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Silencing Synaptic MicroRNA-411 Reduces Voluntary Alcohol Consumption in Mice

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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/6J mice; *Faah* mRNA and *Ppard* mRNA; GluA2; miR-411; two-bottle choice ethanol drinking

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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.

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 and Volkow, 2005; Robison and Nestler, 2011). These changes likely result from abnormal expression of a plethora of genes in the brain (Warden and 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 (Baingana et al., 2015; World Health Organization, 2014).

MicroRNAs are small non-coding RNAs that regulate the translation of many genes (Ambros, 2001; Lee and Ambros, 2001), and are referred to as ‘master regulators’ of cellular gene expression (Miranda, 2014; Miranda et al., 2010). Alcohol-induced changes in microRNAs have been associated with development of cellular tolerance to alcohol (Pietrzykowski et al., 2008; Ron and 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 and 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 two 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 NIH 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 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- μ l microsyringe (model #1701, Hamilton, Reno, NV) and a 30-gauge needle. Compounds (described below) were diluted to 0.5 nmol/ μ l in ACSF and injected in a 1- μ l volume over a period of two minutes. Dosing was based on previous studies (Darcq et al., 2015; Li et al., 2013). After injections, the syringe was left in place for another two minutes before retraction. Incisions were closed with tissue adhesive (Vetbond, #70200742529, 3M, 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 above 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 one 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 two 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 LNA inhibitor – antagomiR (Exiqon, Vedbaek, Denmark) and a microRNA mimic (Dharmacon, Lafayette, CO), respectively, or artificial cerebrospinal fluid (ACSF, 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; mimic-203-3p (GUGAAAUGUUUAGGACCACUAG). AntagomiRs were HPLC purified and 5'-TYE-563 labeled, and mimics were HPLC purified and 5'-DY547-psngr labeled.

Chronic alcohol consumption

Seven to 10 days after cannulation, mice underwent four weeks of a two-bottle choice paradigm with continuous (24 h) 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 four weeks of consumption 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- μ l syringe, and 1 μ l 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 four 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 one-week recovery period, and were then infused with antagomiR-411 or ACSF into the mPFC as described above. 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 above 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 one 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 one 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, washed with an ice-cold saline solution and the mPFC was sliced at coronal levels bregma +1 mm 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 PCR (qPCR)

MicroRNA PCR—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); snoRNA234 (001234, endogenous control).

Following the RT-PCR, 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 $\times$) (#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 Ct 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 five-point, two-fold (microRNA) or three-fold (SnoRNA) dilution series.

mRNA PCR—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); *Hprt* (Mm00446968_m1, endogenous control).

Reactions were carried out as outlined for microRNAs above, except the standard curve was generated from a five-point, ten-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 Ct 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 (Ago2)

Frozen mPFC samples were homogenized and lysed using 1× 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-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 one hour at 4°C. Beads were separated on a magnet, washed with 0.05% and 0.02% Tween-20 in 1× PBS and resuspended in 50 µl of elution buffer. Samples were treated with 150 µl of Proteinase K buffer (#P5568-1, Sigma Aldrich) for one hour at 42°C. RNA was isolated from the beads and extracted as described above. RT-PCR and qPCR were run as described above 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: $Ct = (Ct_{input} - 3.32) - (Ct_{Ago2-IP \text{ or rat IgG}})$; enrichment = 100×2^{Ct} ; 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 PBS and 4% paraformaldehyde (PFA). Brains were harvested, postfixed for 24 hours in 4% PFA at 4°C, cryoprotected for 24 hours in 20% sucrose in PBS at 4°C, placed in molds containing 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 min at 25°C), washed in PBS (3 × 5 min at 25°C), blocked with 10% donkey serum (30 min at 25°C), treated with primary antibodies at 4°C overnight, washed in PBS (3 × 10 min at

25°C), incubated with fluorescence-conjugated secondary antibodies (2 h at 25°C) and rinsed with PBS (3 × 10 min 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-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-GFAP, clone N206A/8 (1:300, Neuromab, Davis, CA); 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); donkey anti-guinea pig CF 405M (Sigma Aldrich).

Brain sections were mounted on slides using sterile 0.2% gelatin and mounting media (with or without DAPI; Vector Laboratories, Burlingame, CA) and cover-slipped. Images were captured (without saturating the signal) using a Zeiss Axiovert 200M 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 NMDA type subunit associated protein 1 (GRINA) and gamma-aminobutyric acid type B receptor subunit 1 (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 (CTCF) = 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 three 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 and 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 mPFC

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, Leiter 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 four weeks in a two-bottle choice procedure (Figure 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: $t_{65}=0.799$, $P=0.427$; Figure 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 (Figure 1B). These results suggest that the reduction in levels of miR-411 following chronic alcohol 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 (Figure 2A). Treatments were designed to produce loss (antagomiRs) or gain (mimics) of microRNA function. LNA 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: $F_{2,39}=4.84$, $P=0.013$; Figure 2B). Preference for alcohol over water was also reduced following treatment with antagomiR-411 (RM-ANOVA: $F_{2,39}=3.383$, $P=0.044$; Figure 2C). There were no changes in total fluid consumption (Figure 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 (Figure 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 (Supplemental Figure 1).

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 (Supplemental Figure 2A–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 (Figure 3A), preference for saccharin over water (Figure 3B) or total fluid consumption (Figure 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 introduced 15% alcohol using a two-bottle choice procedure (Figure 4A). Pretreatment with antagomiR-411 in naïve mice did not change alcohol consumption (Figure 4B) or preference (Figure 4C). Although there was a treatment $\times$ time interaction effect for total fluid intake (RM-ANOVA: $F_{7,161}=3.216$, $P=0.0032$), there was no main effect of treatment ($F_{1,23}=0.025$, $P=0.8767$; Figure 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 et al., 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 (Supplemental Figure 3A–D).

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

We used qPCR to measure the levels of miR-411 in alcohol-naïve 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: $F_{1,25}=8.33$, $P=0.0079$) (Figure 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 (RISC) complex and subsequent regulation of mRNA stability (Czech and 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 ($F_{1,19}=28.1$, $P<0.0001$) and alcohol consumption ($F_{1,19}=14.99$, $P=0.0011$) (Figure 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 (Supplemental Figure 4A–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) (Most et al., 2016; Paraskevopoulou et al., 2013). 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: $t_7=4.863$, $P=0.0018$; Figure 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 (Sloan et al., 2017; Spagnolo et al., 2016) 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 to 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 revealed that antagomiR-411 increased *Ppard* mRNA levels compared to 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 et al., 2013), human PFC (Lewohl et al., 2011), rat PFC (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-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 above, 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 (Filipowicz et al., 2008; Valencia-Sanchez et al., 2006). 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 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 (Blednov et al., 2015; Ferguson et al., 2014).

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 and 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 and 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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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 qPCR. Samples are from alcohol-consuming (EtOH, N=7–8) compared with control (H2O, N=7–8) mice. Results are shown as fold-change Ct 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 (IP) and qPCR. Samples are from alcohol-consuming (EtOH, N=5) compared with control mice (H2O, N=7–8). IP 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.

E.

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 PFC, 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 $\pm$ 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.

A. **B.**

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 $\pm$ standard error of the mean.

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 D. 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 $\pm$ standard error of the mean.

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 qPCR. 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 C_t 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 (IP) and qPCR: ACSF-H2O (N=5), AntagomiR-411-H2O (N=7),

ACSF-EtOH (N=6) and AntagomiR-411-EtOH (N=5). IP 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<0.01, *** P<0.001, **** P<0.0001). Data represent mean + standard error of the mean.

Figure 6. AntagomiR-411 treatment increases GLUA2 protein levels

Normalized protein expression for GLUA2, GRINA and GABAB1 calculated with the Corrected Total Cell fluorescence (CTCF) 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.

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, ↑ <i>Faah</i> and <i>Ppard</i> 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