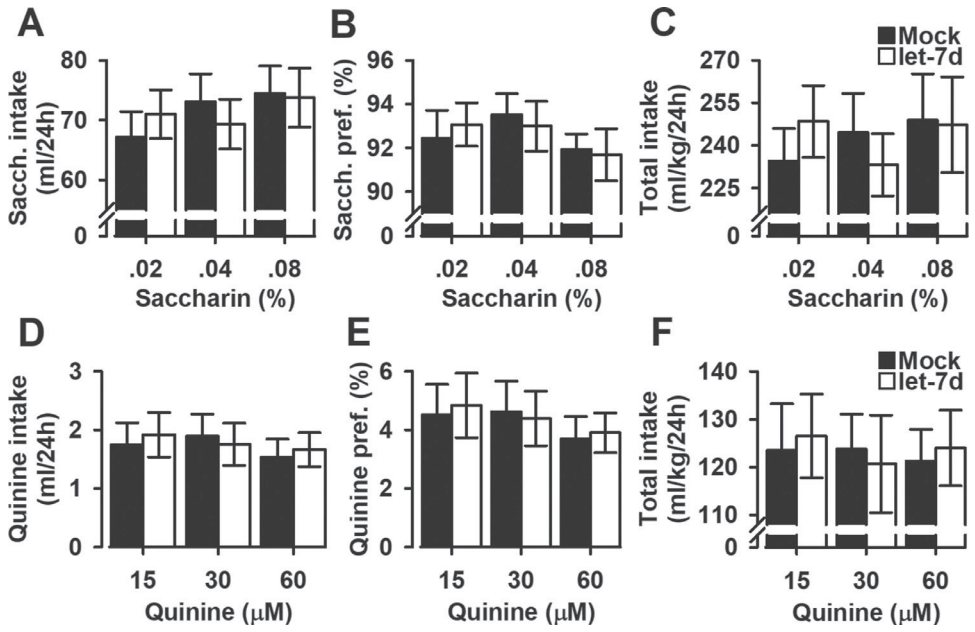

Fig. 3 Effect of Mock and let-7d in the Nacc on tastants' intake and preference. The data are expressed as mean ± SEM for A & D) saccharin and quinine consumption, B & E) saccharin and quinine preference, and C & F) total fluid intake. $^{*}p < 0.001$ and $^{**}p < 0.0001$. For both groups $n = 12$.

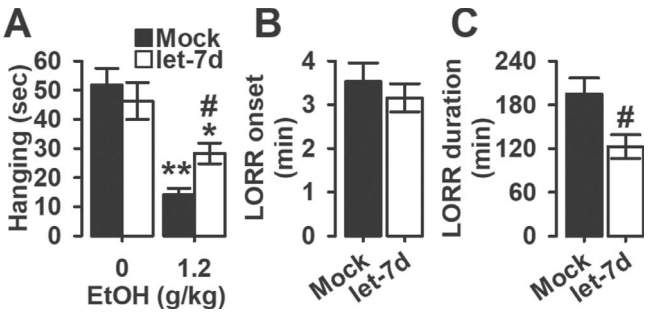

Fig. 4 Effect of Mock and let-7d in the Nacc on the grid hanging (GHT) and LORR tests. The data are expressed as mean ± SEM for A) hanging duration in the GHT, B) LORR onset, and C) LORR duration in the LORR test. $^{*}p < 0.05$ and $^{**}p < 0.001$ indicate significant differences between 0 and 1.2 g/kg ethanol. $\#p < 0.05$ indicates significant differences between Mock and let-7d For both groups $n = 12$.

Table 1 Blood ethanol concentrations (BECs) in Mock and let-7d rats. The data are expressed as mean ± SEM for the blood ethanol levels in 0.5, 1, 2, and 3 h after an acute injection of ethanol (1.2 g/kg). $^{*}p < 0.05$, $^{**}p < 0.0001$ indicates significant differences between 1 and 2 h compared to 0.5 h respectively. For each group $n = 12$.

Time (h)	Mock	let-7d
0.5	16.66 ± 1.80	24.36 ± 1.92
1	178.73 ± 12.21**	168.54 ± 10.12**
2	99.76 ± 12.71*	114.34 ± 16.52*
3	40.86 ± 6.12	44.63 ± 7.48
AUC	587.10 ± 28.38	617.77 ± 31.89

3.3. let-7d overexpression impaired ethanol-induced motor coordination and sedation responses but not blood ethanol concentration (BEC)

As depicted in Fig. 4A, saline injection did not interfere with the latency to fall in the GHT. Prior to ethanol administration, both experimental groups demonstrated a similar degree of motor strength and remained on the wire for a comparable amount of time ($F_{(1,22)} = 0.402$, $p = 0.533$) and both groups showed no significant differences in variances (Leven test: $F_{(1,22)} = 0.082$, $p = 0.788$). However, the ability of the rats to hang from the grid was significantly affected following ethanol administration (1.2 g/kg) with a significant increase in the time spent on the grid by let-7d-overexpressing rats compared to the Mock controls ($F_{(1,22)} = 11.534$, $p = 0.003$).

To assess the sedative/hypnotic effects of ethanol, Mock and let-7d-overexpressing rats were injected with 3 g/kg ethanol, then were tested for their ability to recover an upright posture. At this dose of ethanol, we found that Mock and let-7d-overexpressing rats showed similar LORR onset ($F_{(1,22)} = 0.456$, $p = 0.507$), as both groups lost their reflex ability at approximately the same time following ethanol injection (Fig. 4B). However and as displayed in Figure 4C, let-7d-overexpressing rats recovered their reflex capacity much faster than the Mock controls ($F_{(1,22)} = 6.876$, $p = 0.016$).

To test whether accumbal let-7d overexpression affected ethanol absorption and metabolism, BEC (mg/dL) has been measured after acute ethanol (1.2 g/kg) injection and the

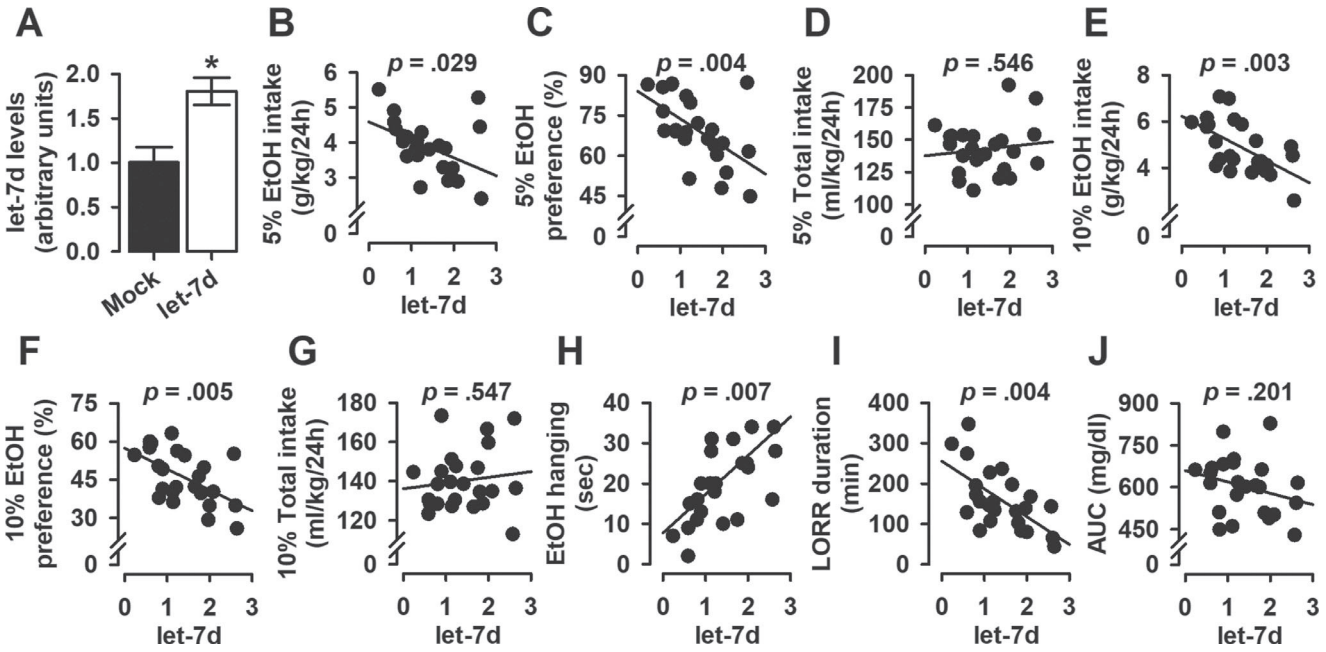

Fig. 5 let-7d quantification, in Mock and let-7d rats, as measured by qRT-PCR and Pearson correlations. A) for the qRT-PCR, the data are expressed as mean ± SEM for relative let-7d levels. $^{*}p < 0.005$. For both groups $n = 12$. For the Pearson correlations, the data represent simple scatter regression between let-7d levels with the B & E) ethanol consumption; C & F) ethanol preference; and D & G) total fluid intake from the TBC drinking test when rats had access to the 5% and 10% ethanol solutions respectively. Also, for the Pearson correlations, the data represent simple scatter regression between let-7d levels with the H) hanging duration in the GHT following ethanol injection; I) LORR duration, and J) AUC from the blood ethanol concentrations.

results are depicted in Table 1. A one-way repeated measures ANOVA was conducted to examine the effects of treatment (Mock vs. let-7d) and time (0.5, 1, 2, and 3 h) on BEC and the results indicated that BECs decreased significantly over time ($F_{(3,66)} = 89.239$, $p < 0.0001$). However, BECs did not significantly differ between the two experimental groups ($F_{(1,22)} = 0.639$, $p = 0.433$), and no treatment × time interaction was found ($F_{(3,66)} = 0.512$, $p = 0.676$), suggesting that the behavioral differences between Mock and let-7d-overexpressing rats did not result from potential differences in ethanol clearance.

3.4. let-7d expression correlated negatively with ethanol intake and preference

At the end of the behavioral experiments, the brains were removed, the Nacc subregion was dissected out, and let-7d expression levels were assessed using quantitative qRT-PCR. The one-way ANOVA indicated that, compared to Mock controls, let-7d levels were significantly upregulated in the accumbal tissue from let-7d-overexpresssing rats ($F_{(1,22)} = 12.008$, $p = 0.002$) (Fig. 5A).

Using the let-7d expression levels obtained by qRT-PCR from the two experimental groups, a simple linear regression (Pearson) analysis was performed to examine the correlation between let-7d transcription levels and parameters of ethanol-related behaviors. It should be emphasized that no correlation was found between let-7d levels in the Nacc with ethanol consumption and preference when rats had access to the 2.5% ethanol solution (Data not shown). However, the correlation coefficient (r) for the difference between let-7d expression and ethanol consumption, from the 5% ethanol solution, was -0.445 and the two parameters correlated significantly ($F_{(1,22)} = 5.446$, $p = 0.029$; Fig. 5B). Also, let-7d expression correlated negatively ($r = -0.560$) with ethanol preference ($F_{(1,22)} = 10.069$, $p = 0.004$; Fig. 5C), but not with the total fluid intake ($F_{(1,22)} = 0.377$, $p = 0.546$; Fig. 5D). Similarly, when rats had access to the 10% ethanol solution, we found a strong negative correlation ($r = -0.586$) between let-7d expression with ethanol intake ($F_{(1,22)} = 11.515$, $p = 0.003$; Fig. 5E), and preference ($F_{(1,22)} = 9.492$, $p = 0.005$; $r = -0.549$; Fig. 5F), but not with the total fluid intake ($F_{(1,22)} = 0.374$, $p = 0.547$; Fig. 5G).

In the GHT, let-7d correlated positively ($r = 0.538$) with the rats' ability to hang from the wire grid when injected with ethanol (Pearson's test: $F_{(1,22)} = 8.970$, $p = 0.007$; Fig. 5H), but not with saline ($F_{(1,22)} = 0.009$, $p = 0.924$; data not shown). Also, let-7d expression did not correlate with the LORR onset ($F_{(1,22)} = 0.850$, $p = 0.367$; data not shown), but it correlated negatively ($r = -0.627$) with its duration ($F_{(1,22)} = 14.218$, $p = 0.001$; Fig. 5I). Finally, there was no significant correlation between let-7d, and the AUC obtained from the BECs data ($F_{(1,22)} = 1.737$, $p = 0.201$; Fig. 5J).

We should emphasize that there was not a direct correlation between let-7d expression and tastants' consumption and preference (Suppl. Figs. 1 & 2).

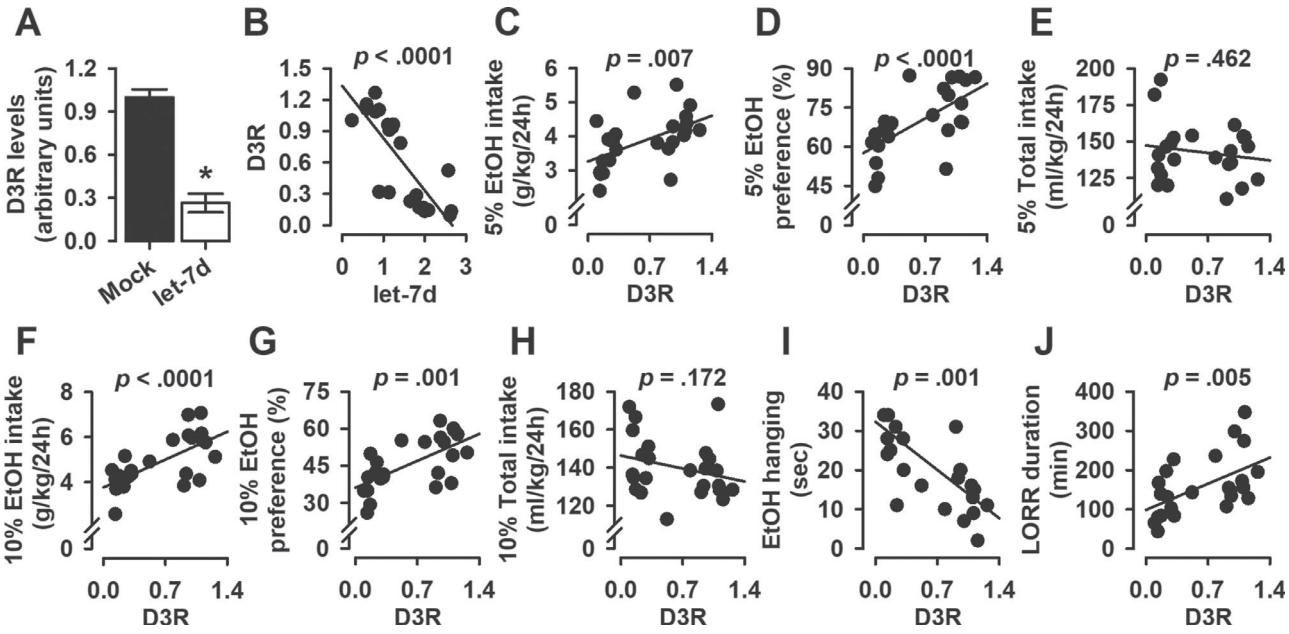

Fig. 6 D3R quantification, in Mock and let-7d rats, as measured by qRT-PCR and Pearson correlations. A) for the qRT-PCR, the data are expressed as mean ± SEM for relative D3R levels. $^{*}p < 0.0001$. For both groups $n = 12$. For the Pearson's correlations, the data represent simple scatter regression between D3R mRNA levels with the B) let-7d transcription levels. Also, between D3R mRNA with C & F) ethanol consumption, D & G) ethanol preference, and E & H) total fluid intake from the TBC drinking test when rats had access to the 5% and 10% ethanol solutions respectively. Also, the data represent simple scatter regression between D3R mRNA expression with the I) hanging duration in the GHT following ethanol injection, and J) LORR duration.

3.5. D3R mRNA expression correlated positively with ethanol intake and preference

D3R mRNA levels in Mock and let-7d-overexprerssing rats were then also quantified, and the one-way ANOVA analysis indicated that let-7d overexpression resulted in a significant and robust down-regulation of accumbal D3R mRNA ($F_{(1,22)} = 72.795$, $p < 0.0001$) (Fig. 6A). Most importantly, the Pearson's analysis showed a significant negative correlation ($r = -0.812$) between let-7d and D3R expression levels ($F_{(1,22)} = 42.462$, $p < 0.0001$; Fig. 6B).

The correlations between D3R mRNA and ethanol-related behaviors were also evaluated, and the results indicated that, like let-7d expression levels, no correlation was found between accumbal D3R mRNA with ethanol consumption and preference when rats had access to the 2.5% ethanol solution (Data not shown). However, when rats were presented with the 5% ethanol solution, the Pearson test revealed a significant positive correlation ($r = 0.535$) between D3R mRNA with ethanol consumption ($F_{(1,22)} = 8.842$, $p = 0.007$; Fig. 6C), and preference ($F_{(1,22)} = 16.892$, $p < 0.0001$; $r = 0.659$; Fig. 6D), but not with the total fluid intake ($F_{(1,22)} = 0.561$, $p = 0.462$; Fig. 6E). Likewise, when the 10% ethanol solution was available, the Pearson analysis showed a significant positive correlation ($r = 0.662$) between D3R mRNA with ethanol consumption ($F_{(1,22)} = 17.176$, $p < 0.0001$; Fig. 6F), and preference ($F_{(1,22)} = 16.379$, $p = 0.001$; $r = 0.653$; Fig. 6G), but not with the total fluid intake ($F_{(1,22)} = 1.993$, $p = 0.172$; Fig. 6H).

In the GHT, D3R mRNA correlated negatively ($r = -0.618$) with the rats' ability to hang from the wire grid when injected with ethanol ($F_{(1,22)} = 13.612$, $p = 0.001$; Fig. 6I), but not with saline ($F_{(1,22)} = 0.744$, $p = 0.398$; data not shown). Also, D3R mRNA correlated positively ($r = 0.557$) with the LORR duration ($F_{(1,22)} = 9.895$, $p = 0.005$; Fig. 6J) but not with its onset ($F_{(1,22)} = 0.015$, $p = 0.903$; data not shown). Finally, there was no significant correlation between D3R mRNA and the AUC obtained from the BECs data ($F_{(1,22)} = 0.089$, $p = 0.769$; data not shown). It should be highlighted that there was not a direct correlation between accumbal D3R mRNA expression and tastants' consumption and preference (Suppl. Figs. 3 & 4).

These findings suggest that rats with high accumbal let-7d levels are most likely to have low D3R mRNA levels inducing reduced ethanol consumption and preference as well as ethanol-induced sedation but increased motor coordination.

4. Discussion

The principal findings in this study are that 24 h voluntary ethanol intake was significantly reduced after lentiviral-mediated injection of let-7d into the Nacc, a brain site documented as central for ethanol reward. While, the data show that accumbal let-7d elicited no response when animals were given either saccharin or quinine, let-7d overexpression improved motor coordination and resistance to ethanol-induced sedation. Finally, accumbal D3R mRNA negatively correlates with let-7d expression. These findings open new avenues for future in vitro and in vivo studies aimed at investigating the functional role of let 7d-D3R association in addiction-related behaviors.

A growing body of literature suggests that miRNAs may be involved in cocaine intake and escalation (Eipper-Mains et al., 2011; Im et al., 2010), nicotine stimulation (Huang and Li, 2009), as well as morphine self-administration (Jia et al., 2019). In the present study the role of let-7d in voluntary alcohol intake was determined in rats using a continuous access two-bottle choice drinking paradigm. To the best of our knowledge, this is apparently the first study to report on the effects of let-7d overexpression on voluntary alcohol intake in rats. Our initial observation was that let-7d overexpression in the Nacc decreased ethanol consumption and preference. This is not likely a result of an overall suppression of drinking behavior as let-7d selectively reduced ethanol intake and preference with no effect on total fluid intake. In addition, the present study shows that saccharin and quinine intake and preference were not different between the two experimental groups, suggesting that reduced alcohol consumption is not due to alterations in taste sensitivity and/or neophobia. Neither did the blood alcohol concentrations differ between the let-7d-injected rats and controls, suggestive of similar alcohol kinetics and peripheral clearance.

Increasing evidence has revealed that miRNAs may play a central role in chronic drug exposure-triggered neuroadaptive processes [for review see (Pietrzykowski, 2010)]. miRNAs were profiled, using miRNA array analysis on the prefrontal cortex (PFC) of postmortem brain tissue from human alcoholics, and it has been reported that approximately 35 miRNAs were significantly up-regulated in the alcoholic group compared with controls (Lewohl et al., 2011). Also, when Tapocik and colleagues examined miRNAs' expression changes in the rat medial PFC, after a history of alcohol dependence, they found that 41 miRNAs were significantly altered after chronic alcohol exposure (Tapocik et al. 2013). Both studies suggest that in both humans and rodents an important alteration in miRNA expression patterns, at least in the PFC, following a history of dependence could result in impaired gene expression. Still, the molecular mechanisms by which chronic alcohol abuse results in these changes in miRNAs and gene expression remain unidentified.

The likely connection between ethanol intake and differential miRNAs expression was reported by the following few studies: in adult mammalian brain, ethanol is found to upregulate miR-9 and to down-regulate the expression of its target gene, the alpha subunit of the large-conductance calcium- and voltage-activated potassium channel, which is a protein related to alcohol addiction (Pietrzykowski et al., 2008). Another study reported that, compared to control mice, lentiviral-mediated silencing of neurokinin-1 receptor, using artificial miRNA, decreased the voluntary alcohol consumption (Baek et al., 2010). In addition, our present results are in line with a recent study showing that miR-382 was down-regulated in alcohol-treated rats and that miR-382 overexpression significantly attenuated voluntary intake and preference for alcohol (Li et al., 2013). Similarly, silencing of miR-411 by infusing antagomiR-411, into the PFC of female C57BL/6J mice, reduced alcohol consumption and preference, without affecting total fluid consumption, or saccharin consumption (Most et al., 2019). Taken together, our findings build upon this literature by showing that modulating miRNA expression in the brain could be an effective alternative to regulate the expression of specific behavior-related genes.

The results described in the present study showed that let-7d overexpression was associated to a reduction of the duration of ethanol LORR and motor impairment. Multiple potential behavioral mechanisms could account for decreased ethanol intake in the two-bottle choice paradigm. For example, rats can decrease consumption of ethanol because of increased aversive properties of alcohol as well as of decrease (or increase) in ethanol reward [for review see (Chester and Cunningham, 2002)]. Alcohol hypnotic/sedative properties might also explain differences in ethanol consumption. Some reports have shown a negative correlation between LORR and voluntary ethanol intake (Hodge et al., 1993; Spanagel et al., 2002), but this is not always the case (Blednov et al., 2003; Boehm et al., 2003). Because ethanol LORR is mediated by multiple molecular mechanisms, it is likely that distinct molecular mechanisms are responsible for ethanol-induced hypnotic effects and voluntary ethanol intake. For instance, 23 candidate genes and at least four quantitative trait loci on different mouse chromosomes were associated to the transmissible component of hypnotic sensitivity to ethanol in mice (MacLaren et al., 2006; Markel et al., 1997). In addition, genotypic differences in duration of ethanol-induced LORR can reflect differences in ethanol clearance and pharmacokinetics (Radcliffe et al., 2005). However, in the current study let-7d overexpression had no effect on ethanol clearance. We hypothesize that the faster recovery of let-7d overexpressing rats from motor incoordination may be due to development of ethanol tolerance, although this is purely speculative. Any comparison of rodents' models to human alcoholism is fundamentally hypothetical, and it is imperative to recognize that the levels of alcohol consumption achieved in our study do not produce intoxication.

In a different level, mechanisms that could logically be proposed to explain the findings reported here might include effects on D3R. In order to determine the potential role of let-7d in voluntary alcohol intake and preference, the effect of let-7d on the expression of accumbal D3R mRNA in rat was determined. Both gain-of-function and loss-of-function approaches have demonstrated that let-7d was a strong regulator for the expression of D3R (Bahi and Dreyer, 2018). Therefore, and as expected, it has been found that D3R mRNA expression was significantly inhibited by overexpression of let-7d These findings are in line with previous studies from our laboratory, using computational examination and experimental approach, which have demonstrated that D3R might be a direct target gene of let-7d (Chandrasekar and Dreyer, 2009;2011). Based on these findings, it is therefore possible, albeit speculative, that D3R might be a downstream target molecule for let-7d-mediated effect on ethanol intake and preference.

D3R is a critical modulator of alcohol abuse [for review see (Heidbreder and Newman, 2010). It is well-established that, via D3R, DA is a central modulator of accumbal activity that plays critical roles in many drugs of abuse including alcohol. We have shown in a previous study that, like the D3R agonist 7-OH-DPAT, D3R overexpression exacerbated alcohol intake (Bahi and Dreyer, 2018). In contrast, like the D3R selective antagonist SB-277011-A, D3R knockdown using lentiviral-mediated siRNA expression, attenuated voluntary

alcohol consumption (Bahi and Dreyer, 2014), which agrees with other findings using SB-277011-A in C57BL/J6 mice (Rice et al., 2015). Similarly, ethanol intake was insignificant in D3R knockouts but robust in wildtype littermates, both in the two-bottle choice and drinking in the dark models (Leggio et al., 2014). Interestingly, treatment with D3R antagonists, SB-277011-A and U99194A, suppressed ethanol intake in wildtype but was ineffective in D3R knockouts (Leggio et al., 2014) confirming that DA signaling via D3R is critical for ethanol-related reward and consumption and may represent a novel therapeutic target to tackle alcoholism in humans. One could argue that quantification of let-7d and D3R expression levels were done after completion of the behavioral tests. This raises the possibility that the changes of D3R mRNA in the Nacc may be the result of the lentiviral injection, the alterations in behavior, or both. Also, the current data should be interpreted with caution because of the potential variability in the accuracy of the lentiviral infusions. We have dissected out the NAcc tissue for RT-PCR quantification, but we can't rule out the spread of the vector that was infused. In fact, given that dorsal striatum and septum can mediate some of the behavioral effects observed in the current study, and are adjacent brain regions to the NAcc, it is possible that the effects observed might be due to any effect but of let-7d miRNA in the NAcc specifically. Therefore, more studies are needed to tease apart these possibilities.

A great number of genes that can potentially affect alcohol-drinking behavior, in humans as well as in laboratory rodents' animals, was identified during the last decade using genomics and behavioral genetics approaches. Nevertheless, it is still not fully understood how these genes are controlled and clustered under the conditions of alcohol use and abuse. Control of these genes' expression by fine-tuned mechanisms of gene regulation, in which miRNAs are highly involved, may represent a significant molecular mechanism responsible for alcohol abuse-related neuropathophysiology (Li et al., 2013; Miranda et al., 2010). However, the in-depth effect of distinct/individual miRNA on alcohol use and abuse and its target genes still needs to be documented and systematic exploration of these possibilities is well beyond the scope of this study.

In summary, the present study demonstrates that accumbal let-7d overexpression is effective in reducing alcohol intake. Findings in this report extend our previous results, which showed that let-7d reduces cocaine-induced CPP in rats and improves measures of anxiety- and depression-like behaviors in mice. These data are consistent with the notion that let-7d is an important regulator and effector in alcohol intake via D3R, its direct target gene. Taken together, these findings provide a heuristic basis for better understanding the molecular and neurophysiological basis of alcoholism and highlight miRNAs as possible innovative therapeutic targets for alcohol addiction.

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Contributors

AB designed the study and performed the experiments. AB and JLD managed the literature searches and analyses. AB undertook the statistical analysis, and AB and JLD wrote the first draft of the manuscript. All authors contributed to and have approved the final manuscript.

Conflict of interest

The authors have no financial interests that might be perceived to influence the results, or the discussion reported in this article.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version.

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