Addiction Biology

SSA SOCIETY FOR THE STUDY OF ADDICTION

ORIGINAL ARTICLE

doi:10.1111/adb.12625

Silencing synaptic MicroRNA-411 reduces voluntary alcohol consumption in mice

Dana Most[1,2], Nihal A. Salem[3], Gayatri R. Tiwari[1], Yuri A. Blednov[1], R. Dayne Mayfield[1] & R. Adron Harris[1,2]

Waggoner Center for Alcohol and Addiction Research, The University of Texas at Austin, Austin, TX USA[1], Institute for Neuroscience, University of Texas at Austin, Austin, TX USA[2] and Texas A&M Institute for Neuroscience and Department of Neuroscience and Experimental Therapeutics, College of Medicine, Texas A&M University, College Station, TX USA[3]

ABSTRACT

Chronic alcohol consumption alters the levels of microRNAs and mRNAs in the brain, but the specific microRNAs and processes that target mRNAs to affect cellular function and behavior are not known. We examined the *in vivo* manipulation of previously identified alcohol-responsive microRNAs as potential targets to reduce alcohol consumption. Silencing of miR-411 by infusing antagomiR-411 into the prefrontal cortex of female C57BL/6J mice reduced alcohol consumption and preference, without altering total fluid consumption, saccharin consumption, or anxiety-related behaviors. AntagomiR-411 reduced alcohol consumption when given to mice exposed to a chronic alcohol drinking paradigm but did not affect the acquisition of consumption in mice without a history of alcohol exposure, suggesting that antagomiR-411 has a neuroadaptive, alcohol-dependent effect. AntagomiR-411 decreased the levels of miR-411, as well as the association of immunoprecipitated miR-411 with Argonaute2; and, it increased levels of *Faah* and *Ppard* mRNAs. Moreover, antagomiR-411 increased the neuronal expression of glutamate receptor AMPA-2 protein, a known alcohol target and a predicted target of miR-411. These results suggest that alcohol and miR-411 function in a homeostatic manner to regulate synaptic mRNA and protein, thus reversing alcohol-related neuroadaptations and reducing chronic alcohol consumption.

Keywords Ago2, C57BL/6 J mice, Faah mRNA and Ppard mRNA, GluA2, miR-411, two-bottle choice ethanol drinking.

Correspondence to: R. Dayne Mayfield, Waggoner Center for Alcohol and Addiction Research, University of Texas at Austin, 2500 Speedway, Austin, TX 78712, USA. E-mail: dayne.mayfield@austin.utexas.edu; dayne.mayfield@mail.utexas.edu

INTRODUCTION

Alcohol use disorder (AUD) has devastating consequences on both physical and mental health. Chronic drug and alcohol abuse cause widespread neuroadaptations in the brain, producing reorganization of synaptic structure and function (Kalivas & Volkow 2005; Robison & Nestler 2011). These changes likely result from abnormal expression of a plethora of genes in the brain (Warden & Mayfield 2017). Given the genetic complexity of AUD, it is not surprising that only a few pharmacotherapies have been identified for treating dependence and preventing relapse (World Health Organization 2014; Baingana *et al.* 2015).

MicroRNAs are small noncoding RNAs that regulate the translation of many genes (Ambros 2001; Lee & Ambros 2001) and are referred to as 'master regulators' of cellular gene expression (Miranda *et al.* 2010; Miranda 2014). Alcohol-induced changes in microRNAs have been associated with development of cellular tolerance to alcohol (Pietrzykowski *et al.* 2008; Ron & Barak 2016), cellular reward mechanisms (Li *et al.* 2013), regulation of alcohol consumption and preference (Li *et al.* 2013; Tapocik *et al.* 2014), episodes of binge drinking (Darcq *et al.* 2015), withdrawal (Tapocik *et al.* 2014) and alcohol-induced conditioned place preference (Chandrasekar & Dreyer 2011). The extensive and coordinated alcohol-induced changes in gene expression may be driven by changes in microRNAs (Mayfield 2017). Alternatively, alcohol-induced changes in mRNA expression may drive changes in microRNA expression (Nunez *et al.* 2013). Regardless of the driving force, the

co-expression of microRNAs with a network of alcohol-responsive mRNAs supports key regulatory roles for microRNAs (Mamdani *et al.* 2015; Most *et al.* 2016).

We reported that chronic alcohol consumption in mice alters the synaptic expression of mRNA networks (Most *et al.* 2015) and microRNAs (Most *et al.* 2016) in the amygdala. Interestingly, the majority of these changes were found in synaptic fractions and not in total tissue homogenates. The relationship between alcohol, microRNAs and mRNAs and their impact on alcohol-related behaviors is a rapidly developing research area with the potential to advance new treatment strategies for AUD. Here, we examined the functional significance of a previously identified alcohol-sensitive synaptic microRNA on alcohol drinking in female mice. These results provide evidence for a homeostatic process where miR-411 regulates excessive alcohol drinking by altering the mRNA synaptic profile.

MATERIALS AND METHODS

Animals

Adult C57BL/6J female mice obtained from The Jackson Laboratory (Bar Harbor, ME) were housed at the University of Texas Austin Animal Resources Center. Mice given a minimum of 2 weeks to acclimate to group housing. Food and water were provided *ad libitum* and monitored daily, as were the temperature and light/dark cycles of the rooms. All procedures were approved by the Institutional Animal Care and Use Committee at the University of Texas at Austin and adhered to the National Institutes of Health Guidelines.

Stereotaxic surgery and microRNA manipulation

Mice were anesthetized by isoflurane inhalation and placed in a stereotaxic apparatus (model 1900, David Kopf Instruments, Tujunga, CA). The skull was exposed, and bregma and lambda were visualized. A digitizer attached to the micromanipulator of the stereotaxic apparatus was used to locate coordinates relative to bregma [for medial prefrontal cortex (mPFC): anteroposterior +2.0 mm, mediolateral ±0.3 mm, dorsoventral −2.0 mm]. Burr holes were drilled in the skull above the injection site (using a drill equipped with a #75 carbide bit, David Kopf Instruments). Injections were performed using a 10-µl microsyringe (model #1701, Hamilton, Reno, NV) and a 30-gauge needle. Compounds (described in the succeeding discussions) were diluted to 0.5 nmol/µl in artificial cerebrospinal fluid (ACSF) and injected in a 1-µl volume over a period of 2 minutes. Dosing was based on previous studies (Li *et al.* 2013; Darcq *et al.* 2015). After injections, the syringe was left in place for another 2 minutes before retraction. Incisions were closed with tissue adhesive (Vetbond, #70200742529, 3 M, Maplewood, MN).

Compounds were either injected into the mPFC at the time of surgery (mice intended for molecular studies), or cannulae were inserted followed by treatment infusions at a later time (mice intended for behavioral studies). For experiments requiring cannulation, a cannula was placed in the same coordinates described earlier and secured using dental cement from the Ortho-Jet Package (#1334PT, Lang Dental, Wheeling, IL), following manufacturer's instructions. The skull was covered with a thin layer of liquid foil for approximately 1 minute. Ortho-Jet liquid was then mixed with Ortho-Jet powder using the disposable brushes. When the mixture became homogenous, it was gently applied to the skull, making sure to partially cover the cannula pedestal. The acrylic gel was allowed to cure for approximately 2 minutes and then polished with a layer of Jet Seal. The cannula guide (26 gauge, Plastics One, Roanoke, VA) was inserted 1.5 mm into the mPFC and capped with a 2-mm long dummy cannula (33 gauge, #8IC315DCS5SP, 0.008 inch, Plastics One). Following surgery, 5 mg/kg of Metacam (#11247402, Animal Health International, Westlake, TX) was administered, and mice were placed in a recovery cage to recover from anesthesia. Each guide cannula contained a 5-mm pedestal cut with the cannula extending 1.5 mm below the pedestal (#8IC315GS5SPC, 26 gauge, Plastics One, Roanoke, VA). Infusion cannulae (#8IC315IS5SPC, 33 gauge, Plastics One) were 2 mm long, thus projecting 0.5 mm beyond the tip of the guide.

Loss and gain of function treatments targeting the mPFC consisted of a microRNA locked nucleic acid inhibitor—antagomiR (Exiqon, Vedbaek, Denmark) and a microRNA mimic (Dharmacon, Lafayette, CO), respectively, or ACFS (Harvard apparatus, Holliston, MA). The following antagomiRs and mimics were used in this study: antagomiR-411-5p (GCTATACGGTCTACT); antagomiR-203-3p (GGTCCTAAACATTTCA); antagomiR-92a-3p (CGGGACAAGTGCAAT); antagomiR-137-3p (GCGTATTCTTAAGCAA); antagomiR-187-3p (TGCAACACAAGACACG); mimic-411-5p (UAGUAGACCGUAUAGCGUACG); and mimic-203-3p (GUGAAAUGUUUAGGACCACUAG). AntagomiRs were high-performance liquid chromatography purified and 5'TYE-563 labeled, and mimics were high-performance liquid chromatography purified and 5'DY547-psngr labeled.

Chronic alcohol consumption

Seven to 10 days after cannulation, mice underwent 4 weeks of a two-bottle choice paradigm with continuous (24 hours) access to one bottle of 15% (v/v) ethanol (referred to as alcohol) and one bottle of water, as previously described (Most *et al.* 2015). Levels of alcohol consumed were similar to previously recorded levels (Most *et al.* 2015). Bottle positions were alternated daily for the first

4 weeks of consumption, and then the preferred location was determined over the next 2–4 days (Blednov *et al.* 2012). The preferred bottle location was kept constant during baseline drinking measurements (2–4 days) and for the remainder of the drinking study.

Synthetic microRNA infusions in mice consuming chronic alcohol

After measuring baseline levels of alcohol consumption, treatments were infused through the cannulae into the mPFC. Briefly, mice were carefully scruffed, and cannulae were uncapped. Injectors (2 mm) connected to tubing were inserted into the cannulae. The tubing was connected to a 10-µl syringe, and 1 µl of treatment was infused for 45 seconds. Then, the dummy cannulae were reinserted, and the mice were returned to their home cages. Treatment-induced effects on alcohol consumption, preference and total fluid intake were recorded.

Saccharin consumption

Mice underwent a two-bottle choice paradigm using 0.0165% saccharin and water for 4 weeks. AntagomiR-411 or ACSF was then infused into the mPFC, and effects on saccharin consumption and total fluid intake were recorded.

Synthetic microRNA infusions in alcohol-naïve mice

Mice were cannulated, allowed a 1-week recovery period and were then infused with antagomiR-411 or ACSF into the mPFC as described earlier. Three days later, alcohol was introduced for the first time in a two-bottle choice paradigm as previously described. Bottle positions were alternated daily throughout this experiment. Because antagomiR-411 did not alter acquisition of alcohol drinking or consumption, we used the same mice for anxiety-related testing.

Anxiety-related behaviors

Open field test

After consuming alcohol for 16 days, mice from the experiment described earlier received a second injection of antagomiR-411 or ACSF. A day after injection, locomotor activity was measured using the open field test. Mice were transported to the testing room during their light cycle 1 hour before testing began. Each mouse was individually placed in the open field and allowed to freely explore the field while their activity was recorded in bins of 5 minutes for 15 minutes using the Opto-microvarimex animal activity meter (Columbus Instruments, Columbus, OH), consisting of six light beams placed along the width of the cage at 2.5-cm intervals, 1.5 cm above the floor. The field was covered with a heavy plastic lid with holes for ventilation. After each mouse was tested, the field was cleaned with 10% alcohol and allowed to air dry before the next mouse was tested. Time, distance and number of entries into the different sections of the field were recorded.

Elevated-plus maze

Three days later, mice were evaluated for treatment-induced anxiolysis using the elevated-plus maze. Mice were transported to the testing room during their light cycle 1 hour before testing began. Each mouse was individually placed on the central platform of the maze facing an open arm and allowed to freely explore the maze for 5 minutes, during which the following measurements were recorded: number of open arm entries, number of closed arm entries, total number of entries, time spent in open arms and time spent in closed arms. A mouse was considered to be on the central platform or any arm when all four paws were within its perimeter. After each mouse was tested, the field was cleaned with 10% alcohol and allowed to air dry before the next mouse was tested.

RNA isolation

Immediately following the behavioral tests, mice were sacrificed by cervical dislocation during the light phase of the light/dark cycle. Brains were removed and washed with an ice-cold saline solution, and the mPFC was sliced at coronal levels bregma +1 to +2.5 mm. The mPFC was microdissected, snap frozen in liquid nitrogen and stored at −80°C. Total RNA was extracted at a later time (miRNeasy Mini Kit, #217004, Qiagen, Hilden, Germany) and DNAse treated (RNase-Free DNase Set, #79254, Qiagen), following manufacturer's guidelines. Yields and purity were assessed using NanoDrop 8000 and Qubit Fluorometric Quantitation (Thermo Fisher Scientific, Grand Island, NY), and quality was determined using the Agilent 2200 TapeStation (Agilent Technologies, Santa Clara, CA). RNA integrity numbers were above 7.5.

Quantitative real-time polymerase chain reaction

MicroRNA polymerase chain reaction

To measure microRNA levels, RNA was reverse transcribed (TaqMan MicroRNA Reverse Transcription Kit, #4366596, Thermo Fisher Scientific) using a primer pool per manufacturer's instructions. The following microRNAs and Taqman Assay IDs were used: hsa-miR-411-5p (001610); hsa-miR-203-3p (000507); mmu-miR-92-3p (000430); mmu-miR-137-3p (001129); mmu-miR-187-3p (001193); and snoRNA234 (001234, endogenous control).

Following the real-time polymerase chain reaction, quantitative polymerase chain reaction (qPCR) was

performed in triplicate in 20-µl reactions by combining the following: 10 µl of TaqMan Universal Master Mix II, No AmpErase UNG (2×) (#4440040, Thermo Fisher Scientific), 7 µl of Nuclease-free water, 2 µl of RT cDNA product (for a total of 40 ng cDNA input per reaction) and 1 µl of Taqman MicroRNA assays. Relative microRNA expression was determined using the ΔΔCt method per manufacturer's instructions and as outlined elsewhere (Hellemans *et al.* 2007). Reactions were carried out in a CFX384 Real-Time PCR Detection System (Bio-Rad, Hercules, CA). MicroRNA assays were tested in triplicate using a standard curve generated from a 5-point, two-fold (microRNA) or three-fold (SnoRNA) dilution series.

mRNA polymerase chain reaction

To measure mRNA expression levels, samples were reverse transcribed into cDNA using a High-Capacity cDNA Reverse Transcription Kit (#4366596, Thermo Fisher Scientific) and amplified using TaqMan Universal PCR Master Mix, no AmpErase UNG (#4368814, Thermo Fisher Scientific) with gene-specific primers. The following genes and Taqman assay IDs (Thermo Fisher Scientific) were used: *Aldh3a2* (Mm00839320_m1); *Faah* (Mm00515684_m1); *Glud1* (Mm00492353_m1); *Glua2(Gria2)* (Mm00442822_m1); *Grm7* (Mm01189424_m1); *Maob* (Mm00555412_m1); *Ppard* (Mm00803184_m1); *Tlr4* (Mm00445273_m1); and *Hprt* (Mm00446968_m1, endogenous control).

Reactions were carried out as outlined for microRNAs mentioned earlier, except the standard curve was generated from a 5-point, 10-fold dilution series. Baseline and CT values were automatically calculated with default parameters. qPCR was performed in triplicate using 6 ng of each cDNA. All target gene replicates were run on the same plate to avoid between-plate variation. Each RNA sample was evaluated for the presence of genomic DNA by comparing *Gapdh* (#Mm99999915_g1, Thermo Fisher Scientific) Ct values from RT+ and RT− reactions. In all qPCR experiments, samples were tested in triplicate, and RNA expression levels were determined using the ΔΔCt method per manufacturer's instructions and as outlined elsewhere (Hellemans *et al.* 2007) relative to the endogenous control (snoRNA234 for the microRNA experiments and *Hprt* for the mRNA experiments) and normalized to the control group.

Immunoprecipitation of Argonaute

Frozen mPFC samples were homogenized and lysed using 1X RIPA lysis buffer supplemented with protease inhibitors (Complete Mini, #11836153001, Roche, Basel, Switzerland) and RNAse inhibitors (RNaseOut, #10777019, Thermo Fisher Scientific). Ten percent of each lysate was kept for subsequent input RNA isolation. Ninety percent of each lysate was combined with 8 µl of rat anti-argonaute (Ago2) antibody (SAB4200085, Sigma Aldrich, St. Louis, MO) or rat IgG isotype control (for negative control, #02–9602, Thermo Fisher Scientific), incubated overnight, combined with 50 µl of pre-blocked Dynabeads Protein G (#10003D and #10007D, Thermo Fisher Scientific) and incubated for 1 hour at 4°C. Beads were separated on a magnet, washed with 0.05% and 0.02% Tween-20 in 1X PBS and resuspended in 50 µl of elution buffer. Samples were treated with 150 µl of Proteinase K buffer (#P5568–1, Sigma Aldrich) for 1 hour at 42°C. RNA was isolated from the beads and extracted as described earlier. Real-time polymerase chain reaction and qPCR were run as described earlier in the microRNA section. Immunoprecipitation data were analyzed using the percent input method (relative to input RNA levels), and enrichment over input values were calculated using the following equations: ΔCt = (Ct input − 3.32) − (Ct Ago2-IP or rat IgG); enrichment = 100 * 2^ΔCt; association over negative IgG pulldown = values higher than the negative control were calculated by subtracting the negative control value from that of the sample; values below that of the negative control are reported as not associated with Ago2.

Immunohistochemistry and image analysis

Mice were euthanized and transcardially perfused with phosphate-buffered saline (PBS) and 4% paraformaldehyde. Brains were harvested, postfixed for 24 hours in 4% paraformaldehyde at 4°C, cryoprotected for 24 hours in 20% sucrose in PBS at 4°C, placed in molds containing Optimal Cutting Temperature (OCT) compound (VWR, Radnor, PA) and frozen using isopentane. Brains were equilibrated to −14°C in a cryostat (Thermo Fisher Scientific) for 30 minutes, and 30-µm coronal sections were taken from the mPFC and placed in sterile PBS. Sections were treated with 0.1% Triton X-100 (2 × 10 minutes at 25°C), washed in PBS (3 × 5 minutes at 25°C), blocked with 10% donkey serum (30 minutes at 25°C), treated with primary antibodies at 4°C overnight, washed in PBS (3 × 10 minutes at 25°C), incubated with fluorescence-conjugated secondary antibodies (2 hours at 25°C) and rinsed with PBS (3 × 10 minutes at 25°C). The following primary antibodies and dilutions were used: rabbit polyclonal anti-GLUA2 (1:50, Abcam, Cambridge, United Kingdom); rabbit polyclonal anti-GRINA (1:100, GeneTex, Irvine, CA); rabbit polyclonal anti-gamma-aminobutyric acid type B receptor subunit 1 (GABAB1) (1:50, Santa Cruz, Dallas, TX); guinea-pig polyclonal anti-NEUN (1:1000, EMD Millipore, Burlington, MA); mouse monoclonal anti-NEUN, clone A60 (1:500, Neuromab); mouse monoclonal anti-glial fibrillary acidic protein (GFAP), clone N206A/8 (1:300, Neuromab, Davis,

CA); and goat polyclonal anti-IBA (1:300, Abcam). The following secondary antibodies were used (1:1000 dilution): donkey anti-mouse Alexa 488 (Thermo Fisher Scientific); donkey anti-rabbit Alexa 488 (Thermo Fisher Scientific); donkey anti-mouse Alexa 568 (Thermo Fisher Scientific); donkey anti-rabbit Alexa 568 (Thermo Fisher Scientific); donkey anti-rabbit Alexa 350 (Thermo Fisher Scientific); and donkey anti-guinea pig CF 405 M (Sigma Aldrich).

Brain sections were mounted on slides using sterile 0.2% gelatin and mounting media (with or without 4',6-diamidino-2-phenylindole; Vector Laboratories, Burlingame, CA) and cover-slipped. Images were captured (without saturating the signal) using a Zeiss Axiovert 200 M Fluorescent Microscope (Zeiss, Thornwood, NY) at a 20× and 63× magnification, imported into the ImageJ software package, and composite images were split into individual channels and overlaid. Two sets of control experiments were performed to test specificity: (1) omission of primary antibodies and (2) omission of secondary antibodies. No immunostaining was detected under either of these conditions.

To assess cell-type specificity of antagomiR-411 transfection, sections were stained with Neun, Iba or GFAP antibody. The percent transfection per cell type was calculated by counting 100 Neun/Iba/GFAP-positive cells on the unmerged channel image and then using the merged channel image to count the percent of antagomiR-411-positive cells within those (N = 4–5 per group). AntagomiR-411 was found in 98% of Neun-positive neurons and in 86% of Iba-positive microglia cells (data not shown). We did not detect transfection of antagomiR-411 in GFAP-positive astrocytes.

We measured the effects of antagomiR-411 on glutamate receptor AMPA-2 subunit (GLUA2), glutamate ionotropic receptor N-methyl-D-aspartate type subunit associated protein 1 (GRINA) and GABAB1 proteins, by tracing the desired protein (specifically in NeuN-positive cells) on the merged channel image, and measuring the area, integrated density and mean gray value of the protein on the individual channel image. Cells (35–50) were counted per brain slice (N = 4–5 per group). For a normalized comparison between images, values were corrected to the background intensity using the equation for corrected total cell fluorescence = Integrated density − (Area of selected cell × mean fluorescence of background readings).

Experimental design and statistical analysis

We used the Shapiro–Wilk normality test and found that the majority of the datasets in any given analysis were normal; thus, parametric tests were used in this study. qPCR and immunohistochemistry data were analyzed using Student's t-tests or two-way ANOVA followed by Holm–Sidak post hoc tests. Outliers were determined using the Grubbs test, and values were removed. The behavioral data were analyzed using repeated-measures ANOVA (RM-ANOVA) followed by Holm–Sidak's post hoc analysis or using unpaired t-tests with Welch's correction. Missing values in the behavioral data were replaced with the average values obtained from individual mice across the subsequent 3 days, or if one of the last three values was missing, the previous value was used. Corrected P-values < 0.05 were considered statistically significant. MicroRNA target predictions were performed using the DIANA-microT-CDS tool (Paraskevopoulou *et al.* 2013) and miRDB (Wong & Wang 2014). Simple calculations were performed using Microsoft Excel 2013 (Microsoft). All statistics and graphics were performed using Prism (GraphPad, La Jolla, CA).

RESULTS

Chronic alcohol consumption reduces levels of microRNAs in mouse medial prefrontal cortex

We hypothesized that alcohol-responsive synaptic microRNAs in the mPFC are involved in the neuroadaptations induced by chronic alcohol consumption and that specific microRNAs can be manipulated to reduce excessive alcohol drinking. In order to select microRNAs for *in vivo* manipulation, we first evaluated the effects of alcohol on microRNAs in the mPFC that were previously identified as alcohol-sensitive in amygdalar synaptoneurosomes [e.g. miR-411, miR-203, miR-137 and miR-187 (Most *et al.*, 2016)]. miR-92a was also chosen given that many of its family members and precursors are sensitive to alcohol (Most *et al.* 2016).

ACSF-infused mice were given access to 15% alcohol for more than 4 weeks in a two-bottle choice procedure (Fig. 2a), and levels of miR-411, miR-203, miR-92a, miR-137 and miR-187 were measured in mPFC using qPCR. miR-411, miR-203, miR-92a and miR-137 were reduced following chronic alcohol drinking compared with controls (unpaired t-tests, miR-411: $t_{65} = 3.83$, $P = 0.0003$; miR-203: $t_{65} = 4.05$, $P = 0.0001$; miR-92a: $t_{65} = 3.09$, $P = 0.0029$; miR-137: $t_{65} = 2.57$, $P = 0.012$; miR-187: $t_{65} = 0.799$, $P = 0.427$; Fig. 1a). The amount of alcohol consumed in the last session (last drinking day) was significantly correlated with the levels of miR-411 (Pearson $r = 0.796$, $P = 0.0181$) but not with the levels of miR-203, miR-92a, miR-137 or miR-187 (data not shown). The levels of miR-411 associated with Ago2 were reduced following chronic alcohol consumption, as measured by Ago2 immunoprecipitation and qPCR (unpaired t-test $t_9 = 3.64$, $P = 0.005$). However, there were no changes in levels of Ago2 associated with miR-203, miR-92a, miR-137 or miR-187 (Fig. 1b). These results suggest that the reduction in levels of miR-411 following chronic alcohol

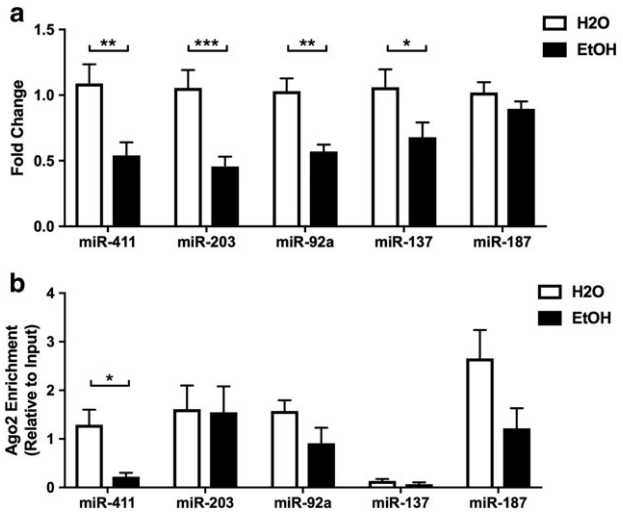

Figure 1 Alcohol decreases the levels of miR-411, miR-203, miR-92a and miR-137 and the levels of miR-411 associated with Ago2. (a) Effects of alcohol on miR-411, miR-203, miR-92a, miR-137 and miR-187 levels measured by quantitative polymerase chain reaction. Samples are from alcohol-consuming (EtOH, N = 7–8) compared with control (H2O, N = 7–8) mice. Results are shown as fold-change ΔΔCt values relative to H2O. (b) Effects of alcohol on Ago2 association with miR-411, miR-203, miR-92a, miR-137 or miR-187, measured by immunoprecipitation and quantitative polymerase chain reaction. Samples are from alcohol-consuming (EtOH, N = 5) compared with control mice (H2O, N = 7–8). Immunoprecipitation data are shown relative to input microRNA levels. For all panels, significance was determined using unpaired t-tests followed by Holm–Sidak post hoc tests (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$). Data represent mean + standard error of the mean

consumption is mediated through decreased association of miR-411 with Ago2.

In vivo silencing of miR-411 decreases alcohol consumption

Mice consumed 15% alcohol in a two-bottle choice procedure, and then fluorescently labeled microRNA treatments were infused into the mPFC (Fig. 2a). Treatments were designed to produce loss (antagomiRs) or gain (mimics) of microRNA function. Locked nucleic acid miRNA antisense inhibitors act by steric blockade to silence endogenous microRNA function; whereas, microRNA mimics are double-stranded RNA oligonucleotides that act to mimic mature microRNAs function. ACSF infusions served as controls. We tested three different cohorts of mice: (1) treatment with antagomiR-411 or mimic-411; (2) treatment with antagomiR-203 or mimic-203; and (3) treatment with antagomiR-92a, antagomiR-137 or antagomiR-187.

Alcohol consumption was markedly reduced following treatment with antagomiR-411 compared with mimic-411 or ACSF treatment (RM-ANOVA: $F_{2,39} = 4.84$, $P = 0.013$; Fig. 2b). Preference for alcohol over water was also reduced following treatment with antagomiR-411 (RM-ANOVA: $F_{2,39} = 3.383$, $P = 0.044$; Fig. 2c). There were no changes in total fluid consumption (Fig. 2d). Infusion of mimic-411 did not alter alcohol consumption, preference or total fluid intake. Figure 2e shows that there were more occurrences of decreased alcohol drinking in the antagomiR-411 group (left tail of antagomiR-411 curve) compared with the ACSF group (Fig. 2e).

Mimic-411 increases levels of miR-411

We considered that the lack of behavioral effect with mimic-411 might simply be due to cellular degradation of mimic-411. However, levels of miR-411 were increased in response to mimic-411 in both control and alcohol consuming animals (Fig. S1).

In contrast to antagomiR-411, there were no changes in alcohol consumption, preference or total fluid intake following treatment with antagomiR-203, mimic-203, antagomiR-92a, antagomiR-137 or antagomiR-187 (Fig. S2A–F).

Silencing of miR-411 has no effect on preference for saccharin

We next examined the effects of antagomiR-411 on saccharin consumption. There were no changes in saccharin consumption (Fig. 3a), preference for saccharin over water (Fig. 3b) or total fluid consumption (Fig. 3c). Although miR-411 treatment decreased preference for alcohol, it did not alter the preference for saccharin.

Silencing of miR-411 has no effect on acquisition of alcohol drinking in alcohol-naïve mice

Because antagomiR-411 decreased alcohol consumption and preference when given to mice exposed to chronic alcohol, we tested antagomiR-411 in alcohol-naïve mice to determine if pretreatment could prevent acquisition of alcohol consumption. We infused antagomiR-411 or ACSF into the mPFC of alcohol-naïve mice and then

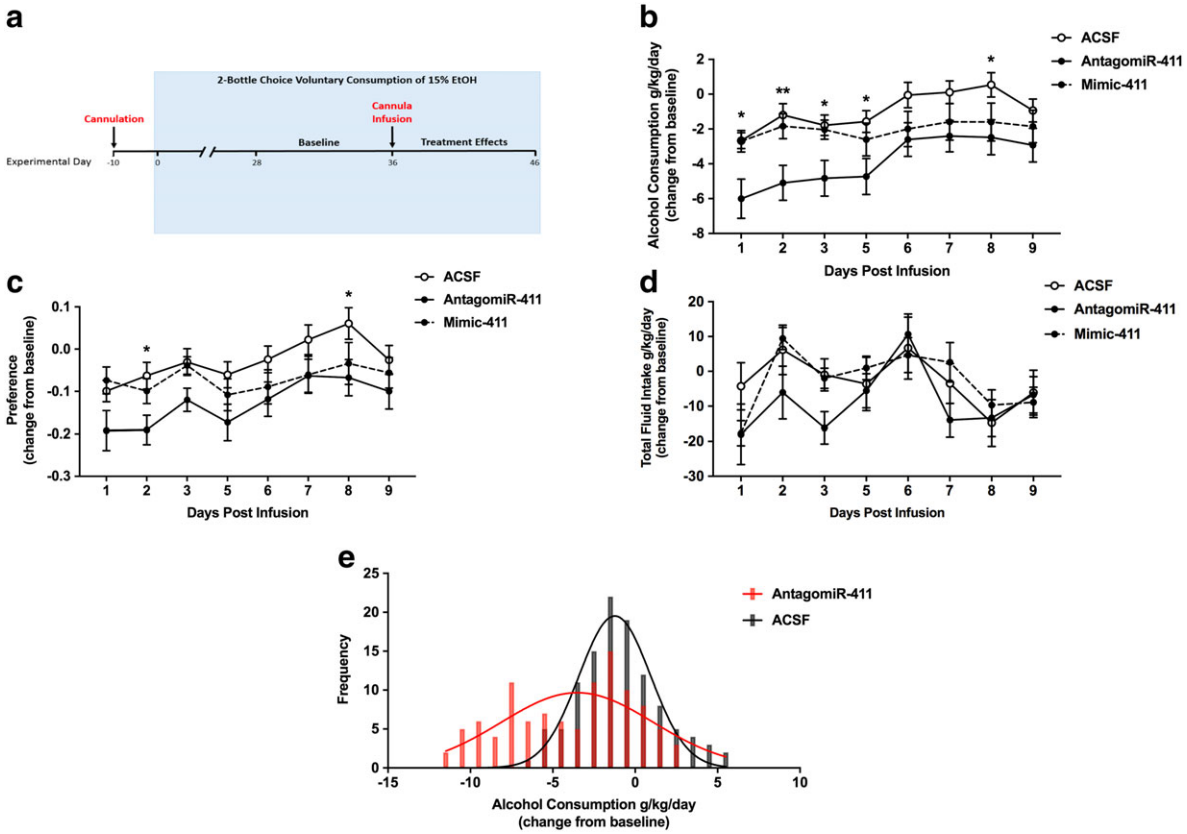

Figure 2 AntagomiR-411 treatment decreases alcohol consumption in mice exposed to chronic alcohol. (a) Timeline of cannulation and the two-bottle choice alcohol (15%) consumption procedure. Following chronic alcohol consumption, treatments were infused into the prefrontal cortex, and drinking effects were measured for 6–10 days. Effects of antagomiR-411 (N = 13), mimic-411 (N = 15) or ACSF (N = 14) on (b) alcohol consumption, (c) preference for alcohol over water and (d) total fluid consumption. Data are presented as change from baseline per day (Day X—baseline). Significance was determined using repeated-measures ANOVA followed by Holm–Sidak post hoc tests (*$P < 0.05$, **$P < 0.01$). Data represent mean ± standard error of the mean. One data point was determined to be an outlier and was not included in the analysis. (e) Histogram of the change in consumption levels following treatment with antagomiR-411 or ACSF, collapsed throughout the days post-infusion

introduced 15% alcohol using a two-bottle choice procedure (Fig. 4a). Pretreatment with antagomiR-411 in naïve mice did not change alcohol consumption (Fig. 4b) or preference (Fig. 4c). Although there was a treatment × time interaction effect for total fluid intake (RM-ANOVA: $F_{7,161} = 3.216$, $P = 0.0032$), there was no main effect of treatment ($F_{1,23} = 0.025$, $P = 0.8767$; Fig. 4d). Thus, pretreatment with antagomiR-411 did not prevent acquisition of alcohol consumption when given to alcohol-naïve mice.

Silencing of miR-411 has no effect on anxiety-related behaviors

Stress is known to alter alcohol consumption (Lopez, Anderson, & Becker 2016), and we studied the effects of miR-411 silencing on two different anxiety-related behaviors. The elevated-plus maze and open field tests were performed using alcohol-consuming mice after infusion of antagomiR-411 (N = 10) or ACSF (N = 8). There was no effect of antagomiR-411 on these behaviors (Fig. S3A–D).

AntagomiR-411 reduces miR-411 and miR-411/Ago2 levels

We used qPCR to measure the levels of miR-411 in alcohol-naive and alcohol-consuming mice compared with ACSF treatment. Both antagomiR-411 treatment ($F_{1,25} = 25.4$, $P < 0.0001$) and alcohol consumption ($F_{1,25} = 4.99$, $P = 0.035$) decreased the levels of miR-411, and there was an interaction between the two treatments (two-way ANOVA: $F_{1,25} = 8.33$, $P = 0.0079$) (Fig. 5a). A second, independent analysis of a separate set of samples showed similar results—levels of miR-411 were reduced following treatment with antagomiR-411 (two-way ANOVA: $F_{1,27} = 28.2$, $P < 0.0001$) and alcohol ($F_{1,27} = 6.16$, $P = 0.0196$), although the interaction between them was not significant in this instance ($F_{1,27} = 0.12$, $P = 0.734$).

MicroRNAs bind with Ago2 during the formation of the RNA-induced silencing complex and subsequent regulation of mRNA stability (Czech & Hannon 2011). We next examined if alcohol consumption altered the association of miR-411 with Ago2 and found that Ago2-associated miR-411 levels were reduced by both antagomiR-411 treatment

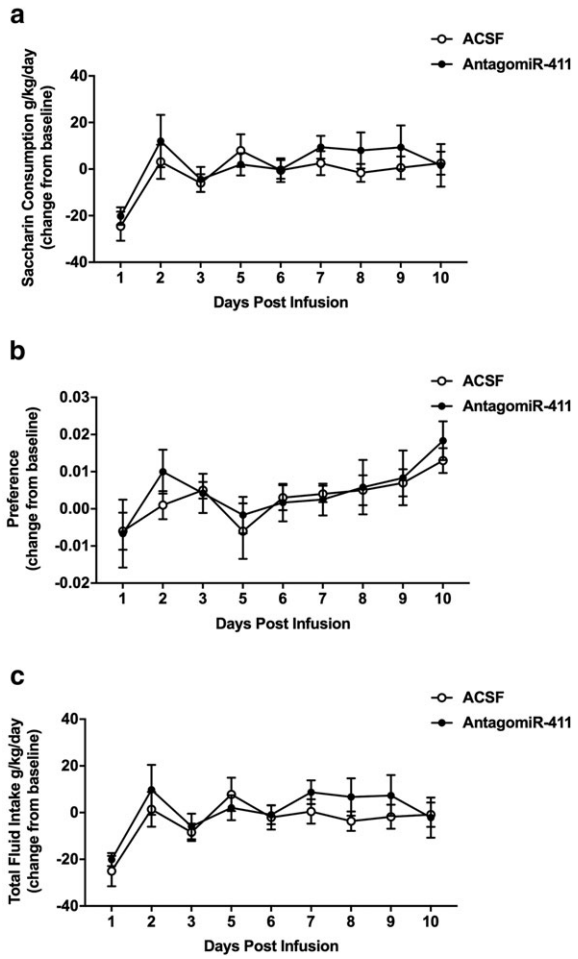

Figure 3 Antagomir-411 treatment does not change saccharin consumption. (a–b) Effects of antagomir-411 (N = 12) or ACSF (N = 10) on (a) saccharin consumption, (b) preference for saccharin over water and (c) total fluid intake. Data are presented as change from baseline per day (Day X—baseline). For all panels, significance was determined using repeated-measures ANOVA followed by Holm–Sidak post hoc tests. Data represent mean ± standard error of the mean

($F_{1,19}$ = 28.1, $P < 0.0001$) and alcohol consumption ($F_{1,19}$ = 14.99, P = 0.0011) (Fig. 5b). There was an interaction between the treatments on the association of miR-411 with Ago2 (two-way ANOVA: $F_{1,19}$ = 13.9, P = 0.0014).

AntagomiR-411 did not affect the levels of miR-203, miR-92a, miR-137 and miR-187, or their association with Ago2 (Fig. S4A–B).

Functional effect of antagomiR-411: increase in a target protein

Previous studies showed that alcohol decreases GLUA2 protein levels (Jin *et al.* 2014; Santerre *et al.* 2014). In addition, we showed that chronic alcohol consumption reduces synaptic levels of *Glua2* mRNA in the amygdala (Most *et al.* 2015; Most *et al.* 2016). *Glua2* is also a predicted target of miR-411 (DIANA-microT-CDS tool) (Paraskevopoulou *et al.* 2013; Most *et al.* 2016). To determine if the antagomiR-411-induced reduction in alcohol consumption is mediated through GLUA2, we measured these protein levels after treatment with antagomiR-411. Levels of GLUA2 protein were increased, specifically in NEUN-labeled cells (unpaired t-tests: t_7 = 4.863, P = 0.0018; Fig. 6). In contrast, two other alcohol-responsive proteins, GRINA and GABAB1 (Most *et al.* 2015) that are not predicted targets of miR-411, were not altered by treatment with antagomiR-411. These results suggest that miR-411, a synaptic microRNA that may decrease GLUA2 protein levels in the mPFC, plays a role in regulating alcohol consumption and preference in alcohol-consuming mice.

AntagomiR-411 regulates levels of predicted mRNA targets

We used qPCR to measure the levels of predicted mRNA targets of miR-411. Some of the predicted targets were sensitive to antagomiR-411 treatment but were not sensitive to alcohol [e.g. aldehyde dehydrogenase 3 family member A2 (*Aldh3a2*), glutamate dehydrogenase (*Glud1*), monoamine oxidase B (*Maob*) and toll like receptor 4 (*Tlr4*); data not shown]. Although we showed that antagomiR-411 increased neuronal GLUA2 protein levels, the levels of *Glua2* mRNA were not altered by antagomiR-411 ($F_{1,33}$ = 2.2, P = 0.15) or alcohol ($F_{1,33}$ = 0.56, P = 0.46).

Some mRNAs were sensitive to both antagomiR-411 treatment and alcohol consumption. One example was fatty acid amide hydrolase (*Faah*), a predicted target of miR-411 (DIANA-microT-CDS tool) (Paraskevopoulou *et al.* 2013) and a well-known alcohol-sensitive transcript in humans (Spagnolo *et al.* 2016; Sloan *et al.* 2017) and rodents (Zhou *et al.* 2017). Significant main effects were observed for both antagomiR-411 treatment ($F_{1,32}$ = 6.92, P = 0.01) and alcohol consumption ($F_{1,32}$ = 8.87, P = 0.01); however, the treatment effect interaction was not significant ($F_{1,32}$ = 2.06, P = 0.16). Post hoc analysis revealed that antagomiR-411 significantly increased *Faah* mRNA levels compared with control (qPCR fold change of 1.35, Holm–Sidak post hoc P = 0.041). No antagomiR-mediated changes in *Faah* mRNA were found in alcohol-consuming mice in the post hoc analysis.

In addition, peroxisome proliferator activated receptor delta (*Ppard*), a downstream participant in the *Faah* pathway (Fu *et al.* 2003; Yan *et al.* 2007), was sensitive to antagomiR-411 treatment and alcohol consumption. Significant main effects were observed for both antagomiR-411 treatment ($F_{1,31}$ = 45.5, $P < 0.0001$) and alcohol consumption ($F_{1,31}$ = 11.2, P = 0.002). A significant interaction between treatments was also observed ($F_{1,31}$ = 4.57, P = 0.04). Post hoc analysis

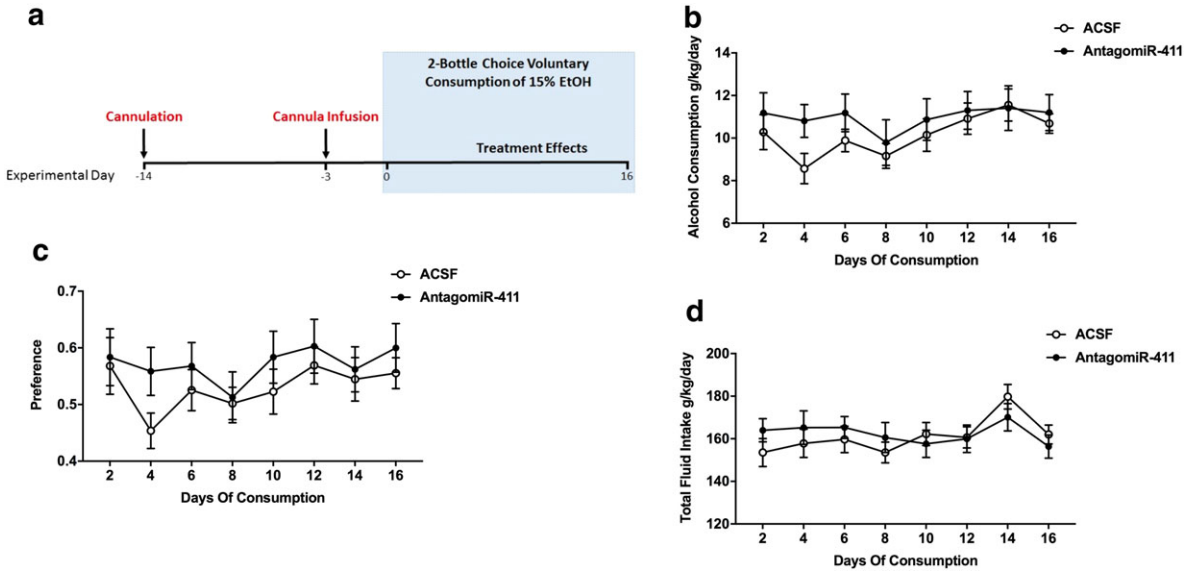

Figure 4 AntagomiR-411 treatment in alcohol-naïve mice does not change alcohol consumption. (a) Timeline of cannulation and the two-bottle choice alcohol (15%) consumption procedure. (b–d) Effects of antagomiR-411 (N = 14) or ACSF (N = 11) on (b) alcohol consumption, (c) preference for alcohol over water and (c) total fluid consumption. Data are presented as two-day averages. Significance was determined using repeated-measures ANOVA followed by Holm–Sidak post-hoc tests. Data represent mean ± standard error of the mean

revealed that antagomiR-411 increased *Ppard* mRNA levels compared with control (qPCR fold change of 1.67, Holm–Sidak post hoc P = <0.0001), and alcohol consumption reduced the antagomiR-mediated changes in *Ppard* mRNA levels (qPCR fold change of 1.34, Holm–Sidak post hoc P = 0.0141); however, levels were not reduced to control levels. These results suggest that chronic alcohol consumption decreases *Faah* and *Ppard* mRNA levels and also reduces antagomiR-411-mediated increases in expression.

DISCUSSION

We showed that chronic alcohol consumption decreased the levels of miR-411 and increased GLUA2 protein in mouse mPFC. Silencing of miR-411 decreased alcohol intake and preference in mice exposed to chronic alcohol and increased levels of some miR-411 targets but did not alter preference for saccharin or anxiety-related behaviors. In contrast, silencing of other alcohol-sensitive microRNAs (miR-203, miR-92a, miR-137 or miR-187) did not alter alcohol consumption. Importantly, we show that antagomiR-411 reduced alcohol drinking in chronic alcohol-exposed mice but did not affect the acquisition of drinking in mice without a history of alcohol exposure. These results suggest that miR-411 is involved in the neuroadaptations related to long-term alcohol use (see Table 1 for a summary of the molecular and behavioral effects of miR-411 manipulation in the mPFC). We chose to use C57BL/6J female mice because they consistently consume larger quantities of alcohol than do male mice in the two-bottle choice paradigm because; thus, improving the likelihood of measuring of small magnitude treatments effects. Further studies are warranted to determine the treatment response to antagomiR-411 in male mice.

AntagomiR-411 decreased alcohol consumption in alcohol-consuming mice

Alcohol decreased the levels of miR-411, miR-203, miR-92a and miR-137 in the mPFC. These microRNAs were studied based on previous findings in cultured cells (Van Steenwyk, Janeczek, & Lewohl 2013), human prefrontal cortex (Lewohl *et al.* 2011), rat prefrontal cortex (Tapocik *et al.* 2013) and mouse amygdala (Most *et al.* 2016). Chronic alcohol consumption did not alter levels of miR-187, and as expected, manipulating miR-187 had no effect on alcohol consumption. Of the alcohol-sensitive microRNAs, only manipulation of miR-411 altered alcohol drinking. This selectivity may be due to the alcohol-induced decreases in miR-411/Ago2 association, which is required to alter drinking. This explanation is supported by the fact that Ago2 has endonuclease activity that facilitates splicing of the miR-411 target mRNAs (rather than inhibition of mRNA translation mediated by other Ago proteins).

Mimic-411 did not alter alcohol consumption

Although antagomiR-411 decreased chronic alcohol consumption and preference, treatment with mimic-

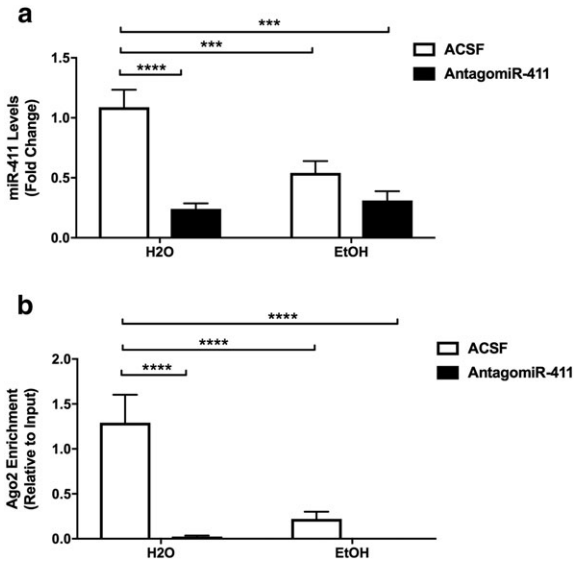

Figure 5 AntagomiR-411 treatment decreases levels of miR-411 and miR-411 associated with Ago2. (a) Effects of antagomiR-411 and alcohol on miR-411 levels measured by quantitative polymerase chain reaction. Samples were taken from alcohol-consuming (EtOH) and control (H2O) mice after treatment with antagomiR-411 or ACSF: ACSF-H2O (*N* = 8), AntagomiR-411-H2O (N = 8), ACSF-EtOH (N = 8) and AntagomiR-411-EtOH (N = 5). Data were quantified using the ΔΔCt method (relative to ACSF-H2O levels). SnoRNA-234 was used as an endogenous control. (b) Effects of antagomiR-411 and alcohol on Ago2 association with miR-411 measured by immunoprecipitation and quantitative polymerase chain reaction: ACSF-H2O (N = 5), AntagomiR-411-H2O (N = 7), ACSF-EtOH (*N* = 6) and AntagomiR-411-EtOH (N = 5). Immunoprecipitation data are shown relative to input microRNA levels. For all panels, significance was determined using unpaired *t*-tests followed by Holm–Sidak post hoc tests (***$P < 0.001$, ****$P < 0.0001$). Data represent mean + standard error of the mean

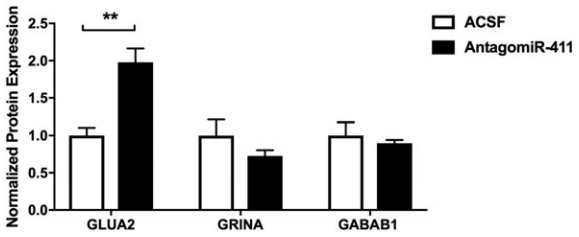

Figure 6 AntagomiR-411 treatment increases glutamate receptor AMPA-2 subunit (GLUA2) protein levels. Normalized protein expression for GLUA2, glutamate ionotropic receptor N-methyl-D-aspartate type subunit associated protein 1 (GRINA) and gamma-aminobutyric acid type B receptor subunit 1 (GABAB1) calculated with the corrected total cell fluorescence method. Values were calculated using immunohistochemistry-stained prefrontal cortex slices from alcohol-consuming mice. Significance was determined using unpaired *t*-tests followed by Holm–Sidak post hoc tests (**$P < 0.01$). Data represent mean + standard error of the mean

411 did not alter drinking. We note that female C57BL/6J mice display high levels of alcohol drinking, perhaps exhibiting a ceiling effect that would limit further increases in consumption. Another potential factor is that key mRNA targets for miR-411 may be present in low levels (i.e. downregulated by alcohol or bound by other microRNAs/binding proteins), such that increasing expression of miR-411 may not produce a functional response. This theory goes hand-in-hand with the 'counter-adaptivity' hypothesis, in which alcohol directly reduces the expression of the mRNA targets for miR-411, which in turn reduces miR-411 expression to prevent further decreases in target proteins.

AntagomiR-411 reduced GLUA2 protein but not *Glua2* mRNA levels

Discrepancies between protein and mRNA levels have been reported previously (Greenbaum *et al.* 2003; Kaller *et al.* 2011). In our study, levels of GLUA2 protein, but not the corresponding mRNA, were increased by antagomiR-411. As noted earlier, Ago2 has endonuclease activity, allowing it to splice and degrade mRNA; in contrast, other Ago proteins only slow down the translation of a specific mRNA (translational repression), without affecting the overall levels (Valencia-Sanchez *et al.* 2006; Filipowicz, Bhattacharyya, & Sonenberg 2008). AntagomiR-411-mediated increases in GLUA2 protein levels could reflect a decrease in *Glua2* mRNA degradation or decreased translational repression. Because *Glua2* mRNA levels were not altered, this indicates that translational repression was the likely mechanism. Alternately, the interaction between miR-411 and *Glua2* mRNA may be regulated by a different Ago protein.

Alcohol reverses the effects of antagomiR-411 on *Faah* and *Ppard* mRNAs

Chronic alcohol consumption decreased mRNA levels of both *Faah* and *Ppard* while treatment with antagomiR-411 increased these levels. Interestingly, the antagomiR-411-induced increase in mRNA levels was reduced by chronic alcohol consumption, suggesting that these effects are mediated by a common mechanism of action.

Reduced FAAH activity or expression has been linked with increased alcohol consumption. For example, FAAH activity was reduced after alcohol vapor exposure (Vinod *et al.* 2006), FAAH activity and expression were reduced in Finnish alcohol-preferring AA rats (Hansson *et al.* 2007) and a missense mutation in FAAH was associated with drug or alcohol use in humans (Sipe *et al.* 2002). Moreover, manipulation of FAAH activity or expression was shown to modulate alcohol consumption; for

Table 1 Summary of the molecular and behavioral effects of miR-411 manipulation

Behavioral paradigm	*Molecular effects*	*Behavioral effects*
Chronic alcohol consumption	↓ miR-411	Alcohol consumption and preference are maintained
AntagomiR-411 infusion in alcohol-naïve mice	↓ miR-411 ↓ miR-411/Ago2 association	No effect on acquisition of alcohol consumption
Mimic-411 infusion in alcohol-naïve mice	↑ miR-411	—
AntagomiR-411 infusion after chronic alcohol consumption	↓ miR-411 ↓ miR-411/Ago2 association ↑ GLUA2 protein, ↑ *Faah* and *Ppard* mRNAs	↓alcohol consumption and preference No effect on total fluid intake
Mimic-411 infusion after chronic alcohol consumption	↑ miR-411	No effect on alcohol and total fluid intake
AntagomiR-411 infusion after saccharin consumption	—	No effect on saccharin and total fluid intake

Faah = fatty acid amide hydrolase, GLUA2 = glutamate receptor AMPA-2 subunit, *Ppard* = peroxisome proliferator activated receptor delta; — = not measured.

example, FAAH knockout mice voluntarily consumed more alcohol than wild-type littermates, and treatment with the FAAH inhibitor URB597 also increased alcohol intake in wild-type mice (Blednov *et al.* 2007). Overall, alcohol reduces FAAH activity or expression while inhibition of FAAH activity increases alcohol consumption, which is consistent with our findings showing that antagomiR-411-induced increases in levels of *Faah* reduces alcohol consumption.

Unlike *Faah*, *Ppard* is not a predicted target of miR-411. However, studies have shown that these mRNAs may interact on a functional level. For example, inhibition of FAAH causes downstream activation of PPARD by increasing endogenous levels of the PPARD agonist oleoylethanolamide (Fu *et al.* 2003; Verme *et al.* 2005). In addition, *Faah* is decreased in the amygdala of mice following treatment with bezafibrate, a pan PPAR agonist (Ferguson *et al.* 2014), suggesting a feedback loop between the two mRNAs. Although we found that *Ppard* was altered by alcohol and is potentially a downstream target of *Faah*, it has not been shown to directly affect alcohol consumption. We previously reported that treatment with a direct PPARD agonist (GW0742) or a pan PPAR agonist (bezafibrate) did not alter alcohol consumption (Ferguson *et al.* 2014; Blednov *et al.* 2015).

Decreased miR-411 levels: a homeostatic adaptation to reduce alcohol consumption

It has been proposed that if alcohol consumption decreases levels of a microRNA, then increasing levels of the particular microRNA (and restoring original levels) could reduce alcohol consumption and reverse the behavior. This relationship between effects of drugs of abuse on microRNA levels and opposing effects of microRNA manipulations on drug consumption has been reported for miR-382 (Li *et al.* 2013), miR-212 (Hollander *et al.* 2010; Im *et al.* 2010), miR-124, let-7d and miR-181 (Chandrasekar & Dreyer 2011). However, our results show a different relationship—chronic alcohol consumption decreased miR-411 levels, and further reducing these levels with antagomiR-411 led to reduced alcohol consumption. This is similar to alcohol studies with miR-30a (Darcq *et al.* 2015), miR-206 (Tapocik *et al.* 2014) and miR-124 (Bahi & Dreyer 2013). These results suggest that alcohol-induced decreases in microRNA levels may not directly impact alcohol drinking but may represent a homeostatic mechanism to decrease consumption.

Drug-induced neuroadaptations and transcriptional changes influence a complex regulatory network. If alcohol decreases certain mRNA levels (e.g. mRNAs targeted by miR-411), the cellular response may be to decrease levels of miR-411 to maintain homeostasis (Nunez *et al.* 2013). This 'counter-adaptivity' explanation is attractive given that antagomiR-411 had no effect on alcohol consumption when administered to alcohol-naïve mice. It will be important to clarify which components of the process are direct targets of alcohol and which may reflect a homeostatic response to alcohol.

SUMMARY

Our findings suggest that miR-411 in the mPFC is important in regulating chronic alcohol consumption. Furthermore, we provide evidence that the antagomiR-411-induced reduction in alcohol consumption is mediated by increasing *Faah* mRNA and GLUA2 protein levels in the mPFC. This study describes a pathway by which changes in a single microRNA can alter key mRNAs and proteins to alter alcohol consumption and highlights

the role of microRNA as potential treatment options for AUD.

Acknowledgements

We thank Jody Mayfield (Waggoner Center for Alcohol and Addiction Research, University of Texas at Austin) for thoughtful critiques and help writing and editing the manuscript. We also thank Shlomo Yeshurun (The Florey Institute of Neuroscience and Mental Health, University of Melbourne) for helpful comments; Mendy Black (Waggoner Center for Alcohol and Addiction Research, University of Texas at Austin) for assisting with the behavioral experiments; and Kelly Jameson and Jason Wang (Waggoner Center, University of Texas at Austin) for their help with troubleshooting the initial molecular experiments.

Funding and disclosure

The work published in this paper represents original research, not previously submitted or published elsewhere. This work was supported by the National Institutes of Health (grants AA022557, AA013520, AA012404, AA020926 and AA020683).

Authors Contribution

RDM, DM and RAH were responsible for the study concept and design. YAB and DM contributed to the acquisition of animal data. NAS and DM performed the Ago2 experiments. RDM, GRT, NAS and DM assisted with data analysis and interpretation of findings. DM drafted the manuscript. RDM and RAH provided critical revision of the manuscript. All authors reviewed content and approved final version for publication.

References

Ambros V (2001) MicroRNAs: tiny regulators with great potential. Cell 107:823–826.

Bahi A, Dreyer JL (2013) Striatal modulation of BDNF expression using microRNA124a-expressing lentiviral vectors impairs ethanol-induced conditioned-place preference and voluntary alcohol consumption. Eur J Neurosci 38:2328–2337.

Baingana F, al'Absi M, Becker AE, Pringle B (2015) Global research challenges and opportunities for mental health and substance-use disorders. Nature 527:S172–S177.

Blednov YA, Benavidez JM, Black M, Ferguson LB, Schoenhard GL, Goate AM, Edenberg HJ, Wetherill L, Hesselbrock V, Foroud T (2015) Peroxisome proliferator-activated receptors α and γ are linked with alcohol consumption in mice and withdrawal and dependence in humans. Alcohol Clin Exp Res 39:136–145.

Blednov YA, Cravatt BF, Boehm SL II, Walker D, Harris RA (2007) Role of endocannabinoids in alcohol consumption and intoxication: studies of mice lacking fatty acid amide hydrolase. Neuropsychopharmacology 32:1570.

Blednov YA, Mayfield RD, Belknap J, Harris RA (2012) Behavioral actions of alcohol: phenotypic relations from multivariate analysis of mutant mouse data. Genes Brain Behav 11:424–435.

Chandrasekar V, Dreyer J-L (2011) Regulation of MiR-124, Let-7d, and MiR-181a in the accumbens affects the expression, extinction, and reinstatement of cocaine-induced conditioned place preference. Neuropsychopharmacology 36:1149–1164.

Czech B, Hannon GJ (2011) Small RNA sorting: matchmaking for argonautes. Nat Rev Genet 12:19–31.

Darcq E, Warnault V, Phamluong K, Besserer GM, Liu F, Ron D (2015) MicroRNA-30a-5p in the prefrontal cortex controls the transition from moderate to excessive alcohol consumption. Mol Psychiatry 20:1219.

Ferguson LB, Most D, Blednov YA, Harris RA (2014) PPAR agonists regulate brain gene expression: relationship to their effects on ethanol consumption. Neuropharmacology 86:397–407.

Filipowicz W, Bhattacharyya SN, Sonenberg N (2008) Mechanisms of post-transcriptional regulation by microRNAs: are the answers in sight? Nat Rev Genet 9:102.

Fu J, Gaetani S, Oveisi F, Verme JL (2003) Oleylethanolamide regulates feeding and body weight through activation of the nuclear receptor PPAR-alpha. Nature 425:90.

Greenbaum D, Colangelo C, Williams K, Gerstein M (2003) Comparing protein abundance and mRNA expression levels on a genomic scale. Genome Biol 4:117.

Hansson AC, Bermúdez-Silva FJ, Malinen H, Hyytiä P, Sanchez-Vera I, Rimondini R, de Fonseca FR, Kunos G, Sommer WH, Heilig M (2007) Genetic impairment of frontocortical endocannabinoid degradation and high alcohol preference. Neuropsychopharmacology 32:117.

Hellemans J, Mortier G, De Paepe A, Speleman F, Vandesompele J (2007) qBase relative quantification framework and software for management and automated analysis of real-time quantitative PCR data. Genome Biol 8:R19.

Hollander JA, Im HI, Amelio AL, Kocerha J, Bali P, Lu Q, Willoughby D, Wahlestedt C, Conkright MD, Kenny PJ (2010) Striatal microRNA controls cocaine intake through CREB signalling. Nature 466:197–202.

Im HI, Hollander JA, Bali P, Kenny PJ (2010) MeCP2 controls BDNF expression and cocaine intake through homeostatic interactions with microRNA-212. Nat Neurosci 13:1120–1127.

Jin Z, Bhandage AK, Bazov I, Kononenko O, Bakalkin G, Korpi ER, Birnir B (2014) Selective increases of AMPA, NMDA, and kainate receptor subunit mRNAs in the hippocampus and orbitofrontal cortex but not in prefrontal cortex of human alcoholics. Front Cell Neurosci 8:11.

Kalivas PW, Volkow ND (2005) The neural basis of addiction: a pathology of motivation and choice. Am J Psychiatry 162:1403–1413.

Kaller M, Liffers S-T, Oeljeklaus S, Kuhlmann K, Röh S, Hoffmann R, Warscheid B, Hermeking H (2011) Genome-wide characterization of miR-34a induced changes in protein and mRNA expression by a combined pulsed SILAC and microarray analysis. Mol Cell Proteomics 10:M111. 010462.

Lee RC, Ambros V (2001) An extensive class of small RNAs in Caenorhabditis elegans. Science 294:862–864.

Lewohl JM, Nunez YO, Dodd PR, Tiwari GR, Harris RA, Mayfield RD (2011) Up-regulation of microRNAs in brain of human alcoholics. Alcohol Clin Exp Res 35:1928–1937.

Li J, Li J, Liu X, Qin S, Guan Y, Liu Y, Cheng Y, Chen X, Li W, Wang S (2013) MicroRNA expression profile and functional

analysis reveal that miR-382 is a critical novel gene of alcohol addiction. EMBO Mol Med 5:1402–1414.
Lopez MF, Anderson RI, Becker HC (2016) Effect of different stressors on voluntary ethanol intake in ethanol-dependent and nondependent C57BL/6J mice. Alcohol 51:17–23.
Mamdani M, Williamson V, McMichael GO, Blevins T, Aliev F, Adkins A, Hack L, Bigdeli T, van der Vaart AD, Web BT (2015) Integrating mRNA and miRNA weighted gene co-expression networks with eQTLs in the nucleus accumbens of subjects with alcohol dependence. PLoS One 10:e0137671.
Mayfield RD (2017) Emerging roles for ncRNAs in alcohol use disorders. Alcohol 60:31–39.
Miranda RC (2014) MicroRNAs and ethanol toxicity. Int Rev Neurobiol 115:245–284.
Miranda RC, Pietrzykowski AZ, Tang Y, Sathyan P, Mayfield D, Keshavarzian A, Sampson W, Hereld D (2010) MicroRNAs: master regulators of ethanol abuse and toxicity? Alcohol Clin Exp Res 34:575–587.
Most D, Ferguson L, Blednov YA, Mayfield RD, Harris RA (2015) The synaptoneurosome transcriptome: a model for profiling the emolecular effects of alcohol. Pharmacogenomics J 15:177–188.
Most D, Leiter C, Blednov YA, Harris RA, Mayfield RD (2016) Synaptic microRNAs coordinately regulate synaptic mRNAs: perturbation by chronic alcohol consumption. Neuropsychopharmacology 41:538–548.
Nunez YO, Truitt JM, Gorini G, Ponomareva ON, Blednov YA, Harris RA, Mayfield RD (2013) Positively correlated miRNA-mRNA regulatory networks in mouse frontal cortex during early stages of alcohol dependence. BMC Genomics 14:725.
Paraskevopoulou MD, Georgakilas G, Kostoulas N, Vlachos IS, Vergoulis T, Reczko M, Filippidis C, Dalamagas T, Hatzigeorgiou AG (2013) DIANA-microT web server v5. 0: service integration into miRNA functional analysis workflows. Nucleic Acids Res 41:W169–W173.
Pietrzykowski AZ, Friesen RM, Martin GE, Puig SI, Nowak CL, Wynne PM, Siegelmann HT, Treistman SN (2008) Posttranscriptional regulation of BK channel splice variant stability by miR-9 underlies neuroadaptation to alcohol. Neuron 59:274–287.
Robison AJ, Nestler EJ (2011) Transcriptional and epigenetic mechanisms of addiction. Nat Rev Neurosci 12:623–637.
Ron D, Barak S (2016) Molecular mechanisms underlying alcohol-drinking behaviours. Nat Rev Neurosci 17:576–591.
Santerre JL, Rogow JA, Kolitz EB, Pal R, Landin JD, Gigante E, Werner DF (2014) Ethanol dose-dependently elicits opposing regulatory effects on hippocampal AMPA receptor GluA2 subunits through a zeta inhibitory peptide-sensitive kinase in adolescent and adult Sprague–Dawley rats. Neuroscience 280:50–59.
Sipe JC, Chiang K, Gerber AL, Beutler E, Cravatt BF (2002) A missense mutation in human fatty acid amide hydrolase associated with problem drug use. Proc Natl Acad Sci 99:8394–8399.
Sloan ME, Gowin JL, Yan J, Schwandt ML, Spagnolo PA, Sun H, Hodgkinson CA, Goldman D, Ramchandani VA (2017) Severity of alcohol dependence is associated with the fatty acid amide hydrolase Pro129Thr missense variant. Addiction Biol Epub doi: https://doi.org/10.1111/adb.12491.
Spagnolo PA, Ramchandani VA, Schwandt ML, Kwako LE, George DT, Mayo LM, Hillard CJ, Heilig M (2016) FAAH gene variation moderates stress response and symptom severity in patients with posttraumatic stress disorder and comorbid alcohol dependence. Alcohol Clin Exp Res 40:2426–2434.
Tapocik JD, Barbier E, Flanigan M, Solomon M, Pincus A, Pilling A, Sun H, Schank JR, King C, Heilig M (2014) MicroRNA-206 in rat medial prefrontal cortex regulates BDNF expression and alcohol drinking. J Neurosci 34:4581–4588.
Tapocik JD, Solomon M, Flanigan M, Meinhardt M, Barbier E, Schank JR, Schwandt M, Sommer WH, Heilig M (2013) Coordinated dysregulation of mRNAs and microRNAs in the rat medial prefrontal cortex following a history of alcohol dependence. Pharmacogenomics J 13:286–296.
Valencia-Sanchez MA, Liu J, Hannon GJ, Parker R (2006) Control of translation and mRNA degradation by miRNAs and siRNAs. Genes Dev 20:515–524.
Van Steenwyk G, Janeczek P, Lewohl JM (2013) Differential effects of chronic and chronic-intermittent ethanol treatment and its withdrawal on the expression of miRNAs. Brain Sci 3:744–756.
Verme JL, Fu J, Astarita G, La Rana G, Russo R, Calignano A, Piomelli D (2005) The nuclear receptor peroxisome proliferator-activated receptor-α mediates the anti-inflammatory actions of palmitoylethanolamide. Mol Pharmacol 67:15–19.
Vinod KY, Yalamanchili R, Xie S, Cooper TB, Hungund BL (2006) Effect of chronic ethanol exposure and its withdrawal on the endocannabinoid system. Neurochem Int 49:619–625.
Warden AS, Mayfield RD (2017) Gene expression profiling in the human alcoholic brain. Neuropharmacology 122:161–174.
Wong N, Wang X (2014) MiRDB: an online resource for microRNA target prediction and functional annotations. Nucleic Acids Res 43:D146–D152.
World Health Organization (2014) "Global status report on alcohol and health, 2014." Management of Substance Abuse Unit. World Health Organization: Geneva, Switzerland.
Yan ZC, Liu DY, Zhang LL, Shen CY, Ma QL, Cao TB, Wang LJ, Nie H, Zidek W, Tepel M (2007) Exercise reduces adipose tissue via cannabinoid receptor type 1 which is regulated by peroxisome proliferator-activated receptor-δ. Biochem Biophys Res Commun 354:427–433.
Zhou Y, Schwartz BI, Giza J, Gross SS, Lee FS, Kreek MJ (2017) Blockade of alcohol escalation and "relapse" drinking by pharmacological FAAH inhibition in male and female C57BL/6J mice. Psychopharmacology (Berl) 1–16.

SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

Figure S1. Mimic-411 increases miR-411 levels.
Figure S2. *In vivo* manipulation of miR-203, miR-92a, miR-187 or miR-137 does not change alcohol consumption.
Figure S3. AntagomiR-411 does not change behavioral responses in the open field and elevated-plus maze tests.
Figure S4. AntagomiR-411 does not affect the levels of miR-203, miR-92a, miR-137 and miR-187 or their association with Ago2.