

# P-Glycoprotein Is a Major Determinant of Norbuprenorphine Brain Exposure and Antinociception

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## ABSTRACT

Norbuprenorphine is a major metabolite of buprenorphine and potent agonist of  $\mu$ ,  $\delta$ , and  $\kappa$  opioid receptors. Compared with buprenorphine, norbuprenorphine causes minimal antinociception but greater respiratory depression. It is unknown whether the limited antinociception is caused by low efficacy or limited brain exposure. Norbuprenorphine is an *in vitro* substrate of the efflux transporter P-glycoprotein (Mdr1), but the role of P-glycoprotein in norbuprenorphine transport *in vivo* is unknown. This investigation tested the hypothesis that limited norbuprenorphine antinociception results from P-glycoprotein-mediated efflux and limited brain access. Human P-glycoprotein-mediated transport *in vitro* of buprenorphine, norbuprenorphine, and their respective glucuronide conjugates was assessed by using transfected cells. P-glycoprotein-mediated norbuprenorphine transport and consequences *in vivo* were assessed by using *mdr1a*(+/+) and *mdr1a*(-/-) mice. Antinociception was determined by hot-

water tail-flick assay, and respiratory effects were determined by unrestrained whole-body plethysmography. Brain and plasma norbuprenorphine and norbuprenorphine-3-glucuronide were quantified by mass spectrometry. *In vitro*, the net P-glycoprotein-mediated efflux ratio for norbuprenorphine was nine, indicating significant efflux. In contrast, the efflux ratio for buprenorphine and the two glucuronide conjugates was unity, indicating absent transport. The norbuprenorphine brain/plasma concentration ratio was significantly greater in *mdr1a*(-/-) than *mdr1a*(+/+) mice. The magnitude and duration of norbuprenorphine antinociception were significantly increased in *mdr1a*(-/-) compared with *mdr1a*(+/+) mice, whereas the reduction in respiratory rate was similar. Results show that norbuprenorphine is an *in vitro* and *in vivo* substrate of P-glycoprotein. P-glycoprotein-mediated efflux influences brain access and antinociceptive, but not the respiratory, effects of norbuprenorphine.

## Introduction

The long-duration opioid buprenorphine has been used for several decades for treating acute and chronic pain and is now also marketed for opioid addiction and withdrawal therapy. Buprenorphine is a partial agonist at the  $\mu$  opioid receptor and an antagonist at the  $\kappa$  opioid receptor. Like other  $\mu$  agonists, it causes analgesia, respiratory depression, miosis, and mood changes, but unlike typical  $\mu$  agonists there is a ceiling effect at higher doses (Cowan et al., 1977; Walsh et al., 1994). Buprenorphine is extensively metabolized in humans, primarily to norbuprenorphine, and both undergo sub-

sequent glucuronidation to buprenorphine-3-glucuronide and norbuprenorphine-3-glucuronide (Zacny et al., 1997; Moody et al., 2009). In humans, peak plasma concentrations of norbuprenorphine are similar to or exceed those of buprenorphine. The relative norbuprenorphine, buprenorphine-3-glucuronide, and norbuprenorphine-3-glucuronide exposures, based on molar area under the plasma concentration versus time curves, are 200, 100, and 600% those of buprenorphine, respectively (Moody et al., 2002).

Norbuprenorphine is pharmacologically active, and norbuprenorphine formation may be a bioactivation pathway, rather than an inactivation pathway, as originally considered (Ohtani et al., 1995). Norbuprenorphine has high affinity for the  $\mu$ ,  $\delta$ , and  $\kappa$  opioid receptors (Huang et al., 2001; Brown et al., 2011). Norbuprenorphine causes marked respiratory depression in animal models (Ohtani et al., 1997; Brown et al., 2011) but has comparatively less antinociceptive effect (Ohtani et al., 1995). In contrast, buprenorphine

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**ABBREVIATIONS:** BBB, blood-brain barrier; P-gp, P-glycoprotein; MDR, multidrug resistance protein; MDCK, Madin-Darby canine kidney;  $P_{app}$ , apparent permeability; MPE, maximum possible effect; B  $\rightarrow$  A, basolateral to apical; A  $\rightarrow$  B, apical to basolateral; HBSS, Hanks' buffered salt solution; HBSSCM, HBSS supplemented with 1 mM CaCl<sub>2</sub>, 0.1 mM MgSO<sub>4</sub>, and 0.1 mM MgCl<sub>2</sub>; LC/MS/MS, liquid chromatography/tandem mass spectrometry.

causes much greater antinociception but has no respiratory effects in animal models (Ohtani et al., 1995). In rats, norbuprenorphine had 1/50th the analgesic potency of buprenorphine but 10-fold greater respiratory depressant potency (Ohtani et al., 1997). Norbuprenorphine-3-glucuronide ( $\kappa$ , but not  $\mu$  or  $\delta$ ) and buprenorphine-3-glucuronide ( $\mu$  and  $\delta$ , but not  $\kappa$ ) also have affinity for opioid receptors and are biologically active, causing mild respiratory and antinociceptive effects, respectively (Brown et al., 2011). The role of norbuprenorphine-3-glucuronide in mediating the effects of norbuprenorphine, norbuprenorphine-3-glucuronide analgesic efficacy, and central nervous system access are unknown. The mechanism of limited norbuprenorphine antinociception, as well as that underlying the differences between norbuprenorphine and buprenorphine, is unknown. These mechanisms have been attributed to either low norbuprenorphine intrinsic analgesic activity or limited brain access of norbuprenorphine (Ohtani et al., 1995).

With the exception of essential nutrients, the movement of endogenous and exogenous substances from the blood to the brain is highly restricted by the blood-brain barrier (BBB). The BBB is formed by a unique suite of tight junction proteins, receptors, and transporters expressed by the endothelial cells that make up the microvasculature of the brain (Abbott, 2005; Engelhardt, 2011). Because of the restrictive nature of the BBB, the vast majority of molecules that bind with high affinity to one or more receptors in the brain have little biological activity in vivo (Pardridge, 1997). P-glycoprotein [P-gp; multidrug resistance protein 1 (MDR1), ABCB1] is one of several transmembrane efflux transporters at the BBB that use ATP hydrolysis to protect the brain from a wide variety of exogenous and endogenous compounds. P-glycoprotein has a broad substrate profile, has been shown to regulate brain access of numerous drugs via active efflux, and determines the pharmacologic effect of several drugs acting in the central nervous system (Kim et al., 1998; Jolliet-Riant and Tillement, 1999; Boulton et al., 2002; Uhr et al., 2003; Dagenais et al., 2004; Wang et al., 2004; Sasongko et al., 2005).

Several opioids have been reported previously in animals and/or humans to be in vitro or in vivo substrates for P-glycoprotein, including loperamide (Wandel et al., 2002; Skarke et al., 2003), morphine (Xie et al., 1999; Wandel et al., 2002; Dagenais et al., 2004), methadone (Dagenais et al., 2004; Kharasch et al., 2004), fentanyl (Wandel et al., 2002; Dagenais et al., 2004), alfentanil (Wandel et al., 2002; Kalvass et al., 2007), and sufentanil (Wandel et al., 2002). Norbuprenorphine has been identified as a substrate for P-gp-mediated efflux by in vitro screening using transfected Madin-Darby canine kidney (MDCK) cells (Tournier et al., 2010).

Mice lacking *Mdr1a* and/or *Mdr1b* have been shown to be useful tools for investigating the in vivo role of P-gp in pharmacology (Doran et al., 2005; van Waterschoot and Schinkel, 2011). Genetic P-gp knockouts have been compared with chemical P-gp knockouts in several studies, and the results have shown that genetic disruption of the P-gp gene product has the same effect as chemically inhibiting the transporter (Polli et al., 1999; Zong and Pollack, 2000). *Mdr1a*( $-/-$ ) mice have been used to determine that P-gp limits opioid-induced analgesia of morphine, methadone, and fentanyl (Thompson et al., 2000; Hassan et al., 2009).

The purpose of the current investigation was to test the hypothesis that limited norbuprenorphine antinociception is

the result of P-gp-mediated efflux and limited brain access, using in vitro and in vivo methods. It evaluated P-gp-mediated transport of buprenorphine, norbuprenorphine, buprenorphine-3-glucuronide, and norbuprenorphine-3-glucuronide in vitro and the antinociceptive and respiratory effects of norbuprenorphine in vivo, using *mdr1a*-deficient and wild-type mice. This research is significant because it identifies norbuprenorphine as an avid substrate of P-glycoprotein, which influences norbuprenorphine brain access, and it provides evidence for blood-brain barrier transport differentially influencing the classic opioid effects of analgesia and respiratory depression.

## Materials and Methods

**Materials.** Buprenorphine, norbuprenorphine, and buprenorphine-3- $\beta$ -D-glucuronide were provided by the National Institute on Drug Abuse (Bethesda, MD). Norbuprenorphine-3- $\beta$ -D-glucuronide was synthesized as described previously (Fan et al., 2011). Buprenorphine, norbuprenorphine, buprenorphine-3- $\beta$ -D-glucuronide, and norbuprenorphine-3-glucuronide used as analytical standards were from Cerrilliant (Round Rock, TX). Dulbecco's modified Eagle's media, heat-inactive fetal bovine serum, and Hanks' buffered salt solution (HBSS) were obtained from Mediatech (Herndon, VA). Bac-off antibiotic was obtained from Cell Systems, Inc. (Kirkland, WA). Cell transport assays were conducted by using the Corning HTS Transwell system (Corning Life Sciences, Lowell, MA). All other reagents were from Sigma-Aldrich (St. Louis, MO).

**In Vitro Transport Assay.** MDCK cells transfected with the gene encoding human MDR1 (P-gp) were obtained from P. Borst (Netherlands Cancer Institute, Amsterdam, The Netherlands). Both the untransfected and transfected cell lines were cultured in Dulbecco's modified Eagle's media containing 10% fetal bovine serum and  $1\times$  Bac-off antibiotic. Cells were seeded onto the Transwell supports at a density of 49,000 cells/well and incubated for 4 days at  $37^\circ\text{C}/95\%$  humidity/ $5\%$   $\text{CO}_2$ .

On the day of the assay the media were aspirated and replaced with HBSS supplemented with 1 mM  $\text{CaCl}_2$ , 0.1 mM  $\text{MgSO}_4$ , and 0.1 mM  $\text{MgCl}_2$  (HBSSCM) and allowed to equilibrate for 30 min at  $37^\circ\text{C}$ . The buffer was then aspirated and replaced with fresh HBSSCM in either the apical (upper) or basolateral (lower) chamber. HBSSCM that contained 2  $\mu\text{M}$  of the test drug was added to the opposite chamber, and the cells were incubated for 2 h at  $37^\circ\text{C}/95\%$  humidity/ $5\%$   $\text{CO}_2$ . The donor and receiver chambers for each well were sampled and analyzed by LC/MS/MS as described below. Monolayer integrity was checked by using lucifer yellow. In brief, 200  $\mu\text{l}$  of a 100  $\mu\text{g}/\text{ml}$  lucifer yellow solution in HBSSCM was added to the basolateral compartment of each well, and 100  $\mu\text{l}$  of HBSSCM (without lucifer yellow) was added to the apical chamber. After incubating the cells for 1 h at  $37^\circ\text{C}/95\%$  humidity/ $5\%$   $\text{CO}_2$  the receiver (apical) chamber and the donor chamber (basolateral) were sampled, and the lucifer yellow-related fluorescence was determined by using a BioTEK SynergyMX plate reader (BioTek Instruments, Winooski, VT). Wells were rejected if the permeability of the lucifer yellow was above 2%.

Apparent permeability ( $P_{app}$ ) for both the MDCK wild-type and MDR1-transfected MDCK cells were calculated by using eq. 1, where  $V_r$  is the volume of buffer in the receiver,  $A$  is the surface area of the Transwell membrane ( $0.14\text{ cm}^2$ ),  $t$  is the incubation time in seconds, and  $[\text{Drug}]_r$  and  $[\text{Drug}]_d$  are the drug concentrations of the receiver and donor wells, respectively at time  $t$ .

$$P_{app} = \frac{V_r}{(A \cdot t)} * \frac{[\text{Drug}]_r}{[\text{Drug}]_d} \quad (1)$$

The directional ratio for each cell line was calculated by dividing the apparent permeability value for the basolateral-to-apical direc-

tion by the apparent permeability value for the apical-to-basolateral direction as in eq. 2.

$$\text{efflux ratio} = \frac{P_{appB \rightarrow A}}{P_{appA \rightarrow B}} \quad (2)$$

The net efflux ratio was calculated by dividing the directional ratio value for the MDR1-transfected cell line by the directional ratio of the untransfected MDCK cells by using eq. 3. A net efflux ratio above 2.0 was the criterion used to define significant substrate-transporter interaction (Polli et al., 2001).

$$\text{Net efflux ratio} = \frac{\text{efflux ratio}_{\text{MDR1}}}{\text{efflux ratio}_{\text{wt}}} \quad (3)$$

The mean and S.D. were calculated from three wells for each direction within the same experiment.

**Animals.** Male CF-1 *mdr1a*(+/+) and *mdr1a*(-/-) mice (29–35 g) were purchased from Charles River Laboratories, Inc. (Wilmington, MA). All mice were group-housed on a 12-h light/dark schedule with ad libitum access to food and water. Experiments were performed in accordance with the National Institutes of Health's *Guide for the Care and Use of Laboratory Animals* (Institute of Laboratory Animal Resources, 1996) and approved by the Animal Care and Use Committee of Washington University School of Medicine (St. Louis, MO).

Previous studies in our laboratory showed that a 1 mg/kg dose of norbuprenorphine in wild-type mice elicited a significant decrease in respiratory rate (Brown et al., 2011). Based on the in vitro indication that norbuprenorphine is a P-gp substrate and the hypothesis that decreased efflux from the brain in a P-gp-deficient mouse may result in increased norbuprenorphine effects, a pilot study was performed to determine the norbuprenorphine dose that would elicit the same respiratory effect in the *mdr1a*(-/-) mice as the 1 mg/kg dose administered to the *mdr1a*(+/+) mice. *mdr1a*(+/+) and *mdr1a*(-/-) mice ( $n = 4$  each) were used in the pilot study. A 0.25 mg/kg dose resulted in a 60% decrease in respiratory rate in the *mdr1a*(-/-) mice, which was the same response seen in the *mdr1a*(+/+) mice administered 1 mg/kg. For all further experiments, these equieffective doses of norbuprenorphine were used.

**Collection of Tissue Samples.** P-gp-competent *mdr1a*(+/+) and P-gp-deficient *mdr1a*(-/-) mice ( $n = 22$  each) were administered norbuprenorphine subcutaneously [1 mg/kg for *mdr1a*(+/+) and 0.25 mg/kg for *mdr1a*(-/-) mice]. At 5, 10, 20, 30, 45, and 60 min postdose respiratory rate and antinociception were assessed. Mice were anesthetized with sevoflurane, and blood was collected by cardiac puncture into heparinized Microtainers (BD Biosciences, San Jose, CA). Blood was centrifuged at 14,000 rpm for 1 min to separate plasma. After exsanguination, whole brains were harvested and flash-frozen. Plasma and brain were stored at  $-20^{\circ}\text{C}$  until analysis.

**Quantitation of Norbuprenorphine, Norbuprenorphine-3-Glucuronide, Buprenorphine, and Buprenorphine-3-Glucuronide.** Brain and plasma were prepared for analysis by LC/MS/MS as described previously (Brown et al., 2011). In vitro samples were prepared by the addition of  $d_5$ -norfentanyl (100 ng/ml) as an internal standard. LC/MS/MS was used to quantify norbuprenorphine and norbuprenorphine-3-glucuronide in brain and plasma and all four compounds in the in vitro assay samples. LC/MS/MS as analysis was performed as described previously (Brown et al., 2011). Brain concentrations were corrected for drug present in brain vasculature, assuming 1% of total brain weight caused by brain vasculature. No correction was applied to plasma drug concentrations.

**Tail-Flick Antinociception Assay.** A tail-flick assay (Montana et al., 2009) was used to test the antinociceptive effect of norbuprenorphine in *mdr1a*(-/-) and *mdr1a*(+/+) mice. Tail-flick latency, defined by the time in seconds for tail withdrawal from a warm-water bath ( $52^{\circ}\text{C}$ ), was measured as described previously (Brown et al., 2011), using an IITC 500 warm-water tail-immersion test analgesia meter (IITC Life Science, Woodland Hills, CA). Baseline tail-

flick latency was determined for each mouse before drug dosing. A cutoff of 10 s was used to prevent tissue damage. Animals not responding within 3 s were excluded from the assay. Maximum possible effect (MPE) was calculated as:  $[(T_1 - T_0)/(T_2 - T_0)] \times 100$ , where  $T_0$  and  $T_1$  represent latencies before and after drug administration, and  $T_2$  is the cutoff time. Tail-flick latency was obtained for each animal after removal from a plethysmograph chamber and record of its respiratory rate.

**Unrestrained Whole-Body Plethysmography.** Measurements of respiratory rate were obtained by using unrestrained whole-body plethysmography (Buxco Research Systems, Wilmington, NC) as described previously (Brown et al., 2011). The plethysmograph consisted of eight animal chambers with orifices for entry and exit of breathing air and a 1-ml syringe permitting calibrations, connected to a differential pressure transducer. The air entry orifice was connected to a source of compressed breathing air. Immediately before each experiment, each chamber was calibrated with 1 ml of room air. Each awake mouse was placed in a chamber immediately after administration of norbuprenorphine. Respiratory parameters were recorded for 5, 10, 20, 30, 45, and 60 min. Respiratory values were converted by Biosystems XA software (Buxco Research Systems).

**Statistical Analysis.** Results are expressed as the mean  $\pm$  S.D. Two-way repeated-measures analysis of variance (time versus group), followed by the Student-Newman-Keuls test, was used to test for significant differences between groups in mouse experiments (Sigma Plot 12.2; Systat Software, Inc., San Jose, CA).

Norbuprenorphine concentration-effect data in *mdr1a*(-/-) and *mdr1a*(+/+) mice were analyzed by nonlinear regression fitting (Sigma Plot 12.2) of the sigmoid  $E_{\text{max}}$  model (eq. 4) to antinociception versus plasma or brain concentrations, where  $E_{\text{max}}$  was defined as 100%, and  $\gamma$  was constrained to be the same for *mdr1a*(-/-) and *mdr1a*(+/+) mice as described previously (Kalvass et al., 2007).

$$\% \text{MPE} = \frac{E_{\text{max}} \cdot C^{\gamma}}{EC_{50}^{\gamma} + C^{\gamma}} \quad (4)$$

## Results

**In Vitro Transport Results.** The well established Transwell assay using MDCK cells transfected with human MDR1 was used to determine whether buprenorphine, norbuprenorphine, or their glucuronide metabolites were substrates for P-gp. Transport activity for each drug was measured in the basolateral-to-apical (B  $\rightarrow$  A) and the apical-to-basolateral (A  $\rightarrow$  B) direction across MDCK parental and MDR1-transfected cell lines (Table 1). In the MDR1-transfected MDCK cells the B  $\rightarrow$  A transport of norbuprenorphine was approximately 20-fold greater than that observed for apical-to-basolateral transport (Table 1). When the ratio was corrected for the influence of the endogenous transporters (by comparison against nontransfected cells) the net efflux ratio was nine. Buprenorphine, buprenorphine-3- $\beta$ -D-glucuronide, and norbuprenorphine-3- $\beta$ -D-glucuronide all had net efflux ratios less than the cutoff of 2.0 used to define a transporter substrate (Table 1). Loperamide, a known P-gp substrate (Polli et al., 2001), was used as a positive control. The net efflux ratio for loperamide was 13.

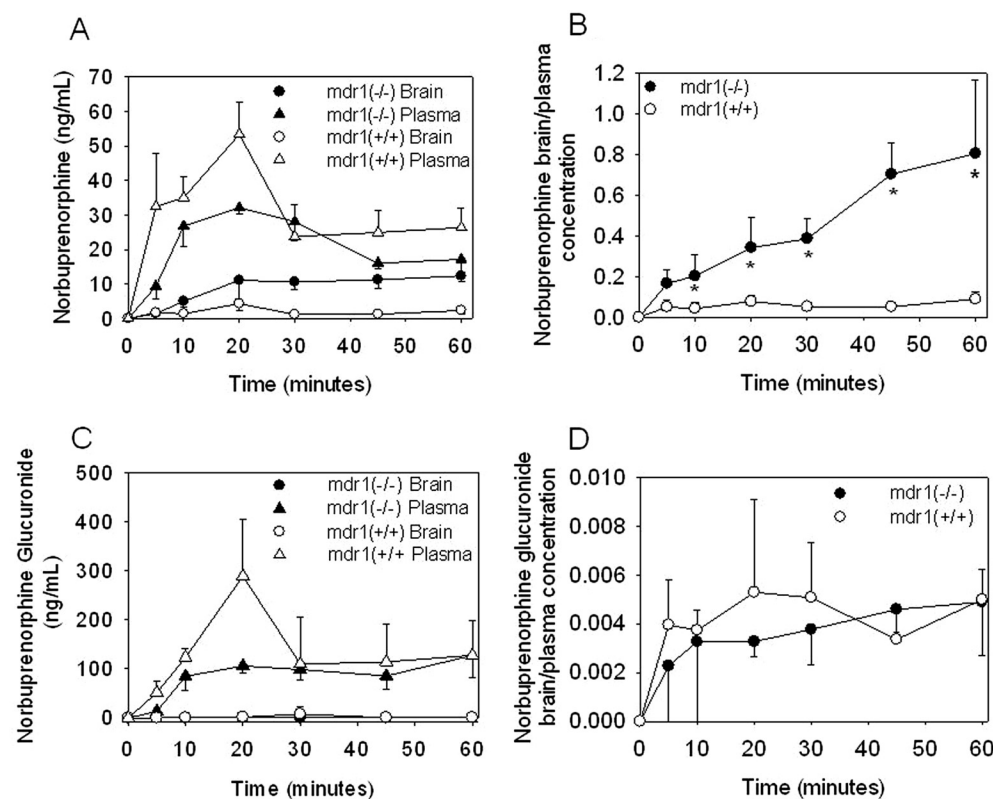
**Disposition of Norbuprenorphine in *mdr1a*(-/-) and *mdr1a*(+/+) Mice.** The brain-to-plasma ratio of norbuprenorphine in the *mdr1a*(+/+) mice was low ( $<0.1$ ) at the 5-min time point and remained essentially unchanged throughout the experiment (Fig. 1). In contrast, the brain-to-plasma ratio in the *mdr1a*(-/-) mice was  $>0.1$  at the 5-min time point and continued to increase over the time course of the experiment, approaching 1 by the 60-min time point (Fig. 1).

TABLE 1

Bidirectional transport across untransfected and MDR1-transfected MDCK cells

Data are presented as the  $P_{app}$ . The efflux ratio for each cell line was determined as  $P_{app(B \rightarrow A)}/P_{app(A \rightarrow B)}$ . The net efflux ratio was calculated as  $\text{efflux ratio}_{MDR1}/\text{efflux ratio}_{MDCK}$ . Compounds are considered P-gp substrates if the net efflux ratio is more than 2.0, as defined under *Materials and Methods*. Results are the mean  $\pm$  S.D. for triplicate wells performed on the same day.

	$P_{app}$		B $\rightarrow$ A/A $\rightarrow$ B		Net Efflux
	MDCK	MDR1	MDCK	MDR1	
	<i>nm/s</i>				
Buprenorphine					
B $\rightarrow$ A	793 $\pm$ 26	821 $\pm$ 153	81.7	45.5	0.6
A $\rightarrow$ B	10 $\pm$ 3	18 $\pm$ 4			
Norbuprenorphine					
B $\rightarrow$ A	211 $\pm$ 5	170 $\pm$ 6	2.2	19.8	9
A $\rightarrow$ B	91 $\pm$ 5	8 $\pm$ 1			
Buprenorphine 3-glucuronide					
B $\rightarrow$ A	166 $\pm$ 1	156 $\pm$ 19	3.8	3.8	1
A $\rightarrow$ B	44 $\pm$ 10	41 $\pm$ 11			
Norbuprenorphine 3-glucuronide					
B $\rightarrow$ A	159 $\pm$ 37	222 $\pm$ 49	1.1	0.9	0.9
A $\rightarrow$ B	197 $\pm$ 12	205 $\pm$ 19			
Loperamide					
B $\rightarrow$ A	144 $\pm$ 17	117 $\pm$ 43	1.8	23.3	13
A $\rightarrow$ B	80 $\pm$ 13	3 $\pm$ 1			



**Fig. 1.** Disposition of norbuprenorphine and norbuprenorphine glucuronide. Concentrations of norbuprenorphine (A and B) and norbuprenorphine-3-glucuronide (C and D) were determined in brain homogenate (circles) and plasma (triangles) by LC/MS/MS. Results are shown for *mdr1a(+/+)* mice (open symbols) and *mdr1a(-/-)* mice (closed symbols). Measured concentrations are shown in A and C; norbuprenorphine and norbuprenorphine glucuronide brain/plasma ratios are shown in B and D. Each data point is the mean  $\pm$  S.D. ( $n = 4$ ). \*, significantly different from *mdr1a(+/+)* controls ( $p < 0.05$ ) by two-way repeated-measures analysis of variance.

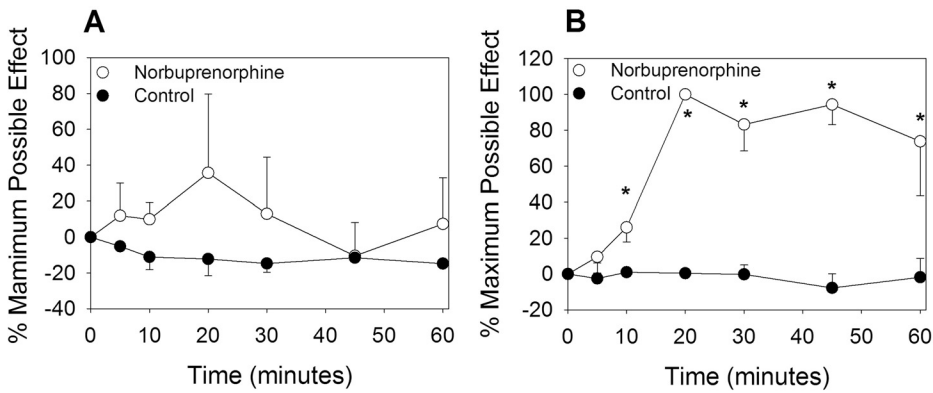
For norbuprenorphine 3-glucuronide there was no significant difference in the brain-to-plasma ratios in the *mdr1a(-/-)* and *mdr1a(+/+)* mice, and the ratio was less than 0.1 throughout the experiment.

The plasma concentrations of norbuprenorphine were 4-fold less in the *mdr1a(-/-)* mice than the *mdr1a(+/+)* mice, consistent with the *mdr1a(-/-)* mice receiving one-fourth the dose of that in the *mdr1a(+/+)* mice (Fig. 1). Plasma concentrations of norbuprenorphine-3-glucuronide were also 4-fold less in the *mdr1a(-/-)* mice than the *mdr1a(+/+)* mice, again consistent with the norbuprenorphine dose difference. Plasma norbuprenorphine-3-glucuro-

nide/norbuprenorphine concentration ratios were similar in the *mdr1a(-/-)* and *mdr1a(+/+)* mice.

**Antinociceptive Effect of Norbuprenorphine in *mdr1a(-/-)* and *mdr1a(+/+)* Mice.** In the *mdr1a(+/+)* mice, norbuprenorphine peak effect was 30% of MPE (Fig. 2). Maximal antinociception occurred 20 min postdosing, and effects returned to baseline at 45 min. In contrast, the *mdr1a(-/-)* mice had markedly increased magnitude and duration of norbuprenorphine antinociception. The effect peaked at 100% MPE at 20 min and only declined to 80% by 60 min (Fig. 2).

Norbuprenorphine concentration-effect curves were constructed for both plasma and brain concentrations. There



**Fig. 2.** Antinociceptive effects of norbuprenorphine in *mdr1a(-/-)* and wild-type mice. Time to withdrawal of the tail from a hot-water (52°C) bath, or tail-flick latency was measured after subcutaneous injection of vehicle or norbuprenorphine [1 mg/kg in the *mdr1a(+/+)* mice; 0.25 mg/kg in the *mdr1a(-/-)* mice]. The percentage of MPE of the *mdr1a(+/+)* mice (A) and the *mdr1a(-/-)* mice (B) was calculated every 15 min for 60 min. Each data point is the mean  $\pm$  S.D. (10/group). \*, significantly different from vehicle control ( $p < 0.05$ ) by two-way repeated-measures analysis of variance.

was a substantial leftward shift of the plasma concentration-effect curve in the *mdr(-/-)* compared with the wild-type mice (Fig. 3). The norbuprenorphine plasma  $EC_{50}$  was  $14 \pm 5$  in the *mdr1a(-/-)* mice compared with  $471 \pm 255$  in the *mdr1(+/+)* mice. In contrast, there was no apparent difference between the two mouse strains in the brain concentration-effect relationship for buprenorphine, which could be described by a common exponential, with the brain  $EC_{50}$  of  $6 \pm 1$  (Fig. 4).

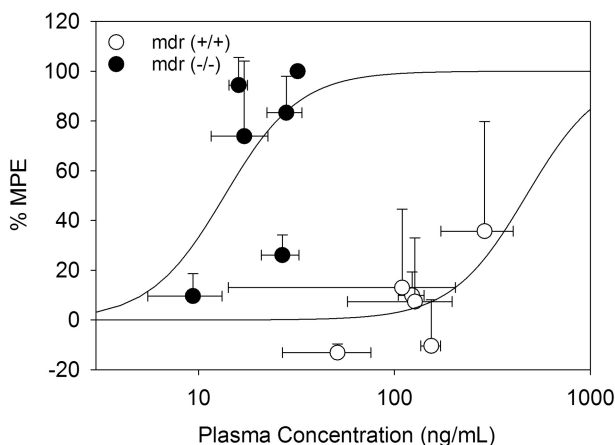
**Norbuprenorphine Respiratory Effects in *mdr1a(-/-)* and *mdr1a(+/+)* Mice.** By experimental design (see *Materials and Methods*) a dose of 0.25 mg/kg norbuprenorphine elicited the same effect in *mdr1a(-/-)* mice as that seen in the *mdr1a(+/+)* mice that received 1 mg/kg (Fig. 5). Respiratory rates decreased approximately 60% from baseline in both groups. There was no change in tidal volume after administration of norbuprenorphine in either *mdr1a(-/-)* or *mdr1a(+/+)* mice.

**Behavioral Observations.** During the course of the experiment, several behaviors were observed in the P-gp-deficient *mdr1a(-/-)* mice that received norbuprenorphine. At 15 min after administration of norbuprenorphine, the *mdr1a(-/-)* mice exhibited Straub tails (Bilbey et al., 1960). The *mdr1a(-/-)* mice exhibited loss of the righting reflex (Oka et al., 1992; Kamei et al., 1996), with the mice unable to right themselves upon being released after the

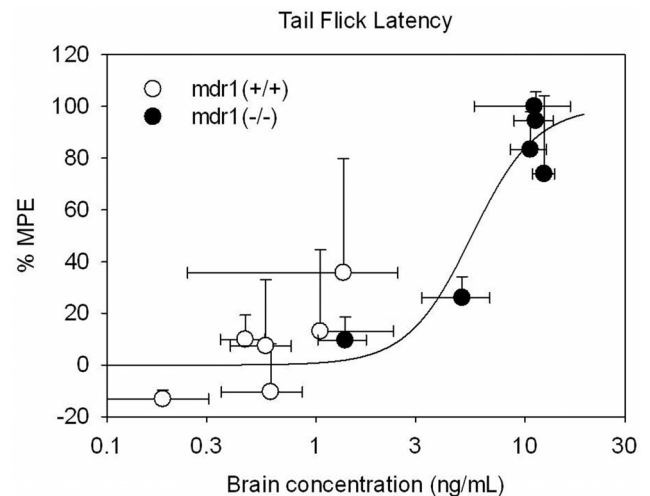
tail-flick procedure. In comparison, the *mdr1a(+/+)* mice did not show Straub tails or loss of righting reflex. Both groups of *mdr1a(+/+)* and *mdr1a(-/-)* mice seemed equally sedated after norbuprenorphine, with minimal movements approximately 15 min after norbuprenorphine dosing.

## Discussion

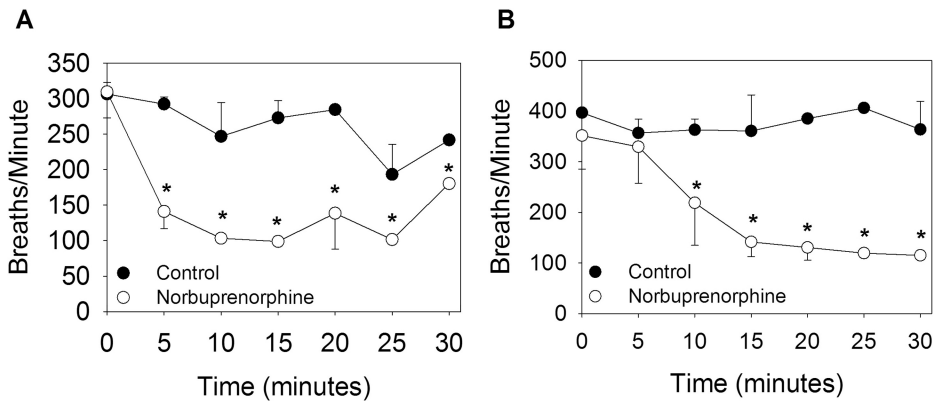
The purpose of this investigation was to evaluate P-gp-mediated transport of buprenorphine, norbuprenorphine, norbuprenorphine-3-glucuronide, and buprenorphine-3-glucuronide in vitro and test the hypothesis that P-gp-mediated efflux limits brain access of norbuprenorphine (and possibly norbuprenorphine-3-glucuronide) and influences norbuprenorphine antinociception. P-gp-transfected MDCK cells were used to screen for P-gp substrates specifically, rather than as a model for blood-brain transport more generally. The major result was that norbuprenorphine was a P-gp substrate both in vitro and in vivo. As measured by the net efflux ratio in transfected cells, P-gp-mediated norbuprenorphine efflux was substantial and approximated that of the unambiguous P-gp substrate loperamide (Polli et al., 2001) (9 versus 13, respectively). P-gp-mediated norbuprenorphine



**Fig. 3.** Relationship between antinociception and plasma norbuprenorphine concentration. The relationship shown is after subcutaneous injections of 0.25 mg/kg norbuprenorphine in *mdr1a(-/-)* mice (●) and 1.0 mg/kg norbuprenorphine in *mdr1(+/+)* mice (○). Data are mean  $\pm$  S.D. ( $n = 4$ ). Lines represent the fit of a sigmoidal  $E_{max}$  model to the effect data.



**Fig. 4.** Relationship between antinociception and brain norbuprenorphine concentration. The relationship shown is after subcutaneous injections of 0.25 mg/kg norbuprenorphine in *mdr1a(-/-)* mice (●) and 1.0 mg/kg norbuprenorphine in *mdr1(+/+)* mice (○). Data are mean  $\pm$  S.D. ( $n = 4$ ). Lines represent the fit of a sigmoidal  $E_{max}$  model to the effect data.



**Fig. 5.** Respiratory rate in *mdr1a*(-/-) and wild-type mice administered norbuprenorphine. Respiratory rate and tidal volume in *mdr1a*(+/+) mice (A) and *mdr1a*(-/-) mice (B) after subcutaneous injection of norbuprenorphine or saline vehicle were measured by unrestrained whole-body plethysmography. Results are mean  $\pm$  S.D. (four per group). \*, significantly different from vehicle control ( $p < 0.05$ ) by two-way repeated-measures analysis of variance. There was no effect of norbuprenorphine on tidal volume.

brain efflux was also observed in vivo. In *mdr1a*(+/+) animals, the norbuprenorphine brain/plasma ratio was negligible and constant. In contrast, in *mdr1a*(-/-) animals the norbuprenorphine brain/plasma ratio increased steadily, approaching unity. This demonstrates that P-gp represents a major barrier to norbuprenorphine entering the brain and elimination of transporter function resulted in increased norbuprenorphine brain concentrations, which essentially equaled plasma concentrations. Thus, absent P-gp-mediated efflux, norbuprenorphine accumulates in the brain until the drug is equally distributed between brain and plasma. The low norbuprenorphine brain concentration in *mdr1a*(+/+) mice probably is caused by a combination of low passive permeability and substantial P-gp-mediated efflux (Ohtani et al., 1995).

P-gp-mediated brain access significantly influenced the pharmacologic effect of norbuprenorphine. Antinociception was greater in both magnitude and duration in *mdr1a*(-/-) compared with *mdr1a*(+/+) mice despite the *mdr1a*(-/-) mice receiving one-fourth the norbuprenorphine dose of the *mdr1a*(+/+) mice. Moreover, the duration of norbuprenorphine antinociception in *mdr1a*(-/-) mice was even greater than that observed after a maximally antinociceptive buprenorphine dose in *mdr1a*(+/+) mice (Brown et al., 2011). There was a substantial shift of the norbuprenorphine plasma concentration-effect curve for antinociception and more than an order of magnitude difference in the plasma  $EC_{50}$  between *mdr1a*(-/-) and *mdr1a*(+/+) mice, yet there was no difference in the brain concentration-effect curves. In addition, some characteristic opioid-induced behaviors were observed after norbuprenorphine in *mdr1a*(-/-) but not *mdr1a*(+/+) mice. The *mdr1a*(-/-) mice administered norbuprenorphine exhibited Straub tails, the tail held erect because of opioid-induced contraction of the sacrococcygenus muscle (Bilbey et al., 1960; Aceto et al., 1969). This has been shown to be mediated through the central  $\mu_2$  opioid receptor subtype (Nath et al., 1994). The *mdr1a*(-/-) mice also exhibited loss of righting reflex, another  $\mu_2$  opioid receptor-mediated effect (Oka et al., 1992; Kamei et al., 1996). Together, these findings strongly support the hypothesis that P-gp regulates brain access of norbuprenorphine, and greater norbuprenorphine brain access in P-gp-deficient mice caused greater antinociceptive and behavioral effects.

Norbuprenorphine is a  $\mu$ ,  $\delta$ , and  $\kappa$  receptor agonist, as shown in vitro (Huang et al., 2001; Brown et al., 2011). Thus, the poor antinociceptive effects of norbuprenorphine in wild-type mice could be caused by limited brain exposure rather

than low intrinsic analgesic efficacy of norbuprenorphine. Although norbuprenorphine was shown to have an analgesic effect one-fourth that of buprenorphine after an intraventricular dose in rats (Ohtani et al., 1995), intraventricular dosing in a wild-type animal may still be accompanied by substantial P-gp-mediated efflux and therefore less overall brain exposure.

Both buprenorphine-3-glucuronide and norbuprenorphine-3-glucuronide are abundant buprenorphine metabolites in humans in vivo (McCance-Katz et al., 2006, 2007) and were found to be biologically active in mice (Brown et al., 2011). The potential contribution of norbuprenorphine-3-glucuronide to norbuprenorphine effects and influence of P-gp deficiency on norbuprenorphine effects were unknown. Therefore, P-gp-mediated norbuprenorphine-3-glucuronide transport was evaluated, along with that of buprenorphine and buprenorphine-3-glucuronide. In vitro, neither buprenorphine, buprenorphine-3-glucuronide, nor norbuprenorphine-3-glucuronide were P-gp substrates. The net efflux ratio was approximately one for each. In vivo norbuprenorphine-3-glucuronide brain/plasma concentration ratios after norbuprenorphine were very small and unaffected by P-gp deficiency, indicating that norbuprenorphine-3-glucuronide has limited brain access, and P-gp does not influence norbuprenorphine-3-glucuronide efflux or brain concentrations in vivo.

Although brain norbuprenorphine-3-glucuronide concentrations were low, they were not zero, indicating that despite the highly hydrophilic nature of this glucuronide, it did pass the blood-brain barrier. Limited but detectable brain access of norbuprenorphine-3-glucuronide is consistent with previous results, which found norbuprenorphine-3-glucuronide in brain after administration of either norbuprenorphine or the glucuronide and a small pharmacologic effect of norbuprenorphine-3-glucuronide (Brown et al., 2011). The determinants of norbuprenorphine-3-glucuronide brain access, including passive permeability and/or active uptake, are not known. Organic anion-transporting polypeptides are expressed in both human and murine brain microvessel endothelial cells (Hagenbuch and Meier, 2004). These transporters can transport glucuronide conjugates of drugs (Hagenbuch and Meier, 2004) and may be responsible, in vivo, for the brain exposure of glucuronides. Further testing is required to determine any possible role for uptake transporters in the brain exposure of norbuprenorphine-3-glucuronide (or buprenorphine-3-glucuronide).

The norbuprenorphine-3-glucuronide results provide additional insights into the mechanism of norbuprenorphine effects. Norbuprenorphine-3-glucuronide was shown previously to have high affinity for  $\kappa$  and nociceptin but not  $\mu$  or  $\delta$  receptors, and, when administered to mice, substantially decreased tidal volume but not respiratory rate (Brown et al., 2011). In the present experiment, brain norbuprenorphine-3-glucuronide concentrations were very low after norbuprenorphine and did not differ between *mdr1a*(+/+) and *mdr1a*(-/-) mice. Respiratory rate but not tidal volume was affected in both *mdr1a*(+/+) and *mdr1a*(-/-) mice. This suggests that norbuprenorphine-3-glucuronide did not contribute to the respiratory effect after a norbuprenorphine dose. Sedation is another effect of both norbuprenorphine and norbuprenorphine-3-glucuronide (Brown et al., 2011). Both the *mdr1a*(-/-) and *mdr1a*(+/+) mice were equally sedated after norbuprenorphine, despite the large differences in brain concentrations of both norbuprenorphine and norbuprenorphine-3-glucuronide between the two strains. Thus, it cannot be concluded whether the sedation observed in this study was attributable to norbuprenorphine, norbuprenorphine-3-glucuronide, or both.

The role of brain access via passive permeability and/or P-gp-mediated efflux and contribution to the respiratory effect of norbuprenorphine is not yet clear. The dose of norbuprenorphine given to the *mdr1a*(-/-) mice was selected to match the respiratory effects of norbuprenorphine in the *mdr1a*(+/+) mice. It is noteworthy that the significant respiratory depression in *mdr1a*(+/+) mice occurred despite very low brain norbuprenorphine concentrations. In addition, the time course of decreased respiratory rate did not parallel the time course of norbuprenorphine brain concentration. Although there was an increase in brain norbuprenorphine corresponding to the decrease in respiratory rate in the *mdr1a*(-/-) mice, there was no change in brain norbuprenorphine in the *mdr1a*(+/+) mice. A plausible hypothesis for the profound norbuprenorphine effect despite such low brain concentrations is high respiratory depressant potency. Although norbuprenorphine potency has been modeled in silico (Yassen et al., 2007), potency of norbuprenorphine respiratory depression remains to be elucidated in vivo. Furthermore, the receptor type involved in mediating norbuprenorphine-induced respiratory depression is still unresolved.

P-gp magnifies the discrepancy between the pharmacologic effects of buprenorphine and norbuprenorphine. Buprenorphine is not a P-gp substrate (Nekhayeva et al., 2006; Hassan et al., 2009; Tournier et al., 2010), and P-gp does not influence the pharmacologic effect of buprenorphine in mice (Hassan et al., 2009). In contrast, norbuprenorphine clearly is a P-gp substrate. Norbuprenorphine has significant respiratory effects in both *mdr1a*(-/-) and *mdr1a*(+/+) mice, but only in the absence of P-gp does norbuprenorphine have a substantial antinociceptive effect. This suggests that the sites or mechanism of action for norbuprenorphine analgesia and respiratory depression may be distinct, and P-gp-mediated efflux restricts norbuprenorphine access to the analgesia effect site but not the respiratory effect site. This could be caused by the differential expression of P-gp in various locations of the blood-central nervous system brain barrier. For example, there is lower P-gp expression in murine endothelial cells of the blood-spinal cord barrier compared with the

blood-brain barrier (Ge and Pachter, 2006). In addition, P-gp influenced blood-brain but not blood-spinal cord morphine distribution in *mdr1a*(-/-) compared with *mdr1a*(+/+) mice (Zong and Pollack, 2000). Thus differences in brain P-gp and norbuprenorphine efflux between *mdr1a*(+/+) and *mdr1a*(-/-) mice may be greater than differences in spinal cord P-gp and efflux, potentially influencing analgesia more than respiratory depression, depending on their respective sites of action.

The influence of P-gp on norbuprenorphine effects can be compared with that of other opioids. The increase in maximum antinociception (MPE) in *mdr1a*(-/-) compared with *mdr1a*(+/+) mice was 60% for norbuprenorphine, but 20, 40, and 50% for methadone (Thompson et al., 2000; Hassan et al., 2009), morphine (Zong and Pollack, 2000), and fentanyl (Hamabe et al., 2006), respectively. In addition, the 8-fold increase in the brain/plasma norbuprenorphine concentration ratio in the absence of P-gp-mediated efflux is greater than that of other opioids. Brain/plasma concentration ratios of morphine, alfentanil, and methadone increased only 2-, 2.4, and 3.5-fold, respectively, in *mdr1a*(-/-) mice compared with *mdr1a*(+/+) mice (Zong and Pollack, 2000; Kalvass et al., 2007; Hassan et al., 2009). Moreover, the more than order of magnitude decrease in plasma  $EC_{50}$  in the absence of P-gp was much greater for norbuprenorphine than for other opioids, such as alfentanil (Kalvass et al., 2007). Thus the degree to which P-gp impedes brain access and antinociception is greater for norbuprenorphine than these other opioids.

The role of P-gp in mediating brain access of norbuprenorphine in humans and the pharmacologic contribution of norbuprenorphine to buprenorphine clinical effects are unknown. If, however, these are clinically relevant, then findings described in this article may explain, in part, clinical observations about buprenorphine (Bruce and Altice, 2006; McCance-Katz et al., 2007). Atazanavir and atazanavir/ritonavir increased plasma buprenorphine and norbuprenorphine concentrations 1.3- to 2.5-fold, albeit not into ranges shown previously to cause side effects (McCance-Katz et al., 2007). Nevertheless, the interaction caused some subjects to become sedated. The adverse side effects after buprenorphine/atazanavir were attributed to inhibition of CYP3A4-mediated buprenorphine metabolism by atazanavir. However, atazanavir is also a P-gp inhibitor (Bierman et al., 2010), and, in light of the findings in the present study, it is possible that atazanavir-enhanced buprenorphine effects could be attributable, at least in part, to P-gp inhibition and increased norbuprenorphine brain access. Tipranavir/ritonavir had no effect on plasma buprenorphine concentrations but diminished norbuprenorphine concentrations to one-fifth of control and had no effect on opioid withdrawal (Bruce et al., 2009). Although this was considered mechanistically unclear, tipranavir and ritonavir are both P-gp inhibitors (Storch et al., 2007) and therefore might have increased brain norbuprenorphine exposure and contributed to obviating withdrawal. Rifampin induced buprenorphine clearance and caused significant withdrawal in opioid-dependent patients (McCance-Katz et al., 2011). Although withdrawal was attributed to diminished buprenorphine concentrations, the decrease in plasma norbuprenorphine concentrations was substantially greater than that of buprenorphine (8- versus 3-fold). Because rifampin induces brain P-gp (Bauer et al., 2006), the combined effects of rifampin to decrease plasma

norbuprenorphine concentrations and up-regulate norbuprenorphine brain efflux could have markedly decreased brain norbuprenorphine exposure and contributed to the withdrawal. Nevertheless, the role of norbuprenorphine and P-gp-mediated brain access in humans remains unknown.

Two limitations of the present investigation merit mention. Buprenorphine was found to bind readily to the polystyrene used for the in vitro Transwell studies, which potentially confounded the accurate determination of the extent of transcellular movement. Because of differences in the surface areas of the apical and basolateral compartments, correction for nonspecific binding was not possible. Therefore, the buprenorphine data should be interpreted carefully. Nonspecific buprenorphine binding has also been reported previously (Tournier et al., 2010). Because of the unexpectedly large effect of P-gp on brain norbuprenorphine access, there was not sufficient overlap of norbuprenorphine brain concentrations between *mdr1a*( $-/-$ ) and *mdr1a*( $+/+$ ) mice to separately model the brain concentration-antinociception data in the two groups, thus they were modeled together.

In conclusion, the limited antinociceptive effect of norbuprenorphine is caused by P-gp-mediated inhibition of brain access. P-gp does not influence the brain disposition of buprenorphine-3-glucuronide or norbuprenorphine-3-glucuronide. These findings add to the complexity of buprenorphine pharmacology.

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#### Authorship Contributions

*Participated in research design:* Brown, Campbell, and Kharasch.  
*Conducted experiments:* Brown, Campbell, Crafford, and Regina.  
*Contributed new reagents or analytic tools:* Holtzman.  
*Performed data analysis:* Brown, Campbell, and Crafford.  
*Wrote or contributed to the writing of the manuscript:* Brown, Campbell, and Kharasch.

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