The role of melanin-concentrating hormone-1 (MCH₁) receptors in the voiding reflex in rats.

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Non-standard abbreviations:

DIRC, distension-induced rhythmic contraction; CSTI, continuous slow transvesicular infusion; MCH, melanin-concentrating hormone; OAB, overactive bladder syndrome; SNAP 7941, ((+)-methyl (4S)-3-[(3-[4-(acetylamino)phenyl]-1-piperidinyl)propyl] amino)carbonyl]-4-(3,4-difluorophenyl)-6-(methoxymethyl)-2-oxo-1,2,3,4-tetrahydro-5-pyrimidinecarboxylate hydrochloride; WAY 100635, N-[2-[4-(2-methoxyphenyl)-1-piperazinyl]ethyl]-N-2-pyridinyl-cyclohexanecarboxamide maleate salt; oxybutynin, 4-diethylaminobut-2-ynyl 2-cyclohexyl-2-hydroxy-2-phenylacetate hydrochloride; SHR, spontaneously hypertensive rat(s); WKY, Wistar Kyoto rat; ANOVA, analysis of variance; PMC, pontine micturition center; CRF, corticotrophin releasing factor.

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Abstract

We have used the selective MCH₁ receptor antagonist SNAP 7941 to investigate the role of the hypothalamic neuropeptide melanin-concentrating hormone (MCH) in the control of voiding in rats. Intravenous administration of SNAP 7941 (3 and 10 mg/kg, i.v.) produced dose-related inhibition of rhythmic, distension-induced voiding contractions in anaesthetized rats. In conscious rats in which repeated voiding cycles were evoked by continuous slow transvesicular infusion of saline, intragastric SNAP 7941 (0.03 – 1 mg/kg, i.g.) produced sustained increases in infusion capacity (max = 220% of basal), comparable to the effects of the 5-HT₁A antagonist WAY 100635 and the muscarinic antagonist oxybutynin. SNAP 7941 produced similar results when administered at a low dose (0.01 nmol) into the lateral ventricle (i.c.v.). The opposite effect was produced when MCH (20 nmol) was delivered i.c.v., resulting in a 34% decrease in apparent bladder capacity with increased urinary frequency. The effect of MCH was blocked by the prior i.g. administration of SNAP 7941 (0.1mg/kg), but oxybutynin (1 mg/kg) was ineffective. Finally, in conscious spontaneously hypertensive rats, SNAP 7941 (0.1 mg/kg, i.g.) produced a 31% reduction in micturition frequency, accompanied by a 36% increase in bladder capacity, with no effect on total volume voided over 6 h. The data indicate that MCH acts via MCH₁ receptors within the CNS to modulate the voiding reflex in rats. The striking effects of the MCH₁ antagonist SNAP 7941 to increase bladder capacity and reduce voiding frequency indicate that MCH₁ antagonists may offer a potential novel approach for treating overactive bladder syndrome.


Introduction

Overactive bladder syndrome (OAB) is a debilitating condition, seriously affecting the lifestyle of persons who suffer from this disorder. It is characterized by urgency and frequency (day time frequency with nocturia), with or without urge incontinence, in the absence of any local or metabolic factors (Abrams et al., 2002). Its etiology is largely unknown. Antimuscarinic agents are the most widely used class of drugs for the treatment of OAB (Hegde et al., 2004; Burgard et al., 2005; Hegde, 2006), however they are associated with considerable adverse effects - e.g., dry mouth, constipation, visual disturbances, and cognitive impairment - which compromise efficacy and patient compliance (Chapple et al., 2005; Scheife and Takeda, 2005). Hence, there exists an unmet need for therapies having improved efficacy with fewer side effects.

The central nervous system plays an important role in the physiology of micturition, and a large body of evidence highlights the impact of spinal and supraspinal mechanisms in the pathophysiology of bladder dysfunction (de Groat et al., 1997; Andersson, 2004; Holstege, 2005). Numerous neurotransmitter systems (e.g., glutamate, GABA, dopamine, 5-HT, CRF, ACh) affect bladder activity as part of the neural circuitry regulating afferent and efferent elements of the voiding reflex (de Groat, 1997; Andersson et al., 2001; Klausner et al., 2005).

Melanin-concentrating hormone (MCH) is a 19-amino acid neuropeptide that plays an important role in the regulation of energy balance and mood (Forray, 2003; Herieu, 2003; Pissios and Maratos-Flier, 2003; Herieu, 2006; Pissios et al., 2006). Its effects are mediated through two types of G-protein coupled receptors: MCH₁ and MCH₂ - the latter
expressed in humans, primates and dogs, but not rodents. In addition to widespread
projections to brain regions important for feeding, reward, arousal and mood (see
Hervieu, 2006), MCH-containing neurons originating in the lateral hypothalamus project
to multiple brain areas (e.g., Barrington’s nucleus, periaqueductal grey, paraventricular
nucleus, median preoptic nucleus, locus coeruleus, bed nucleus of the stria terminalis)
important in the central control of voiding (Valentino et al., 1994; Athwal et al., 2001;
Taniguchi et al., 2002). These areas also exhibit robust immunohistochemical labeling for
the MCH₁ receptor (Hervieu et al., 2000), raising the possibility of a role for MCH in the
regulation of voiding.

In this study we have investigated the role of MCH₁ receptors in the regulation of
voiding in rats by assessing responses to MCH and the selective MCH₁ receptor
antagonist SNAP 7941 (Borowsky et al., 2002) in three models: the distension-induced
rhythmic contraction (DIRC) model in anesthetized rats; the continuous slow
transvesicular infusion (CSTI) model in conscious rats; and natural voiding in non-
instrumented spontaneously hypertensive rats (SHR), a rodent model of hyperactive
voiding.
Methods

Animals. All procedures were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and with the approval of the Institutional Animal Care and Use Committee at Synaptic Pharmaceutical Corporation (now Lundbeck Research USA). Sprague Dawley (Crl:SD, male/female, 250-300 g), Spontaneously Hypertensive (SHR/NCrl, male, 300-400 g) and Wistar Kyoto (WKY/NCrl, male, 300-400 g) rats were obtained from Charles River Laboratories (Kingston, NY, USA) and maintained under standard husbandry conditions (22 ± 2 °C, 35% relative humidity, 12h light:12h dark cycle) with unrestricted access to standard rat chow and drinking water.

Drugs. All test substances were dissolved in normal saline for intravenous (i.v.) or in 20% cyclodextrin for intragastric (i.g.) administration, and were delivered in a volume of 1 ml/kg. SNAP 7941, MCH and Ala11-MCH were prepared in normal saline for intracerebroventricular (i.c.v.) administration and were delivered in a 5 μl volume. MCH (human, rat, mouse) was obtained from Bachem Bioscience (King of Prussia, PA, USA). WAY 100635 (maleate salt) and oxybutynin (HCl salt) were obtained from Sigma-Aldrich (St. Louis, MO, USA). SNAP 7941 (HCl salt) and Ala11-MCH were synthesized at Synaptic Pharmaceutical Corp (now Lundbeck Research, USA).

Surgical Procedures

Distension-induced rhythmic contractions (DIRC) in anesthetized rats. Animals were prepared according to the method of Testa et al. (2001), with minor modifications.
Briefly, female Sprague Dawley rats were anesthetized with urethane (1.2 g/kg, i.p.). The lower abdomen was opened through a midline incision (5-6 cm), and the ureters were separated from the surrounding fatty tissue. The distal ends were ligated to prevent leakage from the bladder, and the proximal ends were cannulated (PE-10 tubing) and externalized to permit uninterrupted urine flow. The abdominal incision was closed with sutures, and the bladder was emptied by applying gentle pressure over the lower abdomen. A cannula of PE-50 tubing was inserted via the external urethra into the bladder (approximately 2.5 cm distance), and secured in place with a 4-0 silk ligature around the external urethral orifice. The cannula was connected to an MLT0699 pressure transducer attached to a PowerLab 8/30 data collection system (ADI Instruments, Colorado Springs, CO) for the measurement of bladder pressure. Saline was introduced into the bladder via an in-line 3-way stopcock.

**Constant slow transvesicular infusion (CSTI) in conscious rats.** To study the effect of peripherally administered compounds on voiding in conscious animals, rats were prepared as follows. Male Sprague Dawley rats were anaesthetized with pentobarbitone sodium (50 mg/kg, i.p). The bladder was exposed through a median abdominal incision, and a polyethylene cannula (PE 50) was introduced into the bladder dome and secured with a purse string suture. At the start of the experiment the cannula was connected to a pressure transducer for the measurement of intravesicular pressure. Another cannula (PE-50) for the intragastric (i.g.) administration of drugs was introduced into the stomach through a paramedian abdominal incision and also secured with a purse string suture. The free end of each cannula was exteriorized subcutaneously at the dorsal neck area. The
surgical wounds were closed with 4-0 silk sutures, and the animal was allowed to recover 2 days with appropriate post-surgical care.

Placement of i.c.v. cannula. To study the effects of test substances administered centrally (i.c.v.), rats underwent a second surgical procedure to position an indwelling cannula into the lateral ventricle. Rats were placed in a stereotaxic apparatus and a midline incision was made to expose the scalp. Tissues covering the scalp were gently retracted, and the area was cleaned with 0.5% hydrogen peroxide followed by saline to provide clear visibility of bone sutures. A small burr hole was drilled and a guide cannula was lowered into the lateral ventricular space, using the coordinates: 1.4 mm lateral, 0.8 mm posterior and 3.4 mm ventral to bregma (The Rat Brain in Stereotaxic Coordinates, Paxinos and Watson, 4th edition). The cannula was secured using dental cement and optical screws, and the wound was closed using tissue adhesive. The animals were provided appropriate post-surgical care and allowed one week recovery. At the end of the experiments, 5 μl of Methylene Blue dye were injected and correct placement of the cannula was confirmed histologically.

Experimental Procedures

Effects of i.v. SNAP 7941 and WAY 100635 in the DIRC model in anesthetized rats. The bladder was emptied by applying light pressure on the lower pelvic area, and the PE-50 cannula was connected to the pressure transducer. Isovolumic, rhythmic voiding contractions were then evoked by filling the bladder with normal saline in increments of 100 μl at 1 min intervals, until attaining repetitive voiding contractions that persisted for ≥ 10 min. SNAP 7941, WAY 100635 or saline (vehicle) were then
administered (1 ml/kg) *via* the jugular vein cannula. To quantify basal contractions and the effects of vehicle and test substances using the same units, we measured “contraction intervals” defined as follows: “basal” – the average frequency of voiding contractions over the 10 min period preceding vehicle injection; “vehicle” – the frequency of contractions for 10 min following vehicle administration; and “test substance” – the single peak-to-peak interval from the last full contraction preceding and the first full contraction following compound administration. For approximately 15% of the rats used in this model contractions could not be evoked, were irregular, or did not resume after a period of drug-evoked cessation; these animals were excluded from the analysis.

**Effect of centrally administered MCH in the DIRC model in anesthetized rats.**

In order to assess the effect of centrally administered MCH on voiding contractions in the DIRC model, volume-induced contractions were evoked in i.c.v.-cannulated rats. After establishing basal contraction frequency, rats received a single i.c.v. injection (5 μl over 1 min) of saline or MCH. Drug effects were measured as described above.

**Effects of i.g. administration of SNAP 7941, oxybutynin and WAY 100635 in the CSTI voiding model.** On the day of the experiment surgically prepared rats were placed in cylindrical Plexiglas restrainers with sufficient room to allow lateral and back and forth movement, and the bladder cannula was connected to a pressure transducer for measurement of intravesicular pressure. After a 20 min acclimation period, repetitive filling-voiding cycles were initiated by a steady infusion of normal saline (room temp) from a syringe pump delivering 100 μl/min. The first series of experiments was aimed at validating the model with compounds shown previously to inhibit voiding. After a period
of ≥ 30 min to determine baseline infusion capacity (the volume of saline infused between micturition episodes), individual rats received a single dose of either vehicle, oxybutynin (1 mg/kg) or WAY 100635 (3 mg/kg). A second set of experiments was performed to determine the effect of SNAP 7941 (0.01 – 30 mg/kg) on voiding, using WAY 100635 (3 mg/kg) as the positive control. All compounds were delivered i.g. (1 ml/kg) rather than by oral gavage, in order to avoid changes in micturition recording resulting from animal handling. Voiding was monitored for 180 min after compound administration. Basal and treatment-related effects on infusion capacity were measured as the average of the final 3 voiding cycles for each evaluation period (usually 30 min, see Fig. 4). When the infusion capacity was too large to produce 3 cycles within the evaluation period, the infusion capacity was taken from the final full cycle within the period. The values are expressed as the percent basal infusion capacity, using each animal as it own control. Animals in which a consistent voiding pattern could not be established (< 5% of rats tested) were excluded from the analysis.

Effects of i.c.v. MCH, Ala^{11}-MCH, and SNAP 7941 in the CSTI voiding model.

To assess the effect of centrally administered test substances on CSTI infusion capacity, animals were prepared as described above. After a period of ≥ 30 min to determine baseline infusion capacity, vehicle (normal saline), MCH (20 nmol), Ala^{11}-MCH (20 nmol) or SNAP 7941 (0.1-10 nmol) was infused (5 μl over 1 min) into the lateral ventricle, and infusion capacity was measured for 200 min. We also assessed the ability of systemically administered (i.g.) oxybutynin (1 mg/kg) or SNAP 7941 (0.1 mg/kg) to antagonize the effects of centrally administered MCH. Infusion capacity was measured for 100 min following antagonist administration, after which the rats received MCH (20
nmol). Infusion capacity was measured for an additional 100 min. Treatment-related effects were measured as described above.

**Natural voiding in conscious spontaneously hypertensive rats (SHR).** To study the effect of drugs on the natural voiding process, SHR were simultaneously dosed and volume-loaded by the oral administration of vehicle (5 ml water), oxybutynin (3 mg/kg), WAY 100635 (3 mg/kg), or SNAP 7941 (0.1 mg/kg) delivered in 5 ml water, and placed in Nalgene metabolic cages. Urine was collected in a container attached to a force transducer (MLT 0201; Radnoti Glass Technology, CA, USA). Urine collected (g) as a function of time was displayed using a Power Lab data acquisition system, and voided volumes were calculated assuming specific gravity = 1. The number of voiding episodes, total volume voided, and volume per voiding episode were measured for 6 hours following drug administration. The effects of SNAP 7941, WAY 100635 and oxybutynin were compared to matched vehicle control animals tested in parallel.

**Statistical Analysis.** All statistics were performed using GraphPad Prism 4.02 software (San Diego, CA). Contraction intervals in the DIRC model were analyzed using 1-way ANOVA (p < 0.05), followed by a Bonferroni’s multiple comparison test of between-group differences. CSTI studies were analyzed using repeated measures 2-way ANOVA (p < 0.05), followed by post-hoc Bonferroni’s multiple comparison test of differences between treatment groups. The effects of compound treatment in the natural voiding model were analyzed using Student’s t-test (p < 0.05).
Results

Effects of i.v. SNAP 7941 and WAY 100635 in the DIRC model in anesthetized rats. Rhythmic, isovolumic voiding contractions were produced in anesthetized rats by incremental distension of the bladder with normal saline. Representative examples of the magnitude and frequency of contractions are illustrated in Fig. 1. These traces also highlight two other general observations: the i.v. injection of saline had no effect on volume-induced contractions; and SNAP 7941 and WAY 100635 tended to produce a transient disruption in contractions, which recovered after some delay to a magnitude and frequency not significantly different from than that prior to treatment. After a 10 minute stabilization period, the average baseline inter-contraction interval was 1.54 ± 0.23 min. The interval was not altered significantly by a saline injection (2.29 ± 0.67 min). One-way ANOVA revealed a significant effect of treatment on the contraction frequency [F(4,17) = 9.69, p < 0.001) (Fig. 2). SNAP 7941 produced a rapid (within 1-2 min) dose-related effect, with a trend to increase the interval at 3 mg/kg (4.52 ± 0.50 min) and a significant increase at 10 mg/kg (9.85 ± 3.2 min; p < 0.01). Similar effects were seen with the reference compound, WAY 100635 (0.3 mg/kg, i.v.), which increased the mean contraction interval to 11.2 ± 2.2 min (p < 0.001). In each case the magnitude of the contractions after recovery was not different from that preceding drug treatment, suggesting that the drugs inhibited the neuronal stimulus for the contraction without affecting the ability of bladder smooth muscle to contract in response to the cholinergic stimulus.
Effect of i.c.v administration of MCH in the DIRC model in anesthetized rats.

The central application of MCH peptide (2 – 20 nmol in 5 μl saline) produced a dose-dependent reduction in the frequency of voiding contractions. A significant overall effect of treatment was found in the one-way ANOVA [F(5,22) = 5.22, p < 0.01]. The basal contraction interval in this series of experiments was 1.67 ± 0.09 min. Contractions were not affected significantly by i.c.v. injection of saline. A significant reduction in the contraction interval was produced by MCH at doses of 5 nmol (0.88 ± 0.15 min; p < 0.05) and 20 nmol (0.94 ± 0.2 min; p< 0.01) (Fig. 3), but the response to a higher dose (50 nmol) was not significant, suggesting a bell-shaped response to MCH in this paradigm.

Effects of i.g. SNAP 7941, oxybutynin and WAY 100635 in the CSTI voiding model in conscious rats. Continuous slow infusion of normal saline at the rate of 100 μl/min resulted in repetitive filling-voiding cycles as shown in Fig. 4. In general, the cycle settled into a consistent rhythm with stable micturition pressures within the first 30 min. The measurement is expressed as “infusion capacity” to differentiate it from a true measurement of “bladder capacity”, which takes into account the residual volume. Mean infusion capacity under basal conditions was 548 ± 60 μl (n=20), although basal capacity for individual rats varied from 158 to 900 μl, depending on the exact position of the surgical ligature in the bladder dome. Therefore, treatment-related effects on infusion capacity were assessed “within subjects” and are represented as a percent of each individual animal’s basal infusion capacity. The mean variability in infusion capacity over 3 h in vehicle-treated group was less than ± 10% of baseline. In the first series of experiments, oxybutynin (1 mg/kg) and WAY 100635 (3.0 mg/kg) produced significant,
sustained increases in infusion capacity: 2-way ANOVA \([F(2,72) = 51.9; \ p < 0.0001]\) (Fig. 5a). A significant effect of oxybutynin was not seen until 90 min after drug administration, whereas WAY 100635 increased infusion capacity significantly within the first 30 min evaluation period. A close inspection of the cystograms showed that oxybutynin produced a transient decrease in micturition pressure in about 40\% of animals, but this effect was gone before the end of the first 30 min observation period. A sustained increase in infusion capacity in response to both compounds persisted throughout the entire 180 minute observation period. WAY 100635 was used as an internal comparator against SNAP 7941.

SNAP 7941 was tested initially at doses of 1, 10 and 30 mg/kg, as these aligned with the doses inhibiting voiding contractions in the DIRC model, as well as doses reported by Borowsky et al (2002) to be efficacious in models of feeding and anxiety. A significant and sustained increase in infusion capacity was seen in response to the 1 mg/kg dose (Fig. 5b), but 10 and 30 mg/kg were ineffective. A test of lower doses revealed a steep dose-response relationship between 0.01 and 0.03 mg/kg (Fig. 5c). Two-way ANOVA applied to the entire data set indicated a significant effect of treatment \([F(4,300) = 35.88, \ p < 0.0001]\). A significant increase in infusion capacity that was sustained throughout the 180 min observation period was produced at doses of 0.03, 0.1 and 1 mg/kg SNAP 7941.

The effect of treatment on micturition pressure was assessed in animals treated with oxybutynin (1 mg/kg), WAY 100635 (3 mg/kg), or SNAP 7941 (0.1 mg/kg). The treatment groups did not differ significantly in their basal micturition pressures (mm Hg): oxybutynin (16.4 ± 1.2); WAY 100635 (18.8 ± 2.1); SNAP 7941 (18.3 ± 2.5).
Furthermore, none of the compounds produced a significant change in micturition pressure (peak – baseline) over 3 h (Fig. 6).

**Effects of SNAP 7941, oxybutynin and WAY 100635 on natural voiding in SHR.**

To facilitate natural voiding, rats were dosed orally with 5ml of water (with or without test compounds) and placed in metabolic cages for collection of urine over 6 h. In experiments to confirm the hyperactive voiding phenotype of SHR, we compared their voiding to that of age/size-matched WKY rats. SHR exhibited a significantly higher voiding frequency (9.8 ± 0.7 voids/6 h, N = 5) relative to control WKY rats (5.2 ± 0.6 voids/6 h, N = 5; p < 0.0001). The corresponding mean volume/void was significantly lower in the SHR (0.25± 0.05 ml) as compared to the WKY rats (0.55 ± 0.15 ml/voids; p < 0.05). No statistically significant difference was observed in the total volume of urine voided over the 6 h period (SHR = 2.3 ± 0.2 ml; WKY = 2.84 ± 0.54 ml).

Relative to vehicle-treated SHR, animals receiving a single oral dose of SNAP 7941 (0.1mg/kg) exhibited a significant reduction in the number of voiding episodes over the 6 h observation period (Fig. 7A). In each case this effect was accompanied by a concomitant increase in the mean volume/void (Fig. 7B). The total volume voided during the 6 h test was not different from vehicle-treated rats (Fig. 7C). Similar significant effects on voiding were seen in response to both oxybutynin (3 mg/kg) and WAY 100635 (3 mg/kg) (Fig. 7A-C).

**Effects of i.c.v. administration of MCH and SNAP 7941 in the CSTI voiding model in conscious rats.** To study the effect of centrally administered compounds, rats were fitted with indwelling i.c.v. cannulae and were allowed 5 d to recover. We
discovered that mean basal infusion volumes were increased in these rats (1538 ± 114 µl; n=15; inter-animal variability: 437 to 2250 µl) relative to those undergoing a single surgery and 2 d recovery (see above).

Low doses of SNAP 7941 (0.1, 1 and 10 nmol) administered i.c.v. caused significant increases in infusion capacity in the CSTI model: 2-way ANOVA \[ F(3,30) = 21.98; p < 0.0001 \] (Fig. 8). Although all doses were effective, a more robust effect was seen at the 10 nmol dose, which produced an increase of approximately 60% that persisted throughout the 180 min observation period.

The effects of centrally administered MCH peptide were evaluated in the series of experiments shown in Fig. 9. Two-way ANOVA revealed an overall significant effect of treatment on infusion capacities \[ F(4,129) = 75.25; p < 0.0001 \]. Central application of MCH (20 nmol, i.c.v.) produced effects in the CSTI model opposite to those of the antagonist. MCH produced a significant reduction in infusion capacity within the first 30 min, reaching a maximum decrease of 34% relative to basal, whereas vehicle injection had no effect (Fig. 9A,B). The effect of MCH diminished gradually over time, but remained significant over the 200 min observation. Ala\textsuperscript{11}-MCH (20 nmol, i.c.v.), an inactive analog of MCH, had no effect on infusion capacity and did not interfere with the subsequent action of MCH (20 nmol injected at the 130 min time point) to rapidly reduce infusion capacity (Fig. 9C). This provides evidence that the effect of MCH is MCH\textsubscript{1} receptor-mediated, rather than a non-specific off-target effect of peptide injection.

To establish further evidence for the involvement of central MCH\textsubscript{1} receptors in the control of micturition, the peptide was administered i.c.v. to rats treated 1 h previously
with an i.g. dose of SNAP 7941 or oxybutynin. As in the previous experiment, SNAP 7941 and oxybutynin each increased infusion capacity significantly (Fig. 9D,E). The magnitude of the effects (~ 33% increase above basal) were somewhat less than those seen in rats that were not implanted with i.c.v. cannulae (~ 60 – 110% above basal), presumably due to the influence of the prolonged surgical recovery on basal infusion capacity (see above). The effect of MCH, delivered i.c.v. at the 100 min time point, was completely blocked in SNAP 7941-treated rats (Fig. 9D). The effect of MCH was not antagonized in oxybutynin-treated rats, where it produced a significant reduction (max 33% decrease) in infusion volume, which persisted throughout the 100 min evaluation period (Fig. 9E).
Discussion

This study demonstrates a role for central MCH₁ receptors in the regulation of voiding in rats. We show in cystometric models in anesthetized and conscious rats that MCH injected into the lateral ventricle stimulates the voiding reflex, whereas blockade of central MCH₁ receptors with the selective antagonist SNAP 7941 inhibits voiding and increases infusion capacity. SNAP 7941 was also shown to increase bladder capacity and decrease voiding frequency in the SHR model of hyperactive voiding. Thus, this study highlights a potential utility of MCH₁ antagonists in the treatment of overactive bladder syndrome.

We investigated the effects of MCH and SNAP 7941 in three voiding models in the rat. The DIRC model in anesthetized rats relies on distension-induced activation of primary afferent sensory neurons within the bladder wall to initiate a spino-bulbo-spinal voiding reflex (see de Groat, 1997). SNAP 7941 produced a dose-related cessation of isovolumic voiding contractions, and when contractions resumed they were similar in magnitude and frequency to those before dosing. The effect was similar to that observed for the 5-HT₁A antagonist WAY 100635, which acts at spinal and supraspinal 5-HT₁A receptors to modulate both afferent and efferent limbs of the voiding reflex (Testa et al., 1999; Kakizaki et al., 2001). The responses to SNAP 7941 were seen at 1 and 10 mg/kg, doses comparable to those reported to be efficacious in rodent models of feeding, anxiety, and depression (Borowsky et al., 2002). The absence of an effect SNAP 7941 on contractility supports the results from our preliminary study, in which MCH and MCH₁-
receptor antagonists failed to modify \textit{in vitro} bladder contractions evoked by either carbachol or electrical field stimulation (data not shown).

In the CSTI model SNAP 7941 at low doses evoked a marked increase in infusion capacity that was well maintained, but higher doses were ineffective, giving rise to an overall bell-shaped dose-response curve (discussed below). Both the magnitude and duration of the effect of SNAP 7941 were comparable to those of WAY 100635 and oxybutynin. It should be noted that the effect of oxybutynin to increase infusion capacity in the present study mimics its clinical profile in the treatment of overactive bladder syndrome (Chapple et al., 2005), however several earlier studies on the effects of oxybutynin in rat cystometry models, measuring only a few voiding cycles, have reported immediate decreases in micturition pressure with no effect on bladder volume capacity (e.g., see Testa et al., 1999; Modiri et al., 2002; Angelico et al., 2005). In our experiments the oxybutynin-evoked reduction in micturition pressure was transient. The delayed response to oxybutynin in this study may result from an immediate peripheral effect to reduce bladder contractility, followed by a delayed and persistent central effect to inhibit the voiding reflex.

SHR have been documented as a genetic model exhibiting symptoms of bladder over-activity, including decreased bladder capacity, reduced volume per void, and an increase in the number of non-voiding bladder contractions (Persson et al., 1998; Steers et al., 1999). The hyperactivity is thought to result from elevations in NGF, leading to increases in both afferent and efferent innervation of the bladder (Clemow et al., 1997). The hyperactive phenotype was confirmed in the present study, in which SHR exhibited a
higher voiding frequency, a reduction in volume per void, and no change in overall volume voided compared to WKY rats. SNAP 7941 significantly reduced the total number of voiding episodes, while producing a significant increase in the volume per void, with no effect on the total volume voided. In these respects the effects of SNAP 7941 were comparable to those of oxybutynin and WAY 100635.

The potency of SNAP 7941 in the two conscious-rat voiding models was significantly higher (ca. 30-100 fold) than in the DIRC model in anesthetized rats, and higher also than reported for rodent behavioral models (Borowsky et al., 2002). The potency differences in the voiding models could relate to the effect of anesthetic in the DIRC model to blunt the sensitivity of MCH circuitry, but this does not explain why SNAP 7941 should be markedly more potent in a voiding model after oral administration than in a behavioral model after oral or i.p. administration. In view of the high affinity of SNAP 7941 for the MCH1 receptor ($K_b = 0.57$ nM vs MCH-evoked IP$_3$ accumulation) and the minimal cross-reactivity profile (Borowsky et al., 2002), it is likely that the potent effects observed in the present model do indeed reflect an MCH$_1$-mediated phenomenon, rather than an off-target effect, the likelihood of which would increase with escalating doses. The bell-shaped nature of the dose-response to SNAP 7941 in the CSTI assay is likely to result from a secondary cross-reactivity associated with higher exposure levels, activating an unidentified mechanism that counteracts the effect of MCH$_1$ receptor blockade.

To further investigate the pharmacological specificity and site of action of SNAP 7941, we employed a series of experiments in which compounds were administered into
the lateral ventricle. In the CSTI model, SNAP 7941 was shown to produce significant increases in infusion volume at i.c.v. doses of 0.1 to 10 nmol. If distributed uniformly throughout the circulation, the minimal effective dose of 0.1 nmol would be equivalent to a systemic dose of ~ 0.0015 mg/kg, significantly below the observed minimal effective dose with intragastric delivery. Therefore, this is suggestive of a central rather than a peripheral site of action for SNAP 7941. Next, we postulated that if the observed effects of SNAP 7941 to inhibit voiding were due to blockade of MCH₁ mediated receptor signaling, then the MCH peptide should stimulate voiding. In the DIRC model the dose-response relationship for i.c.v.-injected MCH appeared bell-shaped, showing a significant increase in voiding contraction frequency with low doses (5, 20 nmol), but no effect of 50 nmol. This may indicate that MCH at low concentration activates receptors at sites readily accessible from the ventricular circulation (e.g. Barrington’s nucleus) to stimulate voiding, while MCH at higher concentrations also diffuses to a second, less accessible population of receptors to activate pathways that can inhibit voiding. The 20 nmol i.c.v. dose of MCH was also effective in the CSTI model, producing a significant reduction in infusion capacity. The effect of MCH in the CSTI model was not mimicked by its inactive analog Ala¹¹-MCH (Gao et al., 2004), which also failed to block the response to MCH injected subsequently. Finally, the MCH-evoked reduction in infusion capacity was blocked completely by i.g. administration of SNAP 7941, but not by an equally effective dose of oxybutynin. Thus, the blockade of the central effect of MCH by SNAP 7941 is most likely due to specific blockade of the MCH₁ receptor, and not functional antagonism.
The data presented indicate that the effects of SNAP 7941 and MCH to modulate voiding are unlikely to involve direct effects on the bladder. Although a spinal site of action cannot be ruled out, the current results are more indicative of a supraspinal, MCH<sub>1</sub> receptor-mediated mechanism. Further studies will be required to determine the neurotransmitters and brain circuits mediating the responses to MCH and MCH<sub>1</sub> antagonists. One region of key interest is the pontine micturition center (Barrington’s nucleus), which acts as a relay switch integrating modulatory signals from higher brain centers with afferent bladder signals to regulate efferent signaling to the bladder (de Groat, 1997). CRF-containing neurons in the PMC project to the spinal cord (Valentino et al., 1995), where CRF evokes a stimulatory effect on micturition (Klausner et al., 2005). In contrast, the CRF antagonist astressin inhibits voiding and improves bladder capacity. MCH is recognized to have a role in stress and anxiety in rodent models, and these states are known to have an impact on voiding (Klausner and Steers, 2004). Among its many effects, MCH has been shown to promote activation of the HPA axis <i>in vivo</i> and to release CRF from hypothalamic explants <i>in vitro</i> (Kennedy et al., 2003). We demonstrated that intra-PVN injection of MCH increases locus coeruleus firing, and that the effect is blocked by the CRF<sub>1</sub> receptor antagonist CP-154526 (unpublished results). Thus, it is tempting to speculate that MCH may have a similar stimulatory effect on the CRF-containing neurons of the PMC, which lies adjacent to the locus coeruleus. Such a circuit would be consistent with the present results with MCH and SNAP 7941.

Overactive bladder syndrome is a debilitating condition, with a large unmet or inadequately met medical need. The current antimuscarinic therapies, although efficacious, exhibit considerable side effects that limit efficacy and patient compliance.
Our observations thus far indicate that MCH₁ antagonists are efficacious in animal models predictive of the clinical efficacy of agents such as oxybutynin. Thus, MCH₁ antagonists may represent a future opportunity for the treatment of overactive bladder syndrome, by offering a comparable efficacy profile without the side effects associated with existing therapies.
References


Footnotes

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Legends for Figures

Fig. 1. Three representative tracings showing the contraction frequency and magnitude of isovolumic bladder contractions in the anaesthetized rat (DIRC model). The effect of intravenous administration of test substances is also shown: A, saline; B, SNAP 7941 (10 mg/kg, i.v.); and C, WAY 100635 (0.3 mg/kg, i.v.).

Fig. 2. Effects of WAY 100635 (0.3 mg/kg, i.v.) and SNAP 7941 (3 and 10 mg/kg, i.v.) on contraction interval in the DIRC model in anesthetized rats. Data represent the mean ± S.E.M. from n = 4 to 7 animals per group. *p <0.05, and ***p<0.001 vs. control group; 1-way ANOVA, followed by Bonferroni post hoc analysis.

Fig. 3. Effect of central (i.c.v.) administration of MCH on the frequency of isovolumic contractions in the DIRC model. Data represent the mean ± S.E.M. from n = 2 to 5 animals per group. *p <0.05, and **p<0.01 vs. basal group; 1-way ANOVA, followed by Bonferroni post hoc analysis.

Fig. 4. Representative tracing showing the stability of CSTI-evoked micturition cycles in conscious rats. Infusion capacity was calculated as the product of the micturition interval X infusion rate as illustrated in the figure. Abscissa: tick marks represent 5 min intervals.

Fig. 5. Comparison of the effects of test substances on infusion capacity in the CSTI model in conscious rats. Bars represent the change in infusion capacity expressed as a percent of baseline (B) for 30, 60, 90, 150, and 180 min after drug administration. The effect of vehicle treatment is shown for each experiment. Doses (mg/kg, i.g.) are
indicated in parentheses. A) oxybutynin (n = 5) vs WAY 100635 (n = 5); B) WAY 100635 (n = 19) vs SNAP 7941 (n = 4 to 8); and C) WAY 100635 (n = 19) vs SNAP 7941 (n = 6 to 7). Data represent the mean ± S.E.M. *p <0.05, **p<0.01, and ***p<0.001 vs. basal group; 2-way ANOVA, followed by Bonferroni post hoc analysis.

Fig. 6. Illustration of the stability of micturition pressure (peak – baseline) over 3 h observation period in rats treated with i.g. doses of oxybutynin (OXY, 1 mg/kg), WAY 100635 (WAY, 3 mg/kg) or SNAP 7941 (0.1 mg/kg). Data represent the mean ± S.E.M. from 5 animals per group.

Fig. 7. Effects of WAY 100635 (3 mg/kg), oxybutynin (3 mg/kg) and SNAP 7941 (0.1 mg/kg) on natural voiding in non-instrumented SHR. Doses were delivered in 5 ml water vehicle and parallel vehicle controls were monitored simultaneously for 6 h after dosing. A) total urinary volume B) voiding episodes and C) volume per voiding episode. Data represent the mean ± S.E.M. from n = 4 to 5 experiments. *p<0.05 and **p <0.01 vs their respective control groups; Students’ t- test.

Fig. 8. Effect of central (i.c.v.) administration of SNAP 7941 (0.1, 1 and 10 nmol) on infusion capacity in the CSTI model in conscious rats, expressed as a percent of baseline (B) for 30, 60, 90, 150, and 180 min after drug administration. Data represent the mean ± S.E.M. of n = 2 to 3 determinations. *p <0.05, **p<0.01, and ***p<0.001 vs. basal group; 2-way ANOVA, followed by Bonferroni post hoc analysis.

Fig. 9. Effects of central (i.c.v.) administration of MCH or the inactive analog Ala11-MCH on infusion capacity in the CSTI model in conscious rats. Vehicle or drugs were
administered after a 30 min measurement of basal capacity. A) Effect of i.c.v. saline. B) Effect of 20 nmol MCH. C) Effect of 20 nmol Ala\textsuperscript{11}-MCH (t=0 min) followed by MCH (20 nmol, t=130 min). D) Effect of MCH (20 nmol, doses at t=100 min) in the presence of SNAP 7941 (SNAP, 0.1 mg/kg; i.g., at t=0 min). E) Effect of MCH (20 nmol, doses at t=100 min) in the presence of oxybutynin (OXY, 3 mg/kg; i.g., at t=0 min). Data represent the mean ± S.E.M. from n = 3 to 11 determinations. *p <0.05, **p<0.01, and ***p<0.001 vs. basal group; 2-way ANOVA, followed by Bonferroni post hoc analysis.
Fig. 2

![Graph showing contraction interval (min) for different treatments: Basal, Veh, 0.3 WAY, 3 SNAP 7941, 10 (mg/kg, i.v.).](image-url)
Infusion capacity = 160s x (100 μl / 60s) = 267 μl
Fig. 6

Micturition Pressure (peak-baseline, mmHg)

- OXY (1)
- WAY (3)
- SNAP 7941 (0.1)

Time (min)

B 30 60 90 150 180
Fig. 8

[Graph showing infusion volume (% basal) over time (min) for Veh and SNAP 7941 (nmol, i.c.v.) at concentrations of 0.1, 1, and 10 nmol. Bars with error bars represent data points, with significance indicated by asterisks (*, **, ***).]
Fig. 9

The figure shows the infusion volume (as a percentage of basal) over time for different conditions and substances. The x-axis represents time in minutes (30, 60, 100, 130, 160, 200), and the y-axis represents the infusion volume (% basal). The conditions are labeled A to E, with each condition having specific treatments:

- **A**: Saline
- **B**: MCH
- **C**: Ala\textsuperscript{11}-MCH
- **D**: SNAP
- **E**: OXY

Each bar represents the infusion volume at different time points, with error bars indicating variability. Significant differences are marked with asterisks: * (p < 0.05), ** (p < 0.01), *** (p < 0.001).