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# Effects of cholinesterase inhibition in supraspinal and spinal neural pathways on the micturition reflex in rats

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## Abstract

**Objective—**To investigate whether activation of brain and spinal cholinergic pathways affects the micturition reflex in rats.

**Materials and Methods—**The effects of intracerebroventricular (i.c.v.) or intrathecal (i.t.) administration of neostigmine as a cholinesterase inhibitor and oxotremorine-M (OXO-M) as a muscarinic acetylcholine receptor (mAChRs) agonist, on the micturition reflex were evaluated by infusion cystometrography (CMG) in urethane-anaesthetized untreated rats or rats pretreated with capsaicin.

**Results—**Neostigmine injected i.c.v increased bladder capacity (BC) and pressure threshold (PT) dose-dependently, with an increase in maximum voiding pressure (MVP) and a decrease in voiding efficiency (VE) at higher doses. Also, neostigmine injected i.t. increased the BC and PT dose-dependently without changing MVP or VE, and these effects were not apparent in capsaicin-pretreated rats. In both routes, atropine as an antagonist of mAChRs, but not mecamylamine as a nicotinic-AChR antagonist, almost completely antagonized the effects of neostigmine. The rank order of potencies of the antagonists for increasing effects of BC induced by 1 nmol of neostigmine was: pirenzepine (an $M_1$ mAChR antagonist) = atropine > 4-DAMP (an $M_3$ mAChR antagonist) ≫ methoctramine (an $M_2$ mAChR antagonist) and tropicamide (an $M_4$ mAChR antagonist) via the i.c.v route; and atropine > methoctramine > pirenzepine > tropicamide and 4-DAMP via the i.t. route, respectively. OXO-M injected via i.c.v and i.t. had the same effects on BC, PT, MVP and VE as neostigmine by i.c.v and i.t., respectively.

**Conclusions—**These results indicate that activation of muscarinic cholinergic mechanisms by the cholinesterase inhibitor in the brain and spinal cord can inhibit the micturition reflex, mainly by affecting afferent pathways. These mAChR-induced inhibitory effects seem to be mediated through $M_1/M_3$ receptor subtypes in the brain, while in the spinal cord, the $M_1/M_2$ receptor subtypes might be involved in inhibitory effects, which are mediated via inhibition of mechanoceptive C-fibre afferent pathways.

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CONFLICT OF INTEREST: None declared.

**Keywords**

neostigmine; brain; spinal cord; micturition reflex; muscarinic receptors

## Introduction

Improvement of storage symptoms, including urge incontinence, by donepezil hydrochloride, an inhibitor of centrally acting cholinesterase (enzymes for acetylcholine, ACh, synthesis and degradation), has been reported in a few isolated cases with Alzheimer's disease [1]. Also, experimental studies suggested that damage of the inhibitory influence of the cortical cholinergic system enhances the micturition reflex in a rat model with detrusor overactivity (DO) after cerebral infarction [2], and that up-regulation of the forebrain cholinergic inhibitory mechanism by intracerebroventricular (i.c.v.) or i.v. injection with donepezil improves DO caused by cerebral infarction [3]. In theory, ACh increase after cholinesterase inhibition might act on muscarinic or nicotinic ACh receptors (mAChRs or nAChRs), or both, in the brain. It was reported that i.c.v. injection with oxotremorine-M (OXO-M), a mAChR agonist, can increase bladder capacity (BC) mediated via excitation of $M_1$ mAChR in awake rats [4,5]. However, injection of OXO-M into the dorsal pontine tegmentum has been reported to reduce BC [5]. It was proposed that two supraspinal muscarinic pathways regulate the bladder activity. The first is an inhibitory pathway that originates from the forebrain, and the second is an excitatory pathway located in the pons and midbrain tegmentum [5]. However, nAChRs are also widely distributed in the brain and spinal cord [6,7]. Recent experimental studies in rats reported that i.c.v.-injected epibathidine or nicotine as a nAChR agonists increased BC [8,9]. Also, nAChR agonists have been reported to induce the release of ACh in the brain [10]. These findings suggest that the nAChRs might act on interneurones and evoke ACh release, leading to activation of mAChRs in the brain.

It was reported that intrathecal (i.t.)-injected OXO-M can increase BC in awake rats [4] and that i.t.-injected nicotine decreased BC in rats mediated via, at least in part, excitation of the glutamate pathway [8]. In pain physiology, the cholinergic system and mAChRs are important for the regulation of nociceptive transmission in the spinal cord. The administration of muscarinic agonists or cholinesterase inhibitors via i.t. injection produce potent analgesia in rodents and humans [11–13]. Cholinesterase inhibitor-induced antinociception is only partially suppressed with mecamylamine, a nAChR antagonist, but fully suppressed with atropine [12].

Therefore, the endogenous ACh mechanism activating nAChRs and mAChRs could be involved in the supraspinal and spinal regulation of the micturition reflex. However, receptor subtypes, especially in spinal mAChRs, are relatively unexplored.

We previously reported that at the spinal level stimulatory effects of nicotine on the micturition reflex involve capsaicin-sensitive C-fibre afferent pathways [8]. However, relationships between spinal cholinergic mechanisms and C-fibre pathways (mechanoceptive or nociceptive) have not been fully investigated. Therefore, we investigated whether the micturition reflex can be modulated by i.c.v. or i.t. injection of neostigmine, with or without mAChR or nAChR antagonists, in control rats and rats treated with capsaicin for C-fibre desensitization.

## Materials and Methods

In all, 262 female Sprague-Dawley rats (220–260 g) were used; all experiments were conducted in accordance with institutional guidelines and approved by the University of Pittsburgh

institutional animal care and use committee. For i.v. injections, a polyethylene tube (PE-50, Clay-Adams, Parsippany, NJ, USA) was inserted into the right jugular vein at the same time as the bladder catheter. For i.t. injections, PE-10 was implanted intrathecally at the level of L6-S1 spinal cord after a laminectomy at the Th11 vertebra under halothane anaesthesia, 3 days before experiments. The volume of fluid within the catheter was kept constant at 6 μL. A single dose of drug was then administered in a volume of 2 μL followed by 7 μL flush with PBS.

For i.c.v injection, the rats were positioned in a stereotaxic frame, a scalp incision was made over the sagittal suture, and a hole was drilled in the right parietal bone to expose the dural surface 1.5 mm and 0.8 mm anterior to the bregma. A sterile stainless-steel catheter (outside diameter 0.3 mm, inside 0.3 mm, length 10.5 mm) was lowered 5.3 mm from the bregma with a micromanipulator. With the aid of a small screw placed in the skull as an anchor, the catheter was fixed to the skull with dental acrylic cement 2 days before cystometrography (CMG). Solutions were injected via an infusion catheter inserted into the larger catheter during CMG. A single dose of drugs was administered in a volume of 2 μL and the infusion catheter was left in place for 1 min after infusion to allow for diffusion of the drug. The injection sites in the spinal cord and the lateral ventricle were confirmed by injection of dye (methylene blue) in every rat at the end of experiments.

The drugs used in the current study were neostigmine bromide as a cholinesterase inhibitor, OXO-M as a mAChR agonist, atropine sulphate as a mAChR antagonist, mecamylamine hydrochloride as a nAChR antagonist, pirenzepine dihydrochloride as an $M_1$ mAChR antagonist, methoctramine tetrahydrochloride as an $M_2$ mAChR antagonist, 4-diphenyl-acetoxy-N-methylpiperidine (4-DAMP) methiodide as an $M_3$ mAChR antagonist, and tropicamide as an $M_4$ mAChR antagonist (all from Sigma Chemical Co., St. Louis, MO, USA). Importantly, mAChR antagonists are only relatively selective, not exclusively specific, for individual subtypes [14]. Therefore, we investigated the dose–response effect of several mAChR antagonists on the micturition reflex to clarify the relative contribution of mAChR subtypes in the brain and spinal cord. For i.v. administrations, drugs were dissolved in saline; for i.t. and i.c.v. administrations, drugs were dissolved in PBS. All drugs were adjusted to pH 7.2–7.4 just before use.

Under urethane anaesthesia (1.0 g/kg, s.c.) a midline abdominal incision was made and a transvesical catheter with a fire-flared tip (PE-50) was inserted into the dome of the bladder for bladder filling and pressure recording. A three-way stopcock was connected to the transvesical tube to monitor bladder pressure during CMG. Physiological saline was infused at room temperature into the bladder at a constant rate of 0.1 mL/min to elicit voiding in untreated rats or rats pretreated with s.c. capsaicin (125 mg/kg), 4 days before the experiments [15]. To evaluate the efficiency of capsaicin, an eye-wipe test was used in each un-anaesthetized rat just before the experiment; a drop of 100 μg capsaicin was placed in the eye and the number of defensive forelimb wiping movements counted. Saline voided from the urethral meatus was collected and measured to determine the voided volume (VV). After constant VVs were collected, bladder infusion was stopped and the residual volume (RV) was measured by withdrawing intravesical fluid through the catheter by gravity. Bladder capacity (BC) was then calculated as the sum of VV and RV, and voiding efficiency (VE) was calculated as (BC – RV)/BC × 100. The intravesical pressure to induce micturition (i.e. pressure threshold, PT) and maximum voiding pressure (MVP) were also measured. The i.c.v administration of neostigmine and OXO-M at high doses apparently increased RV. Therefore, we evaluated the micturition variables in each micturition cycle after i.c.v administration of the drugs. In all experiments, control CMG was recorded for 1–2 h before i.c.v and i.t. administration of vehicle (PBS) or drug solutions.

For the agonist study, we administered drugs at increasing doses (neostigmine, 0.01–3 nmol/rat; OXO-M, 0.01–1 nmol/rat) at 1-h intervals to construct dose-response curves. Preliminary studies indicated that injection of PBS vehicle within three times at 1-h intervals in i.c.v and i.t. routes failed to affect CMG variables in six rats, respectively, indicating the negative volume effects on the micturition reflex. We injected neostigmine cumulatively at 0.01, 0.1 and 1 nmol in one group and at 0.03, 0.3 and 3 nmol in another group in both routes. Also, we injected OXO-M cumulatively at 0.01, 0.1 and 1 nmol in one group and at 0.03 and 0.3 nmol in another group via both routes. In rats pretreated with capsaicin, we injected neostigmine at 1.0 nmol and OXO-M at 0.3 nmol (submaximal dose in both) via the i.t. route.

For the antagonist study, neostigmine (1 nmol) was also tested after pre-treatment (10–20 min) with atropine, pirenzepine, methoctramine, 4-DAMP, tropicamide or mecamylamine at 0.1–30 nmol to evaluate the receptor subtypes involved in the CNS. We calculated the $ID_{50}$ (dose of the antagonist that produced 50% inhibition of the effect of neostigmine (1 nmol) and 95% CI in each antagonist. $ID_{50}$ values were not calculated for antagonist interactions that failed to reach a 50% reversal at the largest dose of antagonist used (30 nmol).

For data comparison, the averaged value of cystometric variables obtained from three micturition cycles were compared before and after drug application. All data values are expressed as the mean (SEM). Results were compared statistically using a one- or two-way ANOVA, with subsequent individual comparisons using Bonferroni/Dunn posthoc test. Student's two-tailed *t*-test was used to compare cystometric variables before and after drug administration in the same rat. A parametric test (unpaired *t*-test) was used to test for differences in the cystometric variables between the different protocol groups. For all statistical tests $P < 0.05$ was considered to indicate significance.

## Results

During control periods, the mean BC, PT, MVP and VE were 0.64 (0.09) mL, 7.2 (1.3) $cmH_2O$, 28.4 (2.3) $cmH_2O$ and 95.2 (2.1)%, respectively (63 rats). The i.c.v. or i.t administration of PBS (vehicle) three times at 1-h intervals did not elicit detectable changes in any CMG variables compared with the control (six rats in each, data not shown). Neostigmine injected i.c.v. (0.01–3 nmol) caused a dose-dependent increase in BC and PT (Figs 1,2). At doses of >0.3 nmol, MVP increased and VE decreased, with an increase in RV (Figs 1,2). Repeated injections of neostigmine showed no desensitization of the effects. Neostigmine injected i.t. (0.01–3 nmol) caused a dose-dependent increase in BC and PT without changing MVP and VE (Figs 1,2). By both the i.c.v. and i.t. routes, the effects of neostigmine appeared immediately and lasted for >1 h (data not shown). Neostigmine injected i.v at 3 nmol slightly increased MVP, but with no significant difference, and failed to affect the BC, PT and VE; this effect did not last for >30 min (five rats, data not shown).

The dose-dependent effects of several antagonists on the increasing effect of neostigmine (1 nmol) on BC (five to six rats at each antagonist dose) by the i.c.v. (Fig. 3) and i.t. (Fig. 4) routes were then investigated. In both routes, atropine, but not mecamylamine, almost completely antagonized the effects of neostigmine, suggesting that the increasing effect of neostigmine on the BC was mediated via excitation of mAChRs in the brain and spinal cord. The $ID_{50}$ and 95% CI for each antagonist in i.c.v. and i.t. routes are shown in Table 1. The rank order of $ID_{50}$ values (nmol) for the antagonist potencies via the i.c.v route was pirenzepine (0.5) = atropine (0.7) ≥4-DAMP (12.1) ≥ ≥ methoctramine and tropicamide. Via the i.t. route, the potency order was atropine (1.7) ≥ methoctramine (16.5) ≥ pirenzepine (28.5) ≥ tropicamide and 4-DAMP.

OXO-M injected via the i.c.v. and i.t. produced almost the same effects as neostigmine by i.c.v. or i.t. (Fig. 5). Both i.c.v. and i.t. OXO-M (0.01–1.0 nmol) caused a dose-dependent increase in BC and PT. Only by the i.c.v. route at doses of >0.1 nmol was MVP increased and VE decreased. OXO-M 1 nmol by i.v. injection slightly increased MVP but with no significant difference, and failed to affect the BC, PT and VE.

The number of wiping responses after instilling one drop of 100 μg/mL capsaicin solution in the eye was 18 (3)/2 min (nine rats) in controls, whereas there was no wiping response in 14 rats with capsaicin pre-treatment. In 14 rats pretreated with capsaicin the mean BC, PT, MVP and VE were 1.14 (0.12) mL, 9.5 (1.6) $cmH_2O$, 27.4 (2.8) $cmH_2O$ and 90.4 (4.3)%, respectively. BC and PT in the capsaicin pretreated rats were significantly ($P < 0.05$) greater than those in control rats. The MVP did not differ between the groups. Neostigmine (1 nmol) injected via i.t. failed to affect micturition variables in eight capsaicin pretreated rats (Figs 1,2). OXO-M (0.3 nmol) injected i.t. also failed to affect micturition variables in six capsaicin pretreated rats (Fig. 5).

## Discussion

The present results indicate that i.c.v. or i.t. injected neostigmine, a cholinesterase inhibitor, increased BC, and these inhibitory effects on afferent micturition pathway were antagonized by atropine, but not by mecamylamine, indicating that an increase in ACh levels in both brain and spinal cord increase the threshold for triggering the micturition reflex through activation of mAChRs, but not nAChRs. These findings were also confirmed by the results of i.c.v. and i.t. administration of OXO-M, a mAChR agonist, as it also produced inhibitory effects on the micturition reflex in the present and other studies [4], and that i.v. injection of OXO-M as a mAChR agonist that crosses the blood–brain barrier suppressed bladder nociceptive responses induced by bladder stimulation after blocking peripheral mAChRs [16]. Overall, these results imply that central inhibitory muscarinic receptors can suppress the processing of sensory inputs from the bladder, thereby increasing BC.

In the present study, neostigmine injected i.c.v. increased BC in urethane-anaesthetized rats, while donepezil hydrochloride (a centrally acting cholinesterase inhibitor) injected i.c.v. has been reported not to affect BC in control (sham-operated) awake rats [3]. It was reported that positive interactions between glutamatergic and cholinergic systems in learning and memory processes probably occur through muscarinic $M_1$ receptors in the brain [17]. It was proposed that two glutamatergic pathways regulate bladder activity. The first is an inhibitory pathway that originates from the forebrain, and the second is an excitatory pathway located at more caudal sites, possibly in the brainstem or in the spinal cord [18]. Urethane has been reported to suppress glutamatergic synaptic mechanisms at various sites in the brain [18]. Therefore, there are possibilities that urethane might suppress the forebrain cholinergic pathway mediated via inhibition of the glutamatergic pathway, and that in the urethane-anaesthetized rats, i.c.v. neostigmine might increase the BC by enhancing forebrain muscarinic pathways.

Our conclusions about mAChR subtypes in the brain and spinal cord relied largely on pharmacological dissection, thus imperfect specificity of mAChR antagonists could have obscured the accurate analysis of the data. Nonetheless, a similar approach has been widely used for identifying and classifying mAChR subtypes. Pirenzepine is a well-recognized $M_1$-selective antagonist [19]. Methoctramine is known to have an affinity two orders of magnitude higher at the $M_2$ receptor than at other subtypes [19]. Similarly, tropicamide has a higher affinity for the $M_4$ subtype than for others [20]. However, 4-DAMP is selective for the $M_3$ receptor when used at appropriate concentrations [19]. In the current study, the $ID_{50}$ of pirenzepine on the increasing effect of neostigmine on the BC via the i.c.v. route was similar to that of atropine, suggesting that the $M_1$ mAChR appears to be the most predominant subtype

in the brain for muscarinic-mediated inhibitory effects on the afferent limb of micturition pathways, as previously reported by other investigators [5]. An autoradiographic study provided evidence of $M_1$ mAChR expression in many forebrain structures, including the cerebral cortex, while a very small fraction of the total number of receptors sites is located in the thalamus and brainstem [21]. In addition, high-dose 4-DAMP partly suppressed the neostigmine-mediated effect on BC. Although 4-DAMP has been described as having a high affinity for $M_3$ receptors, others have noted that it does not seem to differentiate between $M_1$ and $M_3$ receptor subtypes in direct binding or competition studies [22]. Thus, the potential involvement of the $M_3$ receptor subtype cannot be excluded, but the role of this receptor subtype cannot be substantiated in the present study.

Receptor autoradiography and immunocytochemistry studies in the spinal cord have shown that the highest density of mAChRs, pharmacologically identified as $M_1$ to $M_4$, are in the superficial lamina in both rats and humans [23–25]. In the current study, unlike at the supraspinal level, the rank order of $ID_{50}$ for antagonist potency in the spinal cord was atropine 1.7 > methoctramine 16.5 > pirenzepine 28.5 > tropicamide and 4-DAMP, suggesting that $M_2$ as well as $M_1$ receptors might be involved in the spinal mAChR-mediated inhibitory effect on the afferent limb of the micturition pathway. This is in line with the findings that the $M_2$ receptor appears to be the most predominant subtype in the rat spinal cord [24]. However, it was claimed that $M_1$ is not expressed in the dorsal horn [24]. Further investigation by using mAChR ‘knockout’ mice could be done to clarify this discrepancy between expression and function.

In the present study, i.t. neostigmine and OXO-M did not increase the BC in C-fibre-desensitized rats, supporting the hypothesis that activation of mAChRs in the spinal cord increases BC by inhibiting mechanoceptive C-fibre pathways in the spinal cord. TRPV-1 receptors have been reported to exist in the brain stem (sensory nuclei), dorsal horn, dorsal root, trigeminal and nodose ganglia, in addition to peripheral sensory nerves (C-fibre afferents) [26]. The immunoreactivity of $M_2$ receptors in the dorsal spinal cord was also reduced after systemic treatment with a potent capsaicin analogue, resiniferatoxin, suggesting that muscarinic $M_2$ receptors are located, at least in part, on capsaicin-sensitive C-fibre afferent terminals [27]. It was reported that mAChR antagonists have inhibitory effects on C-fibre bladder afferent nerves, thereby, improving BC during the storage phase [28,29]. The present study indicated that mAChR activation in the spinal cord might also be a new strategy for C-fibre-related DO.

There was an increase in MVP with a decrease in VE after neostigmine or OXO-M administration to the brain at higher doses than those required to increase BC and PT. One potential concern was the possibility that drugs administered into the brain could enter the bloodstream and have excitatory effects on the peripheral nervous system, including ganglia and the bladder. Neostigmine was used to reduce this problem because it is a hydrophilic quaternary amine that does not penetrate the blood–brain barrier [15]. We believe that only local effects on the brain and spinal cord were elicited in our experiments, because the effects of i.c.v. and i.t. administration of neostigmine or OXO-M were not duplicated by i.v. administration of the highest dose of each drug, respectively. Another possibility is dysfunction of the urethral outlet to increase outlet resistance and reduce the flow of fluid from the bladder. This effect would be consistent with a previous report indicating that micro-injection of carbachol into the locus coeruleus suppressed urethral sphincter activity [30]. Therefore, activation of mAChRs caused by accumulation of endogenous ACh in the brain might reduce bladder and sphincter coordination during the voiding phase in rats.

In conclusion, the present results indicate that activation of muscarinic cholinergic mechanisms by a cholinesterase inhibitor in the brain and spinal cord mainly affect the afferent limb of

micturition pathways. These mAChRs-induced inhibitory effects seem to be mediated via $M_1$ and $M_3$ receptor subtypes in the brain. By contrast, in the spinal cord, M1 and $M_2$ receptor subtypes might be involved in inhibitory effects, which was mediated via inhibition of mechanoceptive C-fibre afferent pathways. These findings raise the possibility that activation of mAChRs by cholinesterase inhibitors in the CNS could be effective for treating DO in various pathological conditions.

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## Abbreviations

| | |
|---|---|
| (m)(n)ACh(R) | (muscarinic) (nicotinic) acetylcholine (receptor) |
| DO | detrusor overactivity |
| BC | bladder capacity |
| PT | pressure threshold |
| MVP | maximum voiding pressure |
| VE | voiding efficiency |
| VV | voided volume |
| RV | residual volume |
| i.c.v. | intracerebroventricular |
| i.t. | intrathecal |
| OXO-M | oxotremorine-M |
| CMG | cystometrography |

| | |
|---|---|
| 4-DAMP | 4-diphenyl-acetoxy-N-methylpiperidine |
| $ID_{50}$ | dose of the antagonist producing 50% inhibition |

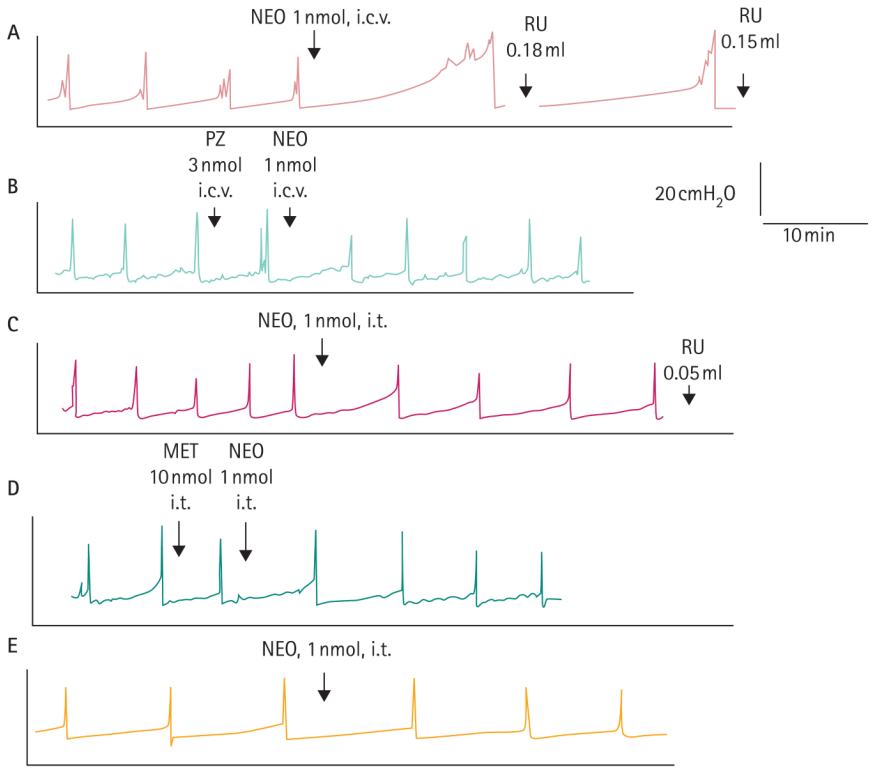


**FIG. 1.**

Effects of i.c.v. (**A,B**) and i.t. (**C–E**) applications of neostigmine (NEO, 1 nmol) with or without pretreatment with a muscarinic $M_1$ receptor antagonist, pirenzepine (PZ, 3 nmol) or a muscarinic $M_2$ receptor antagonist, methoctramine (MET, 10 nmol) on bladder activity in urethane-anaesthetized rats with or without pretreatment with capsaicin (**E**). Arrows indicate the timing of drug administration. RU; residual urine.

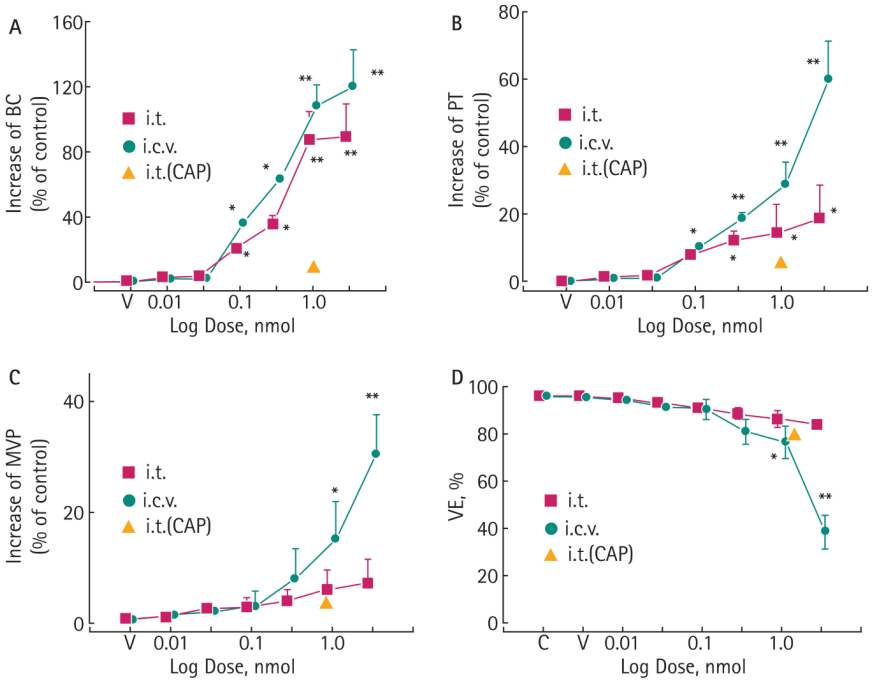


**FIG. 2.**

Dose–response curves showing the effects of increasing doses of neostigmine (NEO, square i.t., six or seven rats in each dose: circle, i.c.v., six or seven rats at each dose; triangle, i.t. in eight rats pretreated with capsaicin, CAP). Abscissa: dose of neostigmine (nmol/rat); C, control (before administration); V, vehicle. Ordinates: (**A**) increase in BC (% of control); (**B**) increase in PT (% of control); (**C**) increase in MVP (% of control); and (**D**) VE (%). Each data point represents the mean (SEM). Individual doses were compared with vehicle treatment using repeated-measures ANOVA (*$P < 0.05$, **$P < 0.01$).

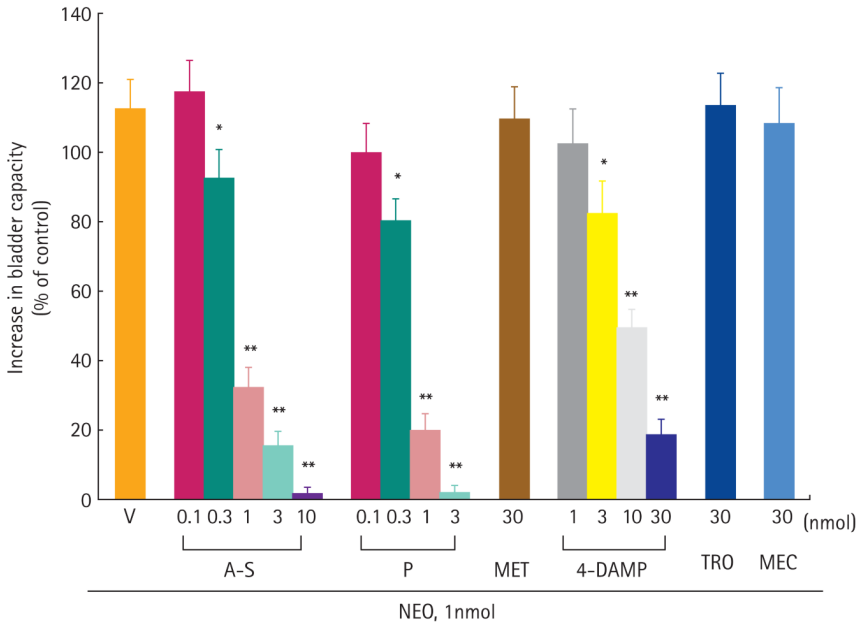


**FIG. 3.**

Effects of i.c.v. pretreatment with atropine sulphate (A-S, 0.1–10 nmol), pirenzepine (P, 0.1–3 nmol), methoctramine (MET, 30 nmol), 4-DAMP (1–30 nmol), tropicamide (TRO, 30 nmol) or mecamylamine (MEC, 30 nmol) on the changes in BC elicited by i.c.v. injected neostigmine (NEO, 1 nmol). V; vehicle (PBS) pretreatment. Each histogram represents the mean (SEM) from five or six different rats. *$P < 0.05$, **$P < 0.01$ vs vehicle treatment.

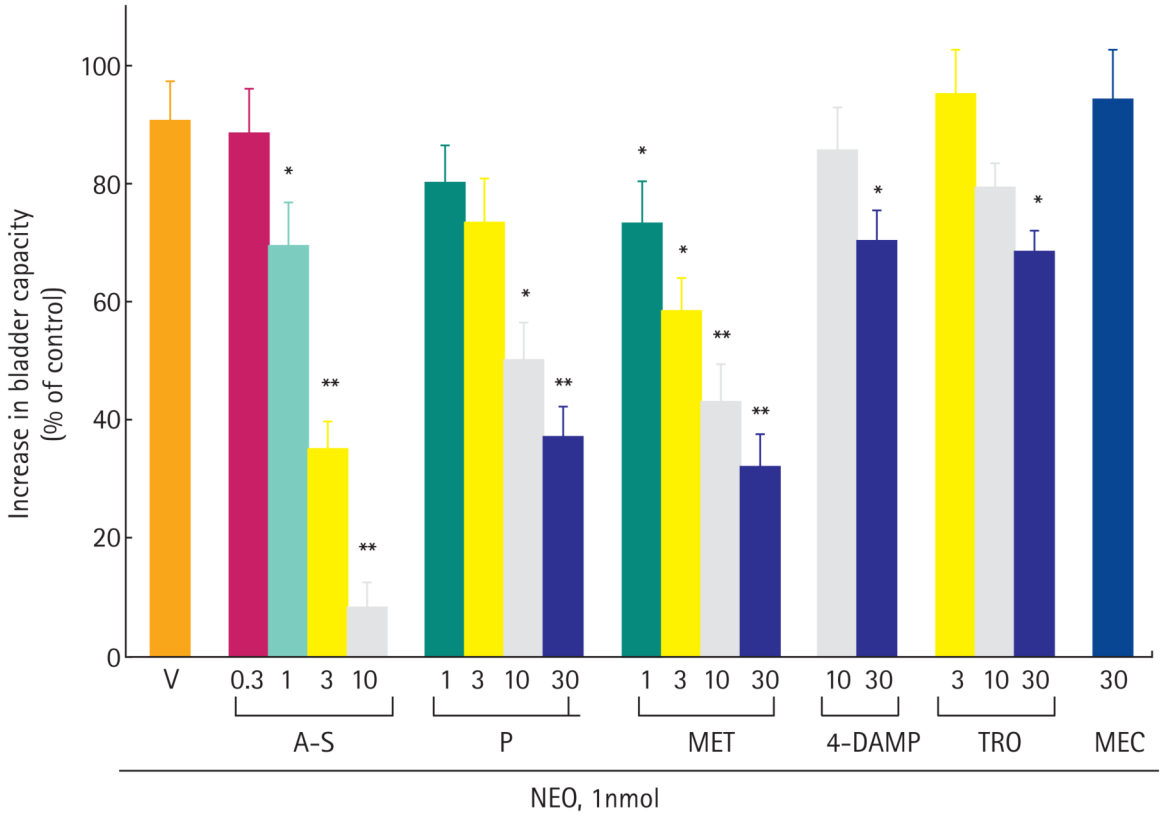


**FIG. 4.**

Effects of i.t. pretreatment with atropine sulphate (A-S, 0.3–10 nmol), pirenzepine (P, 1–30 nmol), methoctramine (MET, 1–30 nmol), 4-DAMP (10–30 nmol), tropicamide (TRO, 3–30 nmol) or mecamylamine (MEC, 30 nmol) on the changes in BC elicited by i.t. injected neostigmine (NEO, 1 nmol). V, vehicle (PBS) pretreatment. Each histogram represents the mean (SEM) from five or six different rats. *$P < 0.05$, **$P < 0.01$ compared with vehicle treatment.

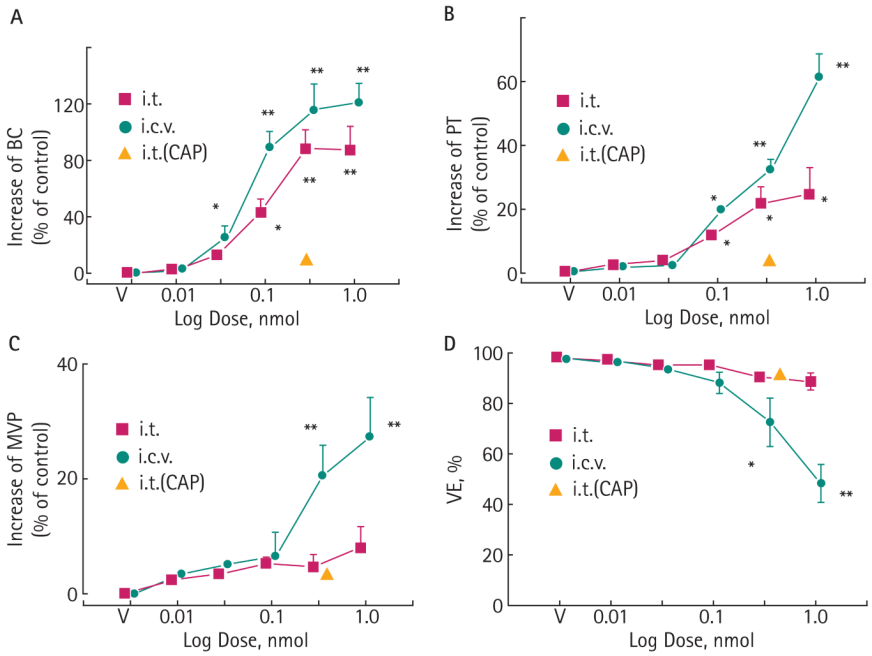


**FIG. 5.**

Dose–response curves showing the effects of increasing doses of OXO-M (square, i.t., six or seven rats at each dose; circle, i.c.v., six or seven rats at each dosel triangle, i.t. in six rats pretreated with capsaicin, CAP). Abscissa: the doses of OXO-M (nmol/rat); V, vehicle. Ordinates: (**A**) increase in BC (% of control)l (**B**) increase in PT (% of control); (**C**) increase in MVP (% of control); and (**D**) VE (%). Each data point represents the mean (SEM). Individual doses were compared with vehicle treatment using repeated-measures ANOVA (*$P < 0.05$, **$P < 0.01$).

## TABLE 1

**The antagonist $ID_{50}$ values, as the median (range), in nmol**

| | Neostigmine (1 nmol) | |
|---|---|---|
| **Antagonist** | **i.c.v.** | **i.t.** |
| Atropine | 0.7 (0.4–1.1) | 1.7 (1.2–2.3) |
| Pirenzapine | 0.5 (0.3–1.0) | 28.5 (15.2–34.5) |
| Methoctramine | >30* | 16.5 (9.7–20.5) |
| 4-DAMP | 12.1 (7.1–18.5) | >30* |
| Tropicamide | >30* | >30* |

*The antagonist failed to produce a reversal of >50% at the largest dose used (30 nmol).