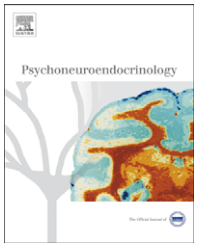
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Multidrug-resistance gene 1-type p-glycoprotein (MDR1 p-gp) inhibition by tariquidar impacts on neuroendocrine and behavioral processing of stress

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Summary
The multidrug-resistance gene 1-type p-glycoprotein (MDR1 p-gp) is a major gate-keeper at the blood–brain barrier (BBB), protecting the central nervous system from accumulation of toxic xenobiotics and drugs. In addition, MDR1 p-gp has been found to control the intracerebral access of glucocorticoid hormones and thus to modulate the activity of the hypothalamic–pituitary–adrenocortical (HPA) system. In view of the implication of glucocorticoids in the control of behavior, we examined how acute pharmacological inhibition of MDR1 p-gp at the BBB by tariquidar (XR9576; 12 mg/kg, PO) impacts the neuroendocrine and behavioral processing of stress in C57BL/6JIcoHim inbred mice.
Inhibition of MDR1 p-gp at the BBB did not alter emotional behavior at baseline. However, mice that were sensitized by water-avoidance stress, a mild psychological stressor, displayed significantly reduced anxiety-related behavior in the elevated plus-maze test when treated with tariquidar. Tariquidar, however, had no effect on stress-coping performance assessed in the forced swim test. Investigating the impact of acute MDR1 p-gp inhibition on the glucocorticoid system, we observed a significant attenuation of the mild stress-induced increase of plasma corticosterone after tariquidar administration. In order to examine whether the anti-anxiety effect of tariquidar in sensitized animals is mediated by glucocorticoids, the animals were treated with corticosterone (1 mg/kg, SC) immediately after exposure to water-avoidance stress. Corticosterone caused a significant anxiolytic-like effect in this stress-related anxiety protocol, whereas tariquidar could not further enhance corticosterone's anti-anxiety effects.
The current data show for the first time that pharmacological inhibition of MDR1 p-gp at

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the murine BBB by tariquidar alters emotional behavior and HPA axis activity. By facilitating the entry of corticosterone into the brain, tariquidar enhances feedback inhibition of the HPA system and in this way improves anxiety-related stress processing. These findings highlight a novel approach to the treatment of stress-related affective disorders in humans.
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1. Introduction

The hypothalamic–pituitary–adrenocortical (HPA) system is a critical mediator of stress responses in humans and rodents, responding to environmental stressors and facilitating adaptation via secretion of steroid hormones from the adrenal glands (cortisol as the major glucocorticoid in humans and corticosterone in rats and mice) (McEwen, 2000). These hormones are potent modulators of the function of neuronal cells and networks that control cognitive processes (de Kloet et al., 2002; Pecoraro et al., 2006) and regulate fear and anxiety-related behaviors in humans and rodents (Holsboer, 2000; Korte, 2001). Within the HPA system, corticosteroids terminate the stress response by an inhibitory feedback action at the level of the pituitary, hypothalamus and limbic system including the hippocampus and amygdala (Herman et al., 2003). This regulatory effect on the HPA axis is mediated by two types of steroid receptor molecules, the high-affinity mineralocorticoid receptor (MR) and the low-affinity glucocorticoid receptor (GR). MR primarily mediates the effects of, and possibly controls, low basal circadian levels of circulating steroids, whereas GR comes into play at circadian peak and stress-induced levels of glucocorticoids, thus mediating negative feedback inhibition of the HPA system (de Kloet et al., 1998).

Apart from regulation by MR and GR, corticosteroid signaling is also modulated by cellular factors and the local environment (Herbert et al., 2006). 11β-Hydroxysteroid dehydrogenases (11β-HSDs), for instance, are enzymes that metabolize glucocorticoids and thus tune the intracellular levels of these hormones that are available for activation of the nuclear corticosteroid receptors (Tomlinson et al., 2004). Mice homozygous for a targeted disruption of the 11β-HSD type 1 gene display a complex pattern of alterations in the circulating versus intracellular levels of corticosterone and, consequently, suffer from an impaired regulation of the HPA system (Holmes et al., 2003). In the context of local steroid bioavailability, there is evidence for a particular role of the multidrug-resistance gene 1-type p-glycoprotein (MDR1 p-gp) in the control of tissue access of glucocorticoid hormones (Ueda et al., 1992; van Kalken et al., 1993; Karssen et al., 2001; Uhr et al., 2002; Webster and Carlstedt-Duke, 2002). The MDR1 p-gp is a transmembrane 170 kDa glycoprotein that is the product of the multidrug-resistance type 1 genes (*mdr1a* and *mdr1b* genes in the mouse and one single MDR1 gene in humans) and belongs to a phylogenetically highly conserved family of ATP-binding-cassette (ABC) transport proteins (Schinkel and Jonker, 2003). The transporter has been shown to secrete naturally occurring metabolites and exogenous substances into the urinary or gastrointestinal tract and to confer treatment resistance to tumor cells by expelling anticancer drugs (Szakacs et al., 2006). In the brain, MDR1 p-gp has been predominantly localized to the luminal surface of capillary endothelial cells forming the blood–brain barrier (BBB) and, possibly, to epithelial cells of the blood–cerebrospinal fluid barrier (Lee et al., 2001; Soontornmalai et al., 2006). Recent findings also suggest that *mdr1* mRNA is expressed in neurons of the rodent hippocampal formation; however, immunohistochemical detection of the functional protein has only been reported after excitotoxin-induced seizures in animal models of epilepsy (Karssen et al., 2004; Volk et al., 2004). While the physiological significance of the transport protein in hippocampal neurons awaits to be elucidated, there is ample evidence for an important role of MDR1 p-gp in the BBB to protect the central nervous system from a wide range of toxic xenobiotics and to inhibit the access of various drugs, e.g., antidepressants and antiepileptics (Loscher and Potschka, 2005).

MDR1 p-gp actively transports corticosteroid hormones across the BBB out of the brain (Uhr et al., 2002) and thus significantly influences the exposure and sensitivity of the brain to glucocorticoids. There is a reciprocal interaction between the corticosteroid and MDR1 p-gp systems, given that modulation of intracellular glucocorticoid levels by MDR1 p-gp alters GR translocation and signaling (Pariante et al., 2004) and the expression of the murine *mdr1b* gene is regulated by steroid hormones in a regulatory feedback loop (Cohen et al., 1991; Altuvia et al., 1993). The first compelling *in vivo* evidence for a sustained impact of MDR1 p-gp function on the HPA axis has recently been provided by Müller et al. (2003). They found that genetic disruption of both the murine *mdr1a* and *mdr1b* gene at the BBB leads to a profound suppression of the HPA axis at the hypothalamic level under basal conditions and following stress.

Since it cannot be definitely ruled out that germline knockout of the *mdr1a* and *mdr1b* genes causes a reprogramming of the stress hormone system and in this way alters acute stress-related responses of the HPA system, the present study pursued a different approach to the elucidation of the interactions between MDR1 p-gp function and HPA axis activity. Specifically, we investigated whether pharmacological inhibition of MDR1 p-gp impacts the neuroendocrine and behavioral processing of stress-related stimuli. We hypothesized that acute inhibition of steroid transport via MDR1 p-gp at the BBB induces cerebral glucocorticoid accumulation following stress when there is a massive release of corticosterone from the adrenal cortex. Increased levels of corticosteroids at hippocampal steroid receptors, in turn, are likely to enhance HPA axis feedback regulation. If this were the case, we reasoned that anxiety-related and stress-coping behavior as well as

the plasma concentrations of corticosterone would be altered.

Pharmacological inhibition of MDR1 p-gp was achieved by tariquidar (XR9576), a potent and highly specific, third generation MDR1 p-gp blocking compound that was developed to restore sensitivity to chemotherapeutic agents in multidrug-resistant cancers (Martin et al., 1999; Mistry et al., 2001). In our study, the behavioral and neuroendocrine effects of tariquidar were examined both under baseline conditions and following exposure to water-avoidance stress (WAS) inducing neuroendocrine and behavioral sensitization. WAS represents a mild psychological stressor that stimulates corticotropin-releasing factor (CRF) synthesis and secretion in the hypothalamus and causes subsequent release of adrenocorticotropic hormone and corticosterone (Bonaz and Tache, 1994; Million et al., 1999).

2. Material and methods

2.1. Experimental animals

All experiments were performed with male C57BL/6JIcoHim (C57BL/6) mice purchased from the Division of Laboratory Animal Science and Genetics, Department of Biomedical Research, Medical University of Vienna (Himberg, Austria). The animals were housed individually in polycarbonate cages under standard laboratory conditions (temperature 21–22 °C) and a 12-h light/12-h dark cycle (lights on between 06:00 and 18:00 h). Food pellets and water were available *ad libitum*. The mice were tested after 2 weeks of adaptation to the new housing environment. At the time of the experiments, they were 9–12 weeks old, with a body weight of 20–25 g. All animals were experimentally naïve.

All experiments were approved by an ethical committee at the Federal Ministry of Education, Science and Culture of the Republic of Austria and conducted according to the Directive of the European Communities Council of 24 November 1986 (86/609/EEC). The experiments were designed in such a way that the number of animals used and their suffering was minimized.

2.2. Drugs

All drug solutions were made up fresh prior to use. Tariquidar (XR9576) was kindly provided by Xenova (Slough, UK) and suspended in a 100% polyethylene glycol (PEG) 200 solution. The drug was administered per os (PO) via a feeding cannula at a dose of 12 mg/kg in a volume of 10 ml/kg. The oral bioavailability of the drug has been reported to achieve 80% (Mistry et al., 2001). Corticosterone (Sigma-Aldrich, Schnelldorf, Germany) was dissolved in saline (0.9% NaCl) and injected subcutaneously (SC) at a dose of 1 mg/kg. ^{3}H-ivermectin B1a was purchased from Biotrend Radiochemicals (Cologne, Germany) and ivermectin from Sigma-Aldrich. ^{3}H-ivermectin (specific activity 20 Ci/mmol) was diluted with carrier ivermectin and suspended in 25% PEG 200 and 75% saline (0.9% NaCl) to a final specific activity of 0.166 μCi/μg. The ivermectin solution was injected intraperitonally (IP) at a dose of 0.2 mg/kg in a volume of 10 ml/kg. On average, radioactivity in the amount of 1 μCi was injected per mouse. Evans blue (Sigma-Aldrich, Schnelldorf, Germany) was prepared as 2% (w/w) solution in saline and injected into the tail vein (2 ml/kg).

2.3. Ivermectin tissue distribution

The ability of tariquidar to inhibit MDR1 p-gp at the BBB was tested by investigating the transport of ivermectin into the brain, given that this antihelminthic drug is a model substrate for MDR1 p-gp (Schinkel et al., 1994). The tissue distribution of ivermectin was measured by a modification of a protocol originally described by Schinkel et al. (1994). Briefly, tariquidar (12 mg/kg) was given PO, followed by IP injection of the ^{3}H-ivermectin/ivermectin solution 2 h afterwards. After another 2 h, animals were deeply anesthetized with an overdose of sodium pentobarbital (Vetanarcol; Intervent, Vienna, Austria) and transcardially perfused with phosphate-buffered saline (pH 7.4). Tissues (blood, brain, liver, gallbladder) were then removed, weighed and homogenized in 4% bovine serum albumin solution. Aliquots of the homogenates were transferred to Ultima Gold counting fluid (Perkin-Elmer, Vienna, Austria), and radioactivity was determined by liquid scintillation counting. The tissue levels of ivermectin were calculated as ng/mg wet weight and ng/ml plasma, respectively. The brain-to-plasma ratio was determined according to Dai et al. (2003) and represents the ratio of disintegrations per minute (dpm) per gram brain tissue over dpm per milliliter of plasma.

2.4. Behavioral assessment and water-avoidance stress

2.4.1. Open field test (OF)

Animals were tested in an open field box (50 cm × 50 cm × 30 cm) made of gray PVC plastic. The arena was evenly illuminated with 80 lx at floor level. The area of the open field was divided into a 36 cm × 36 cm central zone and a surrounding border zone. Mice were individually placed into a corner of the open field, and behavior was measured over a 5 min period with a digital video-tracking system (VideoMot2; TSE, Bad Homburg, Germany). The time spent in and the number of entries into the central zone were recorded as indices of anxiety-related behavior. Horizontal locomotor activity was determined by the total distance traveled during the test session.

2.4.2. Elevated plus-maze test (EPM)

A plus-shaped maze made of gray PVC plastic was used. The device comprised two opposing open arms (30 cm × 5 cm) and two opposing closed arms (30 cm × 5 cm) that had 15 cm high, nontransparent walls. The four arms were connected by a central platform (5 cm × 5 cm). The maze was elevated 70 cm above the floor. The open arms were illuminated with an intensity of 80 lx, the central area with 70 lx and the closed arms with 40 lx. Mice were initially placed in the center of the maze facing one of the open arms and then were allowed to investigate the area for 5 min. Their behavior was recorded by video-tracking (VideoMot2). Entry into an arm was defined when the mouse moved entirely (except the tail) into the arm. The time spent in and the number of entries made into the open and closed

arms as well as the center time were measured to define anxiety-related behavior, and the total distance traveled during the test session was recorded as a locomotion index.

2.4.3. Forced swim test (FST)

Mice were individually placed in a glass beaker (diameter 12.5 cm) containing tap water at 25 °C. The water depth was 11.5 cm which prevented the mice from touching the bottom of the beaker with their paws or the tail. Mice were tested for 5 min and their behavioral activity was scored by a trained observer. The times spent on climbing, swimming, and immobility were recorded to determine active vs. passive stress-coping performance. Mice were considered immobile when floating passively in the water, performing only those movements required to keep their heads above the water level (Cryan et al., 2002).

2.4.4. Water-avoidance stress (WAS)

The stress procedure consisted of placing a mouse on a platform (2 cm × 5 cm × 8 cm) affixed to the bottom center of a white PVC container (61 cm × 41 cm × 21 cm). For experiment 5 (see below), a white PVC container with slightly modified dimensions (54 cm × 36 cm × 21 cm) but the same platform size was used. The container was filled with tap water (30 °C) up to the last 1 cm of the height of the platform. Mice were subjected to this aversive situation for 1 h.

2.5. Plasma corticosterone analysis

Blood samples were taken either by incision of the tail vein before and after exposure to WAS or by decapitation after the final behavioral experiment and collected in EDTA tubes on ice. Blood was centrifuged (15 min, 3000 rpm, 4 °C) to obtain plasma which was stored at −20 °C until assayed for corticosterone by means of a competitive enzyme immunoassay (Assay Designs, Ann Arbor, MI, USA). According to the manufacturer's specifications, the sensitivity of the assay is 27 pg/ml, and the intra- and inter-assay coefficient of variation amounts to 7.7% and 9.7%, respectively.

2.6. Experimental protocols

All behavioral tests and stress sessions were performed between 07:00 and 13:00 h in a sound-attenuated room. Prior to testing, mice were habituated to the testing room for at least 24 h. The mice were naïve to behavioral testing, except in experiment 2, when animals were subjected to a battery of tests.

2.6.1. Experiment 1

In this experiment, the ability of tariquidar to inhibit MDR1 p-gp at the BBB was estimated via its ability to cause ^{3}H-ivermectin accumulation in the brain. To this end, tariquidar or vehicle was given PO, followed by IP injection of ^{3}H-ivermectin 2 h afterwards. After another 2 h, the levels of ^{3}H-ivermectin in the brain and other tissues were determined.

2.6.2. Experiment 2

To examine tariquidar's effects on basal emotional behavior, the animals were tested in a series of behavioral paradigms consisting of the OF, EPM and FST. Two hours before each test, the animals were treated with either vehicle or tariquidar PO. The individual tests were conducted at intervals of 4 days in order to allow for washout of tariquidar. The treatment assignment (vehicle vs. tariquidar) of each animal was changed with each test to avoid pharmacological sensitization or desensitization.

2.6.3. Experiment 3

To study the effect of tariquidar in stressed animals, mice were administered vehicle or tariquidar PO and 2 h afterwards subjected to WAS. The behavioral assessment of the stressed mice in the OF, EPM and FST was performed 24 h later. In order to determine circulating corticosterone levels at baseline and following stress, tail blood was taken 1 h before drug administration and 30 min after the end of the WAS session in one-half of the animals. In addition, trunk blood was collected 30 min after completion of the EPM and FST.

2.6.4. Experiment 4

In a further study of the effect of tariquidar on stress processing, animals received vehicle or tariquidar PO and then were exposed to WAS 2 h later. Twenty-four hours afterwards, the animals were re-subjected to WAS and assessed in the EPM and FST 24 h later on the following day. In addition, trunk blood was taken for corticosterone analysis 30 min after completion of the EPM and FST.

2.6.5. Experiment 5

In order to examine the impact of corticosterone *per se* as well as the interaction between corticosterone and tariquidar on stress-induced modulation of anxiety-related behavior, mice received tariquidar or vehicle PO and, 2 h afterwards, were subjected to WAS. Immediately after WAS exposure, the animals were injected SC either with saline or corticosterone. The next day, the animals were assessed in the EPM, followed by collection of trunk blood for corticosterone analysis 30 min afterwards.

2.6.6. Experiment 6

To estimate the effect of WAS *per se* on BBB integrity (Friedman et al., 1996; Sharma et al., 1996; Ovadia et al., 2001) mice received an injection of the albumin-binding dye Evans blue (2% solution in saline, 2 ml/kg) under a light and short-lasting isoflurane anesthesia immediately after 1 h exposure to WAS. Control animals stayed in their home cages. After an incubation time of 15 min, animals were deeply anesthetized and transcardially perfused with phosphate-buffered saline (PBS; pH 7.4). Brains were then removed, weighed and placed in 0.1 M PBS containing 50% trichloroacetic acid (10 μl/mg tissue). After the tissue had been homogenized and centrifuged (12,000 rpm, 10 min), the concentrations of the albumin–Evans blue complex were spectrophotometrically measured in the supernatant at 605 nm wavelength. The tissue levels of Evans blue were calculated as ng/g wet weight.

2.7. Data presentation and statistical analysis

All data are reported as means ± S.E.M. Two-group comparisons were performed with *t*-tests for independent groups. Statistical comparison of three groups was made by analysis of variance (ANOVA). According to the experimental design, two-factorial ANOVAs were used with *treatment* (vehicle; tariquidar) and *stress* (basal; single WAS; repeated WAS) as between-subject factors. When appropriate, the Newman–Keuls post hoc test was used to identify group differences. Statistical significance was accepted if $p < 0.05$.

3. Results

3.1. Proof of *in vivo* MDR1 p-gp inhibition by tariquidar

In experiment 1, the effect of tariquidar at the MDR1 p-gp transporter *in vivo* was estimated by the tissue distribution of ivermectin, a substrate of reasonable specificity for MDR1 p-gp (Schinkel et al., 1994). Mice received either vehicle ($n = 4$) or tariquidar ($n = 5$), followed by an injection of ^{3}H-ivermectin. As shown in Table 1, tariquidar caused a significant accumulation of ivermectin only in the brain but not in the blood, gallbladder and the liver. The levels of ivermectin in the brain of tariquidar-treated mice were almost three-fold higher than the respective levels in vehicle-treated animals as determined by the brain-to-plasma ratio (Figure 1).

3.2. Lack of effect of tariquidar on basal emotional behavior

In experiment 2, the effects of tariquidar on the behavioral performance of mice in anxiety-related and stress-coping tests were investigated. The animals were first assessed in the OF. As shown in Table 2, no significant differences between vehicle- and tariquidar-treated mice were observed. Thus, the number of entries made into and the time the animals spent in the central area of the open field as well as the total distance traveled were not altered by tariquidar. After a recovery period of 4 days, the animals were subjected to the EPM following readministration of vehicle or tariquidar. Again, there was no significant difference between the treatment groups with regard to the number of entries made into and the time spent on the open or closed arms, the time spent in the center of the maze, and the total distance traveled (Table 2). Four days after the EPM, basal stress-coping performance was assessed in the FST. Tariquidar was without significant impact on the times the animals spent climbing and swimming or were

Table 1 *In vivo* inhibition of ^{3}H-ivermectin transport by tariquidar.

Organ	Treatment	Ivermectin (ng/100 µl)
Plasma	Vehicle	2.9 ± 0.4
	Tariquidar	3.8 ± 0.5
Gallbladder	Vehicle	16.3 ± 1.6
	Tariquidar	27.8 ± 3.9*
Liver	Vehicle	0.4 ± 0.10
	Tariquidar	0.5 ± 0.01
Brain	Vehicle	0.01 ± 0.002
	Tariquidar	0.04 ± 0.003*

Data are presented as mean ± S.E.M.
*$p < 0.05$ tariquidar ($n = 5$) vs. vehicle ($n = 4$).

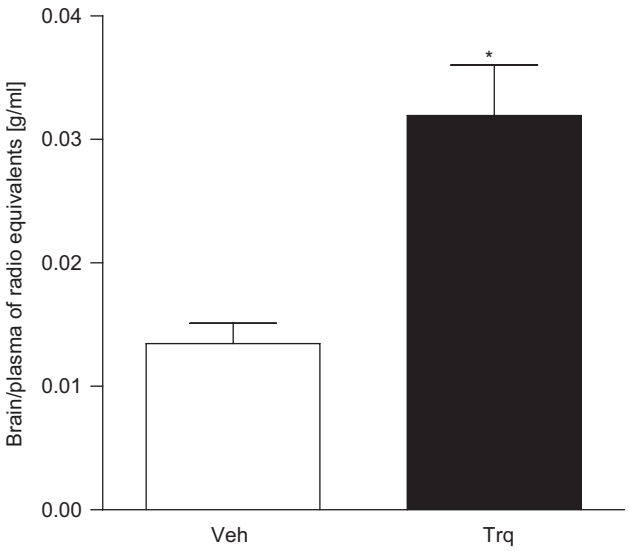


Figure 1 Effect of MDR1 p-gp inhibition by tariquidar (12 mg/kg) on the brain penetration of ^{3}H-ivermectin in mice. The brain-to-plasma ratio of radioequivalents (dpm [disintegrations per minute] in brain [g]/dpm in plasma [ml]) is expressed as means+S.E.M.; *$p < 0.05$, tariquidar (trq; $n = 5$) vs. vehicle (veh; $n = 4$).

Table 2 Basal emotional behavior in mice after tariquidar administration.

Behavioral phenotypes	Vehicle	Tariquidar
Open field test	$n = 10$	$n = 10$
Time center (%)	18.03 ± 2.01	20.23 ± 2.96
Entries center (no.)	25.10 ± 2.79	23.90 ± 1.81
Total distance moved (cm)	2231 ± 103.8	2225 ± 132.5
Elevated plus-maze test	$n = 9$	$n = 10$
Entries open arms (no.)	3.44 ± 0.67	3.20 ± 0.84
Time open arms (%)	7.66 ± 1.34	5.65 ± 1.86
Entries closed arms (no.)	15.33 ± 1.68	16.80 ± 2.68
Time closed arms (%)	77.73 ± 3.04	83.26 ± 3.14
Time center (%)	14.04 ± 2.34	11.09 ± 1.70
Total distance moved (cm)	820 ± 70.24	775 ± 81.21
Forced swim test	$n = 9$	$n = 8$
Time climbing (%)	15.85 ± 2.04	11.77 ± 1.27
Time swimming (%)	14.72 ± 1.23	15.57 ± 1.60
Time immobile (%)	68.54 ± 2.30	72.99 ± 1.99

Data represent means ± S.E.M.

immobile (Table 2). Three animals had to be excluded from behavioral testing and data analysis because they were injured during the PO administration procedure.

3.3. Inhibition of MDR1 p-gp modulates the behavioral response to stress

Experiment 3 was carried out to investigate whether tariquidar alters the behavioral performance of mice exposed to a mild stressor such as WAS. In the OF, vehicle- and tariquidar-treated animals did not differ with respect to the time spent in or the number of entries made into the center of the open field as well as the total distance traveled. Statistical evaluation of the effects of treatment and stress by ANOVA showed, however, that the number of entries into the central zone was significantly increased by the factor stress ($F_{1,36} = 7.41$; $p = 0.010$), whereas no significant effects were found for the factor treatment or the interaction of stress and treatment (Figure 2A). Thus, stressed animals made more entries into the center of the open field than controls. However, stress had no significant effect on the time the animals spent in the center of the open field or the total distance traveled.

Analysis of anxiety-related behavior in the EPM after a single WAS exposure revealed that tariquidar-treated mice spent significant more time on the open arms than vehicle controls (Figure 2B). In addition, a trend ($p = 0.09$) towards more entries of tariquidar-treated animals into the open arms was observed. In contrast, the number of entries into and the time spent on the closed arms as well as the time spent in the center of the maze and the total distance traveled were not altered by tariquidar. Analysis of the time spent on the open arms by ANOVA demonstrated significant effects for the factor stress ($F_{1,364} = 4.31$; $p = 0.046$) and the interaction of treatment and stress ($F_{1,34} = 4.62$; $p = 0.039$), but not for the factor treatment. With respect to the total distance traveled, there was a significant effect for the factor stress ($F_{1,34} = 10.09$; $p = 0.003$), but not for the factor treatment or the factorial interaction (Figure 2C). No other significant effects were uncovered by ANOVA. The protocol of experiment 3 involving a single WAS challenge was extended in experiment 4 to repeated WAS exposure on 2 consecutive days. Statistical analysis of the data obtained

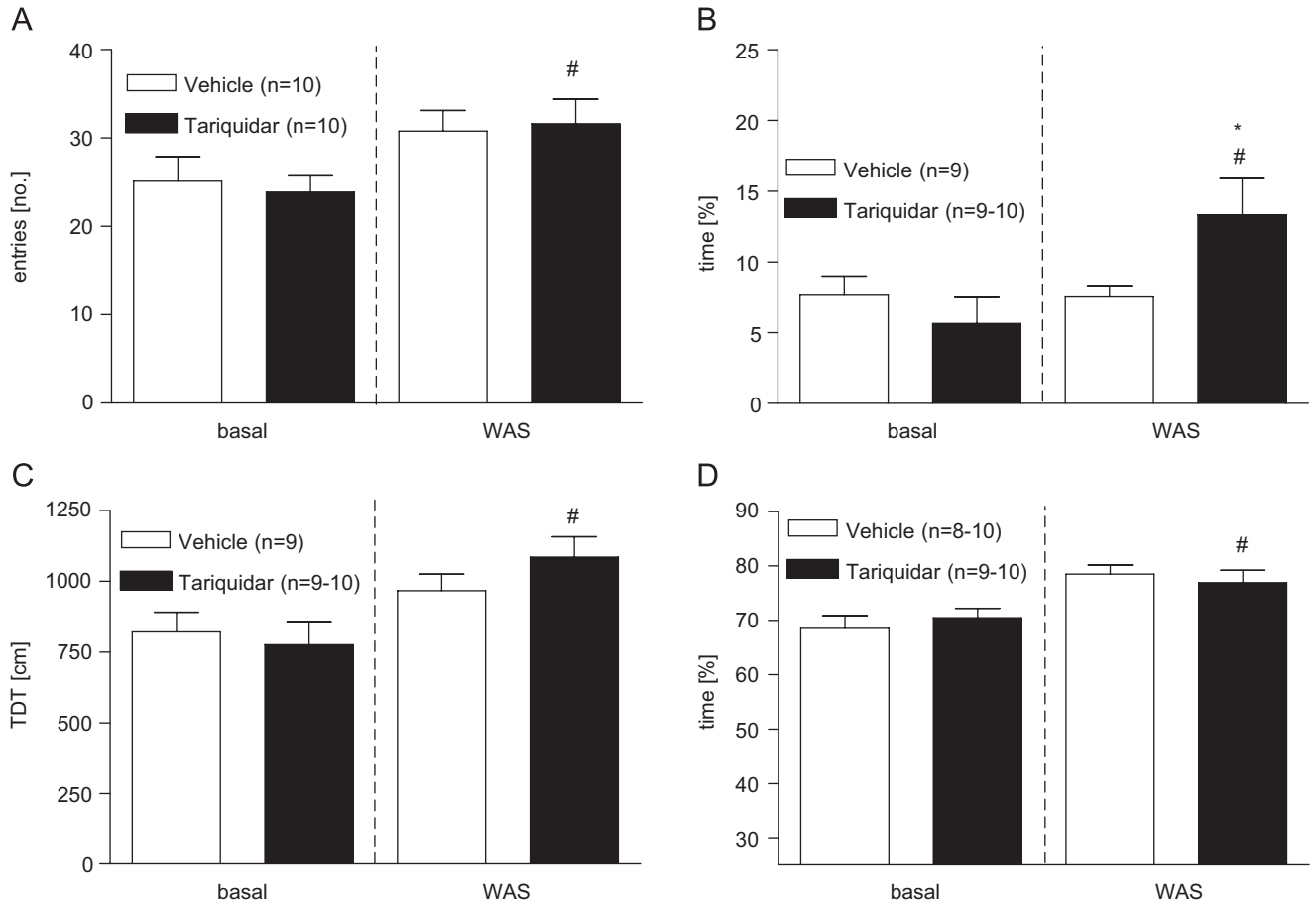


Figure 2 Behavioral responses of mice treated with vehicle or tariquidar (12 mg/kg) under basal conditions and after exposure to water-avoidance stress (WAS). Panel (A) shows the entries the animals made into the central area of the OF, panels (B) and (C) display the time the animals spent on the open arms and the total distance traveled (TDT) in the elevated plus-maze test (EPM), and panel (D) presents the time of immobility in the forced swim test (FST). The time spent on the open arms in the EPM and the time spent immobile in the FST were expressed as a percentage of the total test duration. Results are presented as means+S.E.M.; $^{*}p<0.05$, vehicle versus tariquidar; $^{\#}p<0.05$, stress versus basal (independent of treatment; i.e., significant ANOVA effect for the factor stress).

in experiment 4 showed that neither stress nor treatment with tariquidar had any significant influence on any of the EPM parameters under study (data not shown).

The third behavioral test conducted in experiment 3 was the FST. Following a single WAS exposure, vehicle- and tariquidar-treated mice did not significantly differ in the time they spent while swimming and climbing or were immobile. Evaluation of the impact of treatment and single WAS exposure by ANOVA, however, revealed significant effects for the factor stress regarding the parameters swimming ($F_{1,33} = 4.24$; $p = 0.047$) and immobility ($F_{1,33} = 5.69$; $p = 0.023$). As illustrated in Figure 2D, the time of immobility in stressed mice was longer than in controls. No significant effects for other factors, the factorial interaction and the parameter climbing were observed. Following repeated WAS exposure in experiment 4, none of the FST parameters under study differed between vehicle- and tariquidar-treated mice (data not shown). Similarly, ANOVA for the effects of treatment and repeated WAS challenge did not reveal any significant effects and interaction (data not shown). In experiments 3 and 4, six animals had to be excluded from behavioral testing and data analysis because they were injured during the PO administration procedure.

3.4. Tariquidar impacts on stress-related glucocorticoid release

In experiments 3 and 4, not only the emotional behavior but also the plasma levels of corticosterone were determined. Specifically, the effect of tariquidar on circulating corticosterone was measured 30 min after exposure to WAS, EPM or FST. An ANOVA of the corticosterone levels at baseline and after exposure to WAS showed significant effects for the factors treatment ($F_{1,36} = 8.82$; $p = 0.005$) and stress ($F_{1,36} = 69.57$; $p < 0.001$) and the interaction of treatment and stress ($F_{1,36} = 13.90$; $p = 0.001$). Post hoc analysis revealed that stress caused a significant increase in circulating corticosterone. This stress-induced release of

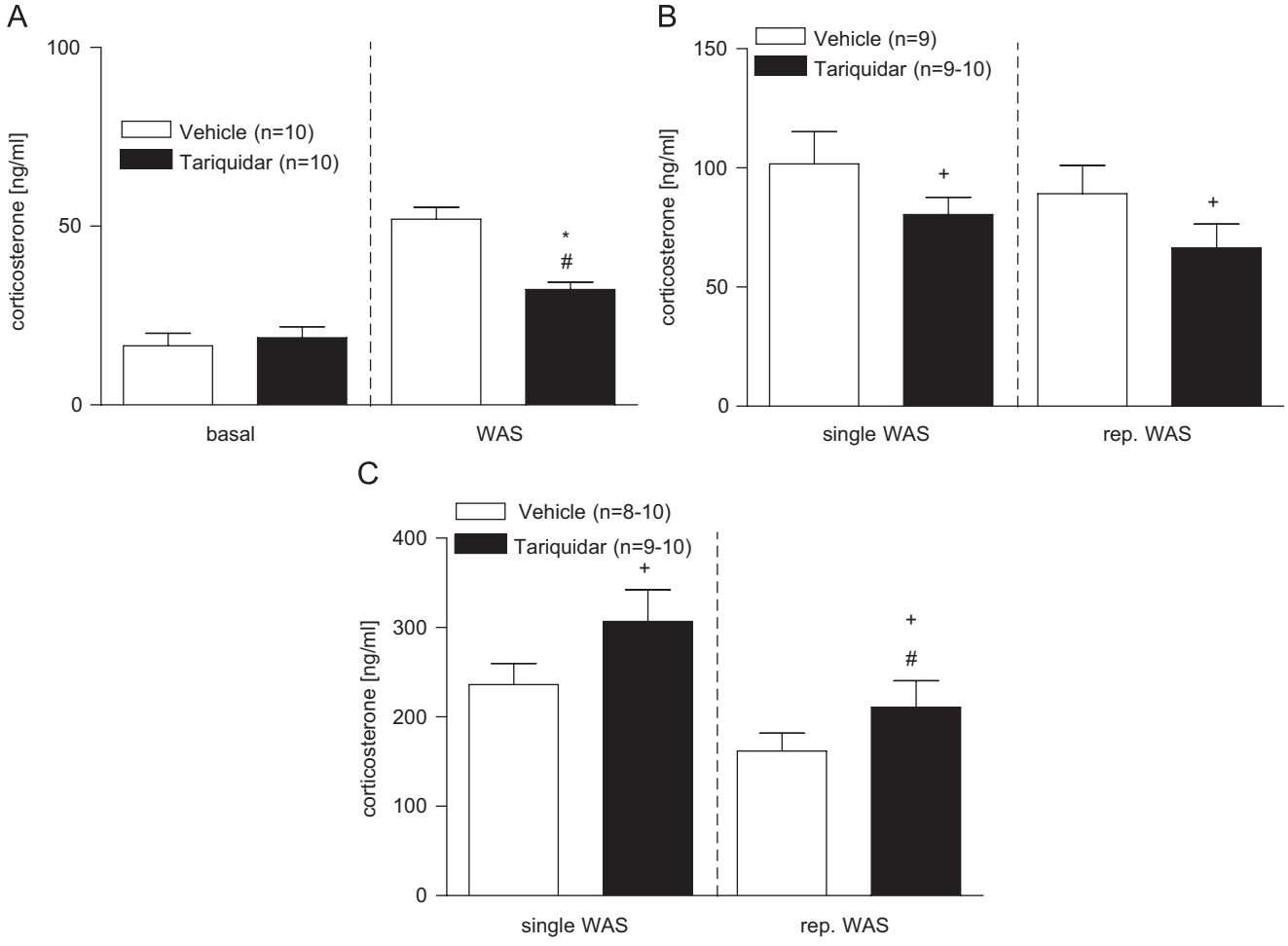


Figure 3 Effects of vehicle and tariquidar (12 mg/kg) on plasma corticosterone levels. Panel (A) presents the corticosterone levels measured at baseline and after single exposure to water-avoidance stress (WAS). Panels (B) and (C) show the corticosterone levels measured after single or repeated (rep.) exposure to water-avoidance stress (WAS) followed by the elevated plus-maze test (EPM) or forced swim test (FST). Tariquidar and its vehicle were administered before exposure to WAS. Results are presented as means+S.E.M.; $^{*}p < 0.05$, vehicle versus tariquidar; $^{\#}p < 0.05$, stress versus basal or single versus repeated WAS (independent of treatment; i.e., significant ANOVA effect for the factor stress); $^{+}p < 0.05$, vehicle versus tariquidar (independent of stress; significant ANOVA effect for the factor treatment).

corticosterone was significantly attenuated by tariquidar (Figure 3A).

ANOVA-based analysis of the influence of tariquidar and single or repeated exposure to WAS on corticosterone release after the EPM demonstrated a significant effect for the factor treatment ($F_{1,33} = 4.14$; $p = 0.050$) but not for the factor stress and the interaction between these factors. As depicted in Figure 3B, mice treated with tariquidar had lower levels of circulating corticosterone than mice treated with vehicle, independently of whether they had been subjected to single or repeated WAS.

The plasma levels of corticosterone measured after the FST were more than double those measured after the EPM (cf. Figure 3B and C). ANOVA-based evaluation of the impact of tariquidar and single or repeated exposure to WAS on corticosterone release after the FST revealed significant effects for the factors treatment ($F_{1,34} = 5.34$; $p = 0.027$) and stress ($F_{1,34} = 7.14$; $p = 0.012$), but not for the interaction between these factors. Figure 2C illustrates that tariquidar enhanced the FST-induced release of corticosterone after exposure to both single and repeated WAS. The corticosterone levels measured in both vehicle- and tariquidar-treated mice subjected to the FST were significantly lower following repeated WAS than after single WAS (Figure 3C).

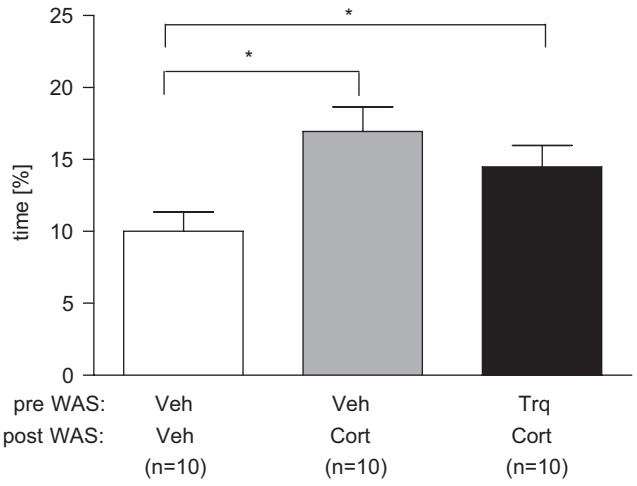


Figure 4 Effects of vehicle (Veh), tariquidar (Tar, 12 mg/kg) and corticosterone (Cort, 1 mg/kg) on behavior in the elevated plus-maze test (EPM). Tariquidar and its vehicle were administered before single exposure to water-avoidance stress (WAS), while corticosterone and its vehicle were administered immediately after the WAS. The time spent on the open arms in the EPM is expressed as a percentage of the total test duration. Results are presented as means+S.E.M.; $^*p < 0.05$ vs vehicle.

3.5. Exogenous corticosterone reduces anxiety-related behavior

Experiment 5 was carried out to investigate whether exogenous corticosterone, administered immediately after exposure to WAS, modulates anxiety-related behavior as assessed in the EPM on the next day and whether such an effect of corticosterone is enhanced by tariquidar. Animals were treated with vehicle or tariquidar before exposure to WAS and received corticosterone or its vehicle immediately after sensitization. Evaluation of the EPM data by ANOVA demonstrated a significant effect for the parameters time spent on the open arms ($F_{2,29} = 5.437$; $p = 0.010$) and total distance traveled ($F_{2,29} = 6.155$; $p = 0.006$). Post hoc testing indicated that mice treated with corticosterone alone as well as mice treated with tariquidar plus corticosterone spent significantly more time on the open arms than the vehicle controls (Figure 4). With respect to the total distance traveled, mice injected with corticosterone alone or tariquidar plus corticosterone also displayed significantly more activity than the vehicle controls (data not shown). However, as animals treated with corticosterone alone or tariquidar plus corticosterone did not enter the open arms more often than the vehicle controls, a confounding effect of the increase in activity (total distance traveled) on the reduction of anxiety-related behavior (prolongation of the time spent on the open arms) can most likely be ruled out.

The effect of exogenous corticosterone, given immediately after exposure to WAS, on the circulating levels of corticosterone measured after the EPM on the following day was also examined. The data of this experiment did not reveal any significant differences in circulating corticosterone between the three treatment groups (means$\pm$S.E.M.; vehicle/vehicle: 153.4$\pm$13.1 ng/ml; vehicle/corticosterone: 153.7$\pm$10.5 ng/ml; tariquidar/corticosterone: 152.7$\pm$8.9 ng/ml; $n = 10$ for each group).

3.6. Was *per se* does not affect BBB integrity

In experiment 6, mice received an IV injection of Evans blue, an albumin-binding dye, immediately after WAS or under control conditions, i.e., mice stayed only in their home cages, to assess the impact of the stress procedure *per se* on structural BBB integrity. The injections were performed under isoflurane anesthesia to limit any further stress imposition. The brain content of Evans blue in control mice was 1.12$\pm$0.29 ng/g wet tissue ($n = 5$) and in the animals exposed to WAS 1.24$\pm$0.51 ng/g wet tissue ($n = 6$). These values did not differ significantly (t-test: $p = 0.86$) from each other excluding a possible interference of this stress procedure with BBB function other than MDR1 p-gp mediated.

4. Discussion

In the present study, we investigated whether blockade of MDR1 p-gp alters the behavioral and neuroendocrine phenotype of mice. To our knowledge, the current data show for the first time that pharmacological inhibition of this membrane transporter at the BBB impacts the anxiety-related behavior in C57BL/6 inbred mice as observed in the EPM, a well established paradigm for anxiety testing. Importantly, the influence of MDR1 p-gp on anxiety-related behavior was seen only when the mice had been subjected to a mild psychological stressor. In addition, our data provide experimental evidence that the changes in anxiety-related behavior caused by inhibition of MDR1 p-gp may be associated with changes in HPA axis activity and in this

aspect extend previous findings by Müller et al. (2003) that MDR1 p-gp modulates the activity of the HPA system.

In the current experiments, tariquidar (XR9576) was used as the pharmacological tool to block MDR1 p-gp. This anthranilic acid derivative is a potent and specific MDR1 p-gp inhibitor that has no affinity for the related transporter multidrug-resistance-associated protein (Martin et al., 1999). Tariquidar is able to increase the activity of a number of cytotoxic agents in tumor cells *in vitro* and *in vivo*, this effect being due to inhibition of MDR1 p-gp which clears the cells of xenobiotic substances (Mistry et al., 2001). In a clinical phase II study of patients with multidrug-resistant advanced breast cancer, tariquidar has been found to partly restore the *in vivo* sensitivity to anthracycline or taxane chemotherapy (Pusztai et al., 2005). Importantly, the drug is well tolerated in humans and rodents, lacking any side effects that would confound behavioral experiments (Stewart et al., 2000). This property of the drug was confirmed in the present study, as we failed to observe any changes in emotional behavior and locomotion under basal conditions. The PO dose of tariquidar used here (12 mg/kg) was chosen on the basis of a previous study that established this dose to have a high bioavailability (80%) and a high efficacy in blocking MDR1 p-gp in the mouse *in vivo* (Mistry et al., 2001).

The utility of tariquidar as an inhibitor of MDR1 p-gp at the BBB was proved by its ability to cause accumulation of IP administered ivermectin in the murine brain. This anthelminthic drug is known to be a good substrate for the MDR1 p-gp transporter as it accumulates in the brain of *mdr1a* knockout mice (Schinkel et al., 1994) and of mice treated with various MDR1 p-gp inhibitors (Marques-Santos et al., 1999). Under normal circumstances, ivermectin is largely excluded from the brain by virtue of MDR1 p-gp which secretes ivermectin into rat brain capillaries *in vitro* (Nobmann et al., 2001). In the current study, acute tariquidar treatment was found to cause a three-fold accumulation of ivermectin in the brain, which attests to an effective inhibition of MDR1 p-gp at the BBB, whereas the transport of ivermectin into the blood, liver and gallbladder compartments was enhanced only to a small or negligible extent.

There is emerging evidence that corticosteroids are relevant to physiological behaviors such as learning and memory (Roozendaal, 2000; Lupien et al., 2005; Cai et al., 2006) as well as to psychopathological conditions such as depression, fear and anxiety (Holsboer, 2000; Korte, 2001). Depending on whether preferentially high-affinity MRs or low-affinity GRs are activated, corticosteroids exert differential effects on cerebral neurons and networks. The type of these effects depends critically on the concentration of the agonists at corticosteroid receptors (de Kloet et al., 1998). For instance, the ratio of MR/GR occupation in the rodent brain determines the direction of glucocorticoid-induced cognitive changes (de Kloet et al., 2002). Long-term potentiation (LTP) in the hippocampus, a model of synaptic plasticity underlying fear learning, is also affected by corticosteroids in a dual manner. Basal levels of corticosterone enhance LTP preferentially via stimulation of the MR, whereas high steroid levels such as those occurring during stress impair hippocampal LTP through GR activation (Joels, 2006; Kim et al., 2006). This differential effect exemplifies the inverted U shape of the dose–response relationship for corticosteroid actions in the brain.

An important consequence of the cerebral yin-yang effects of corticosteroids is that any factors that modify the availability of corticosteroids in the brain will modify not only the magnitude but also the type of action produced. This is a physiologically relevant issue, given that the local concentrations of glucocorticoids are controlled by several factors including, e.g., 11β-HSDs which have a profound impact on HPA system regulation (Tomlinson et al., 2004). Of equal importance is that the corticosteroid concentration in tissues and cells is determined by hormone transport over biological barriers such as the BBB. The BBB represents a selective physical and chemical barrier that insulates the brain from the body fluid. On the molecular level, various ABC transport proteins have been discovered at the BBB, with the MDR1 p-gp being the first described and most extensively studied (Juliano and Ling, 1976; Schinkel and Jonker, 2003). MDR1 p-gp protects the central nervous system from the accumulation of a wide range of toxic xenobiotics and drugs (Loscher and Potschka, 2005). In addition, it serves as an important "gate-keeper" for tissue access of glucocorticoid hormones *in vitro* and *in vivo* (Uhr et al., 2002; Webster and Carlstedt-Duke, 2002). Steroid export, however, depends on substrate specificity and type of transporter. Mice express two isoforms of p-glyocproteins that are encoded by the *mdr1a* and the *mdr1b* genes and function in the same manner as human MDR1 (van de Vrie et al., 1998). Using *mdr1a* knockout mice, Karssen et al. (2001) found that cortisol but not corticosterone, which is the principal glucocorticoid in rodents, is expelled from the brain by this isoform. However, both isoforms of MDR1 p-gp gene are expressed at the murine BBB, and Uhr et al. (2002) provided evidence that it is primarily the p-glyocoprotein encoded by the *mdr1b* that controls the central corticosterone transport in the mouse.

Given that MDR1 p-gp controls the corticosterone concentration in the brain, it is likely that the function of this steroid transporter also has an impact on HPA axis activity. This relationship was first revealed by Müller et al. (2003) who found that mice deficient in both *mdr1a* and *mdr1b* genes display characteristics of a chronically increased negative feedback regulation at the hypothalamic level due to an enhanced brain penetration of corticosterone. Relative to wildtype animals, the mutant mice had lower plasma corticosterone levels at the circadian peak and following acute forced swim stress and were more susceptible to the effect of dexamethasone, a synthetic steroid, to suppress corticosterone release (Müller et al., 2003). Since the ability of CRF to stimulate corticosterone secretion was not altered, the site of HPA axis dysregulation in the mutant mice was localized to hypothalamic or limbic brain areas.

The results of our study support the hypothesis that MDR1 p-gp modulates the activity of the HPA system. Acute pharmacological inhibition of the steroid transporter by tariquidar attenuated the WAS-induced increase of peripheral corticosterone release. Following exposure to the EPM, the plasma levels of corticosterone were likewise decreased by tariquidar. These results are consistent with the concept that MDR1 p-gp inhibition by tariquidar enforces neuroendocrine feedback inhibition of HPA axis activity under stress conditions when the release of corticosterone from the

adrenal cortex is increased. Although not explicitly tested in our study, e.g., by means of *in vivo* CNS-microdialysis, with the MDR1 p-gp at the BBB inhibited, peripheral corticosterone is readily able to access cerebral corticosteroid receptors that are relevant to HPA system regulation. In turn, activation of the central corticosteroid receptors dampens the activity of the HPA axis and consequently leads to a reduction of peripheral corticosterone release. This concept, however, is at variance with the finding that tariquidar-treated mice had higher plasma levels of corticosterone than vehicle controls following exposure to a more pronounced stressor such as the FST. Although this observation likewise attests to a dysregulation of the HPA system, we have at present no explanation for the ''overshoot'' of the HPA system after the FST. Conceivably, this overshoot may reflect an imbalance in the negative feedback signals under tariquidar treatment that derive from different HPA axis regulatory brain areas such as the hippocampus, the amygdala and the paraventricular nucleus of the hypothalamus and converge at anterior pituitary corticotropes (Herman et al., 2003).

On the behavioral level, administration of tariquidar *per se* was without effect on locomotion, anxiety-related and stress-coping behavior in C57BL/6 mice. Acute MDR1 p-gp inhibition by tariquidar combined with WAS exposure, however, led to a decrease of anxiety-related behavior, given that tariquidar-treated stressed animals spent significantly more time on the open arms of the elevated plus maze than the respective vehicle controls. In addition, the psychological stress procedure *per se* induced behavioral activation as indicated by an increase of entries into the center of the OF or an increase of the total distance traveled on the EPM after WAS exposure reflecting stress-induced hyperlocomotion (Strekalova et al., 2005).

Together with our data regarding corticosterone, these observations are in line with the concept that there is a close relationship between behavioral responses of rats and mice in the EPM and neuroendocrine activation resulting in release of corticosterone (Pellow et al., 1985; File et al., 1994; Holmes et al., 1998; Rodgers et al., 1999). Accordingly, the behavioral performance of animals in the EPM is highly sensitive to manipulations of the neuroendocrine stress system, e.g., by adrenalectomy, inhibition of MRs and GRs or glucocorticoid administration (Andreatini and Leite, 1994b; Korte, 2001; Ardayfio and Kim, 2006).

For this reason, we investigated whether administration of exogenous corticosterone immediately after WAS exposure would have a similar influence on the anxiety-related behavior in the EPM as tariquidar treatment before WAS. We reasoned that, if the anxiolytic-like effect of tariquidar in WAS-exposed mice was due to enhanced entry of endogenous corticosterone into the brain and subsequent feedback inhibition of HPA axis activity, administration of an appropriate dose of exogenous corticosterone should reproduce the effect of tariquidar. The dose of corticosterone (1 mg/kg) chosen for this experiment has been reported to result in average plasma concentrations of 43.0 ng/ml corticosterone (Cai et al., 2006), a level that is close to that found after WAS exposure. Our observation that WAS exposure followed by administration of exogenous corticosterone reproduced the anxiolytic-like effect of tariquidar treatment combined with WAS supports the concept that enhanced negative feedback inhibition of HPA axis activity by corticosterone may be related to the concomitant reduction of anxiety-related behavior.

Although tariquidar-induced inhibition of MDR1 p-gp plus administration of exogenous corticosterone was expected to inhibit HPA axis activity and reduce anxiety more profoundly than treatment with exogenous corticosterone alone, tariquidar failed to alter the anxiety score and circulating corticosterone level measured after WAS plus treatment with exogenous corticosterone. One reason for this lack of effect of tariquidar could be the occurrence of adaptation phenomena, given that the effect of tariquidar plus WAS to reduce anxiety was no longer seen when the animals were subjected to WAS twice at a 24 h interval. Likewise, the circulating corticosterone levels measured after EPM and FST were lower after repeated WAS than after a single WAS exposure. Finally, in light of the dose–response relationship for the effects of glucocorticoids on behavior that can take the shape of an inverted U, the (hypothesized) accumulation of endo- and exogenous corticosterone in tariquidar-treated mice may induce a ceiling effect in terms of glucocorticoid-associated anxiolysis. This instance may explain why exogenous corticosterone has in most studies been found to reduce anxiety-related behavior in the EPM (Andreatini and Leite, 1994a, b) and a social interaction test (File et al., 1979) but in another study it was reported to have no effect or even to increase anxiety-related behavior (Calvo et al., 1998). At this stage, we cannot further explain the relationship between intracerebral corticosterone modulation and anxiety-related behavior because we did not test anxiety responses towards different doses of corticosterone.

The complex pharmacology of the cerebral actions of corticosteroids was also reflected by the data obtained with the FST. Exposure to WAS *per se* impaired stress-coping behavior in the FST, whereas tariquidar failed to alter the behavior of the mice in the FST independently of whether or not they were subjected to WAS. In contrast, the circulating levels of corticosterone measured after the FST were significantly elevated by tariquidar. This finding indicates that inhibition of MDR1 p-gp by tariquidar had an impact on HPA axis activity during FST, although this relationship did not translate into a change of the stress-coping behavior in the FST. Our observations are at variance with published findings pointing towards an important role of the glucocorticoid system in the behavioral performance in the FST. For instance, genetic manipulations of the GR or the CRF receptors, adrenalectomy in rodents or the administration of exogenous corticosterone have been shown to modulate immobility behavior (Peeters and Broekkamp, 1994; Brotto et al., 2001; Müller and Holsboer, 2006).

Of note, the shift in HPA system reactivity induced by tariquidar administration may represent only one potential, though plausible, mechanism underlying the anxiolytic-like phenotype after stress sensitization. For instance, endogenous opioid peptides such as β-endorphin or endomorphins 1 and 2 have been demonstrated to be substrates of high affinity for the MDR1 p-gp transporter (King et al., 2001; Oude Elferink and Zadina, 2001) and to be critically involved in the physiological and emotional adaptation to stress (Filliol et al., 2000; Fichna et al., 2007).

In addition, the integrity of the BBB *per se* has been described to be altered under several physiological and

pathological conditions. For instance, acute physical or emotional stress is able to increase BBB permeability in experimental animals, thus causing cerebral influx of substances that are normally excluded by the BBB, e.g., the penetration of Evans blue–albumin complexes into the brain parenchyma (Sharma et al., 1991; Friedman et al., 1996; Ovadia et al., 2001; Esposito et al., 2002). We found that BBB integrity is not compromised by WAS, probably because it is a rather mild stressor. Thus, we can rule out that the stress-related effect of tariquidar on MDR1 p-glycoprotein function is altered or related to any structural disruption of the BBB.

5. Conclusion

In summary, the present findings indicate that acute pharmacological inhibition of MDR1 p-gp at the BBB by tariquidar has an anxiolytic-like effect in mice that have been sensitized by mild psychological stress. This behavioral effect is paralleled by a reduction of circulating corticosterone, which attests to modulation of HPA system activity. Therefore, we suggest that inhibition of MDR1 p-gp increases the corticosterone concentration in the brain, which, in turn, enhances the negative feedback control of the HPA system and improves anxiety-related stress processing. It is of note, however, that this ''model'' is preliminary in nature and needs further experimental validation and a detailed analysis of the underlying neuroendocrine processes.

Nevertheless, our findings may be of clinical relevance, as impairment of corticosteroid receptor signaling and feedback inhibition has been suggested to play a key role in the development of neuroendocrine changes associated with human affective disorders (Holsboer, 2000). In this respect, administration of glucocorticoids to humans has been shown to be of benefit in the treatment of phobias and posttraumatic stress disorder, because these hormones facilitate coping with pathological emotional memory (Aerni et al., 2004; Cai et al., 2006; Schelling et al., 2006; Soravia et al., 2006). Our data highlight an alternative therapeutic approach to corticosteroid receptor modulation in the brain, an approach that targets the corticosteroid-mediated feedback inhibition of the HPA axis via pharmacological facilitation of corticosteroid entry through the BBB.

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Conflict of interest

The authors declare to have no conflict of interest.

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