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# Trace Amine-Associated Receptor 1 Regulation of Methamphetamine Intake and Related Traits

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Continued methamphetamine (MA) use is dependent on a positive MA experience and is likely attenuated by sensitivity to the aversive effects of MA. Bidirectional selective breeding of mice for high (MAHDR) or low (MALDR) voluntary consumption of MA demonstrates a genetic influence on MA intake. Quantitative trait locus (QTL) mapping identified a QTL on mouse chromosome 10 that accounts for greater than 50% of the genetically-determined differences in MA intake in the MAHDR and MALDR lines. The trace amine-associated receptor 1 gene (*Taar1*) is within the confidence interval of the QTL and encodes a receptor (TAAR1) that modulates monoamine neurotransmission and at which MA serves as an agonist. We demonstrate the existence of a non-functional allele of *Taar1* in the DBA/2J mouse strain, one of the founder strains of the selected lines, and show that this non-functional allele co-segregates with high MA drinking and with reduced sensitivity to MA-induced conditioned taste aversion (CTA) and hypothermia. The functional *Taar1* allele, derived from the other founder strain, C57BL/6J, segregates with low MA drinking and heightened sensitivity to MA-induced CTA and hypothermia. A role for TAAR1 in these phenotypes is corroborated in *Taar1* transgenic mice: *Taar1* knockout mice consume more MA and exhibit insensitivity to MA-induced CTA and hypothermia, compared with *Taar1* wild-type mice. These are the first data to show that voluntary MA consumption is, in part, regulated by TAAR1 function. Behavioral and physiological studies indicate that TAAR1 function increases sensitivity to aversive effects of MA, and may thereby protect against MA use.
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## INTRODUCTION

Functional brain circuitry flexibly encodes and responds to rewarding and aversive motivational states and events. Methamphetamine (MA) is highly addictive, but has both rewarding and aversive effects that influence use. Prevention and treatment rely on knowledge of the neural mechanisms that contribute to risk for addiction and to sensitivity to the motivational effects of MA. Replicated sets of mouse lines, bidirectionally selectively bred for high or low MA drinking (MADR), are of particular relevance to the study of genetic risk for human MA use. First, in this genetic model, MA intake is completely voluntary. Second, MA high drinking (MAHDR) mice show increased sensitivity to MA reinforcement in operant intracranial and oral self-administration procedures, whereas MA low drinking (MALDR) mice do not. Third, compared with MALDR mice, MAHDR mice have greater sensitivity to conditioned rewarding effects of MA that are relevant to relapse. Finally, MAHDR mice show little sensitivity to aversive effects of MA in conditioned place and conditioned taste aversion (CTA) assays, whereas MALDR mice exhibit high sensitivity. The genetically-determined, robust sensitivity to aversive effects in MALDR mice likely limits their MA intake (Shabani *et al*, 2011, 2012a,b; Wheeler *et al*, 2009).

In addition to its well-known action as a substrate for neurotransmitter and vesicular monoamine transporters (Fleckenstein *et al*, 2007), MA is an agonist at trace amine-associated receptor 1 (TAAR1) (Bunzow *et al*, 2001; Wolinsky *et al*, 2007). Activation of TAAR1 appears to counteract some effects of MA. For example, pretreatment with the TAAR1 agonist, RO5263397, reduces operant self-administration of MA in rats (Jing *et al*, 2015). Trace amines, such as *p*-tyramine, *β*-phenylethylamine, octopamine, and tryptamine, interact with this G protein-coupled receptor (Borowsky *et al*, 2001; Bunzow *et al*, 2001; Lindemann *et al*, 2005; Wolinsky *et al*, 2007), and TAAR1 modulates monoamine activity, in part, through regulation of neurotransmitter availability and disposition (Revel *et al*, 2011; Xie and Miller, 2008). TAAR1 agonists reduce endogenous firing of dopaminergic (DA), noradrenergic (NE), and serotonergic (5-HT) neurons, and *Taar1* knockout (−/−) mice exhibit greater amphetamine-induced release of these neurotransmitters in the striatum, compared with wild-type (+/+) littermates (Lindemann *et al*, 2008; Wolinsky *et al*, 2007).

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*Taar1* −/− mice also display greater locomotor stimulation to amphetamine and MA (Achat-Mendes *et al*, 2012; Lindemann *et al*, 2008; Wolinsky *et al*, 2007), consistent with the idea that TAAR1 function is important for counteracting some MA effects. However, the role of TAAR1 function in sensitivity to aversive effects of MA has not been examined.

Physiological effects of MA contribute to subjective effects that could impact MA consumption. Acute and chronic MA can induce hyperthermia (Bowyer *et al*, 1994; Sabol *et al*, 2013), but hypothermia may occur at lower doses and 18–20 ° C ambient temperatures (Sabol *et al*, 2013). MA-induced hypothermia may be partly regulated by TAAR1, since other TAAR1 agonists induce hypothermia (Di Cara *et al*, 2011; Fantegrossi *et al*, 2013; Panas *et al*, 2010), and *Taar1* −/− mice do not exhibit hypothermia to doses of the MA-like drug, MDMA, that induce hypothermia in +/+ mice (Di Cara *et al*, 2011). Likewise, *Taar1* −/− mice do not display hypothermia to MA, but the dose of MA used in this published study did not induce hypothermia in +/+ mice (Panas *et al*, 2010), so further study is needed.

*Taar1* is within the confidence interval for a quantitative trait locus (QTL) on mouse chromosome 10 (Figure 1) that accounts for greater than 50% of the genetic variance in MA intake in MADR mice (Belknap *et al*, 2013). DBA/2J (D2) mice possess a unique non-synonymous single-nucleotide polymorphism (SNP, C229A) in *Taar1* (Sanger Mouse Genomes Project SNP browser, 2014; Keane *et al*, 2011; Yalcin *et al*, 2011) that is not present in C57BL/6J (B6) mice. These are the two progenitor strains of the MADR lines. This polymorphism supports investigation of *Taar1* as a viable candidate for a quantitative trait gene (QTG) regulating MA intake.

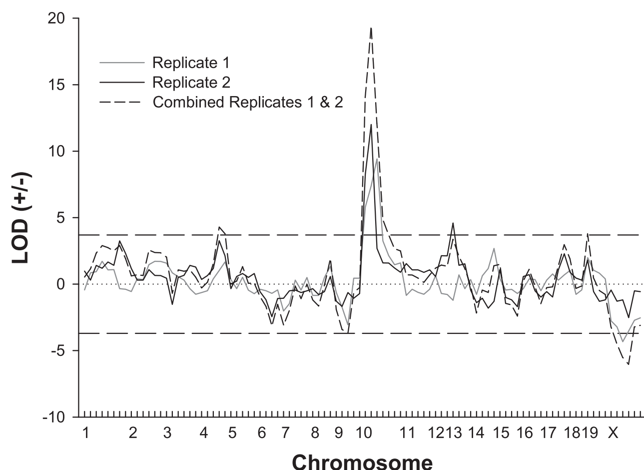


**Figure 1** A quantitative trait locus (QTL) on mouse chromosome 10 has a major effect on MA drinking in MADR mice. The QTL on proximal chromosome 10 (10–40 Mb) explains greater than 50% of the genetic variance in the MA drinking phenotype (Belknap *et al*, 2013). Data are presented as directional genome-wide logarithm of the odds (LOD) scores. Positive and negative LOD scores indicate that higher trait scores are conferred by the D2 and B6 alleles, respectively. Dashed horizontal lines indicate statistically significant support for a QTL at $p < 2 \times 10^{-5}$. Although there are a large number of genes in this interval that have the potential to influence the MA drinking trait, of particular interest based on the literature supporting involvement of opioid, glutamate, and TAAR1 in MA responses, are the mu opioid receptor gene (*Oprm1*), metabotropic glutamate receptor gene (*mGlur1*), and *Taar1*, which reside at 6.76, 10.7, and 23.9 Mb, respectively. Data shown were generated using two independent sets of replicated MADR lines, produced 2 years apart. Also shown are combined data for the independent replications of the QTL study. Figure adapted from Belknap *et al* (2013), with permission.

Here, we present frequency data in MADR mice for the *Taar1* polymorphism found in D2 and B6 mice. We test the hypothesis that voluntary MA consumption is influenced by *Taar1* by measuring MA intake in *Taar1* −/−, heterozygous (+/−) and +/+ littermates. We also test the hypothesis that *Taar1* has a role in sensitivity to a conditioned aversive effect of MA and in the thermal response to MA. In addition, we examine thermal response to ethanol to determine drug specificity of *Taar1* influence. Finally, we examine TAAR1-related 3'-5'-cyclic adenosine monophosphate (cAMP) response to MA in B6- and D2-like TAAR1 isoforms.

These studies provide the first evidence that D2 mice possess a *Taar1* allele that codes for a non-functional TAAR1, and this allele occurs at high frequency in mice that were bred for higher levels of voluntary MA consumption. The absence of TAAR1 function, as found in D2, *Taar1* −/−, and MAHDR mice, increases the risk for MA consumption and decreases the sensitivity to the conditioned aversive and hypothermic effects of MA. These, and published, data suggest that a functional TAAR1 heightens sensitivity to certain aversive and physiological effects of MA that may limit MA use.

## MATERIALS AND METHODS

### Animals

*Methamphetamine drinking selected mouse lines.* The MADR mice were selectively bred from an F2 cross of the B6 and D2 inbred strains. Details of the selective breeding procedures and response to selection have been fully described (Shabani *et al*, 2011; Wheeler *et al*, 2009). Selective breeding was based on amount of the 40-mg/l MA solution consumed in the drinking procedure described below. Three consecutive pairs of MAHDR and MALDR lines have been created, with comparable outcomes, using these procedures.

*Taar1* −/− *mouse breeding and genotyping.* The *Taar1* −/− mice were obtained from the U.C. Davis Knockout Mouse Project (KOMP; www.komp.org). Briefly, chimeric mice were created by injecting BALB/c blastocysts with C57BL/6N ES cells in which the entire *Taar1* coding region was deleted by homologous recombination using VelociGene's Null Allele Bac vector. The chimeras were bred with wild-type B6 mice and their offspring genotyped according to the strategy recommended by KOMP using the following primers: ACTCTTCACCAAGAATGTGG (forward); CCAACAGCGCTCAACAGTTC (reverse, wild-type allele); GTCGTCCTAGCTTCCTCACTG (reverse, null allele). Male and female siblings, heterozygous for the targeted locus, were subsequently bred to produce *Taar1*+/+, +/−, and −/− littermates.

*Animal maintenance and housing.* Before experiment initiation, mice were group-housed in acrylic plastic shoebox cages (28 cm × 18 cm × 13 cm; l × w × h), fitted with wire tops. Cages were lined with Bed-O-Cob (The Andersons, Maumee, OH, USA) or ECOFresh bedding (Absorption Corporation, Ferndale, WA). Mice had free access to rodent

chow (Purina 5001, 4.5% fat content; Animal Specialties, Hubbard, OR) and water at all times except during testing. Colony room temperature was 20–22 °C, and lights were maintained on a 12 : 12 h light:dark schedule, with lights on at 0600 h. Mice of both sexes were used in all studies. Procedures were conducted in accordance with the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals, and were approved by the Veterans Affairs Portland Health Care System Institutional Animal Care and Use Committee.

## Drugs and Reagents

(+)MA hydrochloride was purchased from Sigma (St Louis, MO, USA) and mixed in tap water for consumption or dissolved in sterile physiological saline (0.9% NaCl; Baxter Healthcare Corporation, Deerfield, IL, USA) for injection. All injections were given intraperitoneally at a volume of 10 ml/kg. N-(3-Ethoxy-phenyl)-4-pyrrolidin-1-yl-3-trifluoro-methyl-benzamide (EPPTB) (Liu *et al*, 2014) was first diluted in DMSO, and subsequently diluted into cAMP assay buffer for a final DMSO concentration of 0.1%.

## General Procedures

*Taar1 sequencing.* Genomic DNA from MALDR and MAHDR mice was extracted from ear or tail tissue using QuickExtract DNA Extraction Solution (Epicentre, Madison, WI). *Taar1* DNA was amplified using a Hotstart DNA polymerase kit (Qiagen, Valencia, CA) with sequence specific primers surrounding the SNP-containing region (forward 5′-CACCAACTGGCTCCTTCACT-3′, reverse 5′-CGGTGC TGGTGTGAACTTTA-3′). PCR products were run on a 1.5% agarose gel, and then purified using a QIAquick gel extraction kit (Qiagen, Valencia, CA). Purified DNA was sequenced at the Oregon Health & Science University sequencing core using the forward primer to amplify the *Taar1* gene. Sequences of PCR products were aligned and compared with mouse *Taar1* sequence (NM_053205.1).

*MA two-bottle choice drinking.* Procedures were identical to those used during selection of the MADR lines (Shabani *et al*, 2011; Wheeler *et al*, 2009). MADR or *Taar1* transgenic mice were isolate-housed and habituated for 48 h to drinking from two water-filled drinking tubes. During days 3–10, water was offered *vs* 20-mg/l MA in water for 4 days, and then 40-mg/l MA in water for 4 days. Mice had 24-h access to water, but only 18-h access to MA. The positions of the water and MA tubes were alternated every 2 days to control for side preferences. Body weight was measured every 2 days and used with volume change to calculate MA consumption in mg/kg. Data from the second and fourth days for each MA concentration (the second day after a change in MA tube position) were averaged to provide a measure of intake.

*MA-induced CTA in Taar1 transgenic mice.* Identical procedures for MA-induced CTA have been used to test MADR line mice (Shabani *et al*, 2012b; Wheeler *et al*, 2009). Briefly, *Taar1* +/+, +/−, and −/− mice were isolate-housed and then acclimated to water restriction (2 h of water per day) on days 1–4. On day 5, mice were introduced to the novel taste of a 0.2-M NaCl solution, during a 1-h access period. Conditioning trials then occurred every other day, during which mice were given access for 1 h to the NaCl solution (days 7, 9, 11, 13, and 15), and were then immediately injected with saline or MA (2 mg/kg). This dose of MA was chosen because it induced strong CTA in MALDR mice, but no CTA in MAHDR mice. Water was made available for 30 min, 3 h following injections, to avoid dehydration. On intervening non-conditioning days, water was available for 2 h and no injection was administered.

*MA-induced changes in body temperature.* MADR or *Taar1* transgenic mice were placed in acrylic plastic chambers that isolated the mice from each other and prevented huddling-associated alterations of body temperature (Crabbe *et al*, 1987, 1989). They were allowed to acclimate for 1 h and then baseline (time 0) rectal temperature was measured (Crabbe *et al*, 1987). Mice then received an injection of saline or MA (1, 2, 4, 8, or 16 mg/kg for MADR mice; 2 mg/kg for *Taar1* mice, based on dose-response results) and were returned to the chambers. Temperatures were recorded at 30, 60, 90, 120, and 180 min after injection. A separate set of MADR mice and *Taar1* transgenic mice were identically tested for response to ethanol (with the final reading at 300 min after injection) to examine whether differences in hypothermic response were specific to MA. Doses of ethanol (2 or 4 g/kg) known to induce hypothermia in mice were used (Crabbe *et al*, 1979).

*Cell culture and stable transfection.* HEK-293 cells were cultured as we have previously described (Eshleman *et al*, 1999, 2013). The full-length coding region of the mouse *Taar1* (c-terminus GFP tag) cDNA (OriGene, Rockville, MD) was sequenced to verify that it was consistent with the B6 reference. Plasmid DNA was prepared using the Qiagen miniprep kit (Chatwsorth, CA) and Charge Switch Plasmid maxiprep kit (Invitrogen, Grand Island, NY) after transformation of BL-21 competent *E. coli* cells (Invitrogen). Sequence was verified by *Eco*R I/*Xho* I restriction enzyme digestion. The mutation at position 229 in the mouse *Taar1* gene (D2-like *Taar1*) was created using the QuickChange Lightning Kit (Agilent Technologies, Santa Clara, CA) and the B6 sequence. The mutation was verified by sequencing using the VP1.5 primer (5′-GGACTTTCCAAAATGTCG-3′, OriGene). The B6- and D2-like *Taar1* expression constructs were transfected into HEK-293 cells using Lipofectamine 2000 (Invitrogen; 10 μg DNA/15 cm plate) according to the manufacturer's instructions. Stably transfected cells were selected in 600 μg/ml neomycin (G418) and subsequently analyzed for cAMP accumulation in response to MA.

*cAMP accumulation assay.* Cells expressing the B6- and D2-like constructs were seeded at a density of $2 \times 10^5$ cells/well in 48-well tissue culture plates 2 days before an assay, with culture medium containing 10% FCS. One day before the assay, cells were switched to culture medium containing 10% charcoal stripped FCS and incubated overnight. Experiments were completed in assay buffer as previously described (Watts *et al*, 1998). Seven concentrations of MA ($10^{-8}$ to $10^{-4}$) were added and cells were incubated for 60 min in the presence or absence of 10 μM EPPTB (30 min pre-incubation). cAMP accumulation was measured using a

cAMP EIA kit (Cayman Chemical, Ann Arbor MI), according to the manufacturer's instructions. All experiments were conducted with duplicate determinations.

*Confocal microscopy*. Cells containing the B6- and D2-like constructs were treated and 0.97 μm intervals were analyzed as we have previously described (Keith *et al*, 2012).

*Immunodetection*. Cells were lysed in RIPA lysis buffer containing 1× protease inhibitor (Roche, San Francisco, CA). Protein concentrations of the samples were determined using a BCA kit (Thermo Scientific, Waltham, MA). Samples were loaded on gels using equal amounts of total protein as we have previously described (Shi and Habecker, 2012). The membrane was incubated at 4 °C overnight with mouse anti-turbo GFP (1 : 2000, OriGene) or $\beta$-actin (1 : 2000, Abcam, Cambridge, MA).

### Data Analysis

Behavioral and physiological data were analyzed by repeated measures analysis of variance (ANOVA), with selected line, sex, and dose as between-groups factors, and time as a within-subject factor (repeated measure). There were no interactions involving sex and thus, subsequent analyses excluded sex as a factor. Significant two-way interactions were examined using simple main effect analysis, and the Neuman–Keuls test for *post hoc* mean comparisons was applied, when appropriate. Alpha level was set at 0.05, and statistical analyses were performed using the Statistica 12 software package (StatSoft, Tulsa, OK). The MA dose-response curves for cAMP accumulation were analyzed by ANOVA, with MA dose and receptor type as between-groups factors, followed by Tukey's test for *post hoc* mean comparisons. For western blots, immunodetection analysis was carried out using LabWorks software (UVP, Upland, CA). Confocal microscope images were analyzed with LAS AF (Leica Microsystems CMS GmbH, Wetzlar, Germany). Statistical results are presented in the figure legends.

## RESULTS

### Taar1 Sequence

The D2 strain possesses a non-synonymous allelic variant of the *Taar1* gene (Sanger Mouse Genomes Project SNP browser, 2014; Keane *et al*, 2011; Yalcin *et al*, 2011), as compared with the reference B6 strain. To date, the SNP (C229A) is reported to be unique to the D2 strain; the reference B6 allele is shared by at least 27 additional strains. The SNP causes a substitution from a proline to a threonine residue at amino-acid position 77 (P77T, Figure 2a), which is situated at the cytoplasmic/luminal interface of the second transmembrane domain. We sequenced the *Taar1* gene in the MADR lines and found the D2 allele at a frequency of 1.0 in the 10 MAHDR mice sequenced; every mouse was homozygous for the D2 allele. In the 10 MALDR mice sequenced, both B6 homozygotes and B6/D2 heterozygotes were found (Figure 2b). These data indicate that homozygosity for the D2 allele co-segregates with selection for high MA consumption.

### MA Drinking

Figure 3a shows MA consumption in MADR and *Taar1* +/+, +/−, and −/− mice. Shown for comparison is published MA consumption data for the progenitor B6 and D2 strains (Eastwood and Phillips, 2012). Fold changes were calculated to provide an index of magnitude of difference between genotypes. Data were collected in independent experiments and could not be legitimately included in a single statistical analysis. MAHDR mice consumed 9- and 11.9-fold more MA at the 20- and 40-mg/l concentrations (respectively), compared with MALDR mice. D2 mice consumed 3.8- and 6.6-fold more MA than B6 mice at the 20- and 40-mg/l concentrations (Eastwood and Phillips, 2012). *Taar1* +/+ and +/− mice consumed only small amounts of MA, and *Taar1* −/− mice consumed 3.3- and 6.4-fold more MA at the 20-and 40-mg/l concentrations, compared with *Taar1* +/+ mice. The dose consumed by MAHDR, D2, and *Taar1* −/− mice was significantly greater when MA was offered as a 40-mg/l concentration.

### MA-Induced CTA

MA-induced CTA to the novel NaCl solution was observed in *Taar1* +/+ and +/− mice, but not in *Taar1* −/− mice. Statistical analyses supported significant reductions in NaCl consumption across conditioning trials, only in the MA-treated *Taar1* +/+ and +/− mice (Figure 3b). Saline treatment had no significant effect on NaCl consumption.

### Thermal Response to MA

Figure 4a shows the core body temperature response to multiple doses of MA in replicate two MADR mice. MAHDR mice exhibited a hyperthermic response to all doses of MA (1–16 mg/kg), whereas the primary response in MALDR mice was hypothermia. Similar data were generated in replicate three MADR mice (Supplementary Figure 1). *Taar1* transgenic mice were subsequently tested with 2 mg/kg MA (Figure 4b), because this dose produced a clear difference in hypothermic response in both replicate sets of MADR mice. Data for MADR mice from Figure 4a are reproduced in Figure 4c to facilitate direct comparison. *Taar1* +/+ and +/− mice responded similarly to MALDR mice, showing hypothermia, whereas *Taar1* −/− mice did not experience significant hyperthermia or hypothermia. Therefore, the hypothermic response occurred in mice that possess the B6-like *Taar1* allele. The difference in sensitivity to the hypothermic effect of MA did not generalize to ethanol, as all genotypes showed hypothermia and there were no genotype-dependent differences (Figures 4d,e).

### TAAR1 Function and MA Consuming Mice

To determine differences in function between the B6- and D2-like isoforms of TAAR1, site-directed mutagenesis was used to create the D2 construct found in all MAHDR mice. Both the wild-type and mutant constructs were stably transfected into HEK-293 cells, cells were treated with the TAAR1 agonist, MA, and cAMP accumulation was measured using ELISA. MA elicited a dose-dependent response in cells expressing B6-like TAAR1 ($EC_{50} = 826$ nM), and the

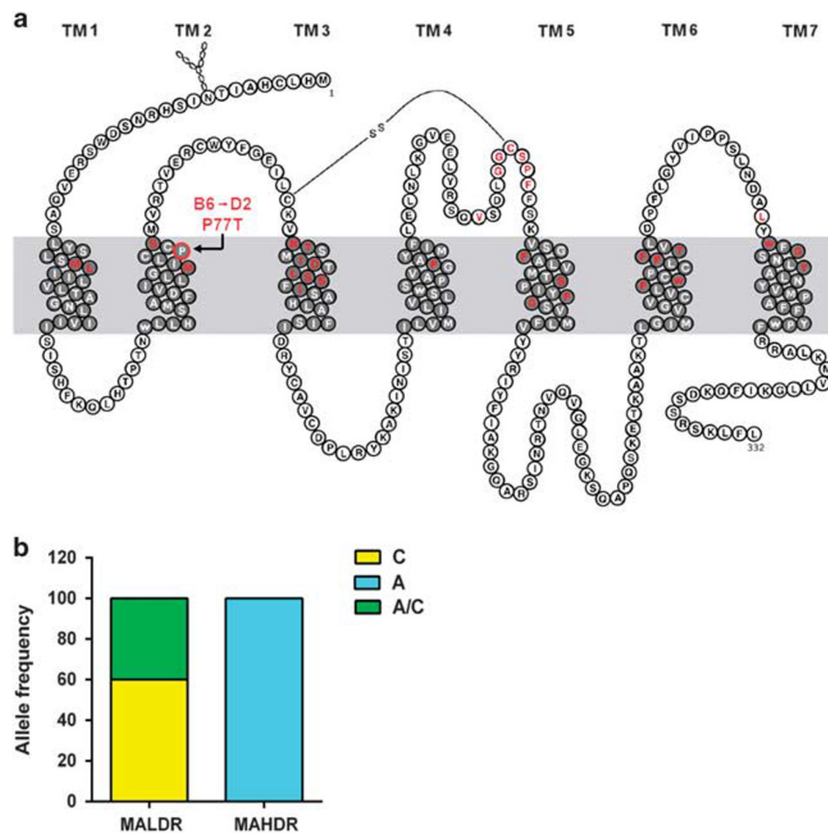


**Figure 2** Schematic transmembrane (TM) topology of mouse TAAR1 (adapted from the human TAAR1) and frequency of B6- and D2-like *Taar1* alleles in MALDR and MAHDR mice. (a) Amino-acid residues incorporated in the transmembrane domains are shaded in gray. Residues comprising the putative ligand binding vector in locations homologous to human TAAR1 are colored red. N-linked glycosylation at N9, as well as the disulfide bridge linking C95 and C181, are indicated according to the annotation in Uniprot entry Q923Y8_TAAR1_MOUSE. Mouse SNP rs33645709 encodes a non-synonymous proline to threonine mutation at amino-acid position 77 (P77T) in D2, compared with B6 mice. Further details are provided in the text. Figure adapted with permission from Lindemann *et al* (2005). (b) Frequency of B6 and D2 *Taar1* alleles in MALDR and MAHDR mice. *Taar1* was sequenced in MALDR and MAHDR mice ($n = 10$/line; replicate 2, selection generation 5). 'A' and 'C' refer to adenine and cytosine, respectively. MAHDR mice are homozygous for the D2 allele at nucleotide 229. This SNP leads to a threonine at amino-acid position 77. MALDR mice are either homozygous or heterozygous for the B6 allele. B6: C57BL/6J; D2: DBA/2J; MALDR: MA low drinking; MAHDR: MA high drinking.

effect was blocked by the TAAR1 antagonist, EPPTB (Figure 5). cAMP accumulation was absent following MA treatment of non-transfected cells and of cells expressing D2-like TAAR1, suggesting that the receptor is non-functional. Immunoblot verified that both the functional B6- and the non-functional D2-like receptors were expressed in transfected HEK-293 cells. Confocal microscopic analysis of GFP-tagged constructs corroborated the immunoblot data, and indicated that both forms of TAAR1 are cytosolic, consistent with previous reports (Bunzow *et al*, 2001; Xie *et al*, 2007).

## DISCUSSION

In the current experiments, MA drinking, sensitivity to MA-induced CTA, and thermal response to MA corresponded with *Taar1* genotype. Homozygous expression of a non-functional isoform of TAAR1 was associated with heightened genetic risk for MA intake. Homo- or heterozygous expression of functional TAAR1 appears to protect against MA consumption, and suggests that the D2 *Taar1* polymorphism is not a dominant negative mutation. However, the functional *Taar1* allele is dominant in its effect on MA intake. The segregation of the D2-like and B6-like alleles in MAHDR and MALDR mice confirms the direction of allele influence predicted by the QTL on chromosome 10 (Belknap *et al*, 2013). Overall, these data provide strong support for *Taar1* as a candidate gene for regulation of MA consumption. However, the MA consumption phenotypes of the MAHDR and MALDR mice were more extreme than those of the non-selectively bred D2/B6 and transgenic mice, as indicated by the fold-difference data. The influence of other genes is supported by this finding and by the finding that the chromosome 10 QTL accounts for about half, not all, of the genetically determined variance in MA intake.

Lower genetic risk for MA consumption was associated with sensitivity to MA-induced CTA and hypothermia. This outcome was observed in two genetic models. Transgenic mice homozygous or heterozygous for a functional *Taar1* allele avoid MA consumption and are sensitive to MA-induced CTA and hypothermia. Similarly, MALDR mice, which are either homozygous or heterozygous for a functional *Taar1* allele, avoid MA consumption and are sensitive to aversive effects of MA (Wheeler *et al*, 2009; Shabani *et al*, 2011, 2012b) and MA-induced hypothermia. This outcome is clear in two replicate sets of MADR lines, which strongly supports

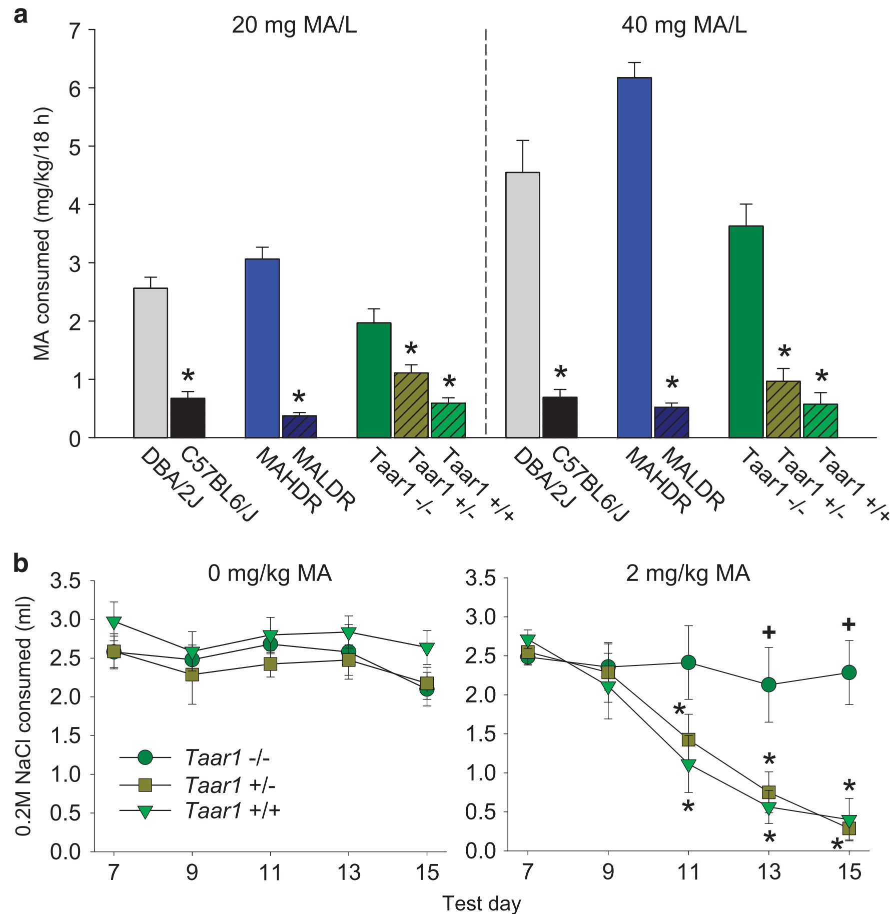


**Figure 3** (a) Methamphetamine (MA) consumption differs by *Taar1* genotype. DBA/2J (D2) mice consume more MA in mg per kg than C57BL/6J (B6) mice, as previously reported (Eastwood and Phillips, 2012). Data shown here support the greater MA consumption of MAHDR mice, compared with MALDR mice. There was a significant line × concentration interaction ($F(1,122) = 121.3$; $p < 0.0001$); MA intake was higher in MAHDR mice for both MA concentrations, but the difference was greater for 40 mg/l MA. *Taar1* −/− mice consumed more MA than *Taar1* +/− or +/+ mice. There was a genotype × concentration interaction ($F(2,63) = 14.4$ $p < 0.001$); MA intake differed for both MA concentrations, but the difference was greater for 40 mg/l MA. *$p < 0.05$ for the difference between the lines or genotypes (D2 *vs* B6, MAHDR *vs* MALDR, *Taar1* −/− *vs Taar1* +/+ and *Taar1* −/− *vs Taar1* +/−) within each concentration. $N = 62$/MADR line (49–54 days old; replicate 3, selection generation 4), and 19–28/transgenic genotype (95–365 days old). B6 and D2 data are shown here with permission (Eastwood and Phillips, 2012). MALDR: MA low drinking; MAHDR: MA high drinking. (b) Sensitivity to MA-induced conditioned taste aversion (CTA) differs by *Taar1* genotype. *Taar1* −/− mice were insensitive to MA-induced CTA at doses that produce CTA in *Taar1* +/+ and +/− mice. There was a significant genotype × treatment × day interaction ($F(8,152) = 2.7$ $p < 0.01$). Subsequent analysis in MA-treated mice identified a significant genotype × day interaction ($F(8,80) = 4.17$ $p < 0.0005$) that was not found in saline-treated mice. Shown are means ± SEM. $^{+}p < 0.05$ for the difference between *Taar1* −/− *vs Taar1* +/+ and *Taar1* −/− *vs Taar1* +/− on specific day; *$p < 0.05$ for the difference in NaCl consumption on the indicated day, compared with consumption on day 7 before conditioning, within genotype, $N = 5$–8/transgenic genotype for saline, and 7–8/transgenic genotype for MA (109–176 days old).

common genetic influence on MA consumption and sensitivity to the aversive and hypothermic effects of MA. Furthermore, combined with data from the *Taar1* transgenic mice, these data suggest *Taar1* as a candidate gene that influences all three traits. Alternatively, *Taar1* may regulate one response that influences the others. For example, *Taar1*-regulated sensitivity to MA-induced hypothermia may cause a reduction in MA consumption or underlie conditioned aversion. This hypothesis could be tested by preventing the hypothermic effect in MALDR mice while measuring MA intake. MADR mice do not differ in locomotor stimulation to 0.5, 2, or 4 mg/kg MA (Shabani *et al*, 2011); therefore, genotype-specific differences in hypothermia at these doses of MA are not likely due to differential locomotor activation by MA. However, unpublished data in the MADR lines indicate greater sensitivity of MAHDR mice, like *Taar1* −/− mice, to the locomotor stimulant effects of some higher doses of MA. Finally, sensitivity to ethanol-induced hypothermia appears to be regulated by genetic factors distinct from those that influence sensitivity to the thermal effects of MA, as the response to ethanol was similar in both the transgenic mice and MADR mice.

Risk for drug use is affected by the balance of positive and negative experiences with a drug (Cruickshank and Dyer, 2009; Davis and Riley, 2010). Considerable attention has been given to positive rewarding effects associated with MA addiction (Beckmann *et al*, 2010; Horton *et al*, 2011; Kamens *et al*, 2005; Mahler *et al*, 2013; Meyer *et al*, 2011; Mizoguchi *et al*, 2004; Shabani *et al*, 2011, 2012a; Wheeler *et al*, 2009), whereas aversive effects that could limit intake have been given less consideration (Harrod *et al*, 2010; Pringle *et al*, 2008; Shabani *et al*, 2011, 2012b; Wheeler *et al*, 2009). Greater

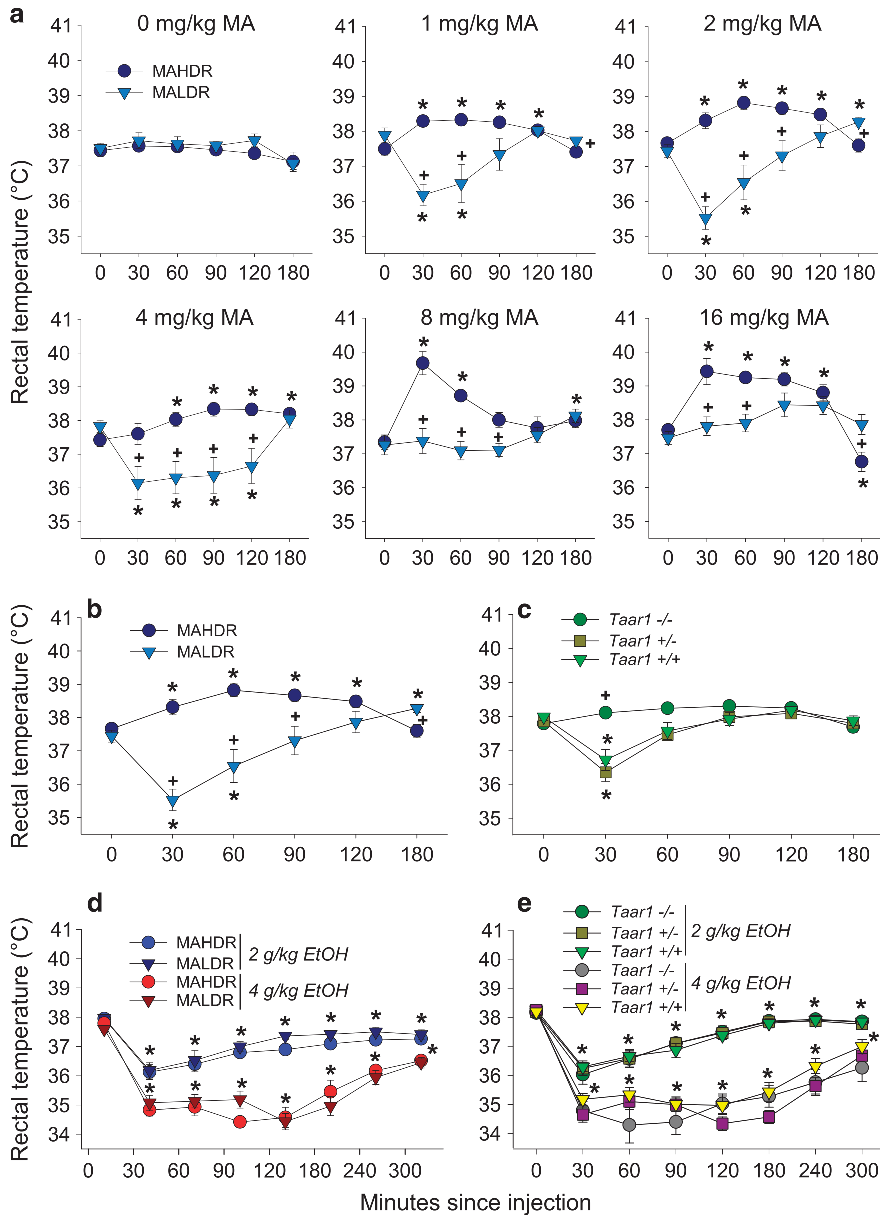


**Figure 4** Methamphetamine (MA)-induced hypothermia differs by *Taar1* genotype. (a) MALDR mice exhibited hypothermia in response to 1, 2, and 4 mg/kg MA, whereas MAHDR mice exhibited hyperthermia in response to all MA doses. There was a significant line × time × dose interaction ($F(25,800) = 3.2$; $p < 0.0001$) and subsequent analysis identified a significant line × time interaction at each dose of MA (F's(5,105–165) = 7.5–22.8; all $p$'s < 0.0001). Significant changes in temperature across time were found in both lines of mice at all MA doses, except in MALDR mice at 16 mg/kg MA. Thermal data in saline-treated mice were similar for the MALDR and MAHDR lines, and there were no significant differences in body temperature from baseline, except for a small reduction at 180 min. $N = 12$/MADR line and dose; 62–99 days old; replicate 2, selection generation 5. (b, c) *Taar1* +/+, and *Taar1* +/− mice exhibit hypothermia in response to 2 mg/kg MA, similar to MALDR mice, whereas the hypothermic response is absent in MAHDR and *Taar1* −/− mice. Data for 2 mg/kg MA in MADR mice are shown in (b) to facilitate comparison with *Taar1* transgenic mice, and are the same data shown in (a). For the *Taar1* transgenic mice (c), there was a significant interaction of genotype × time ($F(10,80) = 3.3$ $p < 0.005$) and significant changes in temperature across time were found for all transgenic genotypes, except *Taar1* −/−. $N = 11$–13/transgenic line and dose (92–290 days old). (d, e) Ethanol-induced hypothermia was induced by both 2 and 4 g/kg ethanol in MADR mice and *Taar1* transgenic mice and no significant differences were found. (d) For MAHDR and MALDR mice, there was a significant time × dose interaction ($F(7,329) = 17.1$; $p < 0.0001$), with greater hypothermia induced by the higher ethanol dose. $N = 12$–13/MADR line; 86–96 days old; replicate 2, selection generation 5. (e) For *Taar1* transgenic mice, there was a significant time × dose interaction ($F(7,581) = 18.0$; $p < 0.0001$), with greater hypothermia induced by the higher ethanol dose. $N = 12$–16/ transgenic line and dose (140–220 days old). Mean comparisons collapsed on genotype identified significant differences in core temperature between the baseline measure and after ethanol treatment. Note: *Taar1* mice were in short supply and before testing for ethanol-induced hypothermia, had been included in another study in which MA was given 5 times at a frequency of every 48 h, and then allowed a 2-week rest interval between the studies. Shown are means ± SEM. $^{+}p < 0.05$ for the difference between the lines or genotypes; $^{*}p < 0.05$ for the difference between baseline temperature and temperature at a given time point within a given genotype, or in the case of ethanol, for the genotypes collapsed. MADR: MA drinking; MALDR: MA low drinking; MAHDR: MA high drinking.

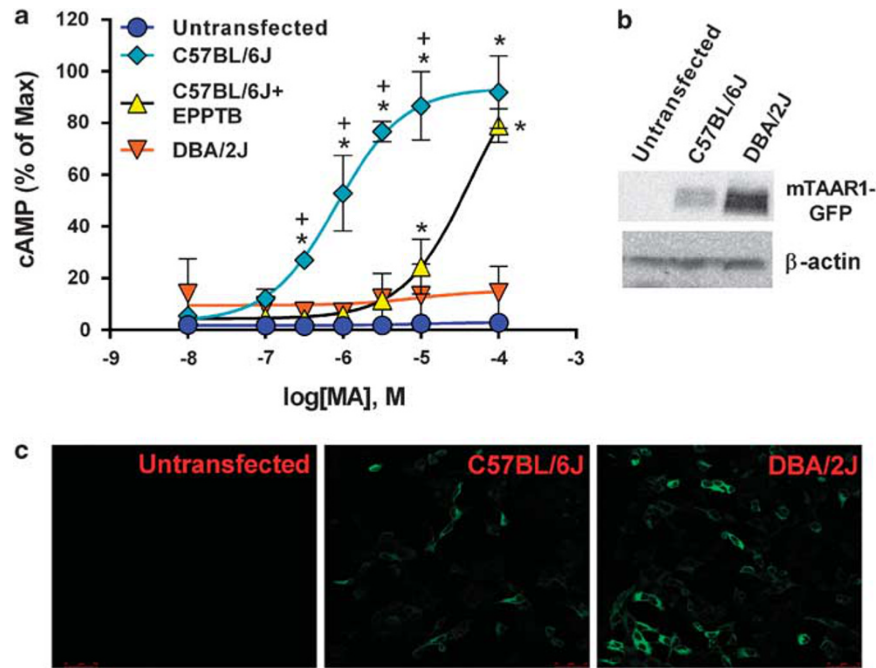


**Figure 5** The B6-like, but not D2-like, isoform of TAAR1 is activated by MA in *Taar1*-transfected cells. HEK293 cells were stably transfected with GFP-tagged B6- or D2-like *Taar1*, and cAMP assays were performed as described in the text. (a) The B6-like isoform of TAAR1 responds to MA stimulation ($EC_{50}$ = 826 nM); however, the D2-like isoform does not, suggesting that the receptor is non-functional. Administration of the TAAR1 antagonist EPPTB ($EC_{50}$ ~ 60 μM) produced a right-ward shift in MA-induced cAMP accumulation in the B6-like recombinant TAAR1. Data shown are the average of at least three independent experiments, each conducted with duplicate determinations. Shown are means ± SEM. There was a significant dose × receptor type interaction ($F(18,56) = 23.4$; $p < 0.0001$). *$p < 0.01$ for the comparison between the indicated group and untransfected control. $^{+}p < 0.01$ for the comparison between the MA alone and MA+EPPTB C57BL/6J TAAR1 groups. (b) Both the functional B6-like and the non-functional D2-like isoforms of TAAR1 were expressed in transfected cells. Untransfected (Untrans) cells did not express TAAR1. β-Actin was measured as a loading control. (c) Confocal images demonstrating expression of the functional B6-like and non-functional D2-like TAAR1 in cells. B6: C57BL/6J; D2: DBA/2J.

sensitivity to the hyperthermic effects of MA did not correspond with reduced voluntary MA drinking. Instead, heightened sensitivity to MA-induced hypothermia was associated with low MA intake and greater sensitivity to MA-induced aversion. MA is an agonist at TAAR1 (Bunzow *et al*, 2001; Reese *et al*, 2014; Wolinsky *et al*, 2007) and the outcome of hypothermia is in agreement with other reports of TAAR1 agonist-induced hypothermia in rodents (Di Cara *et al*, 2011; Fantegrossi *et al*, 2013; Panas *et al*, 2010; Sabol *et al*, 2013). Thus, it appears that TAAR1 mediates MA-induced hypothermia and that the immediate hypothermic effect of MA may have a role in curbing MA intake in MALDR, *Taar1* +/+ and *Taar1* +/− mice. Reduced body temperature alone does not induce CTA in rodents (Misanin *et al*, 1998). However, hypothermia did prolong the associative period during which aversion could be conditioned (Christianson *et al*, 2005; Misanin *et al*, 1998, 2002). Therefore, one possible role of the hypothermic response in MA consumption and MA-induced CTA is that MA-induced hypothermia may increase the association of MA with unpleasant physiological or subjective effects.

MA causes synaptic release of DA and other monoamines, including NE and 5-HT (Fleckenstein *et al*, 2007; Rothman *et al*, 2001). MA de-vesicularizes monoamines, which diffuse into the cytoplasm and can then be reverse transported into the synapse (Fleckenstein *et al*, 2007). Genes encoding the NE transporter (*Slc6a2*), and the 5-HT transporter (*Slc6a4*), but not the DA transporter (*Slc6a3*), are more highly expressed in nucleus accumbens (NAcc) tissue from MAHDR mice than MALDR mice (Wheeler *et al*, 2009). These genes are not located on mouse chromosome 10, and are therefore not candidates for the QTG in that region (Belknap *et al*, 2013). However, *Taar1* is within the QTL interval, and it modulates monoamine levels by altering transporter function in mice and primates (Miller, 2011,2012; Revel *et al*, 2011; Xie and Miller, 2008,2009). Furthermore, *Taar1* −/− mice exhibit lower basal levels and greater amphetamine-induced release of DA in the striatum, compared with +/+ mice (Lindemann *et al*, 2008; Wolinsky *et al*, 2007). Similarly, MAHDR mice, which carry the non-functional version of the TAAR1, also exhibit lower resting DA tone in the NAcc and medial prefrontal cortex (mPFC), and higher MA-induced DA release in the mPFC, but not in the NAcc (Lominac *et al*, 2014). Therefore, DA-related phenotypes may be associated with level of MA intake. On the other hand, differences in 5-HT disposition in MADR and *Taar1* transgenic mice do not correspond. *Taar1* −/− mice have lower basal levels of 5-HT and greater amphetamine-induced 5-HT release compared with +/+ mice (Wolinsky *et al*, 2007), whereas the opposite relationship is seen in MADR mice; MAHDR mice have higher basal levels of 5-HT in the NAcc and show reduced sensitivity to MA-induced increases in 5-HT (Lominac *et al*, 2014). Different brain regions and assay methods could explain discrepancies related to 5-HT. On the other hand, 5-HT may not have a role in genetically-determined differences in MA intake.

Ours is the first report of higher voluntary MA intake in animals with genetic alterations resulting in loss of TAAR1 function. A recent publication involving a pharmacological manipulation of TAAR1 yielded similar results (Jing *et al*, 2015). The TAAR1 agonist, RO5263397, dose-dependently reduced MA self-administration in rats, just as a functional TAAR1 in our studies was associated with reduced voluntary MA intake. In the study in rats, reinstatement of MA seeking was also attenuated by the TAAR1 agonist, whereas the TAAR1 agonist had no effect on reinstatement of sucrose seeking. MADR mice consume similar amounts of saccharin and quinine, indicating that TAAR1 function in this genetic model does not have a role in the consumption of a natural reward or bitter substance (Shabani *et al*, 2011; Wheeler *et al*, 2009). A TAAR1 agonist approach cannot be taken in our mice, because the receptor is non-functional.

The 999-bp mouse *Taar1* on chromosome 10 is phylogenetically related to the 1020-bp human *TAAR1* on chromosome 6 (Lindemann *et al*, 2005), and the 332 amino-acid mouse receptor shares 76% homology with the 339 amino-acid human receptor (Borowsky *et al*, 2001). There are a number of reported synonymous and non-synonymous SNPs in the human *TAAR1* (dbSNP NCBI, 2014), but there are no reported polymorphisms that are shared across the mouse and the human. Some of the reported non-synonymous SNPs in the human *TAAR1* are located in regions that should alter receptor recognition of ligand or receptor function (Pardo *et al*, 1992). A non-functional TAAR1 in mice is associated with higher levels of voluntary MA consumption and reduced sensitivity to aversive effects of MA. It is possible that the TAAR1 limits MA consumption in some humans by conferring sensitivity to aversive effects of MA. Therefore, drugs that stimulate a sub-functional TAAR1 may increase sensitivity to aversive effects of MA and be useful for treating MA addiction.

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The authors declare no conflict of interest.

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