Identification and validation of midbrain *Kcnq4* regulation of heavy alcohol consumption in rodents

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ABSTRACT

Currently available pharmacotherapies for treating alcohol use disorder (AUD) suffer from deleterious side effects and are not efficacious in diverse populations. Clinical and preclinical studies provide evidence that the *Kcnq* family of genes that encode K$_V$7 channels influence alcohol intake and dependence. K$_V$7 channels are a class of slowly activating voltage-dependent K$^+$ channels that regulate neuronal excitability. Studies indicate that the K$_V$7 channel positive modulator retigabine can decrease dopaminergic neuron firing, alter dopamine (DA) release, and reduce alcohol intake in heavy drinking rodents. Given the critical nature of ventral tegmental area (VTA) DA to the addiction process and predominant expression of *Kcnq4* in DA neurons, we investigated the role of midbrain *Kcnq* genes and K$_V$7 channels in the VTA of genetically diverse mice and long-term heavy drinking rats, respectively. Integrative bioinformatics analysis identified negative correlations between midbrain *Kcnq4* expression and alcohol intake and seeking behaviors. *Kcnq4* expression levels were also correlated with dopaminergic-related phenotypes in BXD strains, and *Kcnq4* was present in support intervals for alcohol sensitivity and alcohol withdrawal severity QTLs in rodents. Pharmacological validation studies revealed that VTA K$_V$7 channels regulate excessive alcohol intake in rats with a high-drinking phenotype. Administration of a novel and selective K$_V$7.2/4 channel positive modulator also reduced alcohol drinking in rats. Together, these findings indicate that midbrain *Kcnq4* expression regulates alcohol-related behaviors in genetically diverse mice and provide evidence that K$_V$7.4 channels are a critical mediator of excessive alcohol drinking.

1. Introduction

Current pharmacotherapies for the treatment of alcohol use disorder (AUD) suffer from deleterious side effects and prevent relapse in only a small subset of individuals. Anticonvulsants are a drug class that show promise for treating heavy alcohol (ethanol) drinking and severe alcohol withdrawal associated with AUD (Padula et al., 2013). Emerging evidence indicates that the anticonvulsant retigabine can reduce voluntary alcohol consumption in rodents, especially those with a high-drinking phenotype (McGuier et al., 2016; Rinker et al., 2017). Retigabine is unique among the anticonvulsant class in that it increases the open probability of K$_V$7 channels, which are encoded by the *KCNQ* family of genes and are responsible for generating the M-current in neurons (Blackburn-Munro et al., 2005). In *Drosophila*, loss of K$_V$7 channel function increased tolerance and sensitivity to alcohol's sedative effects (Cavaliere et al., 2012). In addition, genetic variance in *KCNQ* is associated with alcohol intake in rodents and alcohol dependence in individuals with AUD (Edenberg et al., 2010; Kendler et al., 2011; McGuier et al., 2016; Metten et al., 2014; Rinker et al., 2017). Acute alcohol exposure has been shown to reduce M-current in dopamine (DA) neurons in the ventral tegmental area (VTA) (Koyama et al., 2007), and chronic alcohol exposure alters surface trafficking and reduces function of K$_V$7 channels in the nucleus accumbens (NAc) and lateral habenula, respectively (Kang et al., 2017; McGuier et al., 2016). Thus, this converging evidence supports variations in K$_V$7 channels as a mechanism promoting heavy alcohol drinking and

Table 1
Correlations of midbrain *Kcnq4* expression levels from male and female alcohol (EtOH)-naïve BXD recombinant inbred strains of mice with alcohol-related phenotypes. CPP, conditioned-place preference.

GeneNetwork Record ID	Phenotype	Sex	r	P value	Strain pairs	PubMed ID
10074	EtOH (10% v/v) intake	M	−0.5458	0.0435	14	6683363
12964	EtOH (15% v/v) intake	M, F	−0.5699	0.0420	13	7978106
12574	EtOH (15% v/v) intake	M, F	−0.5148	0.0413	16	27793543
10090	CPP - % time in EtOH-paired compartment	M	−0.6766	0.0020	18	7480533
10097	CPP - s/min in EtOH-paired compartment	M	−0.621	0.0059	18	7480533
10081	EtOH withdrawal severity	M	0.907	0.0048	7	6683363
10478	EtOH withdrawal severity	F	−0.5748	0.0198	16	6683363
11975	EtOH-induced ataxia (2.25 g/kg)	M, F	−0.4693	0.0059	33	19958391
11440	Locomotor activity after EtOH (2.25 g/kg)	M	−0.4084	0.0203	32	19958391
11697	Locomotor activity after EtOH (2.25 g/kg)	F	−0.3994	0.0213	33	19958391
12391	Anxiety assay after EtOH (1.8 g/kg)	M, F	−0.3689	0.0347	33	
10087	EtOH-induced hypothermia (2 g/kg)	F	0.4702	0.0364	20	8627539
10086	EtOH-induced hypothermia (3 g/kg)	F	0.5392	0.0142	20	8627539
10085	EtOH-induced hypothermia (4 g/kg)	F	0.4477	0.0478	20	8627539

alcohol-induced functional and behavioral adaptations.

Dopamine release from the VTA to the NAc is a central aspect of the addiction process (Koob and Le Moal, 2005). Although activation of K_V7 channels can attenuate DA synthesis and efflux in the striatum and reduce VTA dopaminergic neurotransmission (Hansen et al., 2006; Jensen et al., 2011; Martire et al., 2007; Sotty et al., 2009), the role that VTA Kv7 channels play in regulating drinking behaviors is unknown. We previously reported that *Kcnq2* and *Kcnq3* lie within multiple alcohol-related QTLs in rodents (McGuier et al., 2016), and *Kcnq* transcript levels in the NAc and prefrontal cortex are negatively correlated with voluntary alcohol drinking in BXD recombinant inbred (RI) strains of mice (Rinker et al., 2017). Whereas *Kcnq2/3* expression is relatively low in DA neurons, *Kcnq4* expression is predominant in DA neurons in the VTA and substantia nigra pars compacta (SNc) (Hansen et al., 2007; Kharkovets et al., 2000). Thus, it is possible that midbrain *Kcnq4* is a critical mediator of alcohol-related behaviors and heavy alcohol drinking.

Here, we performed an integrative functional genomic analysis on midbrain *Kcnq4* expression and alcohol- and DA-related phenotypes in BXD RI strains. We also used pharmacological approaches targeting K_V7 channels in the VTA using a rat intermittent access to alcohol (IAA) model of drinking to validate our genetic findings. Despite its efficacy as adjunct therapy to treat partial onset seizures, production of retigabine was discontinued because of retina and skin pigment changes caused by accumulation of low solubility retigabine dimers after prolonged use (Clark et al., 2015). Retigabine dimers likely form because resonance structures of retigabine are Brandowski's bases that are self-reactive (Dousa et al., 2014). Because K_V7 channels remain a promising target for treating AUD, we explored a novel and selective analog of retigabine (i.e., ML213) with a simplified chemical scaffold that lacks a critical amine group promoting formation of insoluble dimers.

Unlike the broad actions and modest potency of retigabine on $K_V7.2$–7.5 channels, ML213 is an opener of K_V7 channels with selectivity for $K_V7.2$ ($EC_{50} = 230$ nM) and $K_V7.4$ ($EC_{50} = 510$ nM) over other K_V7 channel subtypes and a panel of 68 other ion channels, transporters, and G-protein coupled receptors (Yu et al., 2010). Results from our studies provide evidence that midbrain *Kcnq4* regulates alcohol drinking in genetically diverse mice and pharmacologically targeting these channels reduces excessive alcohol consumption in rats.

2. Materials and methods

2.1. Bioinformatics

To provide evidence for genetic links between *Kcnq4* and alcohol-related behaviors, we performed two integrative bioinformatics analyses using open source databases (www.geneweaver. org and www.genenetwork.org) following our previously reported methods (McGuier et al., 2016; Padula et al., 2015; Rinker et al., 2017). First, we identified QTLs related to alcohol that contain *Kcnq4*. Next, we correlated *Kcnq4* midbrain robust multiarray average (RMA) expression levels in alcohol-naïve male BXD RI strains of mice (N = 37 strains and 129 mice) with alcohol-related phenotypes and phenotypes related to monoaminergic signaling in male and female BXD RI strains. When available, PubMed identification numbers for the original manuscripts describing the phenotypes are reported in Tables 1 and 2.

2.2. Animals and housing

Male Wistar rats were purchased from Harlan (Indianapolis, IN) and housed individually in standard home cages in temperature

Table 2
Correlations of midbrain *Kcnq4* expression levels from alcohol-naïve BXD recombinant inbred strains of mice with components of the monoaminergic system. 5-HIAA, 5-Hydroxyindoleacetic acid; DAT1, dopamine transporter; DOPAC, 3,4-Dihydroxyphenylacetic acid; DRD2, dopamine receptor D2; DRD3, dopamine receptor D3; HVA, Homovanillic acid; NE, norepinephrine; TH, tyrosine hydroxylase; PFC, prefrontal cortex, NAc, nucleus accumbens.

GeneNetwork Record ID	Phenotype	Sex	r	P value	Strain pairs	PubMed ID
12800	5-HIAA level in the medial septal nucleus	M, F	0.7349	0.0378	8	
10234	DAT1 (*SLC6A3*) binding maximum in PFC	M	−0.5351	0.0182	19	11454925
10280	DAT1 (*SLC6A3*) binding maximum in PFC	F	0.5719	0.0259	15	10591541
10281	DAT1 (*SLC6A3*) binding maximum in PFC	M, F	0.5563	0.0252	16	10591541
12798	DOPAC level in the medial septal nucleus in EtOH-dependent mice	M, F	0.8304	0.0107	8	
15146	DOPAC/DA ratio in striatum	M	−0.8495	0.0323	6	23558233
10262	DRD2/DRD3 binding maximum in NAc	F	0.5362	0.0394	15	10591541
12801	HVA level in the medial septal nucleus	M, F	0.8149	0.0137	8	
15161	TH protein level in striatum	M	0.9372	0.0058	6	

and humidity controlled environments with a 12 h light/dark cycle. Food and water were available *ad libitum* throughout the study. All procedures were approved by the Medical University of South Carolina Institutional Animal Care and Use Committee and were in accordance with NIH guidelines for the humane care and use of laboratory animals (2011).

2.3. Two-bottle choice drinking

Intermittent access to alcohol (IAA; 20% *v/v*) or saccharin (0.02% *w/v*) was performed as previously described (McGuier et al., 2016). After reaching baseline consumption, rats were habituated to systemic injections through administration of saline (3 mL/kg, IP). Rats had access to alcohol and saccharin for 7–8 and 4 weeks, respectively, prior to drug administration.

2.4. Intracranial cannula surgery

After six weeks of alcohol consumption in the IAA paradigm, guide cannula were surgically implanted in the VTA of Wistar rats following our standard procedures (McGuier et al., 2016). Bilateral microinjection guide cannula (23 ga O.D.; Plastics One Roanoke, VA) were implanted 2 mm dorsal to the VTA following previously established coordinates (relative to bregma, AP: −5.6 mm, ML: ± 2.1 mm, DV: −7.1, 10°) (Mahler et al., 2013). Rats were allowed 1 week to recover before being given alcohol access.

2.5. Drug treatments

In four separate cohorts, rats received vehicle (10% Tween 80 *v/v* saline) or ML213 (1 or 5 mg/kg in two cohorts and 10 or 20 mg/kg in the other two cohorts; Cayman Chemical, Ann Arbor, MI) 30 min prior to alcohol or saccharin access. Due to its poor solubility (Yu et al., 2010), ML213 was administered systemically rather than into the VTA. Additional cohorts of rats received microinjections beginning 30 min prior to alcohol availability. Injectors were inserted bilaterally to a depth of 2 mm beyond the tip of the guide cannula. Retigabine (5–10 ng in 0.01% DMSO *v/v* aCSF; Axon Med-Chem, The Netherlands), XE991 (0.225 ng in 0.01% DMSO *v/v* aCSF; Tocris, Minneapolis, MN) or vehicle (0.01% DMSO *v/v* aCSF) was delivered in a final volume of 0.5 µl/side at a rate of 0.5 µl/min. Systemic injections of vehicle (10% Tween 80 *v/v* saline) or 7.5 mg/kg retigabine immediately followed microinfusions. At the completion of the study, brains were Nissl stained to confirm cannula placement.

2.6. Locomotor activity

Alcohol-naïve and long-term drinking rats were habituated to a 57 cm × 58 cm × 63 cm opaque acrylic box for 1 h/day for 2 consecutive days starting two weeks after completion of the drinking paradigms. Over the next three consecutive days, rats were treated with vehicle (10% Tween80 v/v saline) or ML213 (1–20 mg/kg, IP) in a Latin square design. Activity was digitally recorded with an overhead video camera under red light illumination. Total movement was automatically scored using EthoVision XT software (Noldus Information Technology, Wageningen, The Netherlands).

2.7. Statistical analysis

Pearson correlations between midbrain *Kcnq4* levels and alcohol- and monoamine-related phenotypes were first calculated in GeneNetwork.org, and the data were exported to Excel. Outliers in *Kcnq4* RMA levels or phenotype data were determined by the Grubbs' test with an alpha of 0.05 and were excluded from subsequent correlation analyses in GraphPad Prism software (version 7.04; GraphPad Software, Inc., La Jolla, CA). To increase power and reduce variance associated with individual differences, a within-subjects design was used for all rat behavioral experiments. A mixed data procedure (PROC MIXED) was used in the statistical software language SAS to analyze all drinking and behavioral data using repeated measures factors following our previous methods (McGuier et al., 2016). Rats were separated into low and high alcohol drinkers using a median split. Responsivity to intra-VTA retigabine and systemic ML213 administration was assessed by calculating the difference between alcohol intake under vehicle and drug conditions using hierarchical linear modeling in SAS (Singer, 1998). In this linear mixed-effects analysis, change in drinking after drug was modeled with fixed effects for baseline drinking, time, and dose, including random effects for dose and rat. All data are reported as mean ± SEM and statistical significance was established with $p < 0.05$.

3. Results

3.1. Kcnq4, alcohol-related behaviors, and dopamine

An integrative functional genomic analysis identified *Kcnq4* in support intervals for alcohol sensitivity and alcohol withdrawal severity QTLs in rodents (GeneWeaver.org gene set ID GS223356 and GS84162; (Bergeson et al., 2003; Radcliffe et al., 2006)). In male and female alcohol-naïve BXD RI strains, midbrain *Kcnq4* transcript levels (but not *Kcnq2*) significantly correlated with alcohol conditioned place preference and alcohol intake (Table 1; Fig. 1A and B). Moreover, *Kcnq4* significantly correlated with the severity of handling-induced convulsions during alcohol withdrawal (Fig. 1C) and other behavioral and physiological measures (e.g., locomotor activity, hypothermia) associated with acute alcohol exposure in BXD RI strains (Table 1). However, not all alcohol-related phenotypes (e.g., alcohol metabolism) correlated with *Kcnq4* midbrain expression levels and no other K^+ channel gene significantly correlated with the majority of phenotypes related to alcohol-seeking and drinking behaviors. The results from the bioinformatics analysis also indicated that midbrain *Kcnq4* levels significantly correlated with NAc expression of D2R/D3R and other components of the monoaminergic system, including positive correlations with 5-HIAA, DOPAC, HVA, and TH protein levels in projection fields of midbrain DA neurons (Table 2).

3.2. Systemic ML213 administration and alcohol drinking

3.2.1. 1 and 5 mg/kg doses of ML213
After Wistar rats established baseline drinking the IAA model (Supplemental Fig. 1A), rats were treated with vehicle and ML213 (1 or 5 mg/kg, ip) in weeks 9 through 11. There was a main effect of dose on alcohol intake, and post-hoc analysis revealed that 5 mg/kg ML-213 significantly reduced alcohol drinking compared rats when treated with vehicle (Supplemental Fig. 1B). Administration of ML213 did not affect water (Supplemental Fig. 1C) or total fluid (Supplemental Fig. 1D) intake. ML213 was then tested in alcohol-naïve rats against home cage saccharin drinking and locomotor activity in an open field. Analysis revealed that 1 and 5 mg/kg ML213 did not affect saccharin intake (Supplemental Fig. 2A), water intake (Supplemental Fig. 2B), saccharin preference (Supplemental Fig. 2C), or total locomotor activity (Supplemental Fig. 2D).

3.2.2. 10 and 20 mg/kg doses of ML213
Because of the modest effects of 5 mg/kg ML213 on alcohol intake and the poor brain penetration (Yu et al., 2010), higher doses of
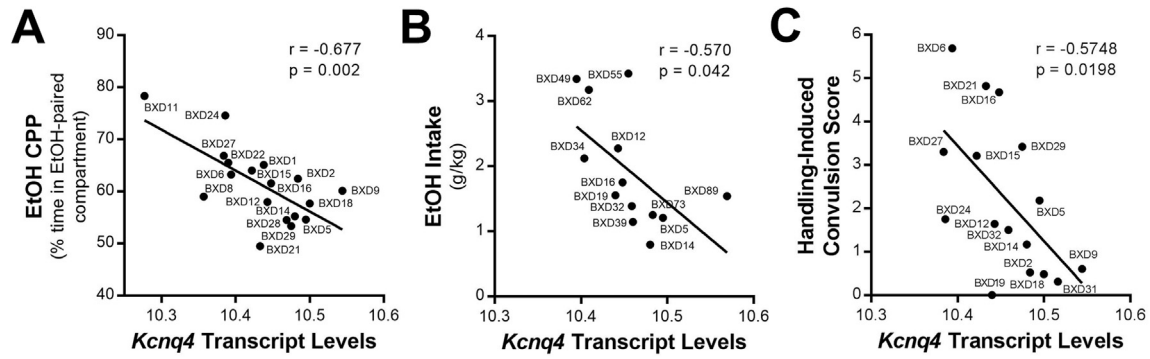
Fig. 1. Midbrain *Kcnq4* transcript expression levels correlate with alcohol (EtOH) drinking and seeking behaviors in BXD recombinant inbred strains of mice. Negative correlations between *Kcnq4* transcript levels in BXD recombinant inbred strains of mice and (**A**) alcohol conditioned place preference (CPP; N = 18 strain pairs), (**B**) alcohol drinking (N = 13 strain pairs), and (**C**) handling-induced convulsions in alcohol-dependent mice (N = 16 strain pairs). GeneNetwork.org data sets: 'VU BXD Midbrain Agilent SurePrint G3 Mouse GE (May12)' for *Kcnq4* (probe sets A_55_P2086198 and A_55_P2086193) and alcohol-related phenotypic data from male and female BXD RI strains.

ML213 were tested in a separate cohort of alcohol drinking rats (Fig. 2A). A significant treatment by time interaction was observed in alcohol-drinking rats (Fig. 2B). Post-hoc analysis revealed that 20 mg/kg significantly reduced drinking at 6 h, whereas both doses of ML213 reduced drinking at 24 h. While ML213 did not affect water intake at either dose (Fig. 2C), total fluid intake was reduced by the 20 mg/kg dose (Fig. 2D). Our previous results showed that retigabine is more effective at reducing consumption in high- (HD), but not low-drinking (LD) rodents (McGuier et al., 2016; Rinker et al., 2017). When analyzed by drinking phenotype, ML213 attenuated alcohol consumption in low- (Fig. 2E) and high-drinking (Fig. 2F) rats. Post-hoc analyses revealed that both doses of ML213 significantly reduced drinking in the LD rats across time, whereas both doses of ML213 significantly reduced drinking in the HD rats at the 24 h time point. A mixed model analysis of the drinking data showed a strong negative linear relationship between intake after vehicle and the change in drinking produced by ML213 treatment (Fig. 2G). In a separate cohort of alcohol-naïve rats, analysis showed that 10 and 20 mg/kg ML213 did not alter consumption of saccharin (Fig. 3A), water intake (Fig. 3B), or saccharin preference (Fig. 3C). In addition, total locomotor activity was not significantly altered by ML213 treatment in alcohol-naïve rats (Fig. 3D). While there is a trend for a reduction in locomotor activity by ML213, the high dose increased activity in the open field apparatus in some of the alcohol-naive rats.
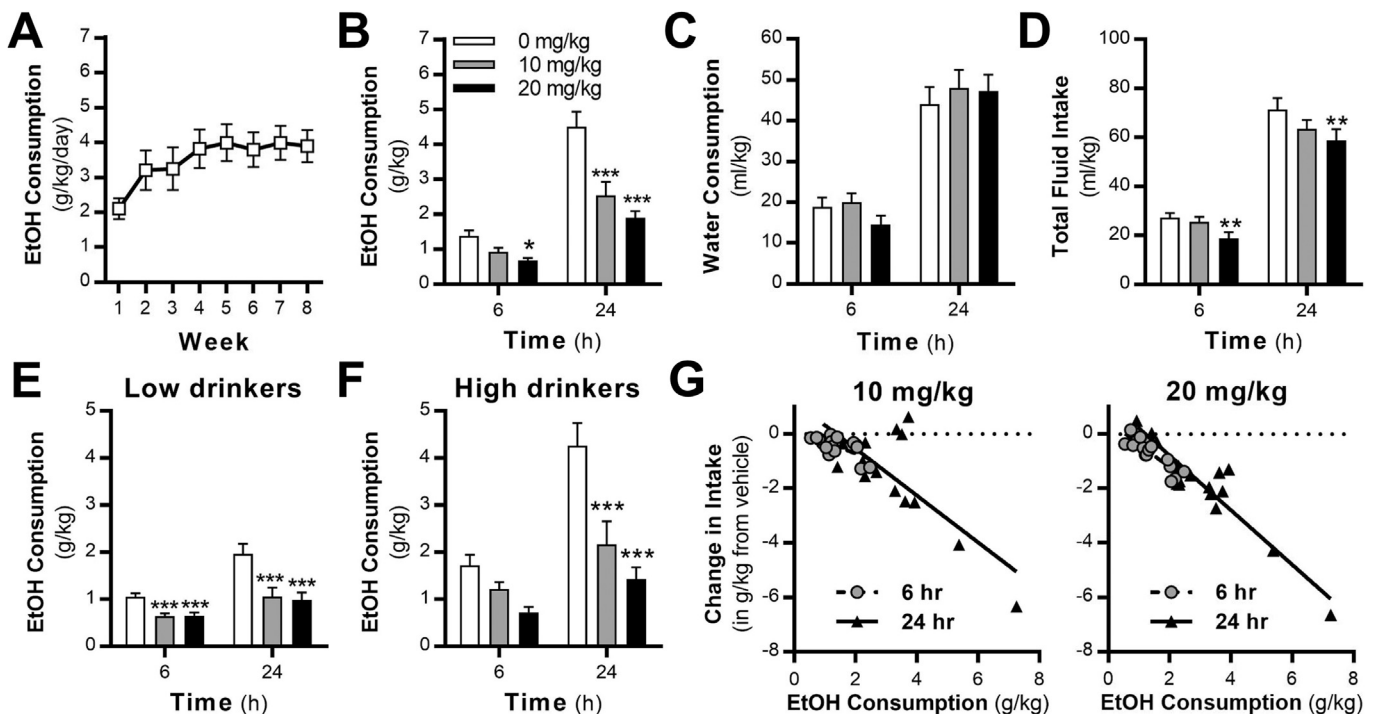

Fig. 2. Systemic administration of ML213 reduced alcohol drinking in rats. (**A**) Weekly average alcohol intake levels across 8 weeks of intermittent access to alcohol (N = 16). Systemic administration of ML213 significantly reduces (**B**) alcohol drinking (interaction: $F_{(2,30)} = 5.12$; P = 0.0112; post hoc, *6 h*: *P = 0.021, 10 mg/kg vs vehicle; *24 h*: ***P < 0.0001, 10 and 20 mk/kg vs vehicle), but not (**C**) water consumption ($F_{(2,30)} = 0.34$; P = 0.7147). (**D**) 20 mg/kg ML213 significantly reduces total fluid intake (main effect of dose: $F_{(2,30)} = 5.46$, P = 0.0095; post-hoc, **P = 0.0025, 20 mg/kg vs vehicle). (**E,F**) Systemic ML213 treatment reduces alcohol intake in rats with low- (main effect of drug: $F_{(2,14)} = 15.8$; P = 0.0003; post hoc, ***P < 0.0004, 10 and 20 mg/kg vs vehicle) and high- (interaction: $F_{(2,14)} = 4.24$; P = 0.0362; post hoc, ***P < 0.0004, 10 and 20 mg/kg vs vehicle at 24 h) alcohol drinking phenotypes. (**G**) HLM analysis showing a negative linear relationship between vehicle drinking values and ML213-induced change in alcohol intake across the 6 and 24 h time points ($\beta = -0.95$; main effect of drug, $F_{(1,30)} = 176.67$, P < 0.0001).
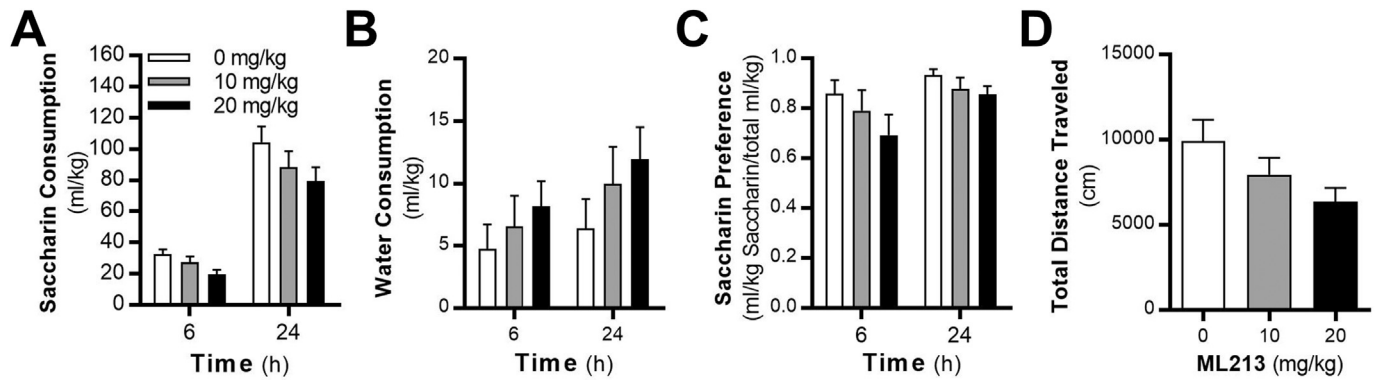
Fig. 3. Systemic administration of 10 and 20 mg/kg doses of ML213 does not affect (**A**) saccharin drinking (F (2,18) = 3.21; P = 0.0641), (**B**) water consumption (F(2,18) = 1.32; P = 0.2915), or (**C**) saccharin preference (F (2,18) = 1.81; P = 0.1929) in alcohol-naïve rats (N = 10). (**D**) Total locomotor activity in alcohol-naïve rats is not affected by ML213 administration (F (2,18) = 2.54; P = 0.107).

3.3. ML213 and locomotor activity in alcohol-drinking rats

To determine if ML213 treatment affects locomotor behavior in rats with a history of alcohol drinking, both cohorts of rats were tested in the open field apparatus two weeks after the last alcohol drinking session. Unlike the alcohol-naïve rats, administration of ML213 significantly reduced total locomotor activity in the open field test (Fig. 4A,D). Post hoc analysis revealed a significant reduction in total distance traveled at the 5, 10, and 20 mg/kg doses of ML213 in the alcohol-drinking rats. The correlations between alcohol drinking and locomotor activity after vehicle injection (Fig. 4B,E) and the correlations between the change in alcohol intake and locomotor activity after ML213 treatment (Fig. 4C,F)

were not significant.

3.4. Microinfusions of retigabine into the VTA and alcohol drinking

3.4.1. Retigabine microinfusions

To validate these genetic findings between midbrain *Kcnq4* levels and alcohol-seeking behaviors, we performed a number of experiments to investigate the role of VTA K_V7 channels in regulating alcohol intake. First, the pan-K_V7 channel positive modulator retigabine was microinfused into the VTA (Fig. 5A) after Wistar rats established baseline drinking the IAA model (Fig. 5B). Baseline drinking was not significantly different before and after the surgery (Fig. 5C). Similar to our study that targeted the NAc core (McGuier
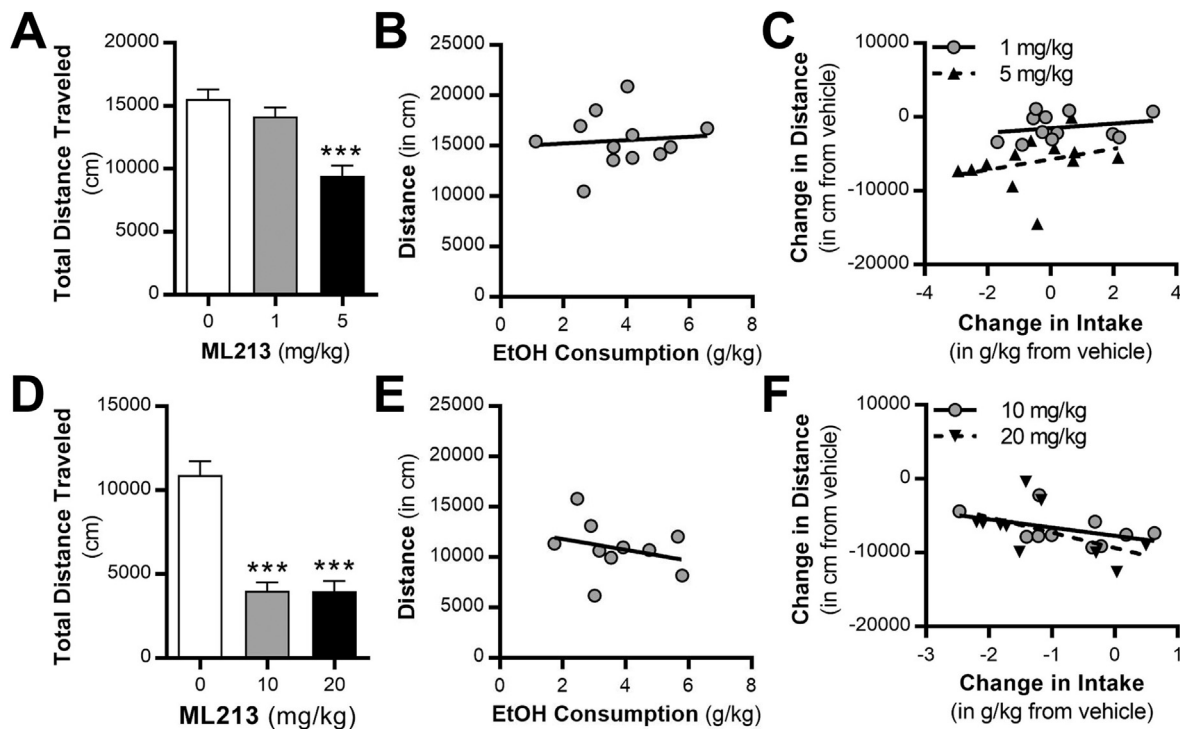

Fig. 4. Systemic administration of ML213 reduces locomotor activity during protracted abstinence in rats with a history of long-term alcohol drinking in the intermittent access model. (**A,D**) ML213 administration reduces total distance traveled in an open field apparatus (*0, 1, and 5 mg/kg*: F (2,22) = 22.42, P < 0.0001; post-hoc test, ***P < 0.0001, 5 mg/kg vs vehicle and 1 mg/kg; *0, 10, and 20 mg/kg*: F (2,18) = 40.61, P < 0.0001; post-hoc test, *P < 0.0001, 10 and 20 mg/kg vs vehicle). (**B,E**) Total distance traveled does not significantly correlate with alcohol intake in vehicle treated rats (*0, 1, and 5 mg/kg*: r = 0.092, P = 0.776; *0, 10, and 20 mg/kg*: r = −0.2971, P = 0.435). (**C,F**) Lack of correlation between the change in distance and alcohol intake in rats treated with systemic ML213 (*0, 1, and 5 mg/kg*: 1 mg/kg: r = 0.248, P = 0.436; 5 mg/kg, r = 0.306, P = 0.334; *0, 10, and 20 mg/kg*: 10 mg/kg: r = −0.460, P = 0.181; 20 mg/kg: r = −0.539, P = 0.108).
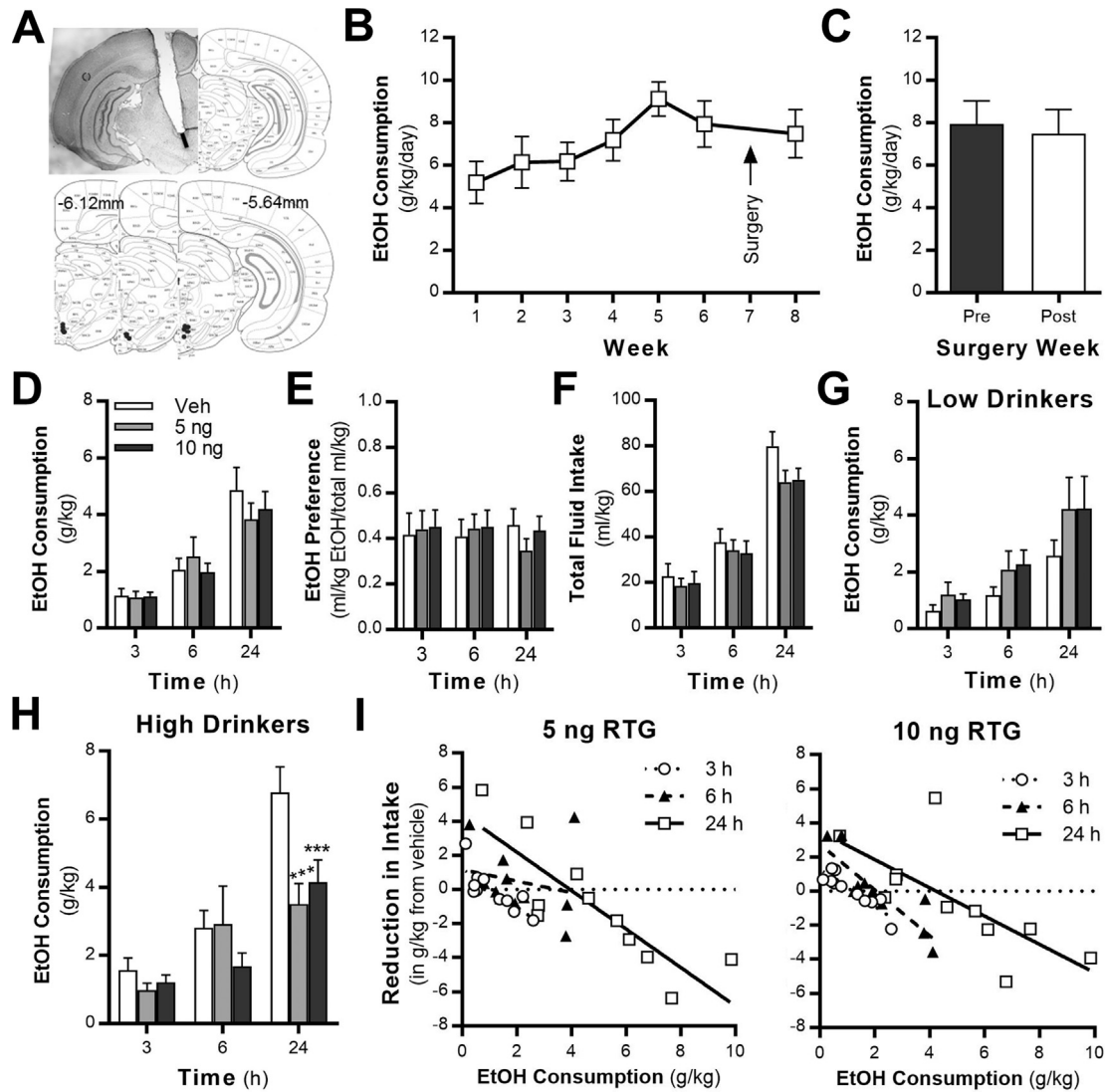
Fig. 5. Retigabine microinfusions into the VTA reduces alcohol intake in high-drinking rats. (**A**) Schematic of localization of guide cannula placement. The black line represents the placement of the injection. The black dots mark the sites of microinfusions. (**B,C**) Weekly average alcohol intake values before and after guide cannula surgery (F (1,11) = 0.06, P = 0.8046; N = 12). (**D,E**) Alcohol consumption (F (2,20) = 0.23, P = 0.8001) and preference (F (2,20) = 0.29, P = 0.7546) in rats that received microinfusions of retigabine into the VTA. (**F**) Total intake of alcohol and water after vehicle and retigabine VTA microinfusions (F (2,20) = 3.15, P = 0.0649). (**G,H**) Retigabine microinfusion reduces alcohol intake in high-, but not low-drinking rats (*low-drinkers*: F(2,8) = 2.72, P = 0.1255; *high-drinkers*: interaction, F (4,20) = 3.04; P = 0.0412; post hoc, 5 ng: ***P = 0.0004 vs vehicle, 10 ng: ***P = 0.003 vs vehicle). (**I**) HLM analysis revealed a negative linear relationship between the reduction in alcohol intake after 5 and 10 ng retigabine microinfusion and the amount of alcohol consumed after infusion of vehicle across time (3, 6, and 24 h time points; β = −0.91; main effect of drug, F (1,41) = 51.84, P < 0.0001).

et al., 2016), retigabine had no effect on alcohol consumption (Fig. 5D), preference (Fig. 5E), or total fluid consumption (Fig. 5F) in the overall population of Wistar rats. Neither dose of retigabine affected alcohol intake in LD rats (Fig. 5G). In the HD group, there was a significant interaction of time and alcohol consumption (Fig. 5H). Post-hoc analysis indicates that both doses of retigabine reduced drinking at the 24 h time point. A mixed model analysis identified a strong negative linear relationship between vehicle treatment and the reduction in intake after intra-VTA retigabine administration across all time points (Fig. 5I).

3.4.2. XE-991 microinfusions

To test if K_V7 channels in the VTA are necessary for the effect of retigabine on alcohol consumption, we microinfused the selective K_V7 channel antagonist XE-991 (0.225 ng) immediately followed by systemic retigabine (7.5 mg/kg). When considering the total cohort of rats, we observed no main effect of drug on alcohol consumption (Fig. 6A), preference (F (3,31) = 0.80; P = 0.5030), or total fluid intake (Fig. 6B). When separated based on drinking phenotype, there was a main effect of treatment on alcohol consumption in LD rats (Fig. 6C). Consistent with our previous data showing that systemic retigabine treatment increases alcohol consumption in rats with a low drinking phenotype (McGuier et al., 2016), post-hoc analysis indicated that retigabine treatment increased intake compared to rats treated with vehicle. Importantly, microinfusion of XE991 into the VTA completely blocked the increase in drinking induced by systemic retigabine in LD rats. There was no difference between XE991 and vehicle microinfusions on any measure, and there was no effect of treatment on total fluid intake in LD rats (Fig. 6D). In HD rats, there was a main effect of treatment on alcohol consumption (Fig. 6E). Post-hoc analysis showed that retigabine reduced drinking compared to vehicle-treated rats, an effect that was blocked when retigabine treated rats received microinfusions of XE991. There was no effect of treatment on total fluid intake
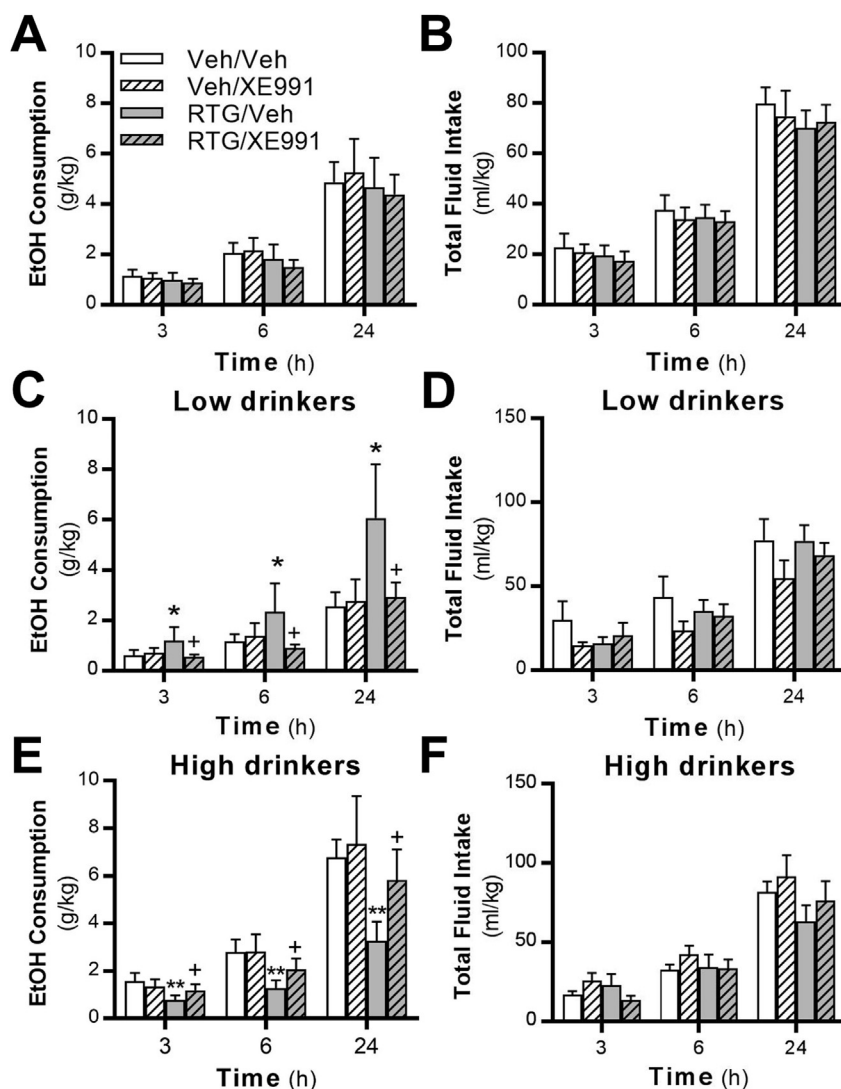
Fig. 6. Blockade of K_V7 channels in the VTA prevents the bidirectional effects of systemic retigabine treatment in low- and high-drinking rats. (**A,B**) Neither systemic retigabine administration nor XE991 microinfusion into the VTA affected alcohol (F (3,31) = 0.74; P = 0.5538; N = 12) or total fluid intake (F (3,31) = 0.59; P = 0.6234) in the total populations of rats. XE991 microinfusion blocked the ability of systemic retigabine treatment to (**C**) increase consumption in low-drinking rats (main effect of drug, F (3,13) = 3.77; P = 0.0388; post-hoc: *P = 0.0147, RTG/Veh vs Veh/Veh; +P = 0.0116, RTG/Veh vs RTG/XE991) and (**E**) reduce alcohol intake in rats with a high-drinking phenotype (main effect of drug, F (3,15) = 5.29; P = 0.0109; post-hoc: **P = 0.0044, RTG/Vehicle (Veh) vs Veh/Veh; +P = 0.0029, RTG/Veh vs RTG/XE991). (**D,F**) Total fluid intake in low- (F (3,13) = 2.27; P = 0.1284) and high-drinking (F (3,15) = 2.32; P = 0.1168) rats was not affected by systemic retigabine administration or XE991 microinfusion.

(Fig. 6F). In sum, systemic retigabine increased alcohol consumption in LD rats and decreased consumption in HD rats, and XE-991 microinfusion into the VTA blocked the actions of retigabine.

4. Discussion

The experiments described here provide genetic and pharmacological evidence that midbrain K_V7 channels are critically involved in the regulation of heavy alcohol drinking in mice and rats. Midbrain *Kcnq4* expression levels negatively correlated with alcohol intake and the rewarding effects of alcohol in BXD RI mouse strains. Consistent with functional links between K_V7 channels and monoaminergic signaling, *Kcnq4* expression correlated with multiple components of the mesocorticolimbic DA circuitry. Systemic administration of the novel $K_V7.2/4$-selective positive modulator ML213 decreased alcohol intake regardless of phenotype without affecting consumption of a sweetened solution. During prolonged abstinence from alcohol drinking, ML213 administration revealed a greater response to the locomotor impairing effects of K_V7 channel activation. We also found that retigabine decreased voluntary alcohol consumption in high-drinking rats, while producing the opposite effect in rats with a low-drinking phenotype. Importantly, blocking K_V7 channels in the VTA prevented the changes in drinking induced by systemic retigabine administration. Together, these experiments provide the first evidence that midbrain *Kcnq4* and VTA K_V7 channels regulate alcohol reward and heavy alcohol drinking.

4.1. Bioinformatics

Our bioinformatics query of midbrain *Kcnq* genes identified *Kcnq4* as a candidate gene that regulates alcohol seeking and reward-related behaviors in BXD RI strains of mice. *Kcnq4* was also correlated with alcohol withdrawal convulsion severity and other behaviors and physiological responses to acute alcohol exposure in genetically diverse strains of mice. Moreover, we identified

multiple links between *Kcnq4* and the monoaminergic system within the mesocorticolimbic DA circuitry. These genetic findings are consistent with a published study showing that $K_V7.4$ channels functionally couple with DA D2-type receptors (Ljungstrom et al., 2003) and co-localize with a subpopulation of serotonin-positive neurons in the dorsal raphe nucleus (Hansen et al., 2008). We found that midbrain *Kcnq4* expression positively correlated with the main metabolites of serotonin and DA in the medial septal nucleus and D2R/D3R receptor and TH expression in the striatum of BXD RI strains. These latter observations are intriguing given that TH activity positively regulates dopaminergic activity (Jones et al., 1998; Salvatore, 2014) and low DA activity correlates with high alcohol drinking in BXD RI strains (Siciliano et al., 2017). Moreover, adaptations in D2-type autoreceptors are an important mechanism underpinning the development of heavy drinking (Karkhanis et al., 2015; Siciliano et al., 2016). These findings stemming from mouse genetic studies suggest that midbrain *Kcnq4* may regulate alcohol intake, possibly through control of monoaminergic signaling in the mesocorticolimbic circuitry.

4.2. VTA microinfusions of retigabine reduce excessive drinking

In a series of microinjection studies to validate the bioinformatics data, we found that K_V7 channels in the VTA regulate excessive voluntary drinking in Wistar rats. VTA microinjection of the pan K_V7 channel positive modulator retigabine reduced alcohol intake in rats with a high-drinking phenotype. Similar to previous evidence (McGuier et al., 2016), systemic retigabine administration produced bidirectional effects on drinking in the low- and high-drinking rats. Importantly, intra-VTA microinfusion of XE-991 blocked retigabine's ability to affect drinking in these two populations. While the functional mechanisms underpinning these bidirectional effects on drinking are unknown and beyond the scope of this study, a recent study described divergent VTA DA neuron firing patterns and functional adaptations in low- and high-drinking mice (Juarez et al., 2017). While VTA DA neuron firing in mice with a high alcohol drinking phenotype was similar to controls, low alcohol drinking mice displayed increased phasic/bursting activity, and phasic, but not tonic, stimulation of VTA DA neurons reduced alcohol intake in high-drinking mice (Juarez et al., 2017). In contrast to these findings, a separate study showed that tonic, but not phasic stimulation of the VTA reduced alcohol intake in high-drinking rats (Bass et al., 2013). Given that both K_V7 channel function and retigabine can regulate dopaminergic neurotransmission, increasing K_V7 channel function with retigabine may alter baseline and anticipatory phasic dopaminergic responses to alcohol in the different drinking populations. It is also possible that retigabine differentially affects firing in low vs high alcohol drinking rats. However, we consider this latter possibility unlikely because retigabine potently reduces tonic and phasic firing of VTA DA neurons (Hansen et al., 2006; Sotty et al., 2009). Despite the unknown neural mechanism driving the bidirectional effects of retigabine on alcohol consumption in rats with different drinking phenotypes, it remains that VTA K_V7 channels contribute to alcohol drinking and alcohol-seeking behaviors and are targets for reducing heavy alcohol intake.

4.3. K_V7 channels and low-drinking rodents

Although retigabine reduces alcohol consumption in rats and mice with a high-drinking phenotype, it elevates intake in low drinkers when administered systemically and has side effects that limit its clinical use. Notably, administration of the novel and selective $K_V7.2/4$ channel positive modulator ML213 reduced drinking in the total population of rats without increasing alcohol drinking in rats with a low-drinking phenotype. ML213 administration did not reduce saccharin intake demonstrating that the effects on intake of rewarding solutions are selective for alcohol. Because ML213 enhances activity of channels containing the $K_V7.2/4$ subunits (Yu et al., 2010), this suggests that the ability of retigabine to increase drinking in low-drinking rats is through modulation of a different K_V7 channel subunit. In addition to the unknown subunit, the locus of the mechanism underpinning the elevation in drinking is unidentified. Whereas systemic administration of ML213 reduced alcohol intake in low-drinking rats, microinjection of retigabine into the NAc (McGuier et al., 2016) or VTA (this study) did not affect consumption in low alcohol drinkers. This suggests that *Kcnq4* channels expressed outside of the VTA may also contribute to the effect of ML213 on alcohol intake. XE991 is known to increase spontaneous firing of VTA DA neurons (Koyama et al., 2007), but microinfusion of XE991 into the VTA did not increase voluntary alcohol drinking at the concentration used in this study, as might be expected. While these mixed results demonstrate a complex role for K_V7 channel regulation of alcohol drinking, our results show that targeting $K_V7.2/4$ channel subtypes with ML213 is a promising strategy for reducing heavy alcohol drinking.

4.4. Locomotor behavior and neuroadaptations

Hypoactivity is commonly observed during acute and protracted withdrawal from chronic alcohol exposure (Broadwater et al., 2011; Zhao et al., 2007), and high doses of K_V7 channel positive modulators can reduce locomotor activity in alcohol-naïve rats (Hansen et al., 2007; Roeloffs et al., 2008). Although we did not directly compare alcohol drinking and non-drinking rats, doses of ML213 that were innocuous in alcohol-naïve rats produced hypoactivity during abstinence in the alcohol-drinking rats. Only the highest dose of ML213 reduced total fluid intake when alcohol was available, and the locomotor response did not correlate with alcohol consumption after vehicle or ML213 treatment. Thus, the locomotor impairing effects of ML213 appear specific to the open field apparatus, suggesting that long-term drinking increases responses to the locomotor-impairing effects of K_V7 channel activation. We demonstrated previously that rats with a history of alcohol drinking showed an enhanced sensitivity to hyperactivity when K_V7 channels were blocked with XE991 (McGuier et al., 2016). One possible explanation is that long-term drinking and protracted withdrawal produce adaptations in the balance of excitatory and inhibitory processes that are unmasked after manipulation of K_V7 channel activity. Alternatively, the augmented behavioral responses to K_V7 channel activation and blockade may reflect neuroadaptations in K_V7 channels in a yet to be identified region or circuitry.

In support of the latter hypothesis, studies have shown that chronic alcohol exposure altered $K_V7.2$ channel surface trafficking in the NAc (McGuier et al., 2016) and reduced K_V7 channel function and expression in the lateral habenula (Kang et al., 2017). Regulation of *Kcnq* transcript levels by alcohol is complex and varies by direction of change, *Kcnq* variant, species, and brain region (Rinker and Mulholland, 2017). For example, *KCNQ2* expression is increased in the amygdala and frontal cortex in postmortem tissue samples from alcoholics, whereas VTA *Kcnq4* expression is not altered in rodent alcohol drinking models (Marballi et al., 2016; McBride et al., 2012, 2013). Importantly, there are SNPs in *KCNQ1/5* that associate with alcohol dependence in humans (Edenberg et al., 2010; Kendler et al., 2011), and chronic retigabine treatment prevented the development of alcohol-induced disruptions in brain EEG rhythms in a rabbit model of dependence (Zwierzynska et al., 2016). Given the emerging evidence for alcohol-induced adaptations in K_V7 channels, it will be important to continue to investigate

the role of K$_V$7 channels in the development of alcohol-seeking behaviors and alcohol dependence and determine the effects of excessive drinking on protein expression levels of K$_V$7 channels in other brain regions, like the VTA and prefrontal cortex.

5. Conclusions

In summary, these data provide several lines of genetic and pharmacological evidence implicating midbrain *Kcnq4* and VTA K$_V$7 channels as modulators of heavy alcohol drinking. Our findings showing that treatment with a subtype selective and novel K$_V$7 channel positive modulator and intra-VTA infusions of retigabine reduced heavy alcohol drinking add to a growing literature that K$_V$7 channels are a target for treating excessive alcohol drinking associated with AUD. These data provide the first evidence that genetic variation in midbrain *Kcnq4* associates with alcohol intake and alcohol-seeking behaviors, as well as multiple components of the monoaminergic system. This class of drugs was well tolerated in non-treatment seeking moderate drinkers (Crean and Tompson, 2013) and should be further investigated as a pharmacotherapeutic approach for treating individuals with AUD.

Author contributions

PJM, NSM, and JAR planned the experiments. PJM, with assistance from SRJ, performed the bioinformatics analysis. NSM, JAR, RC, and DBF performed the behavioral experiments. NSM, JAR, PJM, and MH analyzed the data. NSM, JAR, and PJM wrote the manuscript, and all authors edited the manuscript and provide intellectual input into data interpretation.

Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.neuropharm.2018.05.020.

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