

## **Role of Na<sup>+</sup>,K<sup>+</sup>-ATPase in morphine-induced antinociception**

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## ABSTRACT

We evaluated the modulation by Na<sup>+</sup>,K<sup>+</sup>-ATPase inhibitors of morphine-induced antinociception in tail flick test and [<sup>3</sup>H]naloxone binding to forebrain membranes. The antinociception induced by morphine (1-32 mg/kg, s.c.) in mice was dose-dependently antagonized by ouabain (1-10 ng/mouse, i.c.v.), which produced a significant shift to the right of the morphine dose-response curve. The i.c.v. administration of three Na<sup>+</sup>,K<sup>+</sup>-ATPase inhibitors (ouabain at 0.1-100 ng/mouse, digoxin at 1-1000 ng/mouse and digitoxin at 10-10000 ng/mouse) dose-dependently antagonized the antinociceptive effect of morphine (4 mg/kg, s.c.) in mice, with the following order of potency: ouabain > digoxin > digitoxin. This effect can not be explained by any interaction at opioid receptors, since none of these Na<sup>+</sup>,K<sup>+</sup>-ATPase inhibitors displaced [<sup>3</sup>H]naloxone from its binding sites, whereas naloxone did so in a concentration-dependent manner. The antinociception induced by morphine (5 mg/kg, s.c.) in rats was antagonized by the i.c.v. administration of ouabain at 10 ng/rat, whereas it was not significantly modified by intrathecally administered ouabain (10 and 100 ng/rat). These results suggest that the activation of Na<sup>+</sup>,K<sup>+</sup>-ATPase plays a role in the supraspinal, but not spinal, antinociceptive effect of morphine.

Several authors, including us, have reported an activation of neuronal Na<sup>+</sup>,K<sup>+</sup>-ATPase activity by morphine and endomorphin-1 *in vitro* (Hajek et al., 1985; Nishikawa et al., 1990; Masocha et al., 2002; Horvath et al., 2003), and by morphine *in vivo* (Desaiah and Hoh, 1977; Sharma et al., 1998). Furthermore, it has been demonstrated that electroacupuncture produces antinociception and a stimulation of Na<sup>+</sup>,K<sup>+</sup>-ATPase activity that is blocked by i.p. injection of naloxone prior to electroacupuncture (Lee and Sun, 1984). These results suggest a link between activation of Na<sup>+</sup>,K<sup>+</sup>-ATPase and opioid-induced antinociception. Na<sup>+</sup>,K<sup>+</sup>-ATPase represents the pharmacological receptor of Digitalis and Strophanthus glycosides, which specifically block the activity of this enzyme (Lingrel et al., 1997 and 1998; Wallick et al., 1979). Therefore, if opioid-receptor agonists stimulate Na<sup>+</sup>,K<sup>+</sup>-ATPase activity and this action plays a role in their antinociceptive effects, Na<sup>+</sup>,K<sup>+</sup>-ATPase inhibitors (like ouabain, digoxin and digitoxin) might be able to antagonize such antinociceptive effects. This hypothesis has been little tested and discrepant results have been reported. It has been shown that i.t. administration of ouabain enhanced (Zeng et al., 1999) or did not modify the antinociception induced by i.t. morphine (Horvath et al., 2003), and produced a small antagonism of the effect of low (but not high) doses of i.t. endomorphin-1 (Horvath et al., 2003). The antinociception induced by the i.t. administration of  $\mu$ -opioid agonists is mainly due to the activation of naloxonazine-insensitive ( $\mu_2$ ) receptors (Paul et al., 1989; Pasternak and Letchworth, 1999), whereas the activation of Na<sup>+</sup>,K<sup>+</sup>-ATPase activity by morphine is naloxonazine-sensitive ( $\mu_1$ -mediated) (Masocha et al., 2002). This prompted us to evaluate the effect of i.c.v. administration of several Na<sup>+</sup>,K<sup>+</sup>-ATPase inhibitors (ouabain, digoxin and digitoxin) on the antinociception induced by the s.c. administration of morphine, which produced a naloxonazine-sensitive antinociception (Pick et al., 1991). Since we found a clear antagonism of the



antinociceptive effect of morphine in mice by i.c.v. administered Digitalis glycosides, whereas all the above-mentioned studies that evaluate ouabain-opioid agonists interaction were performed in rats with i.t. administration, we compared the effect of i.c.v. and i.t. ouabain on the antinociception induced by s.c. morphine in rats. Moreover, to discard the possibility that the effect of Digitalis glycosides on morphine-induced antinociception was due to a direct interaction with the opioid receptors we evaluated the effect of these drugs on [<sup>3</sup>H]naloxone binding, a marker of  $\mu$ -,  $\delta$ -, and  $\kappa$ -opioid receptors (Sato and Minami, 1995), to mice forebrain membranes.

## Materials and methods

**Animals.** The experiments were performed in CD-1 Swiss mice (Charles River, Spain) weighing 25-30 g and Wistar rats (animal farm of Szeged University, Hungary) weighing 250-350 g. The animals were housed in temperature-controlled ( $22 \pm 1^\circ\text{C}$ ) rooms with a 12-hour light/dark cycle (light on from 08.00 to 20.00 h) and free access to food and water. All experiments were performed during the same period of the day (8:00 to 14:00 a.m.) to exclude diurnal variations in pharmacological effects. The animals were randomly assigned to treatment groups ( $n = 6-15/\text{group}$ ) and the observer was blind to the treatment administered.

The animals were handled in compliance with EC Council Directive 86/609 for the care of laboratory animals and ethical guidelines for research in experimental pain with conscious animals (Zimmerman, 1983). All procedures were approved by the animal care committees.

**Drugs.** The drugs used in the studies performed in mice and their providers were: morphine HCl (General Directorate of Pharmacy and Drugs, Spanish Ministry of Health), naloxone, ouabain, digoxin, digitoxin (all from Sigma-Aldrich, Spain) and [ $^3\text{H}$ ]naloxone (NEN, MA). The drugs used in the studies performed in rats and their providers were: morphine HCl (ICN Alkaloida, Hungary), ouabain (Sigma-Aldrich, Hungary), ketamine HCl (generous gift from Renate Schwarz, Pfizer Med-Inform, Austria) and xylazine HCl (Bayer, Germany).

**Preparation of mice forebrain  $\text{P}_2$  membranes.** Mice forebrain crude synaptosomal pellets were isolated as previously described in detail (Gonzalez et al., 2001). Briefly, mice forebrains were immersed in tubes containing ice-cold isolation

medium I [320 mM sucrose; 3 mM ethylenediamine tetraacetic acid tetrasodium salt; 10 mM N-2-hydroxyethylpiperazine-N'-ethanesulfonic acid (HEPES), pH 7.4] and were homogenized with a Polytron homogenizer (model PT10-35, Kinematica AG, Switzerland). The homogenates were centrifuged (Avanti 30, Beckman Instruments Inc., Spain) at  $1000 \times g$  for 10 min at 4 °C; the resulting pellets were discarded and the supernatants were centrifuged again under the same conditions. The final supernatant was then centrifuged at  $17000 \times g$  for 20 min to obtain the crude synaptosomal pellet ( $P_2$  pellet). Then the pellet was dissolved in the appropriate incubation medium for binding experiments (see 'Binding experiments') and the protein concentration was determined by a modified version of the Lowry et al. (1951) method.

**Binding experiments.** The  $P_2$  pellet, obtained as described above, was dissolved in Tris 50 mM, pH 7.4. Naloxone was dissolved in Tris 50 mM, pH 7.4, whereas ouabain, digoxin and digitoxin were dissolved in absolute ethanol to make up a 1-mM solution from which further dilutions were made with buffer (Tris 50 mM, pH 7.4). For measuring total binding we incubated in triplicate 20  $\mu$ l of unlabelled drug (0.1 nM – 10  $\mu$ M) or its solvent, 460  $\mu$ l of  $P_2$  membrane fraction (0.6 mg/ml), and 20  $\mu$ l of [ $^3$ H]naloxone (2 nM) at 30°C during 30 minutes as previously described (Freissmuth et al., 1993). For measuring non-specific binding 10  $\mu$ M of unlabelled naloxone was added to the medium. At the end of the incubation period, the reaction was stopped by adding 5 ml of Tris 50 mM, pH 7.4 at 4°C. Each membrane solution was immediately filtered under vacuum through Whatman GF/B glass fibre filters (previously humidified with Tris) with a Brandel cell harvester (model M-12T, Brandel Instruments, UK) and washed twice with 5 ml Tris HCl 50 mM, pH 7.4 at 4°C. The filters were transferred to scintillation counting vials to which 4 ml of liquid scintillation cocktail (Optiphase

Hisafe 2, Wallac Scintillation Products, UK) was added, and left to equilibrate in the dark for 12 hours. The radioactivity retained on the filter was measured using a liquid scintillation spectrometer (Beckman Instruments Inc., Spain) with an efficiency of 52%. Specific binding was calculated by subtracting non-specific binding from total binding.

**Drug treatments and assessment of antinociception in mice.** Morphine was dissolved in ultrapure water (purer than type I in the NCCLS/CAP water quality standards) and injected subcutaneously (s.c.) in a volume of 5 ml/kg. The Na<sup>+</sup>,K<sup>+</sup>-ATPase inhibitors, ouabain, digoxin and digitoxin were dissolved in 1% Tween 80 in ultrapure water and injected intracerebroventricularly (i.c.v.) in a volume of 5 µl/mouse. The control animals received the same volume of vehicles. The s.c. injections were done in the interscapular region. The i.c.v. injections were done in the right lateral cerebral ventricle of mice according to the method described previously (Ocaña et al., 1993). Briefly, the injection site was identified according to the method by Haley and McCormick (1957), and the drug solution was injected with a 10 µl Hamilton syringe with a sleeve around the needle to prevent the latter from penetrating more than 3 mm into the skull. After the experiments were done, the position of the injection was evaluated in each brain, and the results from animals in which the tip of the needle did not reach the lateral ventricle were discarded.

The antinociceptive effect of the treatments was evaluated using a tail flick test as previously described (Ocaña et al., 1993). Briefly, the animals were restrained in a Plexiglas tube and placed on the tail flick apparatus (LI 7100, Letica, S.A, Spain). A noxious beam of light was focussed on the tail about 4 cm from the tip, and the latency to tail flick was recorded automatically to the nearest 0.1 s. The intensity of the radiant heat source was adjusted to yield baseline latencies between 3 and 5 s, this intensity was

never changed and any animal whose baseline latency was outside the pre-established limits was excluded from the experiments. Two baseline tail flick latencies were recorded within 20 min before all injections. At time 0 the animals received an i.c.v. injection of ouabain, digoxin, digitoxin or their solvent and immediately thereafter a s.c. injection of morphine or its solvent. The end of the last injection was considered as time 0; from this time tail flick latencies were measured again at 10, 20, 30, 45, 60, 90 and 120 min after treatment. The cut-off time was 10 s.

The area under the curve of tail flick latency against time (AUC) was calculated for each animal using the GraphPad Prism version 3.00 for Windows (GraphPad Software Inc., USA). The degree of antinociception was determined according to the formula: % antinociception =  $[(AUC_d - AUC_v) / (AUC_{max} - AUC_v)] \times 100$ , where the  $AUC_d$  and  $AUC_v$  are the areas under the curve for drug-treated and vehicle-treated mice respectively, and  $AUC_{max}$  is the area under the curve of maximum possible antinociception (10 s in each determination).

**Drug treatments and assessment of antinociception in rats.** Morphine was dissolved in saline and injected s.c. in a volume of 2 ml/kg. Ouabain was dissolved in 1% Tween 80 in water and injected i.t. or i.c.v. in a volume of 5  $\mu$ l/rat. Control animals received the same volume of the vehicles. For the intrathecal drug administration, the rats were surgically prepared under ketamine-xylazine anaesthesia (72 and 8 mg/kg intraperitoneally, respectively). An intrathecal catheter (PE-10 tubing) was inserted through a small opening in the cisterna magna and passed 8.5 cm caudally into the intrathecal space, as previously described (Yaksh and Rudy, 1976). For i.c.v. drug administration the rats were anaesthetised as described above and placed in a stereotaxic apparatus. A stainless-steel 23-gauge cannula was placed into the right lateral ventricle

and fixed to the skull with dental cement. Coordinates were 0.5 mm caudal and 1.5 mm lateral to the bregma, with the cannula extending 3.5 mm ventral to the skull surface. Injections were performed through a 29-gauge internal cannula which extended 1.0 mm beyond the guide cannula tip. The internal cannula was attached to a Hamilton 10  $\mu$ l syringe with a polyethylene tubing (PE20), allowing the animals to move freely during the injection period. After surgery (both i.t. and i.c.v.), the rats were housed individually, had free access to food and water, and allowed to recover for at least 4 days before use. Rats exhibiting postoperative neurologic deficits were not used. Each animal was studied twice in an experimental series, with an interval of 7-8 days between studies. After experimental use, each rat was killed with an overdose of pentobarbital, and 1% methylene blue was injected to confirm the position of the catheter (i.t.) or cannula (i.c.v.) and probable spread of the injection. If the position was not right, the results obtained in the animal were discarded.

The antinociceptive effect of the treatments was evaluated using a tail flick test as previously described (Horvath et al., 1990). The reaction time was determined by immersing the lower 5 cm portion of the tail in hot water (51.5 °C) until a tail-withdrawal response was observed. The basal latency was  $8.9 \pm 0.32$  s and the cut-off time was 20s. The tail flick latencies were recorded immediately before and at 10, 30, 60, 90 and 120 min after the drug injections of morphine alone or associated to ouabain or its solvent. The area under the curve of tail flick latency against time (AUC) and the degree of antinociception in each animal were calculated as described in the previous section, but using 20 s in each determination to calculate the  $AUC_{max}$ . In the experiments where the effect of ouabain alone was tested, the drug was injected cumulatively (1, 10, 100 and 1000 ng).

**Data analysis.** The values of  $IC_{50}$  (concentration of unlabeled drug that inhibited 50% of specific [ $^3H$ ]naloxone binding),  $ED_{50}$  (dose of morphine that produced half of the maximal antinociception) and  $E_{max}$  (maximum antinociception produced) were calculated from the concentration-response curves or dose-response curves using non-linear regression analysis with the GraphPad Prism version 3.00 computer program (GraphPad Software Inc., USA). Statistical analysis was performed using one-way analysis of variance (ANOVA) followed by Newman-Keuls multiple comparison test or two-way ANOVA followed by Bonferroni post-test, and the differences were considered significant when  $p < 0.05$ . The results in the text and figures are expressed as the means  $\pm$  S.E.M.

## Results

### **Effect of i.c.v. ouabain on the antinociception induced by morphine in mice.**

The analysis of AUC values showed that the administration of morphine (1-32 mg/kg, s.c.) together with the vehicle of ouabain (i.c.v.) produced a dose-dependent increase in the percentage of antinociception in the treated mice (Fig. 1). Using non-linear regression analysis, we fitted the data to a sigmoid curve, which made it possible to calculate  $E_{\max}$  as  $78.40 \pm 2.94\%$  of antinociception and  $ED_{50}$  as  $2.16 \pm 0.20$  mg/kg. The treatment of mice with ouabain (1 and 10 ng/mouse, i.c.v.) produced a dose-dependent antagonism of the ability of morphine to induce antinociception and displaced the dose-response curve of morphine to the right (Fig. 1). The maximum efficacy of morphine was dose-dependently reduced by ouabain ( $E_{\max} = 67.49 \pm 2.19$  and  $63.45 \pm 1.58\%$ , for mice treated with 1 and 10 ng/mouse, respectively). The  $ED_{50}$  of morphine was dose-dependently increased in the animals treated with ouabain ( $ED_{50} = 5.02 \pm 0.34$  and  $6.43 \pm 0.34$  mg/kg, for mice treated with 1 and 10 ng/mouse, respectively).

The s.c. administration of morphine (4 mg/kg) together with the i.c.v. injection of the vehicle of ouabain induced an increase in tail flick latency in a time-dependent manner (Fig. 2, left side). The maximum effect was observed at 30 min and posteriorly decayed progressively. Ouabain (0.1-10 ng/mouse, i.c.v.) dose-dependently antagonized this effect of morphine; the antagonism was particularly evident from 30 to 120 min after morphine administration (Fig. 2, left side). The administration of ouabain (0.1-100 ng/mouse, i.c.v.) together with the solvent of morphine (s.c.) did not significantly modify tail flick latency at any of the times and doses tested (data not shown).

When the area under the curve (AUC) of antinociception along time was analyzed, ouabain (0.1–10 ng/mouse, i.c.v.) antagonized the antinociceptive effect of



morphine (4 mg/kg, s.c.) in a dose-dependent manner (Fig. 2, right side). The maximum antagonism by ouabain was produced at 10 ng/mouse, which reduced the percentage antinociception from  $69 \pm 7.4$  % (morphine + vehicle) to  $20 \pm 8.9$  %. A higher dose of ouabain (100 ng/mouse, i.c.v.) produced a slightly lower antagonism of morphine antinociception (Fig. 2, right side).

**Effects of several Na<sup>+</sup>,K<sup>+</sup>-ATPase inhibitors on the antinociception induced by morphine in mice.** The s.c. administration of morphine (4 mg/kg) together with the vehicle of the Na<sup>+</sup>,K<sup>+</sup>-ATPase inhibitors (i.c.v.), produced a percentage antinociception of  $74.77 \pm 6.75$ % (Fig. 3). The antinociception induced by morphine (4 mg/kg, s.c.) was dose-dependently antagonized by the i.c.v administration of the three Na<sup>+</sup>,K<sup>+</sup>-ATPase inhibitors (ouabain at 0.1 to 100 ng/mouse; digoxin at 1 to 1000 ng/mouse and digitoxin at 10 to 10000 ng/mouse), with the following order of potency: ouabain > digoxin > digitoxin. As seen in Fig. 3 the Na<sup>+</sup>,K<sup>+</sup>-ATPase inhibitors did not antagonize completely the antinociception produced by morphine (4 mg/kg, s.c.). The least percentage of antinociception produced by morphine (4 mg/kg, s.c.) associated with the highest dose of the Na<sup>+</sup>,K<sup>+</sup>-ATPase inhibitor used was  $28.61 \pm 7.08$ % for ouabain (100 ng/mouse, i.c.v.);  $37.08 \pm 5.7$ % for digoxin (1000 ng/mouse, i.c.v.) and  $28.97 \pm 9.46$ % for digitoxin (10000 ng/mouse, i.c.v.). Non-statistically significant differences were found between these values of antinociception. Higher doses of Na<sup>+</sup>,K<sup>+</sup>-ATPase inhibitors were not tested as they caused hyperexcitability and convulsions in some animals.

**Effects of Na<sup>+</sup>,K<sup>+</sup>-ATPase inhibitors and naloxone on [<sup>3</sup>H]naloxone binding to mice forebrain membranes.** Figure 4 shows the competition for [<sup>3</sup>H]naloxone binding sites by naloxone and the Na<sup>+</sup>,K<sup>+</sup>-ATPase inhibitors (ouabain, digoxin and

digitoxin). Naloxone (0.1 nM – 10  $\mu$ M) concentration-dependently displaced [ $^3$ H]naloxone from its binding sites to forebrain membranes, with an  $IC_{50}$  of  $3.58 \pm 0.18$  nM. On the contrary, ouabain, digoxin and digitoxin, even at concentrations of 10  $\mu$ M, did not significantly modify [ $^3$ H]naloxone specific binding.

**Effect of i.c.v. administration of ouabain on the antinociception induced by morphine in rats.** The s.c. administration of morphine (5 mg/kg) together with the i.c.v. injection of the vehicle of ouabain induced an increase in tail flick latency in a time-dependent manner. The maximum effect was observed at 30 min and posteriorly decayed slowly (Fig. 5, left side). Ouabain 10 ng/rat, i.c.v., antagonized this effect of morphine; the antagonism was evident from 30 to 120 min after morphine administration, although only reach statistical significance at 90 and 120 min. On the other hand, a higher dose of ouabain (100 ng/rat, i.c.v.) did not significantly antagonize morphine-induced antinociception at any time (Fig. 5, left side). The administration of ouabain (10 and 100 ng/rat, i.c.v.) together with the solvent for morphine (s.c.) did not modify the tail flick latency at any of the doses or times tested (data not shown).

When the area under the curve of antinociception along time was analysed ouabain (10 ng/rat, i.c.v.) significantly antagonized the antinociceptive effect of morphine (5 mg/kg, s.c.), whereas the highest dose of ouabain used (100 ng/rat, i.c.v.) did not (Fig. 5, right side).

**Effect of i.t. administration of ouabain on the antinociception induced by morphine in rats.** The administration of morphine (5 mg/kg, s.c.) together with the vehicle of ouabain induced an increase in tail flick latency that reach a maximum at 30 min and decreased at 120 min (Fig. 6, left side). Ouabain 10 ng/rat, i.t. significantly

antagonized this effect of morphine only at 60 min after drug administration. On the other hand, a higher dose of ouabain (100 ng/rat, i.t.) had a significant synergistic effect with morphine (5 mg/kg, s.c.) only at 10 minutes after drug administration (Fig. 6, left side). When the area under the curve of antinociception along time was analysed none of the doses of ouabain (10 and 100 ng/rat, i.t.) significantly modified the antinociceptive effect of morphine (5 mg/kg, s.c.) (Figure 6, right side).

The administration of ouabain (10 and 100 ng/rat, i.t.) together with the solvent of morphine (s.c.) did not modify tail flick latency at any of the doses tested (data not shown).

## Discussion

The present study found two main results: 1) Ouabain, digoxin and digitoxin did not have affinity for opioid receptors, but i.c.v. administered antagonized the antinociceptive effect of s.c. morphine in mice. 2) Ouabain administered i.c.v., but not i.t., antagonized the antinociception induced by s.c. morphine in rats.

The i.c.v. administration of several Digitalis glycosides dose-dependently antagonized the antinociceptive effect of morphine in mice. Under the same experimental conditions, these drugs i.c.v. injected (ouabain at 10 and 100 ng/mouse, digoxin at 100 and 1000 and digitoxin at 1000 and 10000 ng/mouse) did not modify the antinociceptive effect of the  $\kappa$ -opioid receptor agonist U50,488H (8 mg/kg, s.c.) (manuscript in preparation). This indicates that the antagonism by Digitalis glycosides of morphine-induced antinociception is specific and not an indiscriminate antagonism of the antinociception induced by any drug.

The antagonism of the antinociceptive effect of morphine by Digitalis glycosides can not be explained by a direct interaction of these drugs with the opioid receptors, since none of these drugs significantly displaced [ $^3$ H]naloxone, a marker of  $\mu$ -,  $\delta$ - and  $\kappa$ -opioid receptors (Satoh and Minami, 1995), from its binding sites to mice forebrain membranes. On the other hand, morphine increases  $\text{Na}^+, \text{K}^+$ -ATPase activity (Nishikawa et al., 1990; Sharma et al., 1998; Masocha et al., 2002) and Digitalis glycosides specifically block the activity of this enzyme (Lingrel et al., 1997 and 1998; Wallick et al., 1979); therefore, the ability of Digitalis glycosides to antagonize morphine-induced antinociception can be due to their ability to block the  $\text{Na}^+, \text{K}^+$ -ATPase activity. In support of this hypothesis, it is interesting to note that the order of potency of the Digitalis glycosides in antagonizing morphine induced antinociception (ouabain >

digoxin > digitoxin) is the same as their order of potency in inhibiting [ $^3\text{H}$ ]ouabain binding in brain membranes (Acuña-Castroviejo et al., 1992). The fact that about 30% of the antinociception induced by morphine in mice was insensitive to Digitalis glycosides might be due to the fact that beside the stimulation of  $\text{Na}^+, \text{K}^+$ -ATPase activity other mechanisms, like the opening of  $\text{K}_{\text{ATP}}$  channels (Ocaña et al., 1993 and 1995; Raffa and Martinez, 1995), the closing of voltage-dependent  $\text{Ca}^{++}$  channels (Del Pozo et al., 1990; Dierssen et al., 1990) or the inhibition of adenylate cyclase (Suh et al., 1995 and 1996), are involved in the antinociceptive effect of morphine. Another possible reason to explain the partial antagonism by Digitalis glycosides of morphine-induced antinociception might be related to the different routes of administration of both drugs. When morphine is administered peripherally (s.c. or i.p.) it is distributed in supraspinal and spinal sites (Miyamoto et al., 1991) and the activation of opioid receptors located in both sites is essential for the production of its antinociceptive effect (Yeung and Rudy, 1980a and 1980b; Roerig and Fujimoto, 1989). When Digitalis glycosides are i.c.v. administered they are expected to be mainly distributed in the supraspinal space. Then, the portion of morphine-induced antinociception not blocked by i.c.v.  $\text{Na}^+, \text{K}^+$ -ATPase inhibitors could have been, at least partially, spinally-mediated. In support of this notion we found that i.t. administration of ouabain did not antagonize the antinociception induced by i.t. morphine in rats (Horwad et al., 2003), nor that produced by s.c. morphine in rats (present study).

In agreement with the data obtained in mice, we observed that the i.c.v. administration of ouabain 10 ng/rat, reduced the antinociception induced by morphine in rats. However, the antagonism of morphine-induced antinociception produced by i.c.v. administration of ouabain was less in rats than in mice. This might be attributed to the species-specific differences of the  $\text{Na}^+, \text{K}^+$ -ATPase  $\alpha$ -subunit (Feschenko et al.,

1997) and variations between species in residues that convey ouabain sensitivity within the first and second transmembrane helices hairpin of the Na<sup>+</sup>,K<sup>+</sup>-ATPase (Lingrel et al., 1997). It could also be speculated that, in the lower degree of antagonism by i.c.v. ouabain in rats compared to mice, a greater relative contribution of spinal versus supraspinal sites of action for s.c. morphine-induced antinociception in rats than mice might have played a role. On the other hand, it is interesting to note that a higher dose of ouabain (100 ng/rat) produce less antagonism of morphine effect than 10 ng/rat. A similar fact was observed in mice (see Fig 2, right side), which suggest that a relatively high dose of ouabain may produce effects additional to those of low doses, that can counteract the antagonism induced by the low doses of ouabain. In fact, the only study that has evaluated previously the effect of i.c.v. ouabain on morphine-induced antinociception found that 100 ng of ouabain did not antagonize the effect of morphine (Calcutt et al., 1971)

On the other hand, ouabain 10 ng/rat i.t. reduced the increase of tail flick latency induced by morphine only at 60 minutes after drug administration, whereas a higher dose of ouabain (100 ng/rat, i.t.) had a synergistic effect on the morphine-induced increase in tail flick latency only at 10 minutes after drug administration. However, both doses of ouabain (10 and 100 ng/rat, i.t.) had no significant effect on the antinociception induced by morphine in rats when a global value of antinociception (AUC of tail flick latency along time) was used. These results demonstrate a difference between the effects of i.c.v. and i.t. administered ouabain on the morphine-induced antinociception. Previous studies have reported that i.t. administered ouabain enhanced (Zeng et al., 1999) or do not modify the antinociception induced by i.t. morphine (Horvath et al., 2003). Therefore, it seems that ouabain antagonized the supraspinal antinociception induced by morphine, whereas it did not significantly antagonize its spinal

antinociception. The supraspinal antinociceptive effects of morphine are mediated mainly through  $\mu_1$  opioid receptors, whilst the spinal antinociceptive effects are mediated through  $\mu_2$  opioid receptors (Paul et al., 1989; Pasternak and Letchworth, 1999). We have demonstrated that  $\mu_1$ -opioid receptors have an important role in the enhancement of synaptosomal  $\text{Na}^+, \text{K}^+$ -ATPase activity induced by morphine (Masocha et al., 2002). Thus, one would expect an antagonism of the antinociceptive effect of morphine by ouabain administered i.c.v. but not i.t.

In conclusion, our results suggest that activation of neuronal  $\text{Na}^+, \text{K}^+$ -ATPase plays a role in the supraspinal, but not spinal, antinociceptive effect of morphine.

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## References

- Acuña-Castroviejo CD, del Aguila CM, Fernandez B, Gomar MD, and Castillo JL (1992) Characterization of ouabain high-affinity binding to rat cerebral cortex. Modulation by melatonin. *Eur J Pharmacol* **226**:59-67.
- Calcutt CR, Doggett NS, and Spencer PS (1971) Modification of the antinociceptive activity of morphine by centrally administered ouabain and dopamine. *Psychopharmacologia* **21**:111-117.
- Del Pozo E, Ruiz-García C, and Baeyens JM (1990) Analgesic effects of diltiazem and verapamil after central and peripheral administration in the hot-plate test. *Gen Pharmacol* **21**:681-685.
- Desaiah D and Ho IK (1977) Effect of morphine on mouse brain ATPase activities. *Biochem Pharmacol* **26**:89-92.
- Dierssen M, Flórez J, and Hurlé MA (1990) Calcium channel modulation by dihydropyridines modifies sufentanil-induced antinociception in acute and tolerant conditions. *Naunyn Schmiedeberg's Arch Pharmacol* **342**:559-565.
- Feschenko MS, Wetzel RK, and Sweadner KJ (1997) Phosphorylation of Na,K-ATPase by protein kinases. Sites, susceptibility, and consequences. *Ann NY Acad Sci* **834**:479-488.
- Freissmuth M, Beindl W, and Kratzel M (1993) Binding and structure-activity-relation of benzo[f]isoquinoline- and norcodeinone-derivatives at  $\mu$ -opioid receptors in the rat cerebral cortex. *Br J Pharmacol* **110**:1429-1436.
- Gonzalez LG, Portillo E, Del Pozo E, and Baeyens JM (2001) Changes in [<sup>3</sup>H]glibenclamide binding to mouse forebrain membranes during morphine tolerance. *Eur J Pharmacol* **418**:29-37.



- Hajek I, Teisinger J, and Sykova E (1985) The effect of opioids and of naloxone on  $\text{Na}^+, \text{K}^+$ -adenosine triphosphatase activity in frog spinal cord membrane fractions. *Neurosci Lett* **59**:291-295.
- Haley TJ and McCormick WG (1957) Pharmacological effects produced by intracerebral injections of drugs in the conscious mouse. *Br J Pharmacol* **12**:12-15.
- Horvath G, Benedek G, and Szikszay M (1990). Enhancement of fentanyl analgesia by clonidine plus verapamil in rats. *Anesth Analg* **70**:284-288.
- Horvath G, Agil A, Joo G, Dobos I, Benedek G, and Baeyens JM (2003) Evaluation of endomorphin-1 on the activity of  $\text{Na}^+, \text{K}^+$ -ATPase using *in vitro* and *in vivo* studies. *Eur J Pharmacol* **458**:291-297.
- Lee DZ and Sun AY (1984) Effect of electroacupuncture on synaptosomal ( $\text{Na}^+ + \text{K}^+$ )-ATPase. *Neurochem Res* **9**:669-678.
- Lingrel JB, Arguello JM, Van Huysse J, and Kuntzweiler TA (1997) Cation and cardiac glycoside binding sites of the  $\text{Na}^+, \text{K}^+$ -ATPase, *Ann NY Acad Sci* **834**:194-206.
- Lingrel JB, Croyle ML, Woo AL, and Arguello JM (1998) Ligand binding sites of  $\text{Na}^+, \text{K}^+$ -ATPase. *Acta Physiol Scand Suppl* **643**:69-77.
- Lowry OH, Rosebrough NJ, Farr AL, and Randall RJ (1951) Protein measurement with the Folin phenol reagent. *J Biol Chem* **193**:265-275.
- Masocha W, Gonzalez LG Baeyens JM, and Agil A (2002) Mechanisms involved in morphine-induced activation of synaptosomal  $\text{Na}^+, \text{K}^+$ -ATPase. *Brain Res* **957**:311-319.
- Miyamoto Y, Morita N, Kitabata Y, Yamanishi T, Kishioka S, Ozaki M, and Yamamoto H (1991) Antinociceptive synergism between supraspinal and spinal sites after subcutaneous morphine evidenced by CNS morphine content. *Brain Res* **552**:136-140.

- Nishikawa T, Teramoto T, and Shimizu S (1990) Effect of morphine on Na<sup>+</sup>,K<sup>+</sup>-ATPase from homogenate of synaptosomes and of synaptic membrane of rat cerebral cortex. *Brain Res* **510**:92-96.
- Ocaña M, Del Pozo E, and Baeyens JM (1993) ATP-dependent K<sup>+</sup> channel blockers antagonize morphine- but not U50488H-induced analgesia. *Eur J Pharmacol* **230**:203-207.
- Ocaña M, Del Pozo E, Barrios M, and Baeyens JM (1995) Subgroups among μ-opioid receptor agonists distinguished by ATP-sensitive K<sup>+</sup> channel-acting drugs. *Br J Pharmacol* **114**:1296-1302.
- Pasternak GW and Letchworth SR (1999) Future opioid analgesics: Targeting the old and the new. *Curr Op CPNS Invest Drugs* **1**:54-64.
- Paul D, Bodnar RJ, Gistrak MA, and Pasternak GW (1989) Different μ receptor subtypes mediate spinal and supraspinal analgesia in mice. *Eur J Pharmacol* **168**:307-314.
- Pick CG, Paul D, and Pasternak GW (1991) Comparison of naloxonazine and β-funaltrexamine antagonism of μ<sub>1</sub> and μ<sub>2</sub> opioid actions. *Life Sci* **48**:2005-2011.
- Raffa RB and Martinez RP (1995) The 'glibenclamide-shift' of centrally-acting antinociceptive agents in mice. *Brain Res* **677**:277-282.
- Roerig SC and Fujimoto JM (1989) Multiplicative interaction between intrathecally and intracerebroventricularly administered mu opioid agonists but limited interactions between delta and kappa agonists for antinociception in mice. *J Pharmacol Exp Ther* **249**:762-768.
- Satoh M and Minami M (1995) Molecular pharmacology of the opioid receptors. *Pharmacol Ther* **68**:343-364.

- Sharma P, Kaur G, Bhardwaj SK, and Kaur G (1998) Role of opioidergic and monoaminergic neurotransmission in the GnRH release mechanism of EBP-primed OVX rats. *Brain Res Bull* **47**:81-86.
- Suh HW, Sim YB, Choi YS, Song DK, and Kim YH (1995) Differential effects of 3-isobutyl-1-methylxanthine injected intrathecally or intracerebroventricularly on antinociception induced by opioids administered intracerebroventricularly in the mouse. *Neuropeptides* **28**:357-363.
- Suh HW, Song DK, Sim YB, Chung KM, and Kim YH (1996) Differential effects of forskolin and phorbol-13-myristate injected intrathecally or intracerebroventricularly on antinociception induced by morphine or beta-endorphin administered intracerebroventricularly in the mouse. *Neuropeptides* **30**:177-185.
- Wallick ET, Lane LK, and Schwartz A (1979) Biochemical mechanism of the sodium pump. *Annu Rev Physiol* **41**:397-411.
- Yaksh JL and Rudy TA (1976) Chronic catheterization of spinal subarachnoid space. *Physiol Behav* **17**:1031-1036.
- Yeung JC and Rudy TA (1980a) Sites of antinociceptive action of systemically injected morphine: involvement of supraspinal loci as revealed by intracerebroventricular injection of naloxone. *J Pharmacol Exp Ther* **215**:626-632.
- Yeung JC and Rudy TA (1980b) Multiplicative interaction between narcotic agonisms expressed at spinal and supraspinal sites of antinociceptive action as revealed by concurrent intrathecal and intracerebroventricular injections of morphine. *J Pharmacol Exp Ther* **215**:633- 642.
- Zeng W, Dohi S, Shimonaka H, and Asano T (1999) Spinal antinociceptive action of Na<sup>+</sup>-K<sup>+</sup> pump inhibitor ouabain and its interaction with morphine and lidocaine in rats. *Anesthesiology* **90**:500-508.

Zimmermann M (1983) Ethical guidelines for investigations of experimental pain in conscious animals. *Pain* **16**:109-110.

## FOOTNOTES

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## LEGENDS FOR FIGURE

**Fig. 1.** Antagonism by treatment with ouabain (1 and 10 ng/mouse, i.c.v.) of the antinociception induced by morphine (1 - 32 mg/kg, s.c.) in a tail flick test in mice. The percentage of antinociception was calculated from the area under the curve of tail flick latency along time (see Methods). Each point represents the mean  $\pm$  S.E.M of the values obtained from 7 to 14 animals. Statistically significant differences in comparison to morphine + vehicle: \*  $p < 0.05$ , \*\*  $p < 0.01$  (two-way ANOVA followed by Bonferroni test).

**Fig. 2.** Antagonism by i.c.v. treatment with ouabain of the antinociception induced by morphine (4 mg/kg, s.c.) in a tail flick test in mice. **Left side:** Time course of the tail flick latency times for various combinations of morphine (4 mg/kg, s.c.) or its vehicle and ouabain (0.1, 1, and 10 ng/mouse, i.c.v.) or its vehicle. Each point represents the mean  $\pm$  S.E.M of the values obtained from 10 to 14 animals. Statistically significant differences in comparison to morphine + vehicle: \*  $p < 0.05$ , \*\*  $P < 0.01$  (two-way ANOVA followed by Bonferroni test). **Right side:** Antagonism by treatment with ouabain (0.1 - 100 ng/mouse, i.c.v.) of the antinociception induced by morphine (4 mg/kg, s.c.). The solid column represents the effect of morphine + ouabain vehicle. The percentage of antinociception was calculated from the area under the curve of tail flick latency along time (see Methods). Each column represents the mean  $\pm$  S.E.M of the values obtained from 10 to 14 animals. Statistically significant differences in comparison to morphine + vehicle: \*  $p < 0.05$ , \*\*  $p < 0.01$  (one-way ANOVA followed by Newman Keuls test).

**Fig. 3.** Antagonism by i.c.v. treatment with different doses of ouabain, digoxin and digitoxin of the antinociception induced by morphine (4 mg/kg, s.c.) in a tail flick test in mice. The percentage of antinociception was calculated from the area under the curve of tail flick latency along time (see Methods). The shaded area represents the mean  $\pm$  S.E.M of the antinociception produced in the animals treated with morphine + vehicle. Each point represents the mean of the values obtained from 6 to 12 animals. Statistically significant differences in comparison to morphine + vehicle: \*  $p < 0.05$ , \*\*  $p < 0.01$  (one-way ANOVA followed by Newman Keuls test).

**Figure 4.** Displacement by naloxone but not by Na<sup>+</sup>,K<sup>+</sup>-ATPase inhibitors (ouabain, digoxin and digitoxin) of [<sup>3</sup>H]naloxone (2 nM) binding to mice forebrain membranes. Each point represents the mean  $\pm$  S.E.M. of the values from 3 independent experiments.

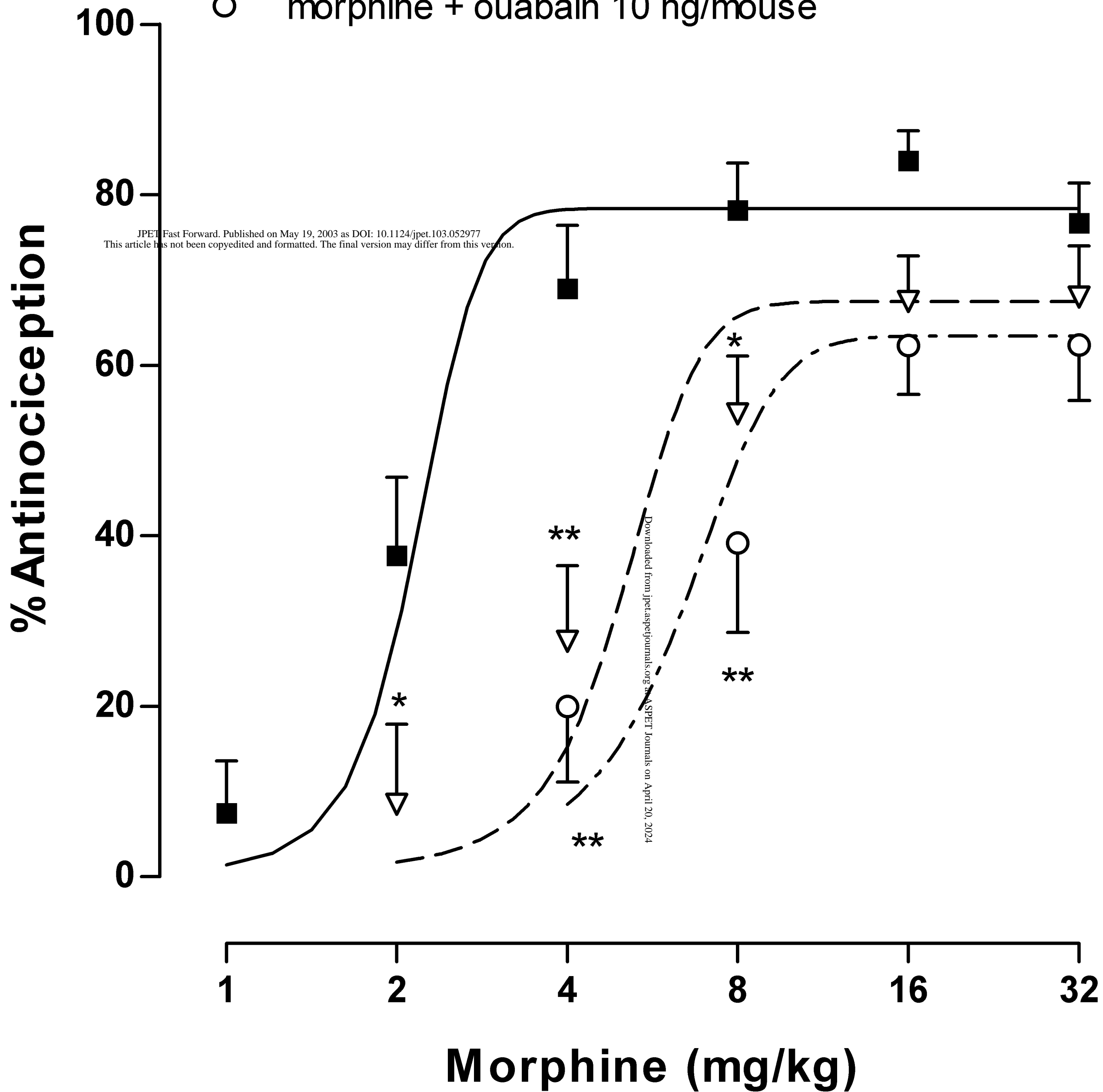
**Fig. 5.** Antagonism by i.c.v. treatment with ouabain of antinociception induced by morphine (5 mg/kg, s.c.) in a tail flick test in rats. **Left side:** Time course of the tail flick latency times for various combinations of morphine (5 mg/kg, s.c.) or its vehicle and ouabain (10 and 100 ng/rat, i.c.v.) or its vehicle. Each point represents the mean  $\pm$  S.E.M of the values obtained from 6 to 10 animals. Statistically significant differences in comparison to morphine + vehicle: \*\*  $p < 0.01$  (two-way ANOVA followed by Bonferroni test). **Right side:** Antagonism by treatment with ouabain (10 and 100 ng/rat, i.c.v.) of the antinociception induced by morphine (5 mg/kg, s.c.). The solid column represents the effect of morphine + ouabain vehicle. The percentage of antinociception was calculated from the area under the curve of tail flick latency along time (see Methods). Each column represents the mean  $\pm$  S.E.M of the values obtained from 6 to

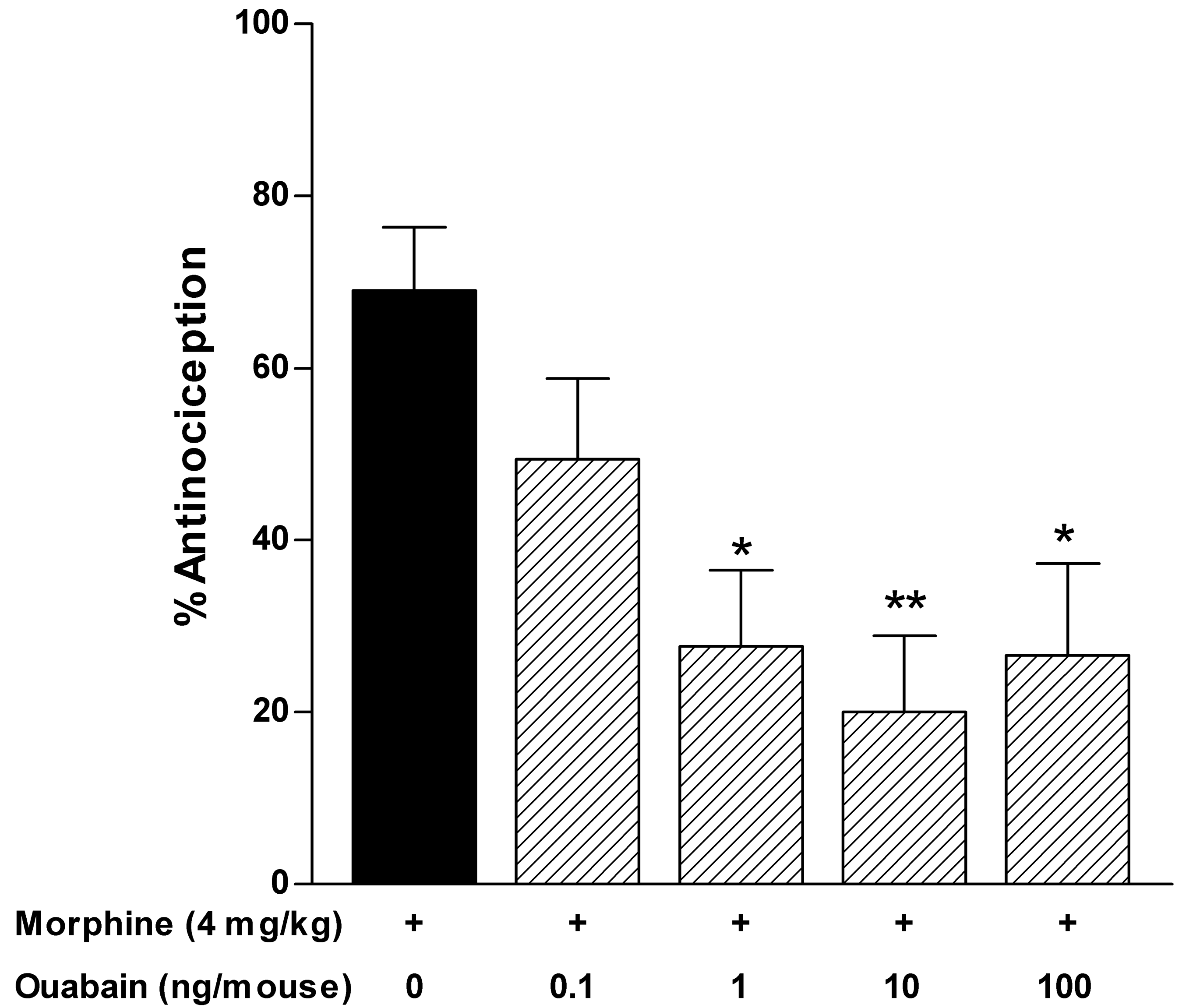
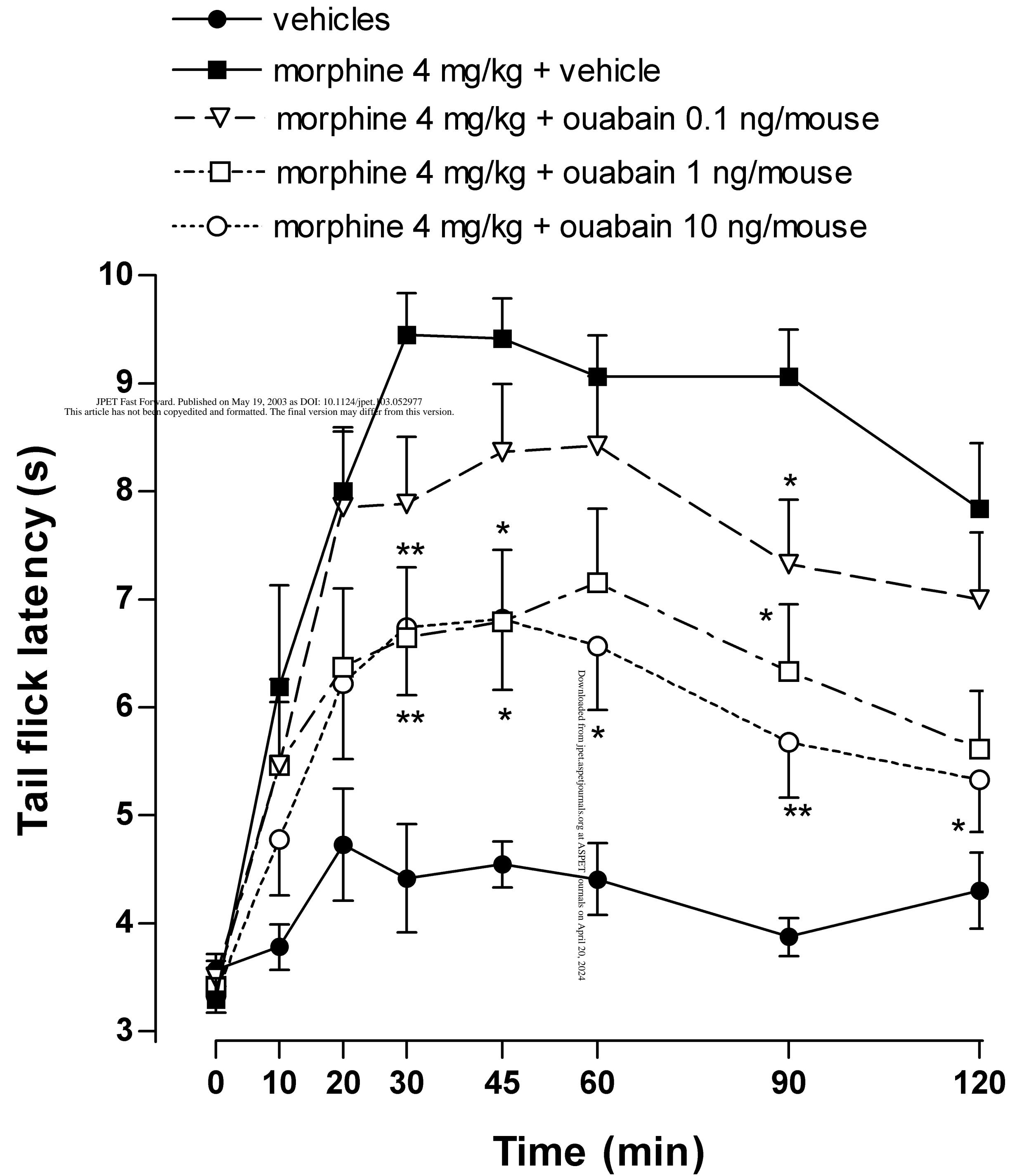
10 animals. Statistically significant differences in comparison to morphine + vehicle: \*  $p < 0.05$  (one-way ANOVA followed by Newman Keuls test).

**Fig. 6.** Effect of i.t. treatment with ouabain on the antinociception induced by morphine (5 mg/kg, s.c.) in a tail flick test in rats. **Left side:** Time course of the tail flick latency times for various combinations of morphine (5 mg/kg, s.c.) or its vehicle and ouabain (10 and 100 ng/rat, i.t.) or its vehicle. Each point represents the mean  $\pm$  S.E.M of the values obtained from 7 to 10 animals. Statistically significant differences in comparison to morphine + vehicle: \*  $p < 0.05$ , \*\*  $p < 0.01$  (two-way ANOVA followed by Bonferroni test). **Right side:** Effect of treatment with ouabain (10 and 100 ng/rat, i.t.) on the antinociception induced by morphine (5 mg/kg, s.c.). The solid column represents the effect of morphine + ouabain vehicle. The percentage of antinociception was calculated from the area under the curve of tail flick latency along time (see Methods). Each column represents the mean  $\pm$  S.E.M of the values obtained from 7 to 10 animals. No statistically significant differences were observed in comparison to morphine + vehicle (one-way ANOVA).



- morphine + vehicle
- ▽ morphine + ouabain 1 ng/mouse
- morphine + ouabain 10 ng/mouse

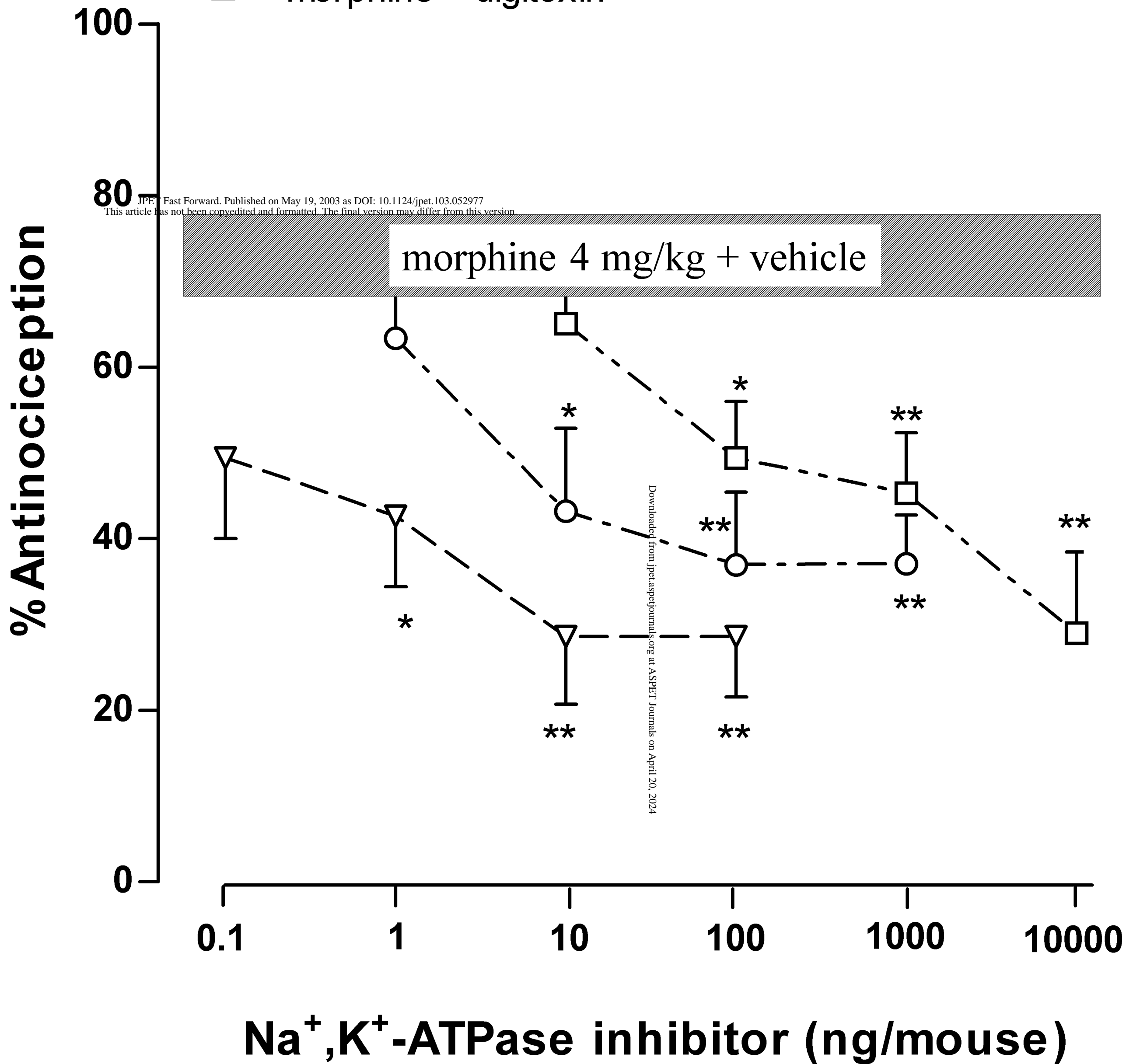




— ▽ — morphine + ouabain

--- ○ --- morphine + digoxin

--- □ --- morphine + digitoxin



**% [<sup>3</sup>H]naloxone specific binding**

100  
80  
60  
40  
20  
0

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-10 -9 -8 -7 -6 -5

**Log [displacing drug] (M)**

- naloxone
- ▽ digoxin
- ouabain
- ◇ digitoxin

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