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Investigation of efflux transport of dehydroepiandrosterone sulfate and mitoxantrone at the mouse blood-brain barrier: a minor role of breast cancer resistance protein

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Running title : A minor role of Bcrp at the mouse BBB

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Abbreviations: BBB, blood-brain barrier; BCRP, breast cancer resistance protein; DHEAS, dehydroepiandrosterone sulfate; DHEA, dehydroepiandrosterone; HPRT, Hypoxanthine phosphoribosyl-transferase; Mdr, multidrug-resistance; Mrp, Multidrug resistance-associated protein; OATP, Organic anion-transporting polypeptide; P-gp, P-glycoprotein

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Abstract

Breast cancer resistance protein (Bcrp/Abcg2) is a new efflux transporter found at the blood-brain barrier (BBB) of humans and pigs. Since it has been hypothesized that Bcrp may act as a new type of efflux transporter at the BBB, we investigated the involvement of Bcrp in the efflux transport of typical substrates, dehydroepiandrosterone sulfate (DHEAS) and mitoxantrone, across the mouse BBB. The expression of Bcrp in mouse brain capillaries was confirmed by quantitative PCR, Western blot and immunohistochemical analysis. The role of Bcrp as an efflux transporter was evaluated using the in situ brain perfusion method in wild-type and P-gp knockout mice, with or without treatment with GF120918, an inhibitor of both Bcrp and P-gp. The increased brain uptake of [^{3}H]DHEAS and [^{3}H]mitoxantrone by GF120918 in wild-type and P-gp knockout mice suggested the existence of a GF120918-sensitive and P-gp independent efflux transporter for DHEAS and mitoxantrone across the BBB. However, the brain uptake of [^{3}H]DHEAS in Bcrp knockout mice was comparable with that in wild-type mice, and the effect of GF120918 was still observed in Bcrp knockout mice. In addition, the brain uptake of [^{3}H]mitoxantrone was also similar in wild-type and Bcrp knockout mice. These results suggest that although BCRP is expressed at the BBB it plays a minor role in active efflux transport of DHEAS and mitoxantrone out of brain and that one or more GF120918-sensitive efflux transporters distinct from BCRP or P-gp contributes to the brain efflux of DHEAS and mitoxantrone.

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Introduction

It is well known that the transport of compounds from the circulating blood into the central nervous system is restricted by the blood–brain barrier (BBB), which is formed by the brain capillary endothelial cells that are characterized by highly developed tight junctions and a paucity of fenestra and pinocytotic vesicles. In addition to these characteristics, efflux transporters expressed in the brain capillaries play an important role in the elimination of endogenous waste products and xenobiotics from the brain, and prevent their accumulation in the central nervous system (Kusuhara and Sugiyama, 2001; Sun et al., 2003). Breast cancer resistance protein (BCRP/*ABCG2*) is a unique ABC efflux transporter that accepts sulfo-conjugated organic anions as well as hydrophobic and amphiphilic compounds as substrates. Bcrp has been shown to restrict the intestinal absorption and fetal penetration of its substrates, such as mitoxantrone and topotecan (Allen et al., 1999; Jonker et al., 2002; Kruijtzer et al., 2002). BCRP has also been found at the luminal side of human and porcine brain capillary endothelial cells (Cooray et al., 2002; Zhang et al., 2003; Eisenblatter and Galla, 2002). Over-expression of human BCRP in immortalized rat brain endothelial cells resulted in enhanced vectorial transport of mitoxantrone, fluorescein, and rhodamine-123 in the abluminal-to-luminal direction (Zhang et al., 2003). Recently, over-expression of Bcrp in Mdr1a P-gp knockout mouse has also been reported (Cisternino et al., 2004). However, it is unclear yet whether BCRP plays a role as an efflux transporter at the luminal membrane of the brain capillaries in vivo, together with P-glycoprotein (P-gp) (Schinkel et al., 1994; Schinkel et al., 1996).

As a neurosteroid, dehydroepiandrosterone sulfate (DHEAS) and its unconjugated form (DHEA) modulates neurotransmission in an excitatory or inhibitory manner via ion-gated channels involving N-methyl-D-aspartate receptors and γ-aminobutyric acid receptors (Schumacher et al., 1997). In rodents, DHEAS and DHEA are also synthesized locally in the brain, and these neurosteroids can be interchanged via sulfotransferase and sulfatase (Baulieu, 1996; Stoffel-Wagner, 2001), and the level of DHEAS is much higher than that of DHEA in the

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brain and plasma of rats (Corpechot et al., 1981). At least in rodents, a compartmental barrier is known to exist for DHEAS between the brain and circulating blood (Biggio and Purdy, 2001) and, in humans, the concentration of DHEAS in brain is known to be much lower than that in blood (Weill-Engerer et al., 2002).

DHEAS is a substrate of several transporters, including Na^+-taurocholate co-transporting polypeptides, organic anion transporting polypeptides (Oatp/OATP) and organic anion transporters (Oat/OAT) (Kullak-Ublick et al. 1998; Hagenbuch and Meier 2003; Hasegawa.et al. 2003). In particular, Oatp2 (*Slco1a4*, OATP1a4) has been reported to be a candidate efflux transporter for DHEAS at the BBB (Asaba et al., 2000). Interestingly, Asaba et al also suggested the existence of primary active efflux transporter(s) for DHEAS in a conditionally immortalized cell line established from mouse brain capillary endothelial cells (TM-BBB4). The net uptake of DHEAS by TM-BBB4 was increased under ATP-depleted conditions (Asaba et al., 2000). This efflux transport system is expected to account for the compartmentalization of DHEAS between brain and the circulating blood and also to be one of the mechanisms of inactivation of DHEAS in the brain to regulate its activity on neurons.

Our previous study using membrane vesicles prepared from BCRP over-expressed P-388 cells clearly showed that DHEAS is an endogenous substrate of BCRP (Suzuki et al., 2003). Therefore, we hypothesized that BCRP plays a role in the luminal excretion of DHEAS at the brain capillaries as an unidentified primary active efflux transporter(s) predicted by Asaba et al. (Asaba et al., 2000). This hypothesis was partially supported by the results of Jonker et al. They showed that GF120918, an acridine derivative known to be an inhibitor of P-gp and BCRP, increased the in vivo oral bioavailability and fetal penetration of topotecan even in multidrug-resistance (Mdr)1a/1b P-gp knockout mice, suggesting a function for BCRP in the intestinal barrier and maternal-fetal barrier (Jonker et al., 2000). In the present study, the expression and localization of Bcrp at the mouse BBB was investigated by real-time quantitative RT-PCR, Western blot analysis and immunohistochemical staining. The brain uptake of DHEAS was

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determined using the *in situ* brain perfusion method, and the effect of GF120918 on brain uptake was examined. The brain uptake of mitoxantrone, a typical substrate of Bcrp, was also investigated in the same manner. Finally, the involvement of mouse Bcrp in the efflux of DHEAS and mitoxantrone across the BBB was evaluated directly using the recently established Bcrp knockout mouse (Jonker et al., 2002).

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Materials and Methods

Reagents and Animals

[^{3}H]DHEAS was purchased from PerkinElmer Life Sciences (Boston, MA). [^{14}C]sucrose and [^{3}H]mitoxantrone were purchased from Moravek Biochemicals (Brea, CA). GF120918 (Elacridar) was a gift from Glaxo Smith Klein (Ware, UK). USP grade propylene glycol was purchased from Sigma-Aldrich (St. Louis, MO). All other chemicals used in the experiments were of analytic grade.

Male Mdr1a/1b P-gp knockout mouse and age matched wild type control mice were purchased from Taconic (Germantown, NY) and male Bcrp knockout mice and age matched wild-type mice of a comparable genetic background were produced as reported (Jonker et al., 2002). All mice (8 ~ 22 weeks) was maintained under standard conditions with a reverse dark-light cycle. Food and water were available ad libitum.

Isolation of mouse brain capillaries

A brain capillary enriched fraction from the mouse brain (BBB-enriched fraction) was isolated according to the reported procedure with slight modification. (Ball et al., 2002; Dallaire et al., 1991). Briefly, large brains were dissected from the heads after perfusion with 0.9% saline and homogenized using a Polytron homogenizer in 0.32 M sucrose solution and centrifuged at 4°C at 2,200 g for 10 min. The pellet was further purified according to the procedures suggested by Dallaire et al. and used as BBB-enriched fraction (Dallaire et al., 1991). All reagents as well as the tissue should be kept on ice or as close to 4 degrees as possible throughout the isolation process to minimize degradation.

The purity of the isolated BBB-enriched fraction was checked by the enhanced alkaline phosphatase activity in the brain homogenate and BBB-enriched fraction (Ball et al., 2002; Dallaire et al., 1991). Isolated brain capillary enriched fraction from mice contained tangled skeins of microvessel, which was confirmed under light microscopy. The alkaline phosphatase

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activity in the BBB-enriched fraction was 18.6-fold greater than that in the brain homogenate. This BBB-enriched fraction was used for further analyses: Western blot and real-time quantitative PCR.

Quantification of transporter mRNA in the brain homogenate and the BBB-enriched fraction

To quantify the expression of Bcrp at the mouse BBB, real-time quantitative PCR was used. Total RNA was isolated from the BBB-enriched fraction and brain homogenate from wild-type FVB mice using an RNeasy Mini Kit® (Qiagen, Valencia, CA) and was converted to cDNA using random primer and avian myeloblastosis virus reverse transcriptase. Real-time quantitative PCR was performed using a QuantiTect® SYBR Green PCR Kit (Qiagen, Valencia, CA) and LightCycler™ system (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions. The primers used in the quantification are listed in Table1. All primers were designed based on the published full sequence of each protein. Hypoxanthine phosphoribosyl-transferase (HPRT) was used as a housekeeping gene for the internal standards and Mdr1a was used as positive control gene for putative transporter at the brain microvessel (Ball et al., 2002). An external standard curve was generated by dilution of the target PCR product, which was purified by agarose gel electrophoresis. The absolute concentration of external standard was measured by PicoGreen® dsDNA Quantitation Reagent (Molecular Probes, Inc., Eugene, OR). To confirm amplification specificity, PCR products were subjected to a melting curve analysis and gel electrophoresis. All gene expressions in each reaction were normalized by the expression of HPRT in same sample (Ball et al., 2002).

Besides Bcrp, the mRNA of Mdr1a, Multidrug resistance-associated protein 1 (Mrp1), Mrp4, and Oatp2 in total brain cortex of wild-type and Bcrp knockout mouse was also measured by real-time quantitative PCR. These transporters have been reported to be expressed in the mouse brain, and this result was used to show the relative expression of these transporters in the

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wild-type mouse brain and to examine the possibility of up and/or down regulation of these transporters in the brain of the Bcrp knockout mouse.

Western blot analysis

The lysates of the BBB-enriched fraction and brain homogenate were subjected to electrophoresis on a 7.5% SDS-polyacrylamide gel. Proteins were transferred to nitrocellulose membranes (Immobilon; Millipore, Bedford, MA) which were blocked with Tris-buffered saline containing 0.05% Tween 20 (TBS-T) and 5% skim milk for 2h at room temperature. After washing with TBS-T, membranes were incubated with anti-BCRP monoclonal antibody (40-fold diluted BXP-53 antibody, from Signet, Dedham, MA) in TBS-T overnight at 4°C and proteins were detected using the ECL system (Amersham, Arlington Heights, IL).

Immunocytochemical analysis of the expression of Bcrp at the mouse BBB.

Brain samples from the wild-type mouse and Bcrp knockout mouse were fixed in 4% phosphate-buffered formalin, embedded in paraffin, sectioned at 4 μm, and stained with hematoxylin and eosin according to standard procedures. For immunohistochemistry, tissues were deparaffinized in xylene and rehydrated. Endogenous peroxidase activity was blocked by 3% (vol/vol) H_2O_2 in methanol for 10 min. Before staining, paraffin sections were pretreated by heat-induced epitope retrieval. Slides were incubated with 5% normal goat serum/PBS for 30 min, and subsequently sections were incubated overnight with a 1:400 dilution of BXP-53 at 4°C. mAb immunoreactivity was detected by the streptavidin-biotin immunoperoxidase (sABC) method by using biotinylated goat anti-rat IgG (Dako, 1:100) as a secondary antibody, and diaminobenzidine substrate for visualization. After counterstaining with hematoxylin, slides were mounted. To investigate the localization of Bcrp in brain microvessels, double immunostaining with antibodies of P-gp (luminal expression) and glucose transporter 1 (Glut1, luminal and abluminal co-expression) was also performed using cryostat sections of wild-type

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mouse brain (10 μm in thickness) (Cooray et al., 2002). Brain sections without fixation were incubated overnight at 4^0C with primary antibody at following concentrations: Bxp-53 (Bcrp, 1:40 dilution in 1% BSA/PBS), C219 (P-gp, 1:40 dilution in 1% BSA/PBS, from Signet, Dedham, MA) and anti-Glit1 (Glut1, 1:40 dilution in 1% BSA/PBS, from Santa Cruz, Santa Cruz, CA). After washing with PBS, sections were incubated with appropriate Alexa Fluor secondary antibodies (Molecular Probes, Eugene, OR) and Topro3 (DNA dye, Molecular Probes, Hilversum, Netherlands) for 1 h and mounted in Vectashield mounting medium® (Vector Laboratories, Burlingame, CA) and visualized under a Zeiss confocal fluorescence microscope.

In situ brain perfusion to determine the brain uptake of Bcrp substrates

The right cerebral hemisphere of the mouse was perfused using the reported method (Takasato et al., 1984, Dagenais et al., 2000; Murakami et al., 2000) with minor modification. In brief, the mouse was anesthetized by intraperitoneal injection of xylazine (Sigma Chemical Co. St Louis, MO, USA; 10 mg/kg) and ketamine (Sankyo Co., Tokyo; 100 mg/kg). The right common carotid artery was exposed and then catheterized with polyethylene tubing (0.2 mm i.d. × 0.5 mm o.d, Natsume, Tokyo, Japan) filled with heparinized saline. The right hemisphere of the brain was perfused with Krebs-bicarbonate buffer (pH 7.4 with 95% O_2 and 5% CO_2 containing 10mM D-glucose) at a flow rate of 1 ml/min (Murakami et al., 2000). The thorax of the mouse was opened and the cardiac ventricle was severed immediately before perfusion. [^{3}H]DHEAS or [^{3}H]mitoxantrone was added to perfusate at a concentration of 1 μCi/ml with carbon-labeled sucrose as a vascular volume marker. Perfusion was terminated by decapitation at selected times (1 and 2 minutes, for [^{3}H]DHEAS; 1.5 minutes for [^{3}H]mitoxantrone). The right hemisphere of the brain was removed from the skull and weighed. Aliquots of the perfusion fluid also were collected to determine tracer concentrations in the perfusate. Brain

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samples were digested in 2 ml 1N NaOH at 55°C and the dual radioactivity associated with the brain was measured in a liquid scintillation counter (LS 6000SE; Beckman Coulter).

In all perfusion experiments, the brain vascular volume (V_{vasc};μl/g) was estimated from the tissue distribution of [^{14}C]sucrose, which is known to diffuse very slowly across the BBB, using the following equation (Dagenais et al., 2000):

$$V_{vasc}=X_{sucrose}/C_{sucrose} \quad (1)$$

where $X_{sucrose}$ (dpm/g) is the amount of sucrose measured in the right brain hemisphere and $C_{sucrose}$ (dpm/ml) is the concentration of labeled sucrose in the perfusion fluid. V_{vasc} is the brain vascular distribution volume of substrate used to check BBB integrity during the experiments (Dagenais et al., 2000).

Brain distributional volume of substrate (V_{brain},μl/g) is calculated as:

$$V_{brain} = X_{brain}/C_{substrate} \quad (2)$$

where X_{brain} is the amount of substrate in the brain (dpm/g) corrected for vascular contamination ($X_{total}-V_{vasc} \times C_{substrate}$) and $C_{substrate}$ is the concentration of substrate in the perfusate (dpm /mL) (Dagenais et al., 2000).

The uptake clearance of substrate (CL_{up}, μl/g) is calculated as the slope of the plot of time vs V_{brain}.

$$CL_{up} = X_{brain}/T/ C_{substrate} \quad (3)$$

where T is the perfusion time (min) (Dagenais et al., 2000).

Effects of Bcrp and P-gp on the brain uptake of [^{3}H]DHEAS and [^{3}H]mitoxantrone

As an inhibitor of Bcrp, GF120918 (10 mg/kg or 20mg/kg, dissolved in a 3:2 mixture of propylene glycol : water), was injected intravenously to mouse (125 μl/25g mice) at 10 minute before the *in situ* perfusion of [^{3}H]DHEAS and [^{3}H]mitoxantrone (Hyafil et al., 1993, Cisternino et al., 2001). Because GF120918 inhibits both P-gp and Bcrp (Allen et al., 1999), the role of Bcrp on the brain uptake of substrates was investigated by comparing the brain uptake in wild-

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type control mice and P-pg knockout mice, with or without treatment with GF120918, respectively, to exclude any confounding effects of P-gp inhibition (Jonker et al., 2000). The role of Bcrp on BBB transport was also examined directly by comparing the brain uptake of [^{3}H]DHEAS and [^{3}H]mitoxantrone in Bcrp knockout mice and wild-type control mice (Jonker et al., 2002). Control groups received only vehicle solution in all experiments.

Statistical Analysis

Data are presented as the mean ± standard error (SE) for 3–10 animals unless specified otherwise. Student's two tailed unpaired t-test and one-way ANOVA followed by the Newman-Keuls multiple comparison test were used to identify significant differences between groups when appropriate. Statistical significance was set at $P < 0.05$.

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Results

The expression of Bcrp at the mouse BBB

The expression of Bcrp at the BBB was suggested by comparing mRNA expression between brain homogenate and capillary enriched fraction by real-time quantitative PCR. The concentration of Bcrp mRNA, which was normalized by that of HPRT, was 5.6-fold higher in the BBB-enriched fraction than that in brain homogenate (5.6±1.3, mean±SD), while that of Mdr1a mRNA was enriched 12-fold in the BBB enriched fraction(12±2, mean±SD).

The protein band (70kD) of Bcrp in the BBB-enriched fraction was clearly detected by Western blot analysis using mAb BXP-53, whereas only weak staining was observed in the brain homogenate, suggesting that the primary localization of Bcrp is at the BBB (Fig. 1A). Furthermore, immunohistochemical analysis of brain sections of wild-type and Bcrp knockout mice showed Bcrp expression only in the brain capillaries of the wild-type mouse (Fig. 1B). Although the absolute staining level does not appear to be very high, Bcrp-related staining of blood capillaries throughout the mouse brain was clearly observed, whereas it was completely absent in Bcrp knockout mouse brain (Fig. 1B). Double immunostaining of Bcrp with P-gp and Glut1 clearly showed that Bcrp is expressed at the luminal side of brain microvessels (Fig. 1C). The Bcrp signal was completely superimposed on that of P-gp expressed at the luminal side of the brain microvessels. However, the Bcrp signal only partially overlapped with that of Glut1 expressed at the abluminal and luminal side of brain microvessel.

The effect of pre-treatment with GF120918 on the brain uptake of [^{3}H]DHEAS and [^{3}H]mitoxantrone

The time-dependent brain uptake of [^{3}H]DHEAS in mice is shown in Fig. 2. The brain uptake increased linearly, and the uptake clearances could be calculated from the slope of the plot of V_{brain} vs time (Dagenais et al., 2000). The brain uptake clearance (CL_{up}) of [^{3}H]DHEAS was 18.0 μl/min/g brain (Fig. 2). Pre-treatment of wild-type mice with GF120918 (10 mg/kg)

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increased the brain uptake clearance of [^{3}H]DHEAS about 2.1-fold (approximately estimated, 38.6 μl/min/g brain). Increasing the dose of GF120918 (20 mg/kg) showed a further increase in the brain uptake of [^{3}H]DHEAS by 3.0-fold (approximately estimated, 54.0 μl/min/g brain)(Fig. 2). The V_{brain} of [^{3}H]DHEAS at 2 min was increased 2.0-fold and 2.8-fold by pre-treatment with GF120918 10 mg/kg and 20 mg/kg, respectively (Fig. 2, $*p < 0.05$).

The brain uptake of [^{3}H]DHEAS (Fig. 3A) in Mdr1a/1b P-gp knockout mice was comparable with that in wild-type mice. However GF120918 (10 mg/kg) increased the brain uptake of [^{3}H]DHEAS even in the Mdr1a/1b P-gp knockout mice (Fig. 3A). The V_{brain} of [^{3}H]DHEAS at 2 min was increased 2.6-fold by pre-treatment with GF120918 (10 mg/kg) in Mdr1a/1b P-gp knockout mice compared with that in the non-treated group.

The brain uptake of [^{3}H]mitoxantrone in wild-type mice and Mdr1a/1b P-gp knockout mice, with or without pre-treatment with GF120918, is presented in Fig. 3B. The brain uptake of [^{3}H]mitoxantrone was increased in Mdr1a/1b P-gp knockout mice (1.8-fold) and wild-type mice following pre-treatment with GF120918 (10 mg/kg, 2.4-fold). GF120918 (10 mg/kg) also increased the brain uptake of [^{3}H]mitoxantrone in Mdr1a/1b P-gp knockout mice (1.6-fold). The inhibitory effect of GF120918 was found to be more potent in Mdr1a/1b P-gp knockout mice for [^{3}H]DHEAS (Fig. 3A, wild-type + GF120918 10mg vs Mdr1a/1b P-gp knockout + GF120918 10mg ; $p<0.05$ Newman-Keuls multiple comparison test), although the reason was unclear. In the case of [^{3}H]mitoxantrone, no significant difference was not between the wild-type + GF120918 10mg group and the Mdr1a/1b P-gp knockout + GF120918 10mg group.

Effect of Bcrp gene knockout on the brain uptake of [^{3}H]DHEAS and [^{3}H]mitoxantrone

The impact of Bcrp on the transport of [^{3}H]DHEAS and [^{3}H]mitoxantrone across the BBB was evaluated directly using the Bcrp gene knockout mouse (Fig. 4A and B). The brain uptake of [^{3}H]DHEAS in Bcrp knockout mice was not different from that in wild-type mice (Fig. 4A). Pre-treatment with GF120918 (10 mg/kg) increased the brain uptake of [^{3}H]DHEAS even

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in Bcrp knockout mice. The V_{brain} of [^{3}H]DHEAS at 2 min was increased about 1.8-fold and 2.2-fold by GF120918 (10 mg/kg) in wild-type and Bcrp knockout mice, respectively.

In addition, the brain uptake of [^{3}H]mitoxantrone in Bcrp knockout mice was no different from that in wild-type mice (Fig. 4B). The V_{brain} of [^{3}H]mitoxantrone at 1.5 min was 144±3 (μl/g brain, n=3) in the wild-type mouse and 161±18 (μl/g brain, n=3) in the Bcrp knockout mouse, respectively, and there was no statistical difference between the two groups (unpaired Student's t-test, $P > 0.05$)

Assessment of BBB Integrity

In all experiments, the physical integrity of the BBB was assessed by [^{14}C]-sucrose, which serves as a brain vascular space marker. The brain vascular space under each set of experiment conditions was not changed by knockout of the Mdr1a/1b gene and pre-treatment with GF120918 (Table 2). A similar brain vascular space was also observed between Bcrp knockout mice and their wild-type controls suggesting that the BBB integrity was not adversely affected by these experiments.

Relative expression of transporters in mouse brain

To estimate the relative expression of these transporters in the wild-type mouse brain and to examine the possibility of up and/or down regulation of these transporters in the brain of the Bcrp knockout mouse, mRNA quantification of known transporters such as Mdr1a, Mrp1, Mrp4, and Oatp2 was carried out using cDNA prepared from mouse brain homogenate (Table 3). Each value represents the mRNA level in the brain homogenate from one single mouse normalized by the mRNA level of HPRT in the same sample. Except for Oatp2, mRNA levels of other transporters in wild-type mouse brain were comparable ($p>0.05$, one-way ANOVA followed by the Newman-Keuls multiple comparison test). The mRNA levels of Oatp2 were statistically different with those of other transporters ($p<0.05$, one-way ANOVA followed by the Newman-

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Keuls multiple comparison test). The mRNA levels of all transporters in the brain of the Bcrp knockout mouse were similar to those in the brain of the wild-type mouse, except for the case of Bcrp. It is interesting that Mrp4 shows abundant expression in the brain homogenate, as much as Mdr1a. Furthermore, the concentration of Mrp4 mRNA was 4.7-fold greater in the BBB-enriched fraction than in the brain homogenate.

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Discussion

In the present study, we examined the involvement of Bcrp in the efflux transport of DHEAS using the *in situ* brain perfusion method to examine the hypothesis that BCRP acts as a functional efflux transporter at the BBB for sulfo-conjugated organic anions. In addition, the role of BCRP at the BBB was also investigated using another typical BCRP substrate, mitoxantrone.

Quantitative real-time PCR showed that the concentration of Bcrp mRNA in the brain was comparable with that of ABC transporters which are expressed in the mouse brain, such as Mdr1a, Mrp1 and Mrp4 (Table 3), and that Bcrp mRNA was enriched in the BBB-enriched fraction, similar to Mdr1a P-gp mRNA. Western blot analysis revealed that Bcrp was clearly detectable in the BBB-enriched fraction, and the band density was greater in the BBB-enriched fraction than in the brain homogenate (Fig. 1A). Furthermore, immunohistochemical analysis revealed that Bcrp was localized at the luminal side of mouse brain capillaries (Fig. 1B and 1C). All these results indicate that Bcrp is expressed and localized at the BBB, suggesting the possibility the Bcrp may play a role in the efflux of its substrates at the BBB.

Involvement of Bcrp in the transport of DHEAS and mitoxantrone at the BBB was investigated by examining the effect of GF120918 on their brain uptake determined using the *in situ* brain perfusion technique in the mouse. Because GF120918 inhibits both P-gp and Bcrp (Allen et al., 1999), the P-pg knockout mouse was used in conjunction with the wild-type mouse to exclude any confounding effects of P-gp inhibition (Jonker et al., 2000). Time-dependent brain uptake of [^{3}H]DHEAS was observed up to 2 min, and treatment with GF120918 increased the brain uptake of [^{3}H]DHEAS in a dose-dependent manner (Fig. 2), whereas it did not affect the distribution volume of sucrose (Table 2). This suggests that the effect of GF120918 is not due to a non-specific effect, such as the destruction of the BBB by opening the tight junctions, but to inhibition of efflux transport at the BBB. Since GF120918 is an inhibitor of both P-gp and Bcrp (Allen et al., 1999), the brain uptake of [^{3}H]DHEAS was also determined in the

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Mdr1a/1b P-gp knockout mouse to exclude the possibility that the effect of GF120918 is due to inhibition of P-gp at the BBB (Fig. 3A). The brain uptake of [^{3}H]DHEAS in Mdr1a/1b P-gp knockout mice was comparable with that in wild-type mice, and the increased brain uptake of [^{3}H]DHEAS by GF120918 was still observed in Mdr1a/1b P-gp knockout mice (Fig. 3A). In the case of another typical substrate of Bcrp, mitoxantrone, GF120918-treatment increased the brain uptake of [^{3}H]mitoxantrone similar to that of [^{3}H]DHEAS (Fig. 3B). Since the brain uptake of [^{3}H]mitoxantrone was increased in Mdr1a/1b P-gp knockout mice compared with that in wild-type mice (Fig. 3B), the effect of GF120918 is partly accounted for by inhibition of P-gp. However, GF120918 was still effective in wild-type and Mdr1a/1b P-gp knockout mice (Fig. 3B). Therefore, in addition to P-gp, it is likely that a GF120918-sensitive transporter other than P-gp is involved in the efflux of mitoxantrone at the BBB. Recently, Cisternino et al., also reported that the brain uptake of mitoxantrone was linear up to 2 min using the in situ perfusion method and its uptake was increased by treatment of GF120918 (Cisternino et al., 2004). This is consistent with our results although there is a discrepancy in the effect of knockout of P-gp on the brain uptake of [^{3}H]mitoxantrone for some, as yet unknown reason (Cisternino et al., 2004)

In order to show that the effect of GF120918 is due to inhibition of Bcrp, the brain uptake of [^{3}H]DHEAS and [^{3}H]mitoxantrone was determined in Bcrp knockout mice (Fig.4A and B). Surprisingly, the brain uptake of [^{3}H]DHEAS and [^{3}H]mitoxantrone was found to be comparable and independent of the Bcrp expression (Fig. 4A and B). Furthermore, treatment with GF120918 still increased the brain uptake of [^{3}H]DHEAS even in Bcrp knockout mice (Fig. 4A). In order to examine the possibility of adaptive up and/or down regulation of transporters in the brain of Bcrp knockout mice, quantitative PCR was carried out. The mRNA levels of Mdr1a, Mrp1, Mrp4 and Oatp2 were similar to those in the brain of wild-type mice (Table 3). This suggests that distinct adaptive alteration of the expression of transporters may not have occurred in the brain of Bcrp knockout mice as far as these transporters are concerned. Taking all these results into consideration, especially the *in situ* analysis using Bcrp knockout mice, the

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contribution of Bcrp to the efflux transport of [^{3}H]DHEAS and [^{3}H]mitoxantrone at the mouse BBB was considered to be minor, if it exists at all, and thus it is suggested that other GF120918-sensitive transporter(s), distinct from Bcrp and P-gp, may account for the efflux of [^{3}H]DHEAS and [^{3}H]mitoxantrone at the BBB. Whether one and the same GF120918-sensitive efflux transporter affects [^{3}H]DHEAS and [^{3}H]mitoxantrone remains to be demonstrated.

Collectively, the present study could not demonstrate any involvement of Bcrp in the efflux transport of the Bcrp substrates, DHEAS and mitoxantrone, at the BBB, although Bcrp is abundantly expressed at the BBB and is likely to play an important role as a detoxification system in the central nervous system together with P-gp. This result is also supported by the recent findings of Herwaarden et al (van Herwaarden et al., 2003). They reported that the hepatobiliary and intestinal elimination of 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine was significantly reduced in Bcrp knockout mice, however, there was no significant change in the brain penetration between wild-type and Bcrp knockout mice. The functional role Bcrp at the BBB remains virtually unknown. Mogi et al, reported that Akt signaling modulates the SP cell phenotype by regulating the translocation of Bcrp between the plasma membrane and intracellular compartment (Mogi et al., 2003). We cannot exclude the possibility that the function of Bcrp at the BBB is also modulated by an unknown mechanism and works only under the certain conditions. Further investigation is necessary to elucidate the role of Bcrp in the detoxification system in the brain. Cisternino et al also demonstrated that the brain uptake of prazosin and mitoxantrone was increased by treatment with GF120918 in Mdr1a single knockout mice (Cisternino et al., 2004). It would be interesting to discover whether the effect of GF120918 on the brain uptake of prazosin is also ascribed to inhibition of Bcrp-mediated efflux.

The present study shows the presence of a GF120918-sensitive efflux transporter for [^{3}H]DHEAS and [^{3}H]mitoxantrone at the BBB. The uptake transporter, Oatp2, is expressed both in the luminal and abluminal membrane of the brain capillaries. According to the in situ study by Dagenais et al (2001), the brain uptake of [D-penicillamine(2,5)]-enkephaline, a peptide

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substrate of Oatp2 in Mdr1a P-gp knockout mice was saturable and inhibited by Oatp2 substrates, suggesting the involvement of Oatp2. Therefore, it is possible that Oatp2 accounts for the luminal uptake of DHEAS at the luminal membrane of brain capillaries as well as efflux from the brain at the abluminal membrane. Although the physiological meaning of luminal Oatp2 remains unknown, this uptake is also considered to be present in humans since the human isoform of Oatp2, OATP-A, has been shown to exhibit similar membrane localization in the brain capillaries (Gao et al., 1999; Gao et al., 2000). Thus, it may be important to limit Oatp2- and OATP-A mediated DHEAS uptake by the GF120918-sensitive efflux transporter at the BBB in addition to facilitating the elimination of locally synthesized DHEAS from the brain in order to regulate the effect of DHEAS on neuronal function. In addition, the efflux transporter may account for the DHEAS compartmentalization between the brain and blood.

Currently, no candidate transporter other than P-gp and Bcrp has been reported to interact with GF120918 (Hyafil et al., 1993; Allen et al., 1999; Evers et al., 2000). Interestingly, quantification of mRNA revealed the abundant expression of MRP4 (*ABCC4*), an ABC transporter classified as a member of the Mrp/MRP(*ABCC*) family, in the brain, compared with other ABC transporters such as P-gp and Mrp1. Furthermore, Mrp4 mRNA was increased in the BBB enriched fraction like Mdr1a and Bcrp. RT-PCR analyses have demonstrated its expression in primary cultured bovine brain capillary endothelial cells and the brain capillary-enriched fraction (Zhang et al., 2000). MRP4 shows broad substrate specificity for a number of compounds including DHEAS as well as cyclic nucleotides, methotrexate, estradiol-17β-glucuronide and prostaglandins (van Aubel et al., 2002; Zelcer et al., 2003). In the revision process of this paper, Leggas et al reported that Mrp4 is localized at the luminal membrane of the brain capillaries, and decreased the efflux rate of topotecan from the brain in Mrp4 knockout mice compared with wild-type mice (Leggas et al, 2004). This recent results may support our speculation, and further investigation is necessary to show that Mrp4 is also involved in the efflux transport of organic anions at the BBB.

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In conclusion, we have demonstrated that a GF120918-sensitive transporter(s) is involved in the efflux of [^{3}H]DHEAS and [^{3}H]mitoxantrone at the BBB, facilitating their elimination from the brain and limiting their uptake by the brain. Although BCRP is abundantly expressed in blood capillaries forming the BBB, we did not find any evidence indicating that Bcrp is a functionally active efflux transporter at the BBB.

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Footnotes.

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Fig. 1. Expression of Bcrp at the BBB.

(A) Western blot of brain homogenate and the BBB-enriched fraction with with anti-mouse BCRP monoclonal antibody (BXP-53). Approximately 70kD Bcrp band in BBB enriched fraction was clearly detected(lanes 4, 5, 6) whereas only faint bands observed in brain (lanes 1, 2, 3). Lanes 1, 4(25μg protein), Lanes 2, 5(50μg protein), Lanes 3, 6(100μg protein).

(B) Immunohistochemical detection of Bcrp in brain sections from wild-type (Left) and Bcrp knockout mouse (Right). A consistent staining of blood capillaries for Bcrp (brown) throughout the mouse brain was observed, suggesting moderate Bcrp expression in wild-type brain capillaries, whereas it was completely absent in Bcrp knockout mouse brain.

(C) Double immunostaining of Bcrp with P-gp and Glut1. P-gp was used a marker of luminal expression and Glut1 was used a marker of luminal and abluminal co-expression: Green - Bcrp, Red - P-gp(Left) or Glut1(Right), Blue color indicates nuclei stained with Topro3. The Bcrp and P-gp signals were completely superimposed, whereas the Bcrp signal only partially overlapped with Glut1 signals, suggesting luminal expression of Bcrp in brain capillaries. Scale bar = 10μm.

Fig. 2. Time dependent and GF120918-sensitive brain uptake of [^{3}H]DHEAS.

Brain uptake is expressed as brain distribution volume, V_{brain} and uptake clearances could be calculated by the slope of plot of V_{brain} vs time profile. ● Control, CL_{up}=18.0 μl/min/g brain, ○ +GF120918, 10 mg/kg, CL_{up}=38.6 μl/min/g brain, ▼ +GF120918, 20 mg/kg, CL_{up}=54.0 μl/min/g brain. (n= 3 ~ 10 per point, * statistically different with control, p<0.05).

Fig. 3. Brain uptake of [^{3}H]DHEAS and [^{3}H]mitoxantrone, which was expressed as brain distribution volume, V_{brain}.

(A) The GF120918-sensitive brain uptake of [^{3}H]DHEAS at 2 min after in situ brain perfusion (V_{brain} at 2 min). The pretreatment of GF120918 increased the brain uptake of [^{3}H]DHEAS,

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whereas knockout of Mdr1a/1b did not. Data are presented as the mean ± SE (n= 3 ~ 10 per point).

(B) GF120918-sensitive brain uptake of [^{3}H]mitoxantrone at 1.5 min after in situ brain perfusion(V_{brain} at 1.5 min). A knockout of Mdr1a/1b and the pretreatment of GF120918 increased the brain uptake of [^{3}H]mitoxantrone. The pretreatment of GF120918 increased the brain uptake of [^{3}H]mitoxantrone even in Mdr1a/1b P-gp knockout mouse. Data are presented as the mean ± SE (n= 4 ~ 6 per point).

Statistical significance was calculated by one-way ANOVA followed by Newman-Keuls multiple comparison tests.

Fig. 4. Brain uptake of [^{3}H]DHEAS and [^{3}H]mitoxantrone in Bcrp knockout mouse.

(A) The brain uptake of [^{3}H]DHEAS in Bcrp knockout mouse was not different with that in wild-type control mouse of same genetic background. And the brain uptake of [^{3}H]DHEAS was increased by treatment of GF120918 in wild-type mouse and even in Bcrp knockout mouse. Data are presented as the mean ± SE (n=3, respectively). Statistical significance was calculated by one-way ANOVA followed by Newman-Keuls multiple comparison tests.

(B) Brain uptake of [^{3}H]mitoxantrone. The brain uptake of [^{3}H]mitoxantrone in Bcrp knockout mouse was not different with that in wild-type control mouse. Data are presented as the mean ± SE (n=3, respectively). Statistical significance was calculated by unpaired t-test.

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TABLE 1

Nucleotide sequences of the primers used in quantitative PCR.

	Forward primer	Reverse primer	Gene Bank Accession No.
HPRT	GCTTTCCCTGGTTAAGCAGTACA	CAAACTTGTCTGGAATTTCAAATC	J00423
Bcrp	AAATGGAGCACCTCAACCTG	CCCATCACAACGTCATCTTG	NM_011920
Mdr1a	TCATTGCGATAGCTGGAGTG	CAAACTTCTGCTCCCGAGTC	NM_011076
Mrp1	AGGCTGGAGCTAAGGAGGAG	CAGCCATGGAGTAGCCAAAT	NM_008576
Mrp4	GGTTGGAATTGTGGGCAGAA	TCGTCCGTGTGCTCATTGAA	XM_139262
Oatp2	ATAGCTTCAGGCGCATTTAC	TTCTCCATCATTCTGCATCG	NM_030687

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TABLE 2

Vascular volume (V_{vasc}, µl/g brain) of mouse brain in each experiment

Vascular volume was determined by co-perfusion of [^{14}C]sucrose for 2 min ([^{3}H]DHEAS) or 1.5 min ([^{3}H]mitoxantrone). Vascular volumes did not differ significantly between any of the experiments (P>0.05, one-way ANOVA). Data are presented as the mean ± SE (n= 4 ~ 10 per point).

Vascular volume	Wild-type mice			Mdr1a/1b P-gp knockout mice		Bcrp knockout mice	
(µl/g brain)	control	GF120918 (10 mg/kg)	GF120918 (20 mg/kg)	control	GF120918 (10 mg/kg)	control	GF120918 (10 mg/kg)
DHEAS	12.8 ± 1.2	16.9 ± 1.9	15.5 ± 0.8	11.3 ± 0.8	12.4 ± 0.5	11.3± 0.5	8.96± 1.26
mitoxantrone	11.9 ± 2.6	11.3 ± 4.3		16.6 ± 1.8	16.7 ± 4.3	12.9 ± 1.3	13.0 ± 1.6

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TABLE 3

The expression of Bcrp, Mdr1a, Mrp1, Mrp4 and Oatp2 in brain homogenate.

All mRNA levels were measured by real-time quantitative PCR and normalized by the concentration of HPRT. These were results from independent cDNA samples from each three wild-type and Bcrp knockout mice, respectively (n=3~4, mean ± SD, ratio to HRPT). BCRP KO and ND represent Bcrp knockout mice and not detected, respectively.

	Bcrp	Mdr1a	Mrp1	Mrp4	Oatp2
Wild-type	18.1 ± 2.3	26.9 ± 5.9	17.4 ± 1.1	27.7 ± 2.2	40.8 ± 7.6
Bcrp KO	ND	28.4 ± 3.3	19.1 ± 7.6	26.9 ± 1.9	44.2 ± 7.2

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Fig. 1

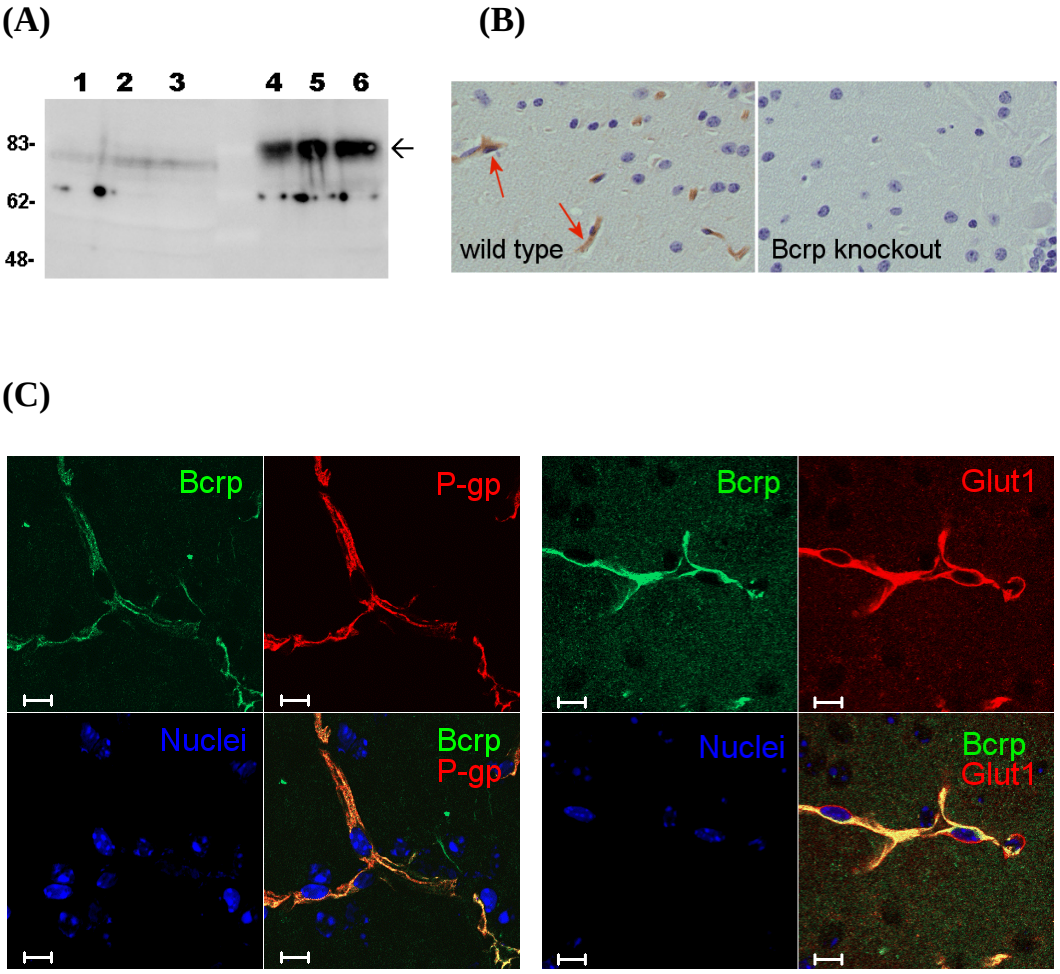

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Fig. 2.

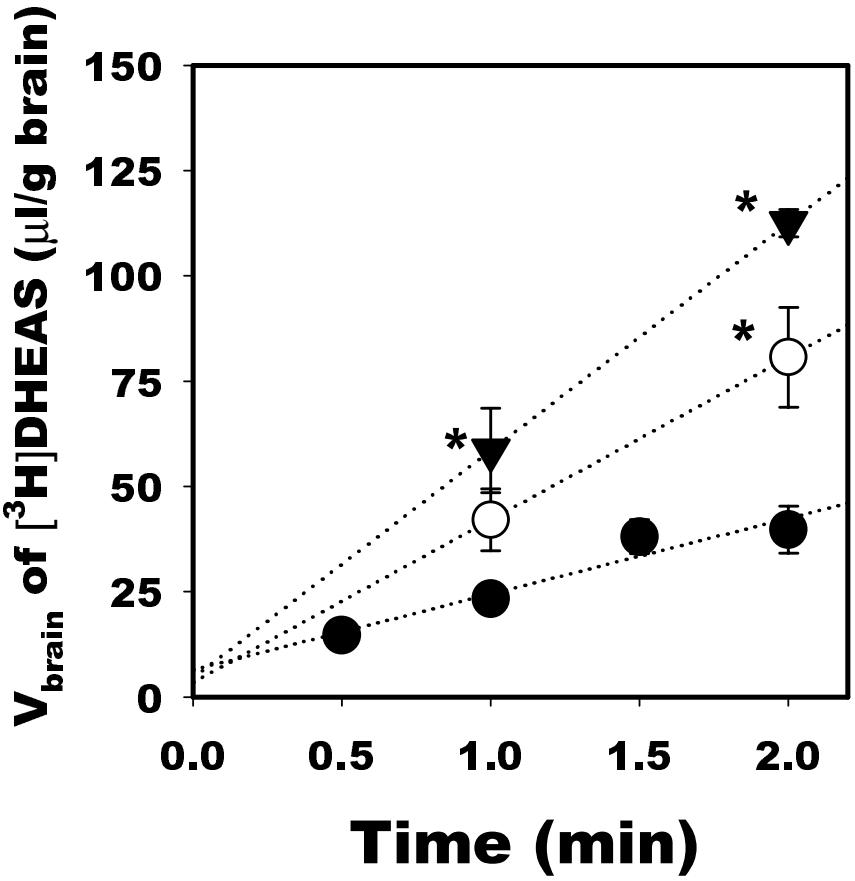

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Fig. 3.

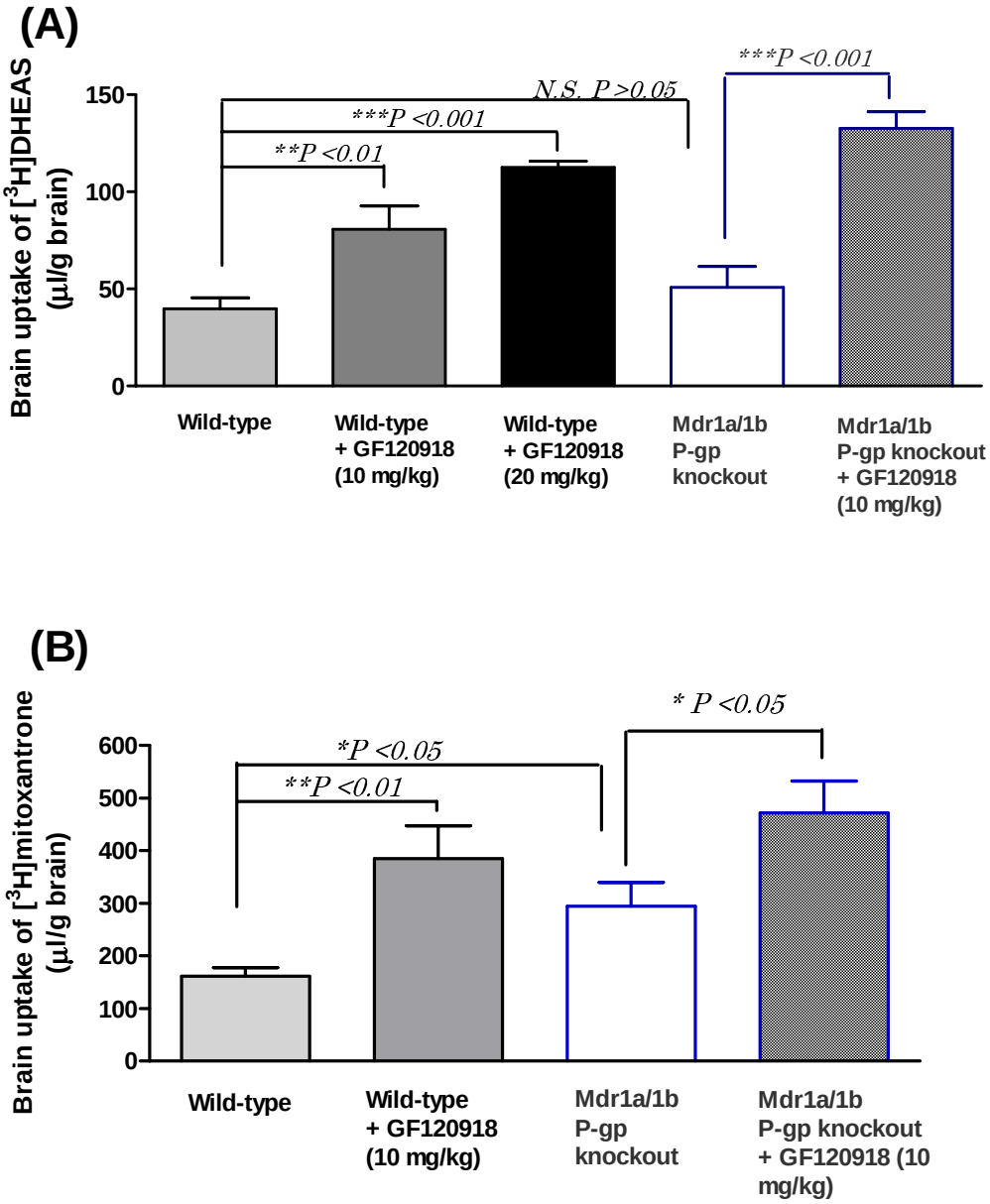

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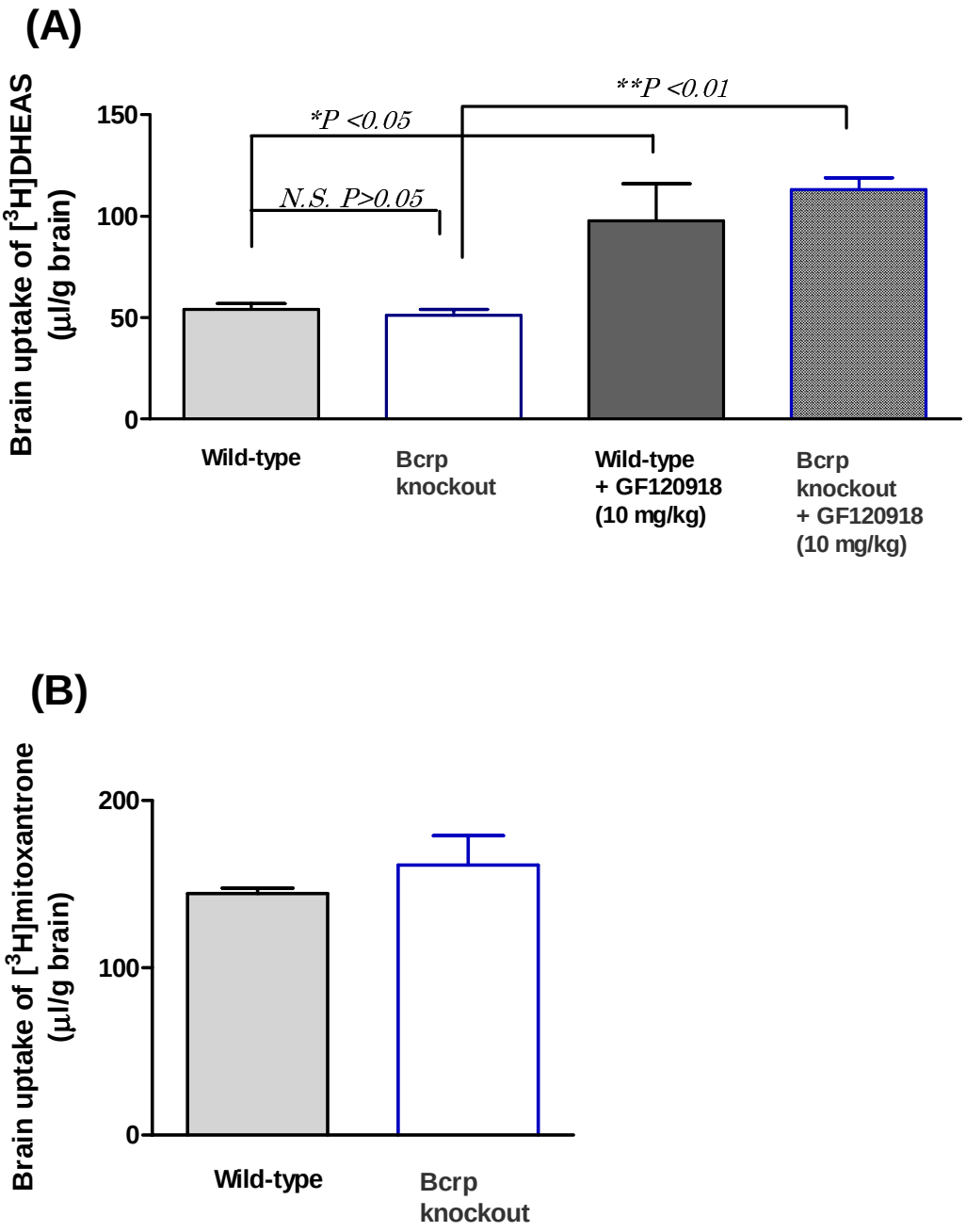
Fig. 4.



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