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Differential in vivo effects of H_3 receptor ligands in a new mouse dipsogenia model

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Abstract

The selective H_3 receptor agonist (*R*)-α-methylhistamine [(*R*)-α-MeHA] stimulates drinking in the adult rat. In the present study, we investigated the role of the H_3 receptor in mediating this behavior in a new dipsogenia model using the CD-1 mouse. In addition, the putative inverse agonists ciproxifan, thioperamide and clobenpropit; the reported antagonist (1*R*,2*R*)-4-[2-(5,5-dimethylhex-1-ynyl)cyclopropyl]imidazole (GT-2331); and the putative neutral antagonist/weak partial agonist proxyfan were evaluated for possible differences in pharmacological activity in this new model. Water intake increased over baseline in a dose-related manner following intraperitoneal administration of 80, 160 or 240 μmol/kg (*R*)-α-MeHA, but this effect was dependent on age (P30 < P60 < P80 = P120). [^{3}H]-*N*-α-methylhistamine binding studies showed no change in H_3 receptor density for the whole mouse brain at these ages. All subsequent studies employed P80 mice dosed with 240 μmol/kg (*R*)-α-MeHA. Ciproxifan (0.001–30 μmol/kg), thioperamide (0.01–10 μmol/kg), clobenpropit (0.1–30 μmol/kg) and GT-2331 (0.03–10 μmol/kg) attenuated drinking dose-dependently, blocking the response completely at the highest doses in each case. In contrast, proxyfan (0.001–10 μmol/kg) only partially attenuated drinking elicited by (*R*)-α-MeHA: coadministration of proxyfan and ciproxifan resulted in an attenuation of ciproxifan's effects. This new dipsogenia model provides the first in vivo behavioral evidence for possible pharmacological differences between three putative H_3 receptor inverse agonists, GT-2331 and proxyfan.

Keywords: Histamine H_3 receptors; Inverse agonist; Ciproxifan; Proxyfan; GT-2331; Dipsogenia; Mouse

1. Introduction

Histaminergic neurons located in the tuberomamillary nucleus of the posterior hypothalamus project diffusely and widely throughout the CNS (Brown et al., 2001) and have been implicated in the regulation of important physiological processes, including feeding and drinking behavior (Doi et al., 1994; Lecklin et al., 1998). Four known histamine receptors, designated as H_1, H_2, H_3 and H_4, mediate histamine's effects and have been identified, to different degrees, both centrally and peripherally (Brown et al., 2001; Coge et al., 2001; Oda et al., 2000; Zhu et al., 2001).

Presynaptic H_3 receptors are found principally in the CNS and regulate the release of histamine, and other neurotransmitters, through a negative feedback mechanism (Arrang et al., 1987; Brown et al., 2001). Activation of H_3 autoreceptors with the potent and selective agonist (*R*)-α-MeHA inhibits the synaptic release of histamine in the CNS in a manner similar to histamine (Arrang et al., 1987). Previous reports also show that (*R*)-α-MeHA elicits drinking in the hydrated adult rat (Clapham and Kilpatrick, 1993; Ligneau et al., 1998). This dipsogenic effect can be elicited following either central (Lecklin et al., 1998) or systemic (Clapham and Kilpatrick, 1993; Ligneau et al., 1998) administration of (*R*)-α-MeHA and can be completely blocked in a dose-dependent manner by coadministration of the standard reference H_3 receptor antagonists thioperamide (Clapham and Kilpatrick, 1993) and ciproxifan (Ligneau et al., 1998), but not by H_1 or H_2 receptor antagonists (Clapham and Kilpatrick, 1993).

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Therefore, we reasoned that this dipsogenic effect might represent a potential model for assessing in vivo central H_3 receptor functional activity.

Recent interest in the role of the H_3 receptor in CNS disorders has led to numerous studies suggesting the potential for clinical intervention by blocking activity at the H_3 receptor in disease states ranging from Alzheimer's disease and schizophrenia to attention deficit/hyperactivity disorder (ADHD) and obesity (Ligneau et al., 1998; Onodera et al., 1998; Passini et al., 2000; Yates et al., 1999, 2000). Indeed, several new H_3 receptor ligands have been described, including the reported H_3 receptor antagonist GT-2331 (Perceptin), currently in clinical development (Tedford et al., 2000) for ADHD. Interestingly, a recent report presented in vitro and ex vivo evidence for constitutive activity of native H_3 receptors in the mouse brain, and demonstrated that H_3 receptor ligands formerly identified solely as antagonists (such as ciproxifan, clobenpropit and thioperamide) can act as inverse agonists under certain conditions. In contrast, proxyfan exhibited properties consistent with neutral antagonism and/or partial agonism (Morisset et al., 2000). However, the potential functional significance of these observations has yet to be assessed in vivo.

To address whether parallel pharmacological differentiation between these putative inverse agonists, reported antagonists, and neutral antagonists/partial agonists could be demonstrated in vivo, and as part of an ongoing effort to identify a new in vivo system capable of providing a sensitive, efficient, reproducible and rapid functional readout of central H_3 receptor blockade, we have developed a model we believe meets these criteria. Accordingly, we present data describing the effects of ciproxifan, clobenpropit, thioperamide, GT-2331 and proxyfan on (*R*)-α-MeHA-induced dipsogenia in the CD-1 mouse.

2. Materials and methods

2.1. Animals

Male CD-1 mice were obtained from Charles River Laboratories (USA) at approximate postnatal days 20, 50, 70, 110 (P20, P50, P70, P110) and maintained at Abbott facilities for 10 days prior to testing at P30, P60, P80 and P120. Additional pregnant CD-1 mice were obtained from Charles River Laboratories for the P10 and P20 mice required for H_3 receptor density and binding affinity studies (see below). Adult mice were housed up to 10 per large colony cage ($52 \times 28 \times 20$ cm) in a dedicated quiet room under conditions of 12 h lights on/12 h lights off (on at 06:00), with food and water available ad libitum. Nestlets were provided on cage/bedding change days to reduce territorial fighting.

Adult male Sprague–Dawley rats (>80 days; Charles River) were also used for initial pilot studies and were housed singly under otherwise identical conditions. For cannula implantation required for intracerebrventricular administration, rats were anaesthetized with sodium pentobarbital 55 mg/kg ip and placed in a David Kopf student stereotaxic instrument (Tujunga, USA) with the skull on an even horizontal plane. Stereotaxic coordinates were: from bregma AP 1.0 mm, lateral 1.6 mm and −4.5 mm deep. Injections were given through a 28-gauge injector cannula, inserted through a cemented guide cannula, using a Harvard syringe pump (Holliston, USA). All experiments were conducted in accordance with Abbott Animal Care and Use Committee and National Institutes of Health Guide for Care and Use of Laboratory Animals guidelines in a facility accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care.

2.2. Chemicals

Ciproxifan, proxyfan and GT-2331 were synthesized, clobenpropit and thioperamide were purchased from Sigma (USA), and (*R*)-α-MeHA was purchased from Tocris (UK). Ciproxifan, thioperamide, clobenpropit, GT-2331 and proxyfan were administered intraperitoneally to mice 5 min prior to (*R*)-α-MeHA, which itself was given intraperitoneally 30 min before testing (5 ml/kg each injection). Saline (0.9% w/v; 5 ml/kg, Abbott Laboratories, USA) was used as a vehicle. To directly compare efficacy, drugs were prepared and administered in units of μmol/kg (mg/kg also given for comparison). In an initial rat study to confirm central activity, thioperamide was administered intracerebroventricularly into the left lateral ventricle, 10 min prior to intracerebroventricular (*R*)-α-MeHA (2.5 μl/min over 2 min for each administration). In an additional rat study, thioperamide was administered intraperitoneally 5 min prior to (*R*)-α-MeHA, which itself was given intraperitoneally 30 min before testing (0.5 ml/kg each injection).

2.3. Dipsogenia test—mouse

After weighing, each mouse received two intraperitoneal injections as outlined above, one on either side of the abdomen, before being returned to their home cage with free access to food but not water. When 30 min had elapsed, mice were transferred from their home cage and placed separately into smaller cages ($23 \times 21 \times 20$ cm) with access to water only. Water was presented through a specially designed 1-ml tube, essentially a modified 1-ml syringe connected to a stainless steel sipper with a 2.5-cm bend that protruded through the back of the cage in a manner analogous to the automated watering system on which these animals were normally maintained. The mice were left alone for a further 30 min after which they were removed to their home cages once more and the amount of water consumed was recorded to the nearest 0.01 ml. Sixteen animals were treated at a time and all studies were carried out between 10 a.m. and 12:30 p.m. or between 3 p.m. and 5:30 p.m. to avoid the midday snacking behavior of rodents at our facilities. A total of 12–16 mice per group were used for all mouse studies.

2.4. Dipsogenia test—rat

After weighing, each rat received either two intraperitoneal (one on either side of the abdomen) or two intracerebroventricular injections as outlined above. Rats treated by the intracerebroventricular route were immediately placed into individual test cages (23 × 21 × 20 cm) for 60 min with access to water only. Water was presented through a specially designed 5-ml tube, essentially a modified 5-ml syringe connected to a stainless steel sipper with a 2.5-cm bend that protruded through the back of the cage in a manner analogous to the automated watering system on which these animals were normally maintained. At the end of the 60-min test period, the amount of water consumed was recorded to the nearest 0.1 ml. Rats treated by the intraperitoneal route were first returned to their home cage with free access to food but not water. When 30 min had elapsed, these rats were then transferred from their home cage and placed into separate cages with access to water only. Water was presented through similar, specially designed 5-ml tubes, as described above. The rats were left alone for a further 10 min after which they were removed to their home cages once more and the amount of water consumed recorded to the nearest 0.1 ml. Group sizes of 12 rats per group were used for both intracerebroventricular and intraperitoneal rat studies.

2.5. H_3 receptor density

Characterization of mouse brain cortical H_3 receptor densities and affinities were determined as described previously (Esbenshade and Hancock, 2000). Briefly, cell membranes from untreated whole mouse brains expressing histamine H_3 receptors were homogenized in cold 50 mM Tris HCl/5 mM EDTA buffer (pH = 7.4) containing 1 mM benzamidine, 2 μg/ml aprotinin, 1 μg/ml leupeptin and 1 μg/ml pepstatin. The homogenate was centrifuged at 40,000 × *g* for 20 min at 4 °C. This step was repeated and the resulting pellet was resuspended in Tris/EDTA buffer in a final volume of three times the wet weight of the tissue. Aliquots were frozen at − 70 °C until needed. On the day of assay, membranes were thawed and diluted with 50 mM Tris/5 mM EDTA buffer to provide a concentration of 0.5 mg protein/tube. For saturation assays, [^{3}H]-*N*-α-methylhistamine (from 0.03 to 3 nM) was incubated with cell membranes either alone or in the presence of 10 μM thioperamide (to determine specific binding) at 25 °C for 30 min and B_{max} and K_D values determined by nonlinear regression analysis of binding isotherms using Prism (Graphpad, USA).

2.6. Spontaneous activity

To assess possible drug effects on spontaneous locomotor activity, additional groups ($n = 6–8$ per group) of P80 mice were treated with vehicle/drug combinations as above and 30 min later placed separately into automated activity monitors measuring 420 × 420 × 400 mm (Accuscan, USA), each utilizing two infrared beam grids–one for assessing horizontal activity and the other for vertical activ-

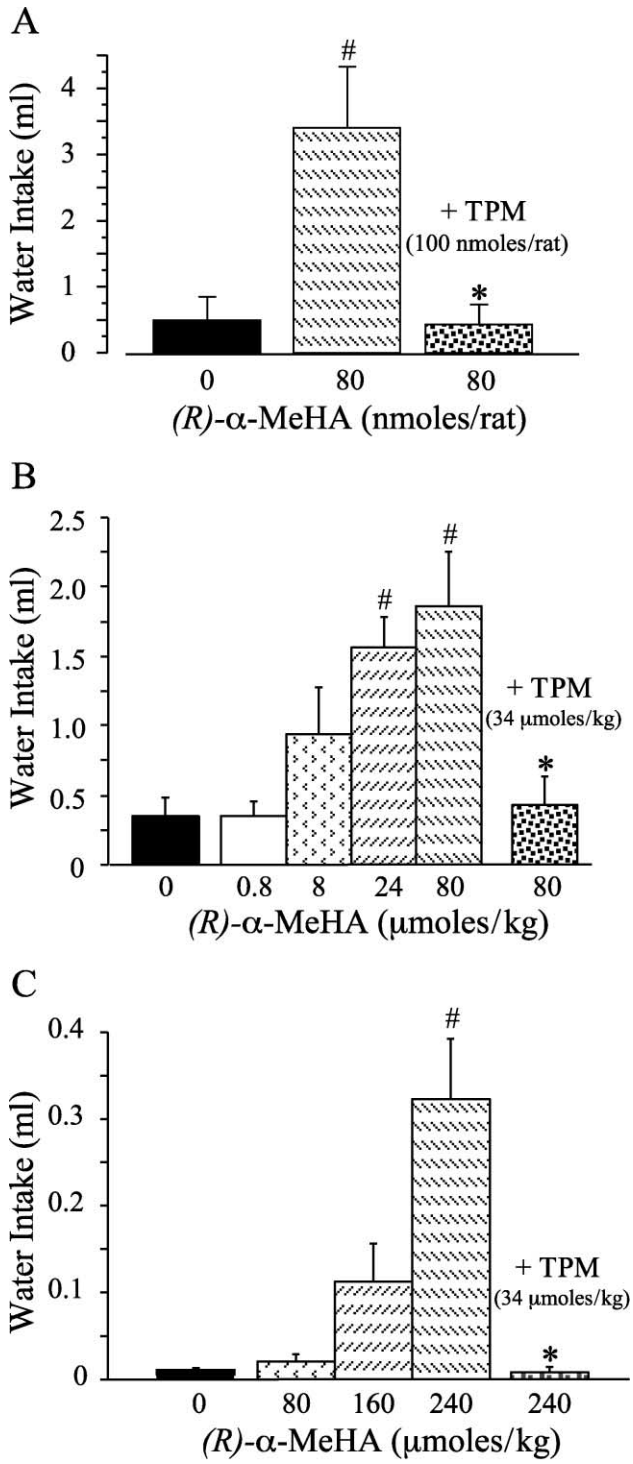


Fig. 1. Effect of central (intracerebroventricular) and peripheral (intraperitoneal) administration of (*R*)-α-MeHA on water intake in the adult rat (A and B, respectively) and the effect of peripheral (intraperitoneal) administration of (*R*)-α-MeHA on water intake in the adult mouse (C). Significant dipsogenia was induced under all three treatment conditions and thioperamide (TPM) completely blocked this effect. All data are represented by means ± S.E.M. ($^{\#}P < .05$ compared with vehicle-treated controls; $^{*}P < .05$ compared with the highest treatment of (*R*)-α-MeHA).

ity. Distance traveled (in centimeters) in the horizontal plane, vertical activity (a measure of rearing frequency) and stereotypy counts (repetitive interruptions of a single infrared beam) were recorded at 10-min intervals for 30 min. Mice were not habituated to the apparatus to retain the element of novelty that likely applies during dipsogenia studies.

2.7. Statistical analyses

Dipsogenia, B_{max} and K_D data were analyzed using a one-way ANOVA followed by Tukey's pairwise comparison post hoc tests. Spontaneous locomotor activity data were analyzed using a repeated measures ANOVA and Tukey's pairwise comparison post hoc test.

3. Results

3.1. Dipsogenia—dose response to (R)-α-MeHA

Initial studies in the rat confirmed previous work that identified a dipsogenia response following central administration of (*R*)-α-MeHA. In the present study, administration of 80 nmol (10 μg) (*R*)-α-MeHA intracerebroventricular elicited a significant increase in water intake in the rat to a level approximately seven times that of vehicle-treated controls (Fig. 1A). This effect was completely blocked with 100 nmol (30 μg) intracerebroventricular thioperamide (Fig. 1A). A significant increase in water intake was also observed in the >P80 rat following intraperitoneal administration of 24 and 80 μmol/kg (3 and 10 mg/kg) (*R*)-α-MeHA, an effect that was completely blocked by 34 μmol/kg ip thioperamide (Fig. 1B). The much higher doses required to elicit the dipsogenia response in the adult (>P80) rat when (*R*)-α-MeHA was given by the intraperitoneal route is also consistent with a centrally mediated effect.

In adult mice (P80), (*R*)-α-MeHA also induced dipsogenia when administered intraperitoneally, reaching statistical significance at 240 μmol/kg (30 mg/kg) when compared with controls (Fig. 1C). Once again, 34 μmol/kg (10 mg/kg) thioperamide completely blocked the dipsogenia response (Fig. 1C).

3.2. Dipsogenia and H_3 receptor density—age-dependent effects

An age-dependent increase in water intake in response to 240 μmol/kg (30 mg/kg) intraperitoneal (*R*)-α-MeHA was noted, with significance from vehicle-treated controls seen only when mice were at least P80 or P120 (Fig. 2A). A similar result was obtained when these data were normalized for body weight (Fig. 2B). The same effect was noted in a smaller study with rats (data not shown). To investigate this further, brain tissue was harvested from mice of various ages for analysis of H_3 receptor density. There was no significant change in B_{max} or K_D values across the age range used for the dipsogenia studies (Fig. 3). However, a significant increase in B_{max} was apparent when compared with brains from much younger mice, indicating an increase in H_3 receptor density between P10 and P20–P120 (Fig. 3). All subsequent studies were carried out using P80 mice.

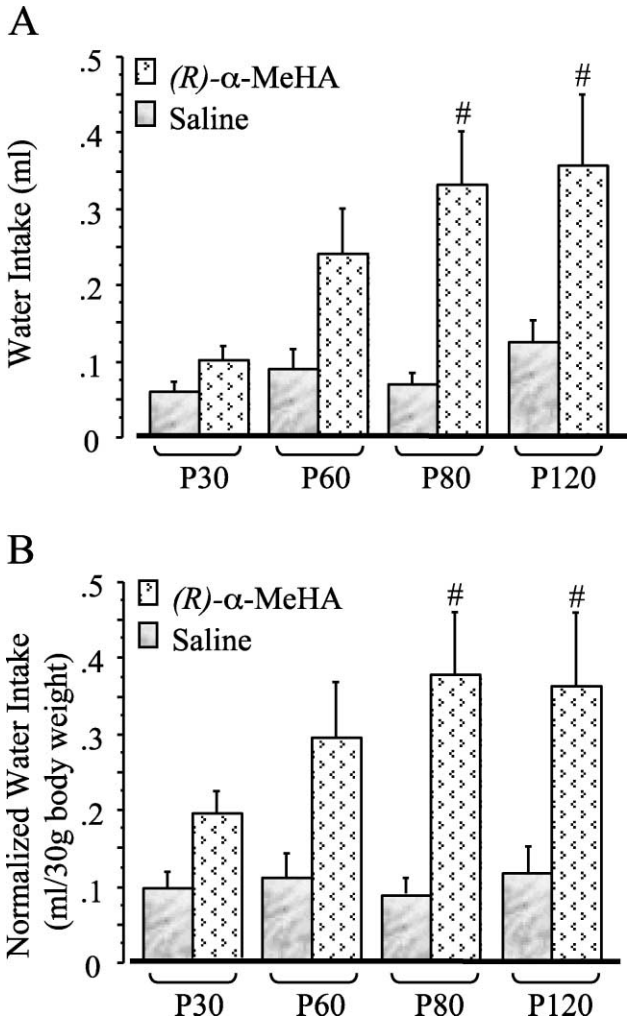
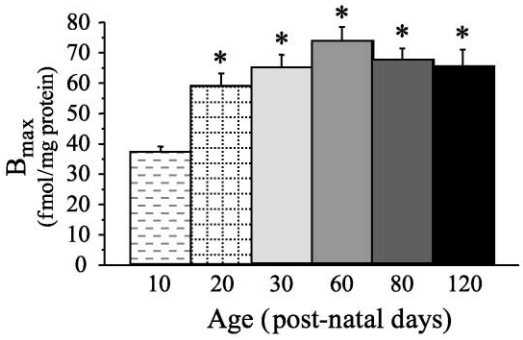


Fig. 2. Age associated effects on dipsogenia induced by intraperitoneal administration of (*R*)-α-MeHA in the CD-1 mouse. Dipsogenia was found to be dependent on age (A) but not body weight (B; normalized and expressed for the average weight of 30 g for a P80 mouse). Data are represented by means ± S.E.M. (#$P < .05$ compared with P30 mice).

3.3. Dipsogenia—effect of H_3 receptor ligands

Ciproxifan completely blocked dipsogenia induced by (*R*)-α-MeHA in the mouse in a dose-dependent manner, reaching significance at a dose as low as 0.03 μmol/kg (0.008 mg/kg) (Fig. 4A). Interestingly, despite this high potency, inhibition of the dipsogenia response by ciproxifan was 'flat' over at least one order of magnitude (compare response over 0.1–1 μmol/kg). A dose of 3 μmol/kg (0.8 mg/kg) was fully efficacious and highly reproducible under the conditions of this model and was used as an internal positive control in all subsequent work; higher doses

A



B

P10	P20	P30	P60	P80	P120	Age (post-natal days)
1.186	1.743	1.460	1.574	1.471	1.564	Mean K_D (nM)
(.968-	*(1.287-*	*(1.203-*	*(1.374-*	*(1.130-*	*(1.164-*	*95 % C.I. (nM)*
1.454)	*2.361)*	*1.771)*	*1.802)*	*1.914)*	*2.101)*	

Fig. 3. Effects of age on H_3 receptor density (A) and binding affinity (B) in the untreated whole mouse brain. Little effect was seen over the age groups used in the previous dipsogenia studies, although a significant increase in B_{max} was seen when these age groups were compared with P10 mice. Data in Panel A are represented by means ± S.E.M. (* $P < .05$ compared with P10 mice). No significant difference was observed for K_D (B).

of 10–30 μmol/kg (2.7–8.1 mg/kg) were not used in this regard to preserve the sensitivity of the model. By itself, ciproxifan had no effect on basal water intake when tested as high as 30 μmol/kg (8.1 mg/kg) (Fig. 4A).

Clobenpropit also dose-dependently attenuated the dipsogenia response, but higher doses were required for full efficacy (Fig. 4B). Similar to ciproxifan, the dose response to clobenpropit was flat over at least an order of magnitude (compare response over 0.3–3 μmol/kg; 0.1–1.1 mg/kg). A dose of 3 μmol/kg (0.8 mg/kg) ciproxifan, used as an internal control in this study, completely blocked dipsogenia induced by (*R*)-α-MeHA (Fig. 4B), compared with 30 μmol/kg (10.5 mg/kg) for clobenpropit. By itself, clobenpropit had no effect on basal water intake when tested at 30 μmol/kg (Fig. 4B).

Thioperamide attenuated (*R*)-α-MeHA-induced dipsogenia in a manner similar to that observed for clobenpropit (compare Fig. 4C with B). Although a significant decrease in water intake was observed at a dose of 0.1 μmol/kg (0.03 mg/kg) when compared with vehicle treated controls, a similar 'flat' dose response curve to that produced by both ciproxifan and clobenpropit was also observed for thioperamide. Full efficacy for thioperamide was achieved at 10 μmol/kg (2.9 mg/kg) compared with 3 μmol/kg (0.8 mg/kg) for ciproxifan, used as an internal standard (Fig. 4C). By itself, thioperamide did not affect basal water intake when tested at 10 μmol/kg (Fig. 4C).

Proxyfan significantly attenuated (*R*)-α-MeHA-induced dipsogenia only at 10 μmol/kg (2.2 mg/kg), the highest dose tested: statistical significance was not attained at any other

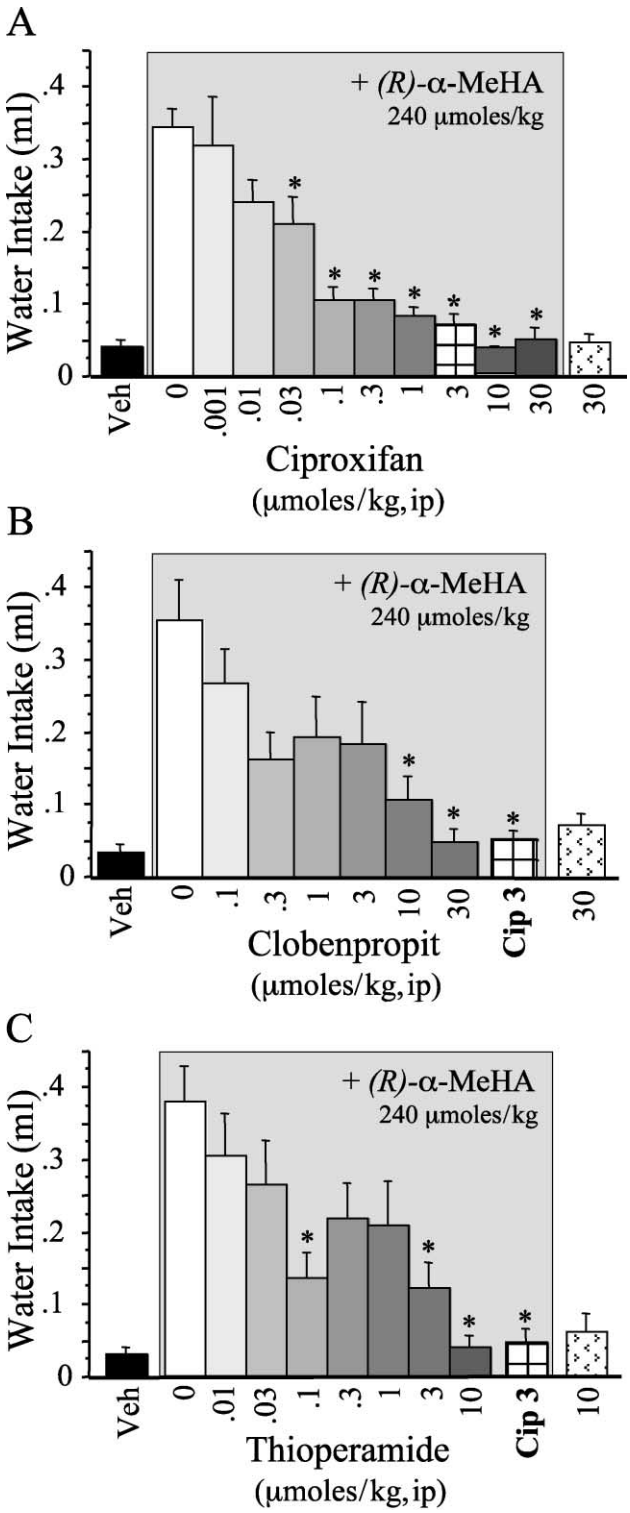
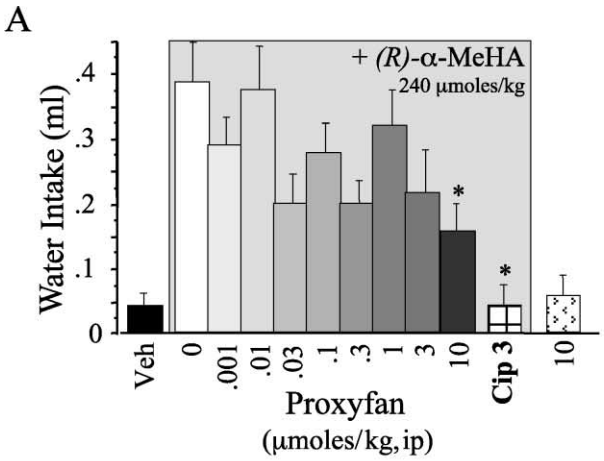
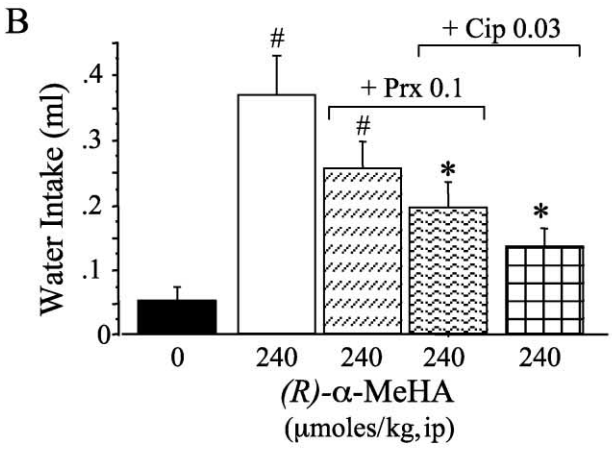
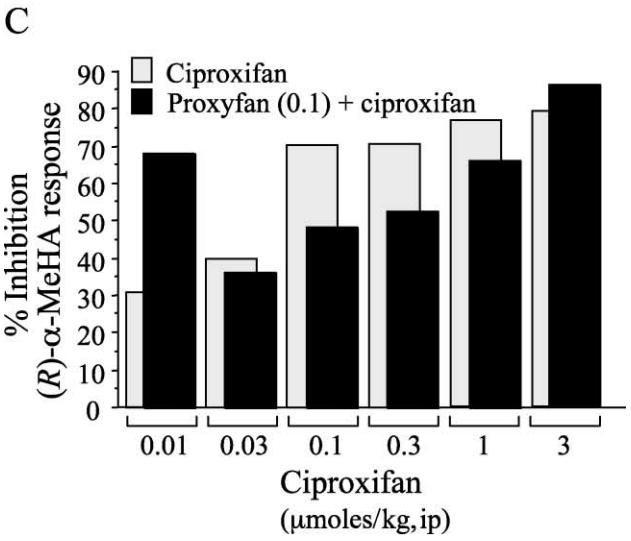


Fig. 4. Effects of three putative H_3 receptor inverse agonists on dipsogenia induced by intraperitoneal administration of (*R*)-α-MeHA in the P80 mouse. All three compounds attenuated dipsogenia in a dose-related manner, with ciproxifan (A) being most potent and clobenpropit (B) the least potent. None had any effect on water intake when administered in the absence of (*R*)-α-MeHA. Ciproxifan, administered at 3 μmol/kg (Cip 3), completely blocked dipsogenia and was used as an internal standard positive control in all other studies. All data are represented by means ± S.E.M. (* $P < .05$ compared with mice treated with vehicle+(*R*)-α-MeHA).

dose (Fig. 5A). The dose response curve was also less robust, with an overall flat appearance over at least two orders of magnitude. Full efficacy was not achieved with proxyfan, in contrast to the full block observed with the ciproxifan internal control group (Fig. 5A). By itself, proxyfan had no effect on basal water intake when tested at 10 μmol/kg.







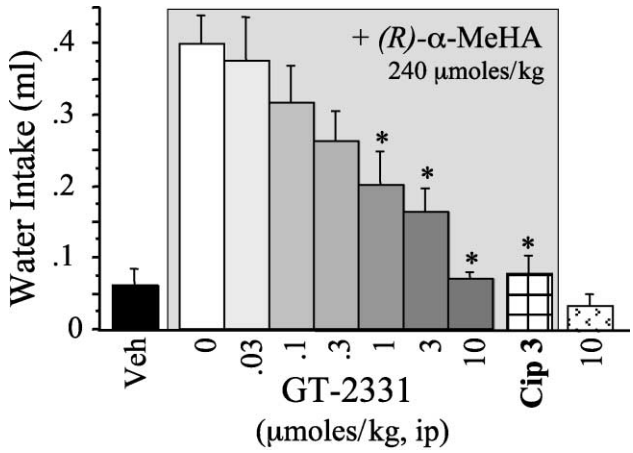


Fig. 6. Effect of GT-2331 on dipsogenia induced by intraperitoneal administration of (*R*)-α-MeHA in the P80 mouse. GT-2331 attenuated dipsogenia in a step-like dose-related manner, reaching significance at the three higher doses tested: it had no effect on water intake when administered in the absence of (*R*)-α-MeHA. The internal positive control ciproxifan administered at 3 μmol/kg (Cip 3) completely blocked dipsogenia. Data are represented by means ± S.E.M. (* $P < .05$ compared with mice treated with vehicle+(*R*)-α-MeHA).

To determine whether proxyfan could influence the attenuation of (*R*)-α-MeHA-induced dipsogenia by ciproxifan in this model, proxyfan (0.1 μmol/kg) and ciproxifan (0.03 μmol/kg) were co administered 5 min prior to (*R*)-α-MeHA. (*R*)-α-MeHA induced a significant increase in water intake as before, and this was significantly attenuated by ciproxifan alone, but not by proxyfan alone (Fig. 5B). However, proxyfan partially attenuated the effects of ciproxifan (Fig. 5B), indicating that proxyfan may be interacting differently at the H_3 receptor level in this model under these conditions when compared with ciproxifan. When 0.1 μmol/kg proxyfan was co administered with ciproxifan over a broader dose range in a subsequent experiment, proxyfan was able to partially attenuate ciproxifan's effects on (*R*)-α-MeHA-induced dipsogenia over several doses, with the notable exception of the lowest dose (0.01 μmol/kg), where an apparent additive effect was observed (Fig. 5C).

In contrast to proxyfan and all other compounds tested, GT-2331 attenuated dipsogenia induced by (*R*)-α-MeHA in a steady, dose-dependent manner (Fig. 6). Significance from vehicle treated controls was attained at 1 μmol/kg (0.2 mg/kg)

Fig. 5. Effect of the putative neutral antagonist proxyfan on dipsogenia induced by intraperitoneal administration of (*R*)-α-MeHA in the P80 mouse. Attenuation of dipsogenia was observed with this compound, only reaching significance at the highest dose tested (A). Proxyfan had no effect on water intake when administered in the absence of (*R*)-α-MeHA. The internal positive control ciproxifan administered at 3 μmol/kg (Cip 3) completely blocked dipsogenia (A). However, coadministration of proxyfan with ciproxifan attenuated the inhibitory effect of ciproxifan (B and C). Data in Panels A and B represent the means ± S.E.M. (* $P < .05$ compared with mice treated with vehicle+(*R*)-α-MeHA; # $P < .05$ compared with mice treated with vehicle + vehicle).

and full efficacy was achieved at 10 μmol/kg (2.2 mg/kg). By itself, GT-2331 at 10 μmol/kg had no effect on baseline drinking activity (Fig. 6).

3.4. Spontaneous activity

Spontaneous locomotor activity was recorded in mice to evaluate any potential confounding effects of drug treatment on general activity and is presented as mean distance (in centimeters) traveled over the full 30-min exposure to the activity apparatus (Fig. 7A and B). A repeated measures ANOVA for vehicle + vehicle-, vehicle + (*R*)-α-MeHA-, ciproxifan+(*R*)-α-MeHA-, clobenpropit + (*R*)-α-MeHA- and thioperamide+(*R*)-α-MeHA-treated mice (Fig. 7A) did not show any significant Group effect [$F(4,25) = 2.359$, $P = .0808$]. This indicated that no overall significant differences existed between these treatment groups when averaged over the 30-min recording period. However, a trend toward a decrease in activity was observed between mice treated with vehicle + vehicle and mice in other treatment groups that received (*R*)-α-MeHA. A significant Time effect [$F(2,50) = 198.644$, $P < .0001$] was observed, indicating that activity decreased with time across groups. This likely reflected a general habituation to the apparatus. No significant Group × Time interaction [$F(8,50) = 0.341$, $P = .9455$] was seen, indicating that no significant differences were observed between treatment groups at discrete periods.

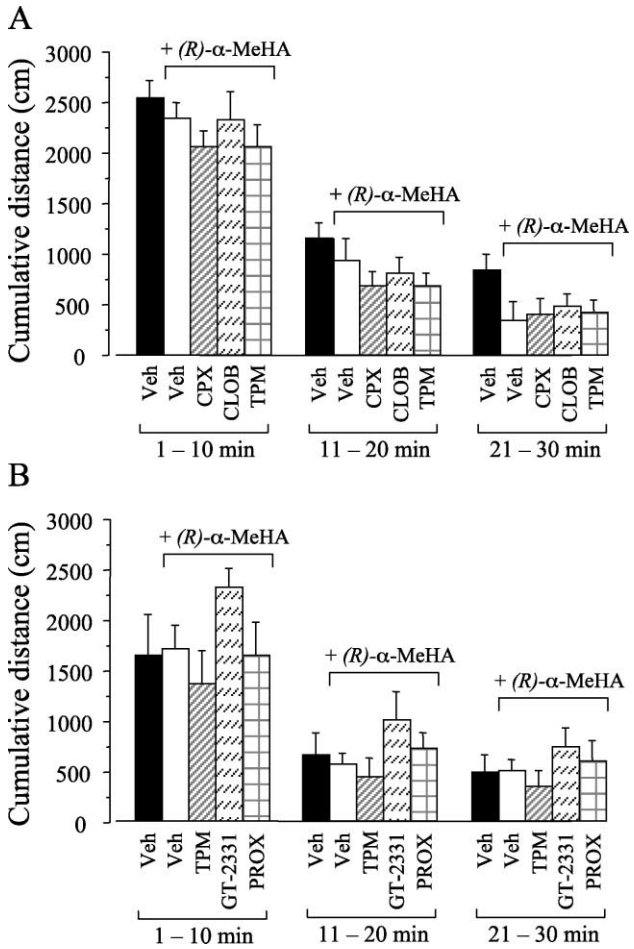


Fig. 7. Spontaneous locomotor activity over a 30-min period, commencing 30 min after administration of dipsogenia-attenuating doses of H_3 receptor ligands. Administration of ciproxifan (CPX, 3 μmol/kg, Panel A) or clobenpropit (CLOB, 30 μmol/kg, Panel A) or thioperamide (TPM, 30 μmol/kg, Panel A) 5 min prior to (*R*)-α-MeHA (240 μmol/kg) did not significantly alter activity when compared with mice treated with vehicle+(*R*)-α-MeHA or vehicle + vehicle. Similarly, in Panel B, administration of TPM (10 μmol/kg,) or GT-2331 (10 μmol/kg) or proxyfan (PROX, 10 μmol/kg) 5 min prior to (*R*)-α-MeHA (240 μmol/kg) did not significantly alter activity when compared with mice treated with vehicle+(*R*)-α-MeHA or vehicle + vehicle. Data are represented by means ± S.E.M.

In a second study, a repeated measures ANOVA for mice treated with vehicle + vehicle, vehicle+(*R*)-α-MeHA, GT-2331+(*R*)-α-MeHA and proxyfan+(*R*)-α-MeHA did not show any significant Group effect [$F(4,27) = 1.173$, $P = .3446$], indicating that no overall differences existed between these treatment groups when averaged over the 30-min recording period (Fig. 7B). A significant Time effect [$F(2,54) = 139.875$, $P < .0001$] was observed, indicating that activity decreased with time across groups. This likely reflected a general habituation to the apparatus. No significant Group × Time interaction [$F(8,54) = 0.341$, $P = .5331$] was seen, indicating that no significant differences were observed between treatment groups at discrete periods.

Similarly, none of the compounds tested had any significant effect on vertical activity (rearing) or stereotypy, although GT-2331 tended to increase both activities (not shown).

4. Discussion

The highly selective H_3 receptor agonist (*R*)-α-MeHA is a potent dipsogen when administered to adult rats, shortening latency to initiate drinking and significantly increasing water consumption over a short period (Clapham and Kilpatrick, 1993; Lecklin et al., 1998; Ligneau et al., 1998). All available evidence points to a central mechanism of action (Lecklin et al., 1998), and our own intracerebroventricular administration studies (this report) with (*R*)-α-MeHA in the adult rat support this concept. However, a role for H_3 receptors in the regulation of water intake in the mouse has not been reported previously. In addition, the in vivo functional consequences of recent in vitro and ex vivo evidence indicating constitutively active H_3 receptors in the mouse CNS (Morisset et al., 2000) and the reclassification of many H_3 receptor ligands are currently unclear.

Here we present the first evidence demonstrating a pronounced dipsogenic response following systemic administration of the H_3 receptor agonist (*R*)-α-MeHA in the CD-1 mouse (Fig. 1C). This effect was highly reproducible, but required a dose approximately three-fold higher than that necessary to produce a similar response in the rat (Fig. 1A).

Additional work controlling for age and body weight demonstrated that older mice ($\geq$P80) exhibited a more pronounced response to (*R*)-α-MeHA when compared with younger P60 or P30 mice (Fig. 2A), even after normalizing for body weight (Fig. 2B).

To further investigate this age-dependent effect, we examined H_3 receptor density in whole mouse brain tissues harvested at six different ages. A significant increase in B_{max} was observed with tissue from P20 and older mice when compared with tissue from P10 animals: mean K_D did not change (Fig. 3). However, no significant difference in B_{max} or K_D was observed between mice from age groups used in our dipsogenia studies. It is possible that H_3 receptor density may have changed with age in specific areas of the mouse brain such as the hypothalamus, where (*R*)-α-MeHA may be mediating its dipsogenia effects. Unfortunately, the relatively small size of this brain region precluded its investigation in this regard. It is also possible that H_3 receptors may be differentially coupled to G proteins/second messenger systems, or that endogenous histamine levels may differ, as a function of age.

H_3 receptor agonist-induced dipsogenia could be differentially blocked in the mouse by the putative inverse agonists ciproxifan, clobenpropit and thioperamide (Fig. 4), the reported antagonist GT-2331 (Fig. 6), and by the putative neutral antagonist/weak partial agonist proxyfan (Fig. 5). The observation that ciproxifan appeared to be more potent in this model than clobenpropit despite similar binding affinities (K_i for clobenpropit in rat cortex = 0.4 nM; K_i for ciproxifan in rat cortex = 0.6 nM, unpublished observations) is likely a consequence of the relatively poor brain penetration previously observed for clobenpropit (Mochizuki et al., 1996). Similarly, the relatively low binding affinity for thioperamide (K_i in rat cortex = 7.5 nM, unpublished observations) is consistent with the apparent lower potency of thioperamide observed in the dipsogenia model when compared with ciproxifan. These binding affinities compare with previously published K_i values of 0.5 nM for clobenpropit, 0.5–0.7 nM for ciproxifan, and 4–10.2 nM for thioperamide in rat striatum or cerebral cortex (Ligneau et al., 2000; Lovenberg et al., 2000). It was not possible to calculate consistent IC_{50} values for dipsogenia because the differences observed in the dose response curves between compounds would necessitate different curve fitting parameters, making direct comparisons problematic.

Interestingly, the dose-related effects of ciproxifan, clobenpropit and thioperamide appeared to have three components: an initial attenuation of dipsogenia at relatively low doses to approximately 50% of the maximum water intake exhibited by (*R*)-α-MeHA-treated controls, followed by a flat response over at least one order of magnitude, and finally a complete block of the dipsogenia response at high doses. Proxyfan (K_i for rat H_3 receptor = 2.8 nM, unpublished observation), in contrast, attenuated the dipsogenia response elicited by (*R*)-α-MeHA by, at best, approximately 40–60% across the dose range tested, the less robust response tending to remain flat across at least two orders of magnitude (Fig. 5A). As expected, by itself proxyfan had no effect on basal water intake under our experimental conditions. It was not possible to test for possible inverse agonist-like effects of ciproxifan, clobenpropit or thioperamide alone because of the low basal level of drinking under our model conditions (inverse agonists might be expected to reduce basal water intake); these compounds had no significant effect on water consumption when administered alone.

The significance of the apparent differences in response when comparing ciproxifan, clobenpropit, and thioperamide with proxyfan is unclear at present but may reflect the recently proposed different pharmacological activity for these compounds at the H_3 receptor (Morisset et al., 2000). To further investigate these effects, we reasoned that if proxyfan was acting as a neutral antagonist or weak partial agonist and ciproxifan as an inverse agonist in our model, proxyfan should not only be able to inhibit the effects of (*R*)-α-MeHA, but should also be capable of attenuating the effects of ciproxifan. Therefore, we co administered a dose of proxyfan that had no significant effect on the dipsogenia response by itself, with a dose of ciproxifan that produced approximately 40% inhibition of the (*R*)-α-MeHA-induced dipsogenia. Indeed, we found that proxyfan could attenuate the inhibitory effects of ciproxifan under these conditions (Fig. 5B). When a similar experiment was conducted over a broader dose range for ciproxifan, proxyfan attenuated the effects of ciproxifan at 0.1, 0.3 and 1 μmol/kg (Fig. 5C), apparently consistent with the proposed different pharmacological activity for these compounds. Interestingly, at the lowest dose of ciproxifan, the effects of both compounds appeared to be additive. The reasons for this are unclear at present, but may represent a summation of competitive antagonism at low doses. However, since the presence of the selective agonist (*R*)-α-MeHA is required to elicit the measured response in this model, precise interpretation of these data is difficult. These interesting results nevertheless lay the groundwork for future experiments in this area.

The effects of GT-2331 in this model appeared to be different from the other four compounds tested, despite a high affinity for the rat cortical H_3 receptor (K_i = 0.25 nM, unpublished observation; K_i = 0.12 nM, Tedford et al., 1999) and good brain penetration (Tedford et al., 1999; unpublished observation). A complete block of the dipsogenia response was observed, but only at the highest dose tested (Fig. 6). Further, there was no evidence of the plateau-like effect observed with ciproxifan, clobenpropit and thioperamide. While it is possible that GT-2331 mouse H_3 receptor binding affinity may differ from the rat, this is unlikely given that the sequence coding the third transmembrane region of the H_3 receptor, differences in which account for distinct inter-species pharmacology (Drutel et al., 2001; Ligneau et al., 2000; Lovenberg et al., 2000;

Stark et al., 2001), is identical in the mouse (see GenBank Accession #AY044153 for mouse sequence) and rat (Lovenberg et al., 2000). However, although GT-2331 is reported as an antagonist (Tedford et al., 1998), we have found that GT-2331 only partially reverses H_3 receptor-agonist mediated inhibition of histamine release from rat cortical synaptosomes ($K_b = 7.5$ nM), but with very low efficacy (Esbenshade et al., 2001). Further, GT-2331 can behave as a partial agonist in the same model ($EC_{50} =$ 12 nM). For comparison, ciproxifan behaved as a functional antagonist ($K_b = 9.1$ nM); inverse agonism, although evident with ciproxifan, was not assessed in this model due to technical considerations. Because of this apparent partial agonism, it is difficult to evaluate GT-2331 as an inverse agonist using in vitro models, making it hard to definitively categorize this compound.

None of the compounds tested in our model had any significant effect on spontaneous locomotor activity when administered in an identical manner to mice at doses effective in the dipsogenia studies, but assessed in an automated activity monitoring system (Fig. 7). However, a trend toward a decrease in activity was observed between mice treated with vehicle + vehicle only and mice in other treatment groups that received (*R*)-α-MeHA. Since the comparison groups of interest all received (*R*)-α-MeHA and no significant difference was observed between any of these groups, this finding is not a cause for concern. It is, therefore, unlikely that any of the dipsogenia-attenuating effects observed were due to an overall change in activity.

In summary, we have shown for the first time a role for the H_3 receptor in the regulation of drinking behavior in the CD-1 mouse. Further, we have demonstrated this to be an age-dependent effect, with the dipsogenia response increasing with age. This effect did not appear to be due to a change in whole mouse brain H_3 receptor density over the age groups tested in the dipsogenia model, although an increase in B_{max} was observed at earlier ages and it is possible that there may be regional changes. (*R*)-α-MeHA-induced dipsogenia could be differentially attenuated or completely blocked in a dose-related manner by ciproxifan, clobenpropit, thioperamide, and GT-2331; proxyfan produced only a partial attenuation and could inhibit the effects of ciproxifan.

The apparent pharmacological differences between these compounds in this dipsogenia model may reflect their recent identification as potential inverse agonists (ciproxifan, clobenpropit, thioperamide), a reported neutral antagonist/weak partial agonist (proxyfan) or an antagonist/putative partial agonist (GT-2331). This model may, therefore, be useful for identifying in vivo functional activity of novel compounds at the rodent H_3 receptor. Further, as additional neutral antagonists or partial agonists are identified, it will be interesting to determine if the differences observed in the current studies will serve to help categorize the in vivo pharmacological effects of such ligands.

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