

## Opiate-induced Analgesia Is Increased and Prolonged in Mice Lacking P-glycoprotein

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**Background:** P-glycoprotein is a transmembrane protein expressed by multiple mammalian cell types, including the endothelial cells that comprise the blood-brain-barrier. P-glycoprotein functions to actively pump a diverse array of xenobiotics out of the cells in which it is expressed. The purpose of this study was to determine if P-glycoprotein alters the analgesic efficacy of clinically useful opioids.

**Methods:** Using a standard hot-plate method, the magnitude and duration of analgesia from morphine, morphine-6-glucuronide, methadone, meperidine, and fentanyl were assessed in wild-type Friends virus B (FVB) mice and in FVB mice lacking P-glycoprotein [*mdr1a/b* (-/-)]. Analgesia was expressed as the percent maximal possible effect (%MPE) over time, and these data were used to calculate the area under the analgesia versus time curves (AUC) for all opioids studied. In addition, the effect of a P-glycoprotein inhibitor (cyclosporine, 100 mg/kg) on morphine analgesia in both wild-type and *mdr* knockout mice was also determined.

**Results:** Morphine induced greater analgesia in knockout mice compared with wild-type mice (AUC 6,450 %MPE min vs. 1,610 %MPE min at 3 mg/kg), and morphine brain concentrations were greater in knockout mice. Analgesia was also greater in knockout mice treated with methadone and fentanyl but not meperidine or morphine-6-glucuronide. Cyclosporine pretreatment markedly increased morphine analgesia in wild-type mice but had no effect in knockout mice.

**Conclusions:** These results suggest that P-glycoprotein acts to limit the entry of some opiates into the brain and that acute administration of P-glycoprotein inhibitors can increase the sensitivity to these opiates. (Key words: Fentanyl; FVB; *mdr1a/1b* (-/-); meperidine; methadone; morphine-6-glucuronide.)

P-GLYCOPROTEIN is a 150-kD transmembrane protein that was first identified in tumor cells by its ability to confer drug resistance to chemotherapeutic agents.<sup>1</sup> Resistance to chemotherapeutic drugs occurs because P-glycoprotein actively transports chemotherapeutic agents out of tumor cells so that intracellular concentrations are below the toxic threshold. Because P-glycoprotein can transport drugs of widely differing chemical structure, cross-resistance to multiple chemotherapeutic agents readily occurs; hence, it is also named multidrug resistance (MDR) protein.

P-glycoprotein is also present at the luminal borders of several normal tissues, including intestinal epithelium, liver canalliculi, renal tubules, and bronchial epithelium.<sup>2-4</sup> In these tissues, P-glycoprotein acts either to secrete xenobiotics (e.g., bile canalliculi, renal tubules) or prevent their absorption (e.g., intestinal epithelium). Importantly, P-glycoprotein has been identified in mouse, rat, bovine, and human brain capillary endothelium where it is believed to be a vital component of the blood-brain barrier.<sup>5-7</sup>

Many clinically relevant xenobiotics are P-glycoprotein substrates, including steroids, cyclosporine, vinca alkaloids, ondansetron, domperidone, verapamil, and digoxin. Of interest to anesthesiologists, the opiates morphine, morphine-6-glucuronide (M-6-G), methadone, meperidine, and loperamide have been shown to be P-glycoprotein substrates in *in vitro* cell culture systems.<sup>8,9</sup> These studies suggest that P-glycoprotein may play an important role in limiting the bioavailability of some opioids in both the brain and spinal cord. Consistent with this hypothesis, Letrent *et al.*<sup>10</sup> have shown that P-glycoprotein inhibition increases morphine analgesia in rats.

The question arises, however, whether P-glycoprotein inhibitors potentiate morphine analgesia solely by inhib-

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iting P-glycoprotein or whether they may affect other molecular process involved in pain perception or suppression. An alternative means of evaluating the role of P-glycoprotein in opiate analgesia is to use recently developed strains of mice that have been genetically engineered to lack P-glycoprotein.<sup>11</sup> Thus, the aim of this study was to determine whether P-glycoprotein limits opioid-induced analgesia *in vivo* by determining the analgesic potency of morphine, M-6-G, meperidine, methadone, and fentanyl in P-glycoprotein-deficient knockout mice and in wild-type mice of the same strain.

## Methods

### Animals

All animal experiments were conducted according to a protocol approved by the Institutional Animal Care and Use Committee of the University of Washington. Wild-type male Friends virus B (FVB) and male *mdr1a/1b* (-/-) mice (25–30 g) on an FVB background were purchased from Taconic farms (Germantown, NY). The mice were housed individually in the same room and were allowed free access to food and water except during experimental testing.

### Drugs and Injections

Morphine (Elkins-Sinn, Inc., Cherry Hill, NJ), M-6-G (Sigma-Aldrich, Inc., St. Louis, MO), methadone (Roxane Laboratories, Inc., Columbus, OH), fentanyl (Janssen Pharmaceutica, Inc., Titusville, NJ), and meperidine (Elkins-Sinn, Inc.) were dissolved or diluted to the desired concentration in normal saline and sterile-filtered immediately before use. All drugs were administered in a volume of 0.1 ml by subcutaneous injection into the abdominal wall. The doses of morphine studied were 1, 5, 10, and 20 mg/kg in the wild-type mice and 1, 3, and 5 mg/kg in the knockout mice. A lower morphine dose was studied in the knockout mice because 5 mg/kg produced 100% of the maximum possible analgesic effect in this group. M-6-G was studied at doses of 1, 3, and 5 mg/kg in both groups of animals. The remaining opioids were studied at a single dose: methadone 5 mg/kg, fentanyl 50  $\mu$ g/kg, and meperidine 50 mg/kg.

### Nociceptive Testing

All animals were tested for thermal analgesia using a standard hot-plate method. Briefly, each mouse was placed on a hot-plate surface (IITC Model 39 D; IITC

Inc., Woodland Park, CA) maintained at  $55 \pm 0.1^\circ\text{C}$ . The mouse was removed from the hot plate as soon as it licked a hind paw, and the time from hot-plate placement to hind-paw lick was recorded. If the animal had not licked a hind paw within 30 s, it was removed from the hot plate to prevent tissue injury, and the latency was recorded as 30 s. In the nonterminal analgesia studies comparing morphine, methadone, meperidine, fentanyl, and M-6-G, each mouse received each opioid buffered with at least a 5-day rest between testing days to avoid opioid tolerance or learning. In addition, the study drug and dosage were selected randomly on each study day to avoid sequential bias. There were eight animals in each group. Animals receiving cyclosporine were killed at the end of the experiment to avoid potential long-term cyclosporine toxicity; therefore, this was the final study for each of these animals.

Predrug (baseline) latency was determined by averaging three separate hot-plate tests at 15-min intervals. Any animal with a predrug latency of greater than 10 s or who exhibited immediate jumping behavior was excluded from further experiments. After injection of the study drug, the hot-plate test was repeated at 15, 30, 45, 60, 80, 120, 135, 150, 180, 240, 300, and 330 min until all mice in the group had returned to baseline latency.

### Effect of P-glycoprotein Inhibition on Morphine Analgesia

At baseline, wild-type and *mdr1a/1b* (-/-) knockout mice (n = 10 in each group) were tested for baseline latency on the hot plate as described previously and were then injected intraperitoneally with either with 0.1 ml normal saline (n = 5 in each group) or 100 mg/kg cyclosporine (Sandoz Pharmaceutical Corporation, East Hanover, NJ; n = 5 in each group) in a volume of 0.1 ml normal saline. Mice were then tested for thermal analgesia as described previously at 15 and 30 min after cyclosporine/saline administration. Immediately after the 30-min hot-plate test, all animals in both groups were injected with morphine doses that would produce comparable analgesia if cyclosporine-induced inhibition of P-glycoprotein resulted in a significant increase in the analgesic efficacy of morphine. These doses were based on the studies described previously; for wild-type mice this dose was 5 mg/kg, and for knockout mice, it was 2 mg/kg. The mice were again tested for thermal analgesia 15 and 30 min after morphine injection.

### *Effect of P-glycoprotein on Morphine Concentration in Brain*

To determine whether P-glycoprotein altered the distribution of morphine into brain tissue, wild-type and knockout mice ( $n = 5$  in each group) were injected subcutaneously with 5 mg/kg morphine sulfate. Thirty minutes after morphine injection, the mice were anesthetized by halothane, and brain and skeletal muscle from one hind leg were immediately removed onto ice and stored at  $-20^{\circ}\text{C}$  for later measurement of morphine concentration.

### *Morphine Analysis*

Morphine extraction from brain and muscle tissue was adapted from the method of Grinstead.<sup>12</sup> Briefly, approximately 0.1 g of brain or muscle tissue was homogenized, and an internal standard (20 ng nalorphine) with 1 ml boric acid/sodium borate buffer (pH 8.9) was added. The aqueous layer was decanted, and the remaining tissue was twice homogenized in 2 ml of 95:5 chloroform:isopropanol, and the solvent was decanted. Then, 0.5 ml of this sample was placed in  $13 \times 100$ -mm screw-cap culture tubes with 1 ml boric acid/sodium borate buffer (pH 8.9) and 4 ml of 97:3 chloroform:isopropanol. Tubes were capped, placed on a reciprocating shaker at 150 rpm for 15 min, and then centrifuged for 10 min at 3,000 rpm. The aqueous layer was removed, and the organic layer was poured into a clean  $13 \times 100$ -mm tube. The organic layer was evaporated to dryness under a stream of nitrogen at  $65^{\circ}\text{C}$  for 5 min. After cooling to room temperature, 50  $\mu\text{l}$  of pentafluoropropionic anhydride was added, and tubes were immediately capped and heated to  $65^{\circ}\text{C}$  for 45 min. Pentafluoropropionic anhydride was evaporated under a stream of nitrogen at  $20^{\circ}\text{C}$ , and the residue was reconstituted in 100  $\mu\text{l}$  ethyl acetate.

Quantitation of morphine was performed using a combined mass spectrometry/gas chromatography unit (Hewlett Packard, Palo Alto, CA) consisting of an HP 5890 II gas chromatography unit, 7673B autosampler port, and 5989A mass spectrometer. The mass spectrometer was operated in the SIM mode, monitoring 414.2  $m/z$  for morphine and 440.2  $m/z$  for nalorphine with a dwell of 200 ms/ion. The gas chromatography unit was equipped with an HP-5 MS column ( $30 \text{ m} \times 0.25 \text{ mm} \times 0.25 \text{ m}$ ) operated with an initial head pressure of 25 psi at  $150^{\circ}\text{C}$  in splitless constant flow mode with vacuum compensation. The injector and transfer line temperatures were maintained at  $280^{\circ}\text{C}$ . The chromatography

unit was programmed for  $150^{\circ}\text{C}$  for 1 min, then increased by  $15^{\circ}/\text{min}$  to  $270^{\circ}\text{C}$  until nalorphine eluted.

A standard curve plotting peak area/peak height ratio *versus* concentration was prepared from control samples containing known concentrations of morphine in blank plasma, and determination of morphine concentration in tissue was calculated from this curve.

### *Data Analysis*

The percentage of maximum possible effect (%MPE) was calculated for each mouse at each opiate dose and time point according to the following formula:

$$\%MPE = \frac{[(\text{postdrug latency} - \text{predrug latency}) / (\text{cutoff latency} - \text{predrug latency})] \times 100}{100}$$

where latency to hind-paw lick was measured in seconds, predrug latency was determined from an average of three predrug determinations, and cutoff latency was selected at 30 s as noted previously. The trapezoidal rule without extrapolation to infinite time, was used to calculate area under the %MPE *versus* time curves (AUCs) for each individual animal. Differences between the two mouse groups for AUCs generated from %MPE *versus* time curves and tissue concentrations of morphine were assessed by unpaired *t* test. In the cyclosporine/morphine study, analysis of variance for repeated measures was used to determine if cyclosporine altered morphine-induced analgesia. All data are expressed as mean  $\pm$  SD.  $P < 0.05$  was considered significant.

## **Results**

### *Analgesia Testing*

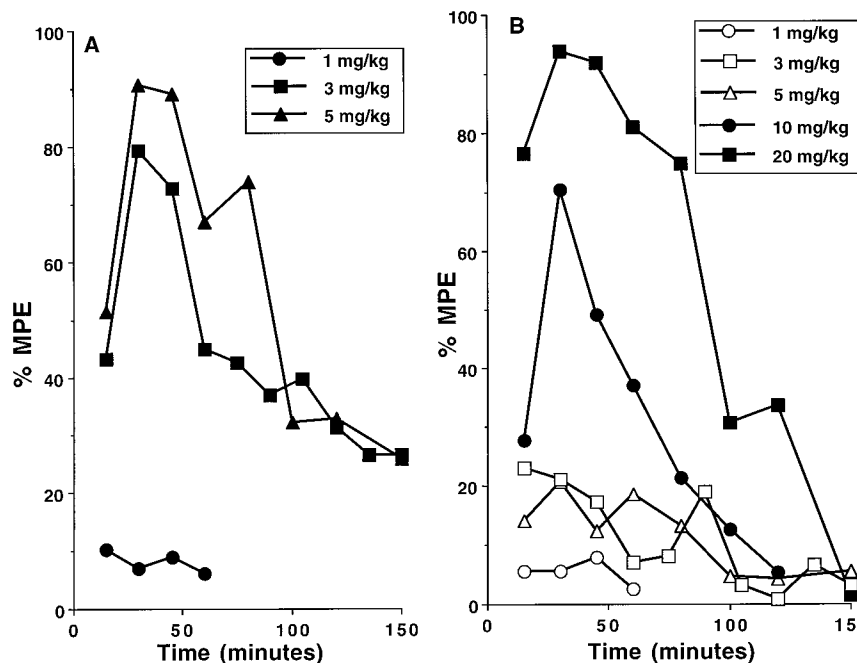
Figures 1-5 show the %MPE/dose-response curves for morphine (fig. 1), M-6-G (fig. 2), methadone (fig. 3), fentanyl (fig. 4), and meperidine (fig. 5). The AUCs derived from these %MPE *versus* time curves were significantly greater in the knockout mice for morphine at doses of 3 and 5 mg/kg, methadone, and fentanyl (table 1). The differences in AUCs for morphine at 1 mg/kg, M-6-G at all doses, and meperidine were not statistically significant (table 1).

### *P-glycoprotein Inhibition*

In P-glycoprotein knockout mice, cyclosporine alone did not alter hot-plate latency. After morphine injection

## OPIOID ANALGESIA AND P-GLYCOPROTEIN

**Fig. 1.** Average percent maximum possible effect (%MPE) versus time for morphine in P-glycoprotein knockout mice (A) and in wild-type mice that express P-glycoprotein (B). The area under these curves was significantly greater for the knockout mice at doses of 3 and 5 mg/kg. SD bars were omitted for clarity.



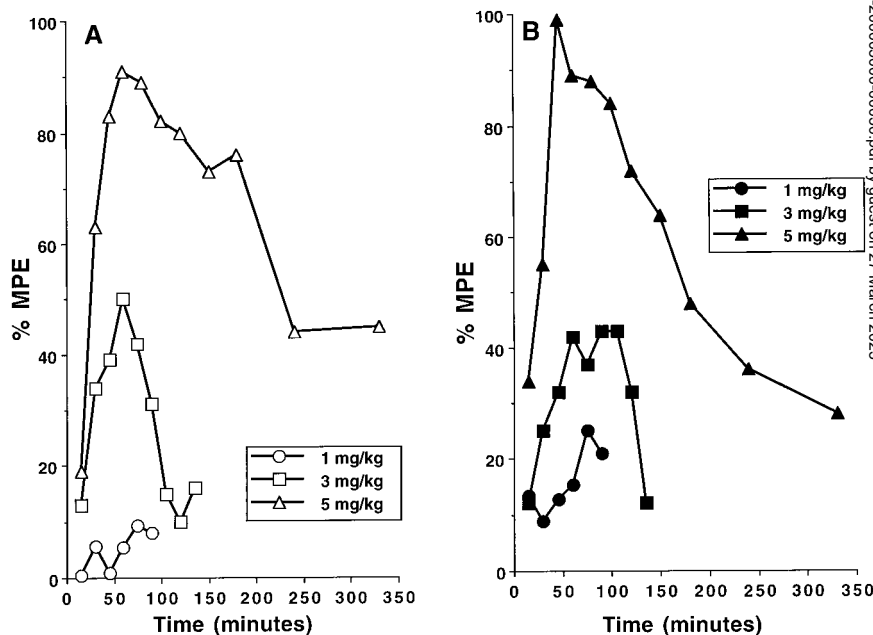
in this group, the animals developed significant analgesia, but there was no difference between animals pretreated with saline and those pretreated with cyclosporine (fig. 6). Similarly, wild-type animals did not develop analgesia after cyclosporine injection alone. However, after morphine injection, only animals that were pre-

treated with cyclosporine developed significant analgesia (fig. 6).

#### Morphine Tissue Concentrations

After 5 mg/kg subcutaneous morphine, the ratio of the concentration of morphine in brain to that in muscle

**Fig. 2.** Average percent maximum possible effect (%MPE) versus time for morphine-6-glucuronide in P-glycoprotein knockout mice (A) and in wild-type mice that express P-glycoprotein (B). The area under these curves was not significantly different between the two groups of mice. SD bars were omitted for clarity.



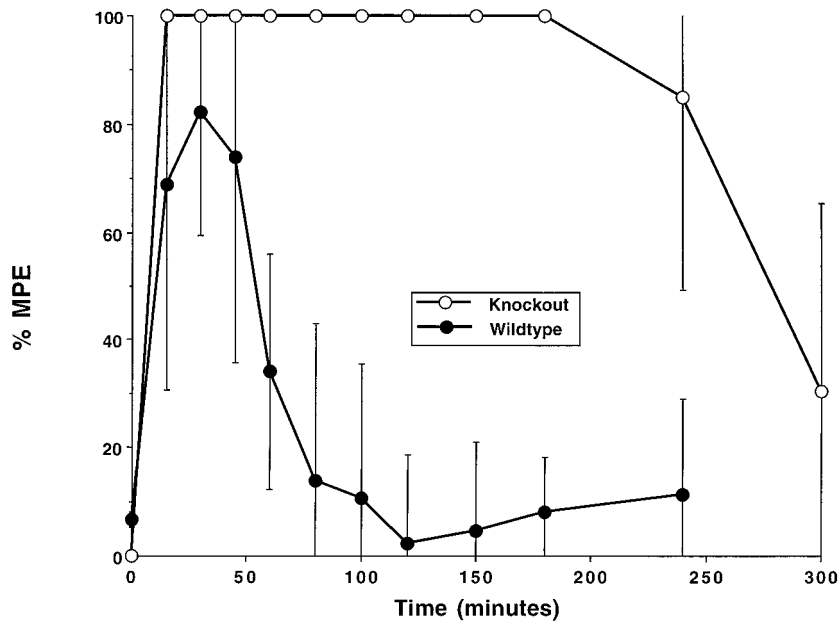


Fig. 3. Average percent maximum possible effect (%MPE) versus time for methadone in P-glycoprotein knockout mice and in wild-type mice that express P-glycoprotein. The area under these curves was significantly different between the two groups of mice.

was  $0.29 \pm 0.13$  in the knockout mice and  $0.06 \pm 0.04$  in the wild-type mice ( $P = 0.0054$ ).

## Discussion

The goal of this study was to determine whether P-glycoprotein plays an important physiologic role in limiting the analgesic efficacy of clinically used opioids. The data clearly indicate that the presence of P-glycoprotein

reduces both the magnitude and the duration of analgesia produced by morphine, methadone, and fentanyl, whereas the analgesic efficacy of M-6-G and meperidine are unaffected. Whether P-glycoprotein plays a similar role in humans is not clear from these data; however, it is notable that P-glycoprotein is also expressed in human brain capillary cells. Thus, humans have the same “machinery” and thus likely display the same physiology.

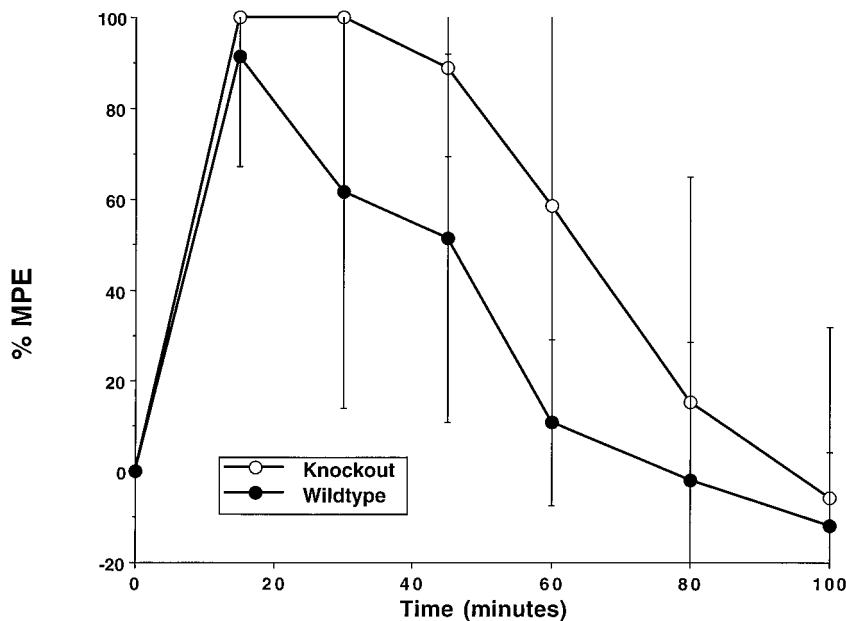


Fig. 4. Average percent maximum possible effect (%MPE) versus time for fentanyl in P-glycoprotein knockout mice and in wild-type mice that express P-glycoprotein. The area under these curves was significantly different between the two groups of mice.

## OPIOID ANALGESIA AND P-GLYCOPROTEIN

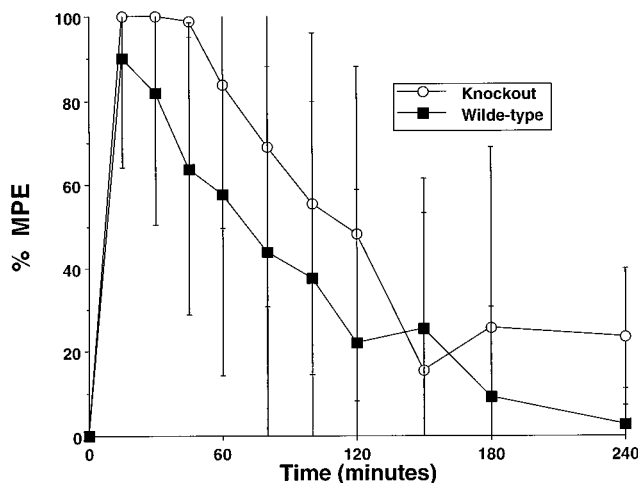


Fig. 5. Average percent maximum possible effect (%MPE) versus time for meperidine in P-glycoprotein knockout mice and in wild-type mice that express P-glycoprotein. The area under these curves was not significantly different between the two groups of mice.

The molecular mechanism by which P-glycoprotein limits the analgesia of some opioids is not precisely defined by this study. However, our data demonstrating that the brain-to-muscle ratio of morphine concentration was higher in P-glycoprotein knockout mice compared with wild-type mice suggest that P-glycoprotein limits morphine entry into brain. This explanation is consistent with the known ability of P-glycoprotein to actively remove xenobiotics from the interior of cells that express it.<sup>13</sup> Thus, we hypothesize that opioids that are P-glycoprotein substrates enter brain capillary endothelial cells and are actively “pumped” back into the plasma against their concentration gradient, thereby limiting their access to the underlying brain. This hypothesis is consistent with the current view that P-glycoprotein is an important functional component of the blood-brain barrier.

Another possible explanation for higher morphine concentrations in the knockout mice is higher morphine plasma concentrations. We did not measure morphine plasma concentrations but did measure morphine concentration in muscle as a control tissue. Because P-glycoprotein does not affect drug accumulation in muscle,<sup>14</sup> morphine concentration in muscle should depend only on the plasma concentration of morphine. Thus, by expressing morphine accumulation in brain as a ratio of the simultaneous brain and muscle concentrations, we have attempted to indirectly account for any intragroup and/or intergroup differences in mor-

phine plasma concentration. Additional evidence that P-glycoprotein does not alter morphine plasma concentrations comes from work by Letrent *et al.*,<sup>10</sup> who demonstrated that P-glycoprotein inhibition did not alter morphine plasma concentrations in rats.

When using knockout animals to study the physiologic role of a specific protein, it is tempting to assume that the only abnormality in the animal is that caused directly by the missing protein. However, it is always possible that absence of the protein during development results in significant “structural” physiologic or behavioral abnormalities that would not be reversed by restoration of the missing protein. However, the *mdr1a/1b* (-/-) mice used in this study are indistinguishable from their parent strain as long as they do not receive xenobiotics that are P-glycoprotein substrates. For example, their fecundity, perinatal survival rates, rate of weight gain, and life span are not different. Thus, we think it is reasonable to assume the differences between the wild-type and the knockout mice stem solely from the absence of P-glycoprotein and not from other underlying physiologic differences between the two groups.

Additional evidence that lack of P-glycoprotein is the sole source of differences between the two groups comes from our studies with the P-glycoprotein inhibitor, cyclosporine-A. Acute P-glycoprotein inhibition markedly increased the analgesic potency of morphine in wild-type mice that normally express P-glycoprotein but had no effect on P-glycoprotein-deficient mice. These findings indicate that cyclosporine itself has no effects on analgesia except for its ability to competitively inhibit P-glycoprotein. In this way, the analgesia studies in P-glycoprotein knockout animals and in P-glycoprotein-inhibited animals are consistent and complementary. It could be argued that cyclosporine increased morphine analgesia by increasing morphine plasma concentrations. However, Letrent *et al.*<sup>10</sup> demonstrated that P-glycoprotein inhibition with the potent P-glycoprotein inhibitor GF120918 did not significantly alter morphine clearance or plasma concentration in rats.

Our *in vivo* findings are in some respects consistent with earlier *in vitro* studies and in some ways are not. All of the opioids studied have been shown to be substrates for P-glycoprotein when studied using *in vitro* cell culture systems. However, it remained to be shown that P-glycoprotein actually altered opiate-induced analgesia *in vivo*. It is therefore interesting to find that meperidine- and M-6-G-induced analgesia are not affected by P-glycoprotein in this mouse model. The reasons for this are unclear, but a likely explanation for the absence of a

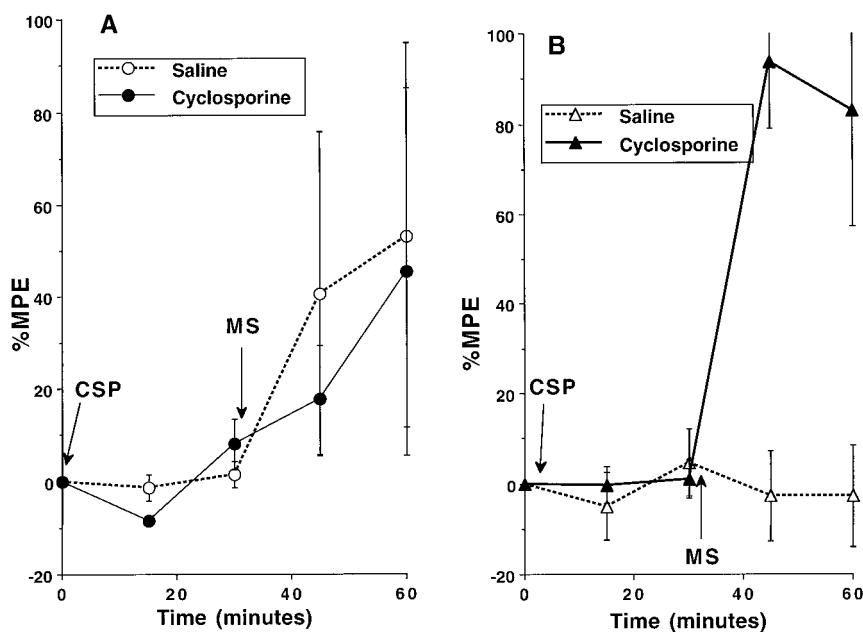
**Table 1. Area under the % MPE versus Time Curves for the Opioids Tested**

Drug	Dose (mg/kg)	Duration of Analgesia Testing (min)	AUC (%MPE * min)		P Value
			Knockout	Wild Type	
Morphine	1	60	434 ± 339	304 ± 349	0.474
	3	150	6453 ± 4359	1610 ± 1299	0.016
	5	180	7924 ± 3956	1732 ± 1373	0.003
Morphine-6-glucuronide	1	90	385 ± 292	823 ± 705	0.159
	3	135	3540 ± 1929	4060 ± 1951	0.625
	5	330	20461 ± 8490	17656 ± 5190	0.490
Fentanyl	0.05	100	6516 ± 1540	3547 ± 1540	0.003
Methadone	5	330	26862 ± 3107	7456 ± 3424	0.001
Meperidine	50	240	11963 ± 5487	8044 ± 5409	0.203

P-glycoprotein effect on M-6-G analgesia is suggested by Huwyler *et al.*,<sup>9</sup> who found that M-6-G uptake by murine leukemia cell line P388 tumor cells that do not express P-glycoprotein was only 0.3-fold greater than in the P-glycoprotein-expressing counterpart cell line MDR-P388. This relatively small difference in M-6-G accumulation suggests that M-6-G may be such a weak substrate for P-glycoprotein that differences in analgesia cannot be easily detected. Low affinity for P-glycoprotein would not seem to explain the fact that meperidine analgesia was not significantly altered by P-glycoprotein because Callaghan and Riordan<sup>8</sup> have shown that the binding affinity of meperidine exceeds that of both morphine and methadone *in vitro*.

It is possible that our study lacks sufficient statistical power to detect a difference in meperidine analgesia

between the two groups. The meperidine results produced a power of 0.3 assuming  $\alpha = 0.05$ . This relatively low power suggests that it is possible that we have made a type II error in concluding that there is no difference between the two groups of mice with respect to meperidine analgesia. However, even if we were to perform sufficient studies ( $n = 32$  in each group to achieve a power of 0.8), the magnitude of the difference in analgesia with meperidine (AUC 49% greater in knockout compared with wild-type mice) was modest compared with that with morphine (AUC 357% greater in knockout compared with wild-type mice at 5 mg/kg) and methadone (AUC 260% greater in knockout compared with wild-type mice at 5 mg/kg). It is not clear why there are differences among drugs in the extent to which they are restricted from the brain by P-glycoprotein. Potential



**Fig. 6. Average percent maximum possible effect (%MPE) versus time for analgesia in P-glycoprotein knockout mice (A) and in wild-type mice (B) after either saline or cyclosporine (CSP) administration followed 30 min later by morphine (MS) administration in all animals. Cyclosporine alone did not produce analgesia in either group. However, cyclosporine pretreatment significantly increased analgesia in wild-type mice but not in P-glycoprotein knockout mice.**

explanations include differences in the affinity with which drugs bind to P-glycoprotein or differences in a drug's inherent blood-brain-barrier permeability.

If these findings are representative of human pharmacology, the ramifications are potentially significant. For example, coadministration of multiple drugs that are P-glycoprotein substrates may competitively inhibit P-glycoprotein, resulting in increased uptake of drugs into tissues such as brain and small intestine and decreased clearance *via* the liver and/or kidneys. Fortunately, most P-glycoprotein substrates are not particularly potent P-glycoprotein inhibitors at clinically relevant concentrations and are therefore not particularly worrisome in this regard. However, some drugs used in supraphysiologic doses (*e.g.*, massive doses of methylprednisolone used to treat acute spinal cord injury) may reach concentrations that are high enough to affect the distribution and/or clearance of opioids such as morphine or methadone. This may become a larger problem in the near future because several potent P-glycoprotein inhibitors are now being investigated as cotherapeutic agents to improve the efficacy of standard chemotherapy regimens.<sup>15,16</sup> Also of concern is the fact that chronic exposure to P-glycoprotein substrates can induce P-glycoprotein upregulation, rendering cells increasingly resistant to drug therapy.<sup>17,18</sup> This raises the possibility that P-glycoprotein upregulation could potentially play a role in phenomena such as opioid tolerance.

In summary, we studied the analgesic efficacy of multiple opioids known to be P-glycoprotein substrates *in vitro*. We found that the analgesic efficacy of morphine, methadone, and fentanyl were increased in animals that lack P-glycoprotein, suggesting that P-glycoprotein plays an important role in limiting access of these drugs to the brain. In contrast, the analgesic efficacy of meperidine and M-6-G were not increased in animals that lacked P-glycoprotein, suggesting that P-glycoprotein does not limit the bioavailability of these opioids within the brain.

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