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Research Article

Studies of Mice with one or Two Copies of G Allele of Human Oprm1 Genetic Variations in Excessive Alcohol Drinking

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Abstract

Endogenous opioid system plays an essential role in regulating alcohol consumption. Genetic deletion of mu-opioid receptor (MOP-r) decreases alcohol intake and reward in mouse models. A single-nucleotide polymorphism (SNP; A118G, rs1799971) in human MOP-r gene (Oprm1) is associate with altered MOP-r activity, resulting in enhanced alcohol consumption and increased risk of developing alcoholism in individuals with at least one copy of G allele (AG and GG). The present study determined the impact of Oprm1 SNP on alcohol drinking using A112G mice (a functionally equivalent SNP in Oprm1 with a similar amino acid substitution in humans) in chronic excessive drinking model. We found that both AG and GG males had greater alcohol intake than AA males, suggesting that increased MOP-r signaling with one or two copies of G allele promoted alcohol consumption. Though AG+GG males showed more sucrose intake than AA males, there was no genotypic difference in saccharin intake. In contrast to males, females did not show any genotypic difference in alcohol, sucrose or saccharin consumption. Pharmacological blockade of MOP-r with low-dose naltrexone reduced intake in AG+GG males, with blunted effects in AA males, confirming that increased MOP-r signaling by G allele contributes to MOP-r mediated modulation of alcohol drinking. However, activation of kappaopioid receptors [KOP-] by nalfurafine only decreased intake in AA males, suggesting altered KOP-r responses by G allele. Together, our data suggest that there is selective involvement of Oprm1 G allele (one or two copies) in excessive alcohol drinking with sex difference and altered opioid receptor responses.

HIGHLIGHTS

- AG and GG males had greater alcohol intake than AA ones.
- AG and GG males showed more intake of sucrose than AA ones.
- There was no genotypic difference in saccharin intake.
- Females did not show any genotypic difference in any consumption.
- Genotypic effects in response to naltrexone and nalfurafine

INTRODUCTION

The endogenous mu-opioid receptor (MOP-r) system consists of a main endogenous ligand beta-endorphin and MOP-r. Many lines of evidence have demonstrated that betaendorphin/MOP-r is involved in the alcohol reinforcement and consumption: (a) Genetic deletion of beta-endorphin or MOP-r decreases alcohol consumption [1-5]; (b) MOP-r antagonists decrease alcohol intake, reduce alcohol-induced reward, and attenuate the motivation to seek and consume alcohol in rodents [6]; and (c) Numerous pharmacological studies provide strong evidence that opioid antagonists decrease alcohol drinking, craving and relapse episodes in human alcoholics [7-9].

A common single-nucleotide polymorphism (SNP, A118G, rs1799971) in human MOP-r gene [Oprm1] has significant effects on the expression and activity of MOP-r. This SNP leads to an amino change from asparagine to aspartic acid at a glycosylation site (N40D) which in turn renders the MOP-r more sensitive to beta-endorphin with increased affinity and potency [10-11], and leads to altered MOP-r expression, maximum binding and signaling, with different results among many studies by different groups using a variety of methods [12-16]. During the last two decades, the Oprm1 A118G SNP has been found to associate with increased genetic vulnerability to alcohol abuse or dependence with altered naltrexone responses in many studies [16-24], but not in other reports [12, 25-35]. Of interest, individuals with one copy of the G allele (AG), similar to the ones with two copies of the G allele (GG), have been reported to have a greater euphoria effect of alcohol drinking and a higher risk of developing alcoholism [20, 22]. Therefore, the human genetic literature suggests that one copy of the G allele (AG) might alter alcohol consumption as profoundly as two copies of the G allele (GG).

To test this hypothesis directly, we designed a specific study to determine the impact of all three different genotypes (namely, AG and GG vs AA) on alcohol drinking in both male and female mice, using A112G mice that express a functionally equivalent SNP in Oprm1 with a similar amino acid substitution in humans

[14]. Firstly, we tested the genotypic effect on alcohol drinking in drinking-in-the-dark (DID) model [4, 36, 37], which allows for limited access (e.g., 4 h/day). C57BL/6J mice become intoxicated and the blood ethanol concentration levels are relatively high during this 4-hour alcohol exposure time after 4 days of subacute alcohol DID, which mimics "binge" drinking to the point of intoxication. Then, we further evaluated the potential genotypic effect in chronic excessive drinking (ED) drinking model, in which the mice had access to voluntary alcohol drinking for 24 hours per day for 3 weeks in a two-bottle freechoice paradigm. C57BL/6J mice, after exposed to 3-week ED, develops high alcohol intake (15- 30 g/kg/day), which constitutes an appropriate mouse model for studying excessive alcohol consumption [4, 38, 39]. Finally, we tested if there were genotypic differences in drinking behaviors in response to MOP-r antagonist naltrexone or kappa-opioid receptor (KOP-r) agonist nalfurafine.

MATERIALS AND METHODS

Animals: As described previously, Oprm1 A112G mice were generated on a C57BL/6 mouse background using site-directed mutagenesis to replace an adenine (A) nucleotide at position 112 with a guanine (G) nucleotide in exon 1 of the Oprm1 gene [14]. All the male and female AG, GG and AA littermates derived from heterozygous A/G parents were used for all experiments. All animals were kept on a 12/12 light–dark cycle at 22°C with food and water available ad 5 | P a g e libitum. At the time the experiments start, all the mice were at the age of 8 weeks. At the end of all the experiments, the mice were genotyped as described previously [14].

The A112G mice were individually housed in ventilated cages fitted with steel lids and filter tops and given ad libitum access to food and water in a stress-minimized facility. Mice were placed on a 12-hour reverse light-dark cycle (lights off at 7:00 am). Animal care and experimental procedures were conducted according to the Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources Commission on Life Sciences 1996). The experimental protocols used were approved by the Institutional Animal Care and Use Committee of the Rockefeller University.

Drugs: Ethanol solutions (15-30% v/v) were prepared from 190 proof absolute ethyl alcohol (Pharmco-AAPER, Brookfield, CT, USA) and diluted in tap water. Sucrose and saccharin purchased from Sigma-Aldrich Inc. (St. Louis, MO) were diluted in tap water at 2-4% concentrations (g/ml) and at 0.1-0.2% concentrations (g/ml), respectively.

The subacute (4 days) drinking-in-the-dark (DID) procedure: The A112G mice accessed alcohol drinking in their home cages with food available in this one-bottle paradigm with alcohol exposure with 1 recording (4 hours in the dark cycle). Based on a previous publication [36], the paradigm with our modifications was as follows [4, 37]: At the time when the mice started individual housing (1 week before the experiments), the water tubes were replaced with those with sipper tubes to acclimate the mice to the sipper tubes (without ball bearings).

During the first day of testing (i.e., day 1), all the AG, GG and AA mice were given access to 15% alcohol for 4 h. Starting at 3 hours after lights off (10:00 am), the water tubes were replaced with alcohol tubes sealed with a rubber stopper on the tope and fitted with a stainless steel straight sipper tube at the bottom. The sipper tubes contained a ball bearing at the end to prevent alcohol leakage. Then they received daily 4-hour access to alcohol for another 3 days. The alcohol tubes were filled with fresh alcohol solution every day, and kept for 4 hours and then were replaced with the water tubes. Alcohol intake values were recorded at 4 hours (to the nearest 0.1 ml). These data were calculated as self-administered alcohol intake (i.e., g / kg).

The chronic (3 weeks) excessive drinking (ED) procedure: After the 4-day DID in the first week, the AG, GG and AA mice in both sexes had intermittent access to alcohol drinking every other day in the home cage for 3 weeks, with food and water available in this two-bottle free choice paradigm. This ED model was like an earlier protocol [38], with some modifications [4, 39, 40]. The procedures were similar to the above DID procedures with the following exceptions: Beginning at 3 hours after lights off, both the alcohol solution and water tubes were provided on home cages. The position of the tubes (left or right side) was randomly changed to avoid the possible side preference. Both the alcohol and water intake values were recorded after 4, 8 and 24 hours of alcohol access, and the data were used to calculate alcohol intake (i.e., g / kg) and relative preference ratio for alcohol (i.e., alcohol intake / total fluid intake). Access to alcohol following the 3-week procedure led to high alcohol intake in the mice [4]. As with the above DID model, we purposely examined alcohol drinking at the beginning of the dark period, the 4- hour time after alcohol access. The alcohol and water intake values were also measured at 8- and 24-hour recording times after alcohol access, to observe alcohol drinking during 24-hour circadian cycle.

Test 1. Genotypic effect on 15% alcohol drinking. The AG, GG and AA mice in both sexes were given access to 15% alcohol in both the Test 1A and Test 1B; Test 2. Genotypic effect on 30% alcohol drinking. The AG, GG and AA mice in both sexes were given access to 30% alcohol in both the Test 2A and Test 2B; Test 3. Genotypic effect on 2-4% sucrose drinking. The exposure procedure was identical to the above ED alcohol tests with the following exception: sucrose solutions replaced alcohol ones. The AG, GG and AA mice in both sexes were given access to 2% sucrose in the Test 3A and then 4% sucrose in the Test 3B; and Test 4. Genotypic effect on 0.1-0.2% saccharin drinking. Similarly, the AG, GG and AA mice in both sexes were given access to 0.1% saccharin in the Test 4A and then 0.2% saccharin in the Test 4B.

Effects of genotype on alcohol (15%) drinking in ED drinking model with single injection of naltrexone or nalfurafine in A112G male mice: The primary objective of the following experiments was to determine whether there was a potential genotypic difference in drinking behavior in the EA mice following MOP-r blockade (naltrexone at 1 mg/kg) [Test 5] and KOP-r activation (nalfurafine at 3 or 10 μg/kg) [Test 6]. Separate groups of male mice were used for each compound. On the test day, 15% alcohol was presented after a single injection of the compound or vehicle, and then alcohol and water intake values were recorded at 4-hour time point. The range of naltrexone and nalfurafine doses was based on our recent publications using the same ED model in C57BL/6 mice: (1) naltrexone at 1 mg/kg sub-effective dose did not reduce alcohol intake; and (2)

nalfurafine at 10 μg/kg, but not 3 μg/kg, decreased alcohol intake [39]. The rationale behind the selective use of males only in these experiments came from the above studies, in which genotypic differences had an effect on 15% alcohol drinking in males, but not females, at 4-hour time point.

Data analysis: In the DID experiments, group differences on alcohol intake were analyzed using 2-way ANOVA with repeat measures for genotype (AG+AG vs AA) and time (1, 2, 3, 4 days). In the ED experiments, group differences on alcohol (or sucrose, saccharin) intake or preference ratio across three different genotypes in each sex were analyzed using two-way ANOVA with repeat measures for genotype (AG+AG vs AA) and for time (0-4h vs 4-8h interval). Based on the published findings that Individuals expressing at least one copy of the G allele have a greater risk of developing alcoholism [e.g., 20], our a priori hypothesis that there was a genotypic effect (AG+GG vs AA) was tested with 2-way ANOVAs followed by NewmanKeuls post-hoc or planned comparison tests. In the experiments with naltrexone or nalfurafine, group differences were analyzed using 2-way ANOVA for treatment (vehicle vs drug) and for genotype (AG+GG vs AA). The accepted level of significance for all tests was $p < 0.05$. All statistical analyses were performed using Statistica (StatSoft Inc, Tulsa, OK).

Results 1

No genotypic difference on 15% alcohol intake (g/kg) in DID model among AG, GG and AA males or females: The objective of this DID experiment was to determine whether 4-hour limited access to alcohol for 4 days will lead to stable alcohol intake with potential genotypic difference among AG, AG and AA mice in each sex. During 4 days of alcohol (15%) drinking, there was no genotypic difference over the 4-day exposure (Table S1).

Genotypic differences on 15% alcohol drinking in ED model among AG, GG and AA males, but not females: To further assess a potential genotypic difference in alcohol drinking, the mice in both sexes were exposed to the 2-bottle "alcohol (15%) vs. water" free choice regimen after the above DID. After exposed to alcohol for 3 weeks, all the AG, AG and AA mice were tested twice (designated Test A and Test B) with 15% alcohol in this 24-hour long-access model with 3 time points.

In Test 1A in males, two-way ANOVA with repeat measure revealed a significant effect of genotype [$F(1, 22) = 6.8$, $p<0.01$], and planned comparison analysis (AG+GG vs AA) showed that both the AG and GG mice had more alcohol intake than the AA mice at 0-4 hours [$p<0.05$], but not at 4-8 hour (Figure 1 A). In the following Test 1B in males, there was a similar genotypic effect: two-way ANOVA with repeat measure revealed a significant effect of genotype [$F(1,22)=7.6$, $p<0.01$]; and during the first 4-hours, the AG and GG mice drank more alcohol than the AA mice [Newman-Keuls post-hoc test, $p<0.05$] (Figure S1). In both the tests, however, total daily average alcohol consumption was not significantly altered by genotypes, as shown in Tables 1A and 1B. There was no significant difference in alcohol preference ratio in either test among the 3 genotypes at any time points (Figure 1 and Figure 2; Tables 1A and 1B).

In contrast to males, females did not show any genotypic difference on alcohol intake or preference in either Test 1 or Test 2 at any time points (Figure 1 and Figure S1, Tables 1A and 1B).

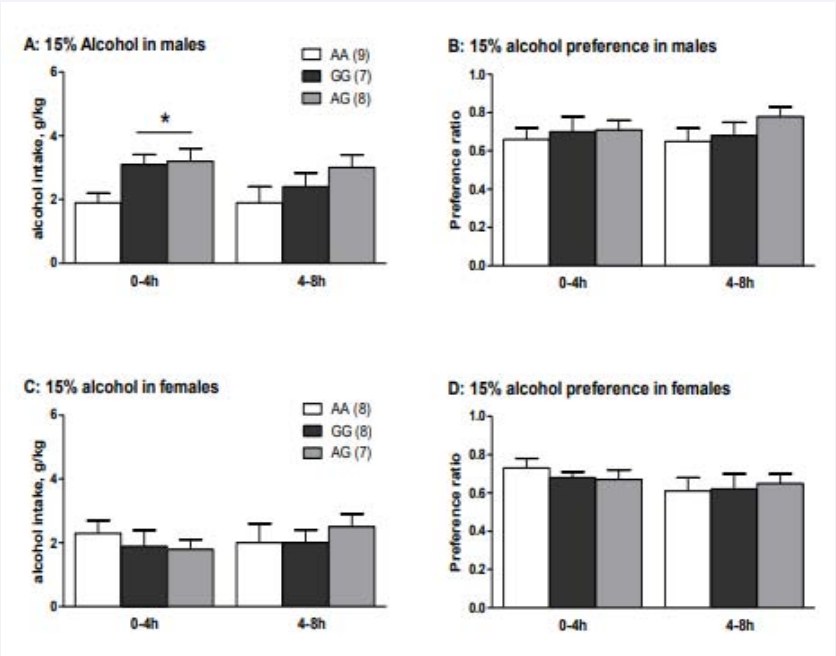

Figure 1 In Test 1A, genotypic difference on 15% alcohol intake (g/kg) after chronic (3 weeks) excessive drinking among AG, GG and AA male (but not female) mice. Mice exposed to the 2-bottle "15% alcohol vs. water" choice regimen and then alcohol and water intake values were recorded after 4 and 8 hours of alcohol access on the test day. Data are presented as mean + SEM. (A) Intake with a genotypic difference: *p<0.05 vs. AA mice in males at 0-4hour interval.

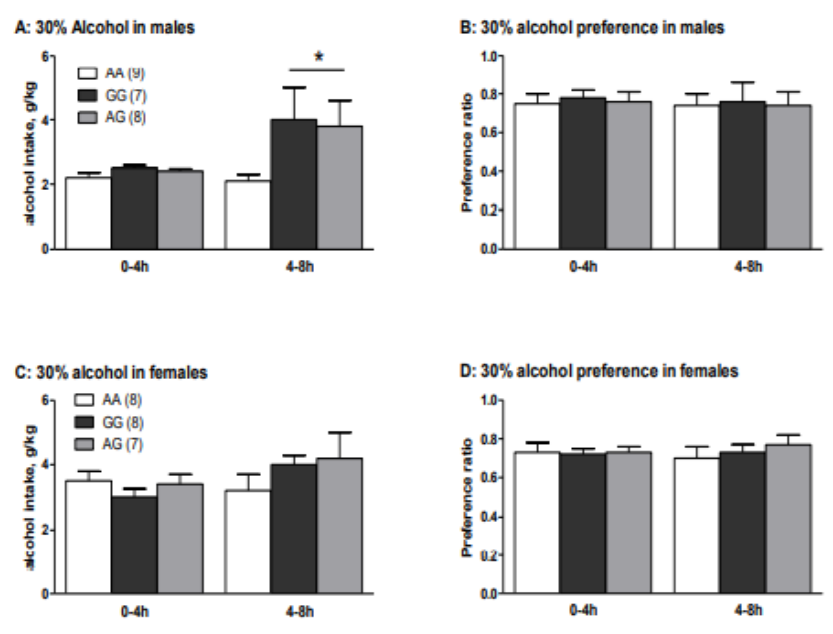

Figure 2 In Test 2A, genotypic difference on 30% alcohol intake (g/kg) after chronic (3 weeks) excessive drinking among AG, GG and AA male (but not female) mice. Mice exposed to the 2-bottle "30% alcohol vs. water" choice regimen and then alcohol and water intake values were recorded after 4 and 8 hours of alcohol access on the test day. Data are presented as mean + EM. (A) Intake with a genotypic difference: *p<0.05 vs. AA mice in males at 4-8hour interval.

Test 1A (15% alcohol)

	Male			Female		
	AA (n=9)	GG (n=7)	AG (n=8)	AA (n=8)	GG (n=8)	AG (n=7)
Intake, g/kg	14 ± 0.8	12 ± 2.1	14 ± 1.2	22 ± 1.5	19 ± 1.5	23 ± 1.1
Preference ratio	0.76±0.03	0.61±0.10	0.70±0.06	0.72±0.04	0.60±0.06	0.62±0.04

Test 1B (15% alcohol)

	Male			Female		
	AA (n=9)	GG (n=7)	AG (n=8)	AA (n=8)	GG (n=8)	AG (n=7)
Intake, g/kg	15 ± 1.7	14 ± 1.5	16 ± 0.9	25 ± 1.6	22 ± 2.2	24 ± 3.1
Preference ratio	0.79±0.04	0.65±0.06	0.69±0.07	0.71±0.04	0.67±0.04	0.69±0.08

Table 1 No genotype difference on 24-hour 15% alcohol intake (g/kg) or preference (preference ratio) after chronic (3 weeks) long-access alcohol excessive drinking among AG, GG and AA male and female mice. Mice exposed to the 2-bottle "15% alcohol vs. water" choice regimen. Data are presented after 24 hours of alcohol drinking in Test 1A and Test 1B.

Genotypic differences on 30% alcohol drinking in ED model among AG, GG and AA males, but not females: After 15% alcohol tests, all the AG, AG and AA mice were tested twice with 30% alcohol. In Test 2A in males, two-way ANOVA with repeat measure revealed a very significant effect of genotype [F (1, 22)=21, p<0.001]. Newman-Keuls post-hoc test (AG+GG vs AA) 10 | P a g eshowed that both the AG and GG mice had more intake than the AA mice at 4-8 hours [p<0.01], but not at 0-4 hours (Figure 2 A), which was different from 15% alcohol drinking (Figure 1 A). In the following Test 2B, a similargenotypic effect was observed: (1) a significant effect of genotype [two-way ANOVA with repeat measure, F (1,22)=6.1, p<0.05]; and (2) during the 4-8 hours, the AG and GG mice drank more than the AA mice [Planned comparison (AG+GG vs AA), p<0.05] (Figure S2). In both the tests, however, total daily average alcohol intake was unaltered by genotypes (Tables 2A and Table 2B). For alcohol preference ratio, there was no significant difference in either test among the 3 genotypes at any time (Figure 3, Tables 2A and 2B).

In contrast to males again, females did not display any genotypic difference on alcohol intake or preference in either Test 3 or Test 4 at any time points (Figure 2 and Figure S2, Tables 2A and 2B).

Genotypic difference on sucrose (2% and 4%) drinking model among AG, GG and AA males, but not females. After exposed to alcohol, the mice exposed to sucrose drinking. As alcohol is a caloric reinforcer, the specificity of the genotypic difference on alcohol intake was firstly tested on sucrose intake (another caloric reinforcer).In Test 3A (2% sucrose) in males, two-way ANOVA with repeat measure revealed a marginally significant effect of genotype [F(1,22) =6.4, p<0.05], and planned comparison analysis (AG+GG vs AA) showed that both the AG and GG mice

Test 2A (30% alcohol)

	Male			Female		
	AA (n=9)	GG (n=7)	AG (n=8)	AA (n=8)	GG (n=8)	AG (n=7)
Intake, g/kg	21 ± 0.7	22 ± 2.3	19 ± 2.5	31 ± 3.0	29 ± 2.5	30 ± 1.9
Preference ratio	0.55±0.03	0.55±0.06	0.52±0.08	0.61±0.08	0.54±0.06	0.58±0.05

Test 2B (30% alcohol)

	Male			Female		
	AA (n=9)	GG (n=7)	AG (n=8)	AA (n=8)	GG (n=8)	AG (n=7)
Intake, g/kg	23 ± 1.8	22 ± 3.4	21 ± 2.8	33 ± 1.8	30 ± 0.9	32 ± 0.9
Preference ratio	0.60±0.05	0.61±0.04	0.58±0.04	0.67±0.05	0.62±0.03	0.59±0.04

Table 2 No genotype difference on 24-hour 30% alcohol intake (g/kg) or preference (preference ratio) after chronic (3 weeks) long-access alcohol excessive drinking among AG, GG and AA male and female mice. Mice exposed to the 2-bottle "30% alcohol vs. water" choice regimen. Data are presented after 24 hours of alcohol drinking in Test 2A and Test 2B.

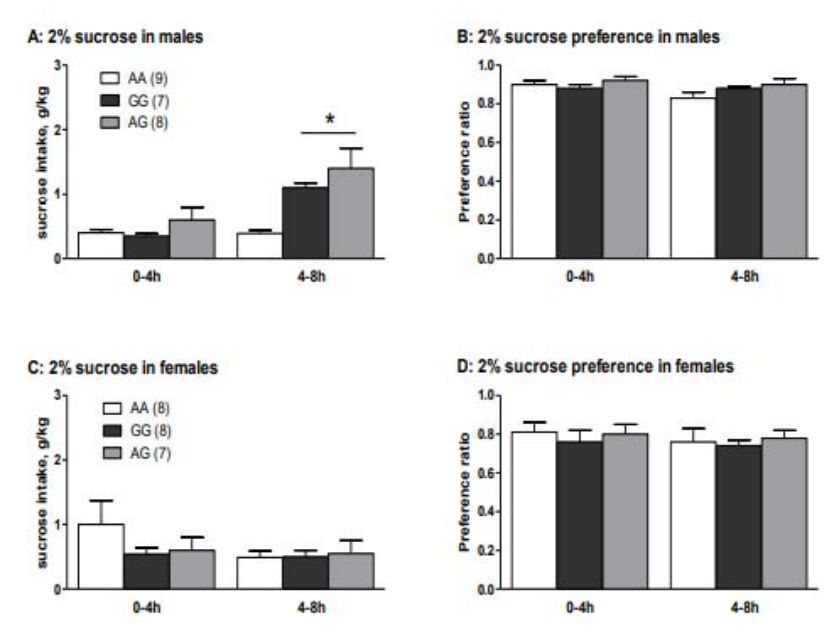

Figure 3 In Test 3A, genotypic difference on 2% sucrose intake (g/kg) after chronic (3 weeks) excessive drinking among AG, GG and AA male (but not female) mice. Mice exposed to be 2-bottle "2% sucrose vs. water" choice regimen and then sucrose and water intake values were recorded after 4 and 8 hours of sucrose access on the test day. Data are presented as mean + SEM. (A) Intake with a genotypic difference: *p<0.05 vs. AA mice in males at 4-8hour interval.

had more intake than the AA mice at 4-8 hours [p<0.05], but not at 0-4 hour (Figure 3 A). In the following Test 3B (4% sucrose) in males, there was a similar genotypic effect: two-way ANOVA with repeat measure revealed a significant effect of genotype [F (1, 22) =10.2, p<0.01]; and during the second 4-hours, the AG and GG mice drank more sucrose the AA mice [Planned comparison (AG+GG vs AA), p<0.01] (Figure 4 A). In both the tests, however, total daily average sucrose consumption was not significantly altered by genotypes, as shown in (Table 3A (2%) and Table 3B (4%). There was no significant difference in sucrose preference ratio in either test among the 3 genotypes at any time points (Figure 4; Tables 3A and 3B).

In contrast, there was no any genotypic difference in females on alcohol intake or preference in either Test 5 or Test 6 at any time points (Figure 3 and Figure 4, Tables 3A and 3B).

No genotypic difference on saccharin (0.1% and 0.2%) drinking among AG, GG and AA males or females: The specificity of the genotypic difference on alcohol consumption was further tested on saccharin intake (non-caloric reinforcer). The mice were therefore exposed to saccharin drinking after sucrose. During 0.1% and 0.2% drinking tests (Test 4A and Test 4B, respectively), there was no genotypic difference in either sex on saccharin intake or preference at any time points (Figures 5 and 6, Tables 4A and 4B).

Genotypic difference in the effect of MOP-r antagonist naltrexone on alcohol ED drinking between AG+GG and AA males: (Figure 7) For alcohol intake, 2-way ANOVA showed significant effects of genotype [F (1, 22) =8.4, p<0.01] and naltrexone treatment [F (1, 22)=5.3, p<0.05], with a significant interaction between the naltrexone treatment and genotype [F (1,22)=5.7, p<0.05]. Newman-Keuls post-hoc tests revealed that:

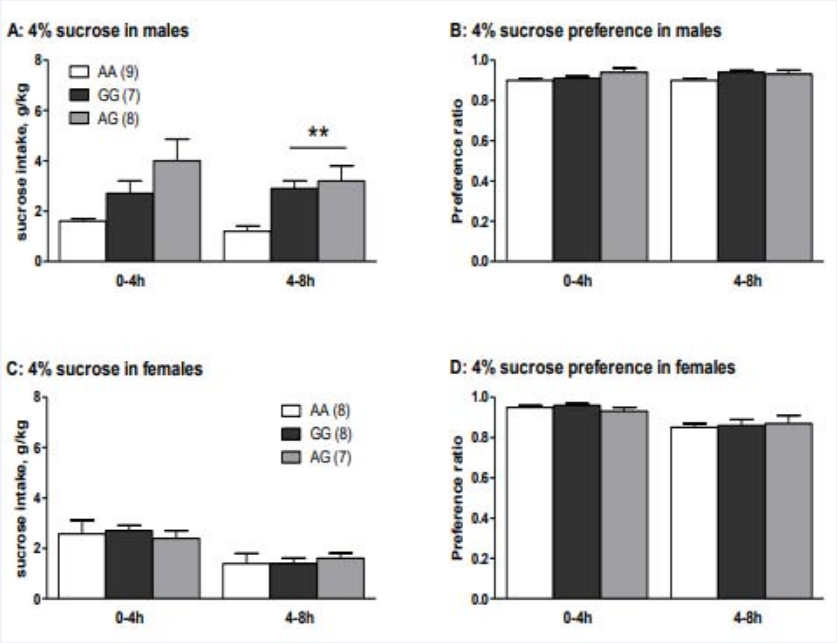

Figure 4 In Test 3B, genotypic difference on 4% sucrose intake (g/kg) after chronic (3 weeks) excessive drinking among AG, GG and AA male (but not female) mice. Mice exposed to the 2-bottle "4% sucrose vs. water" choice regimen and then sucrose and water intake values were recorded after 4 and 8 hours of sucrose access on the test day. Data are presented as mean + SEM. (A) Intake with a genotypic difference: **p<0.01 vs. AA mice in males at 4-8hour

Test 3A (2% sucrose)

	Male			Female		
	AA (n=9)	GG (n=7)	AG (n=8)	AA (n=8)	GG (n=8)	AG (n=7)
Intake, g/kg	5.5 ± 0.17	4.9 ± 0.9	5.7 ± 0.4	6.2 ± 1.0	6.5 ± 0.4	6.0 ± 0.5
Preference ratio	0.94±0.01	0.94±0.03	0.96±0.01	0.86±0.07	0.91±0.02	0.93±0.01

Test 3B (4% sucrose)

	Male			Female		
	AA (n=9)	GG (n=7)	AG (n=8)	AA (n=8)	GG (n=8)	AG (n=7)
Intake, g/kg	12 ± 0.5	12 ± 0.5	15 ± 1.9	20 ± 1.6	19 ± 0.8	21 ± 1.1
Preference ratio	0.79±0.04	0.78±0.08	0.79±0.05	0.90±0.02	0.89±0.03	0.84±0.04

Table 3 No genotype difference on 24-hour sucrose intake (g/kg) or preference (preference ratio) after chronic long-access alcohol excessive drinking among AG, GG and AA male and female mice. Mice exposed to the 2-bottle "2% or 4% sucrose vs. water" choice regimen. Data are presented after 24 hours of sucrose drinking in Test 3A and Test 3B.

(1) both the AG and GG males had more intake than the AA males after vehicle [AG+GG vs AA, p<0.05]; and (2) naltrexone treatment significantly decreased alcohol intakes in the AG and GG males [Vehicle 12 | P a g evs. Naltrexone, p < 0.05], with no effect on the AA males. There was no significant effect of eithergenotype or naltrexone on alcohol preference ratio (data not shown).

Genotypic difference in the effect of KOP-r agonist nalfurafine on alcohol ED drinking between AG+GG and AA males: (Figure 8). At a low doses (3 μg/kg), there was no significant effect of nalfurafine on 15% alcohol drinking in any genotypes (Table S2). With nalfurafine at 10 μg/kg, two-way ANOVA showed a significant effect of genotype on alcohol intake [F (1,28)=10.2, p<0.01], with a significant genotype x nalfurafine treatment interaction[F(1,28)=8.2, p<0.01]. Newman-Keuls post-hoc tests showed that both the AG and GG males had more intake than the AA males after vehicle [AG+GG vs AA, p<0.05] or after nalfurafine treatment [AG+GG vs AA, p<0.01]. Planned comparison test revealed a significant difference between the vehicle and nalfurafine in the AA mice only (p<0.05). There was no significant effect of either genotype or nalfurafine on alcohol preference ratio (data not shown).

DISCUSSION

No genotypic difference in subacute (4 days) and limited-access (4 hours/day) DID model: Our first objective in this study was to investigate the potential effect of the Oprm1 SNP A112G genotypic variants on alcohol "binge" consumption in both male and female mice. As C57BL/6J mice become intoxicated over the 4 days of DID "binge" drinking [4, 36, 37], the AG, GG and AA mice with C57BL/6J background were offered 15% alcohol for 4 hours per day for 4 days. We found that all the mice had similar alcohol intake in each sex in this DID model, with no genotypic difference.

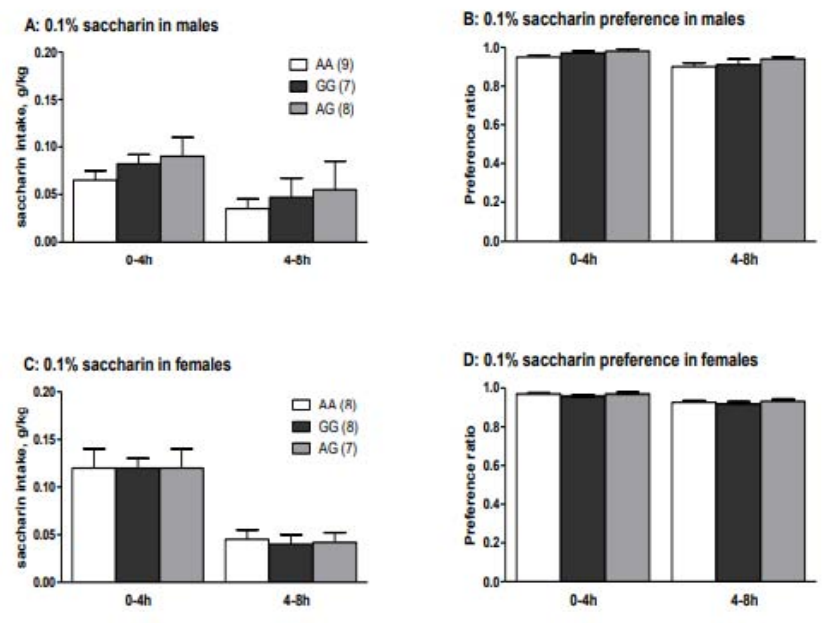

Figure 5 In Test 4A, no genotypic difference on 0.1% saccharin intake (g/kg) after chronic (3 weeks) excessive drinking among AG, GG and AA mice. Mice exposed to the 2-bottle "0.1% saccharin vs. water" choice regimen and then saccharin and water intake values were recorded after 4 and 8 hours of saccharin access on the test day. Data are presented as mean + SEM.

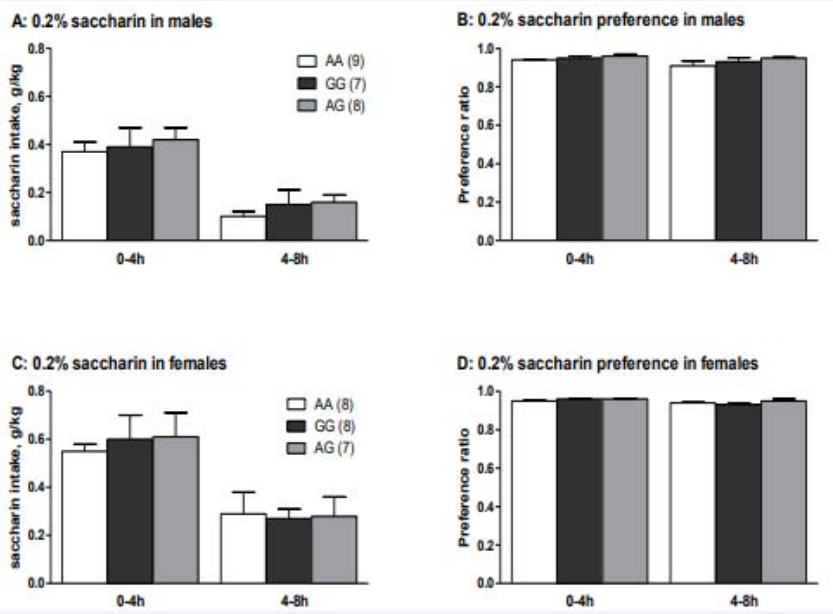

Figure 6 In Test 4B, no genotypic difference on 0.2% saccharin intake (g/kg) after chronic (3 weeks) excessive drinking among AG, GG and AA mice. Mice exposed to the 2-bottle "0.2% saccharin vs. water" choice regimen and then saccharin and water intake values were recorded after 4 and 8 hours of saccharin access on the test day. Data are presented as mean + SEM.

Genotypic difference in chronic (3 weeks) long-access (24 hours/day) ED model: As human studies suggest that the Oprm1 A118G SNP is associated with increased alcohol consumption and increased vulnerability to alcohol dependence [20, 22], we further assessed whether the Oprm1 genotypic difference would be associated with vulnerability to excessive alcohol drinking. After their DID experience, all the AG, GG and AA mice were further exposed to the ED regimen. We found that after chronic excessive drinking, both the AG and GG male mice displayed higher alcohol consumption than the AA male mice with both 15% and 30% concentrations at early exposure times (i.e. after 4 or 8 hours of alcohol access). Our data is consistent with early reports showing that the GG mice had more alcohol intake than the AA mice in "humanized" mice expressing the

Oprm1 A118G SNP, with different alcohol drinking paradigms [41, 42]. In either 15% or 30% alcohol drinking tests, both the AG and GG males showed similar alcohol preference to the AA mice when the alcohol and water choice were available.

It has been found that the G allele leads to elevated MOP-r signaling activity [10]. Therefore, our results on alcohol excessive drinking behavior suggest that the G allele-induced increase in MOP-r/beta-endorphin activity may play a role in promoting excessive alcohol drinking in male AG and GG mice. As the G allele has enhancing effects on dopamine release and MOP-r activity [22, 43], the increase in alcohol intake in male AG and GG mice could be attributed to the enhancement of the rewarding effects of alcohol through both the MOP-r and dopamine pathways. Our finding observed in A112G mice (a functionally equivalent SNP in human Oprm1 A118G) is consistent with some human studies showing that the Oprm1 A118G polymorphism is associated with increased vulnerability to alcohol abuse [16-24] and with mouse studies in "humanized" mice expressing the Oprm1 A118G SNP [41, 42].

Of note, on all excessive alcohol drinking behaviors measured with all the threegenotypes side by side, it was notable that there were significantly greater alcohol intakes in the males with one or two copies of the G allele [namely AG or GG], when compared with the maleswith the AA allele (Figures 1 to 2). As no significant genotypic difference was found between the AG and GG males, our result further suggests no obvious gene-dosage effect in the A112G mice, which is similar to what we observed in FAAH C385A knock-in mice [37]. This result is also consistent with human genetic studies showing that the individuals with at least one copy of the G allele have a greater alcohol-stimulated dopamine release in the striatum [22], a greater euphoria effect of alcohol consumption [19] and a higher risk of developing alcoholism [20]. With one or two copies of the G allele, there is a greater therapeutic response to naltrexone treatment in alcoholics [18, 25], including a longer delay in a return to heavy drinking [18] and fewer relapse rates [23].

Genotypic difference on sucrose, but not saccharin, intake: One group recently studied "binge" eating using Oprm1 A112G SNP mice, and did not observe any genotypic effect on sweet-fat food consumption [44]. In the present experiment, however, it was surprising to observe that the AG and GG males had more sucrose intake at both 2% and 4% concentrations, when compared with the AA males (Figures 3 and 4). Similar to alcohol, the increase in sucrose intake in both the AG and GG males could be attributed to the enhancement of the rewarding effects of sucroseby the G allele on MOP-r and dopamine activities. The specificity on alcohol and sucrose (both caloric reinforcers) was suggested by the lack of any Oprm1 genotypic difference in saccharin intake (palatable non-caloric reinforcer) (Figures 5 and 6). Though the precise mechanisms are unknown, it is very possible that the G allele had a selective effect on caloric reinforcers (alcohol and sucrose), but not non-caloric reinforcers such as saccharin, in our mouse drinking models.

Sex difference: Consistent with previous mouse studies (e.g., [4, 38, 39, 45]), we confirmed sex differences in alcohol drinking, with relatively higher alcohol intake in females (Table 1). However, in all the experiments with both males and females side by side, it was unexpected that there was only genotypic effect in males on alcohol intake in both the DID and ED models. In contrast to females, there was also only genotypic effect on sucrose intake in male mice (Figures 3 to 4). Profound sex differences in both the A112G mice and human A118G carrier mice on several behaviors have been found previously [14, 42, 46, 47]. Though many groups have shown that hormonal variations during the estrous cycle may not affect alcohol intake in female rodents [45], it has been found that estrogen 17beta-estradiol regulates alcohol consumption in female mice [48]. As a decreased alcohol intake in mice with a lack of beta-endorphin or MOP-r was greater in female mice [2-4], one potential interaction may occur between POMC/MOP-r and estrogens/estrogen receptors which could be involved in alcohol drinking in a sex-sensitive manner [49, 50]. Therefore, it is very possible that in male mice, the G allele-promoted MOPr/beta-endorphin activity is blunted by estrogens in female mice. However, at this time we cannot provide a reasonable explanation on the sex differences in response to different A112G genotypes on alcohol intake, with limited information on comparison of mouse sex differences.

We did not measure the blood alcohol concentration [BAC] levels in either male or female A112G mice and could not provide any information on whether there was any sex effect on alcohol metabolic rates or not, which may contribute to the sex difference in the sensitivity to the Oprm1 A112G SNP. However, no genotypic differences in the BAC in either male or female 16 | P a g e mice were found in "humanized" mice expressing the Oprm1 A118G SNP, indicating no pharmacokinetic differences among the genotypes in either sex [41, 42].

Test 4A (0.1% saccharin)

	Male			Female		
	AA (n=9)	GG (n=7)	AG (n=8)	AA (n=8)	GG (n=8)	AG (n=7)
Intake, g/kg	0.32±0.01	0.33±0.03	0.38±0.02	0.41±0.06	0.39±0.01	0.40±0.05
Preference ratio	0.90±0.01	0.90±0.01	0.92±0.02	0.95±0.01	0.93±0.01	0.93±0.01

Test 4B (0.2% saccharin)

	Male			Female		
	AA (n=9)	GG (n=7)	AG (n=8)	AA (n=8)	GG (n=8)	AG (n=7)
Intake, g/kg	0.89±0.07	0.91±0.08	0.90±0.05	1.4±0.10	1.4±0.19	1.5±0.18
Preference ratio	0.91±0.02	0.92±0.01	0.93±0.02	0.94±0.01	0.95±0.01	0.93±0.02

Table 4 No genotype difference on 24-hour saccharin intake (g/kg) or preference (preference ratio) after chronic long-access alcohol excessive drinking among AG, GG and AA male and female mice. Mice exposed to the 2-bottle "0.1% or 0.2% saccharin vs. water" choice regimen. Data are presented after 24 hours of drinking in Test 4A and Test 4B.

Genotypic differences in the effect of naltrexone and nalfurafine between AG+GG and AA males: Finally, we purposely investigated whether low-dose naltrexone could affect alcohol drinking in the AG and GG male mice with increased MOP-r signaling activity, and observed a significant reduction of alcohol intake with naltrexone at 1 mg/kg in the AG+GG males (Figure 7), though the same naltrexone treatment had no effect on alcohol drinking in the AA males. Our results agree with one earlier study showing that voluntary alcohol intake in response to naltrexone was sensitized in "humanized" mice expressing the Oprm1 A118G SNP [41]. It is, therefore, further confirmed that enhanced MOP-r signaling activity by one or two copies of Oprm1 G allele contributes to the MOP-r mediated increases of alcohol drinking. For comparison with naltrexone, we also tested a selective KOP-r agonist nalfurafine and found that the AG+GG males displayed a blunted response to nalfurafine anti-dipsogenic effect on alcohol intake in comparison with the AA males (Figure 8), suggesting a disruption of KOP-r function by the Oprm1 G allele. The potential mechanisms are unknown, and further studies are needed.

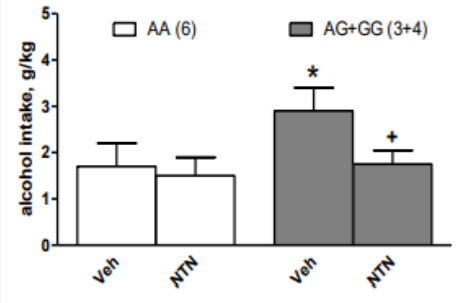

Figure 7 Genotypic differences in the effect of MOP-r antagonist naltrexone (1 mg/kg) on 15% alcohol intake between male AG+GG and AA mice after chronic (3 weeks) excessive drinking. Mice exposed to the 2-bottle "15% alcohol vs. water" choice regimen and then alcohol and water intake values were recorded after 4 hours of alcohol access on the test day. Data are presented as mean + SEM. Genotypic difference: *p<0.05 vs. AA mice after vehicle [Veh]; Naltrexone [NTN] effect: +p<0.05 vs. Veh-treated AG+GG mice.

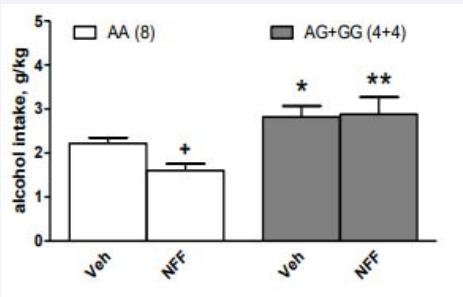

Figure 8 Genotypic differences in the effect of KOP-r agonist nalfurafine (10 μg/kg) on 15% alcohol intake between male AG+GG and AA mice after chronic (3 weeks) excessive drinking. Mice exposed to the 2-bottle "15% alcohol vs. water" choice regimen and then alcohol and water intake values were recorded after 4 hours of alcohol access on the test day. Data are presented as mean + SEM. Genotypic difference: *p<0.05 vs. AA mice after vehicle [Veh]; **p<0.01 vs. AA mice after nalfurafine [NFF]; Nalfurafine effect: +p<0.05 vs. Veh-treated AA mice.

In summary, using A112G mice, we observed an impact of Oprm1 A118G on alcohol drinking in a long-access excessive, but not limited-access "binge", alcohol drinking, in male mice only. Specifically, both the AG and GG males had greater alcohol intake than the AA males after excessive drinking, with a sensitized response to naltrexone, suggesting that increased MOP-r signaling with one or two copies of the G allele increased alcohol consumption. Consistently, human studies have demonstrated that the individuals with at least one copy of the G allele have a greater risk of developing alcoholism.

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