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Brain Region-Specific Regulation of Histone Acetylation and Efflux Transporters in Mice

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Abstract

Multidrug resistance protein 1 (MDR1) and breast cancer resistance protein (BCRP) protect the brain by restricting the passage of chemicals across the blood-brain barrier. Prior studies have demonstrated the epigenetic regulation of MDR1 and BCRP in cancer cells treated with histone deacetylase (HDAC) inhibitors that enhance histone acetylation and gene transcription. In the present study, we tested the *in vivo* effects of two HDAC inhibitors, valproic acid (VPA, 400 mg/kg) and apicidin (5 mg/kg), on Mdr1 and Bcrp transporter in brain regions of adult, male mice injected ip daily for 7 days. VPA increased Mdr1 protein expression in striatum (70%) and Bcrp protein in midbrain (30%). Apicidin enhanced striatal Mdr1 protein (30%) and hippocampal Bcrp protein (20%). Transporter induction correlated with increased histone H3 acetylation in discrete brain regions. In conclusion, HDAC inhibitors up-regulate transporter proteins *in vivo*, which may be important in regulating regional xenobiotic disposition within the brain.

Keywords

HDAC inhibitor; Mdr1; P-glycoprotein; Bcrp; Abcb1; Abcg2; transporter; brain

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INTRODUCTION

Within the brain, multidrug resistance protein 1 (MDR1) and breast cancer resistance protein (BCRP) transporters are located predominantly at the blood-brain barrier (BBB), but also in brain parenchymal cells including microglia, astrocytes, and neurons [1–4]. Human MDR1 (rodent Mdr1a and 1b) and BCRP (rodent Bcrp) play protective roles at the BBB, regulating the passage of compounds from the circulation into the brain [5]. The function of both transporters in brain parenchymal cells is relatively unknown, but evidence suggests that they similarly protect against xenobiotic exposure and cellular damage [6, 7].

Neuroactive drugs and toxicants are transported by MDR1 and BCRP. In patients with major depression, a significant association between a loss-of-function *MDR1* polymorphism and postural hypotension induced by nortriptyline, a MDR1 substrate, has been observed [8]. In addition, MDR1 and BCRP also transport neurotoxic endogenous chemicals, including amyloid-β, as well as exogenous pesticides [9–15]. Therefore, understanding the mechanisms by which BBB transporters are regulated is important for neuronal protection and drug efficacy in the brain.

Pharmacological inhibition of histone deacetylase (HDACs) can up-regulate MDR1 and BCRP transporters in an array of cancer cell lines[16–21]. HDAC inhibitors (HDACi) promote histone acetylation, open DNA for transcription factors to bind, recruit RNA polymerase, and initiate gene transcription [22–25]. HDACi such as VPA are FDA-approved for treating seizure disorders and mania,[26] whereas other HDACis are under investigation as pharmacotherapy for patients with stroke, Parkinson's disease, depression, and schizophrenia [27–32]. It is therefore important to test the ability of HDACi to alter the *in vivo* expression of MDR1 and BCRP in the brain.

In the present study, we evaluated the ability of two HDACi, VPA and apicidin, to alter the expression of Mdr1 and Bcrp transporters in different brain regions of adult, male C57BL/6 mice. These HDACi have been shown to up-regulate efflux transporter expression and activity in various brain cell lines including glioblastoma A172 and U87 cells and human brain microvascular endothelial hCMEC/D3 cells [33, 34], and were therefore selected for this *in vivo* study. Our results indicate that HDACi significantly increased Mdr1 and Bcrp protein expression in parallel with enhanced histone H3 acetylation in discrete brain regions of mice.

MATERIALS AND METHODS

Chemicals and Reagents

VPA and apicidin were purchased from Cayman Chemical (Ann Arbor, MI) and Sigma-Aldrich (St. Louis, MO), respectively. All other chemicals were purchased from Sigma-Aldrich unless otherwise specified.

Animal Treatment

Male C57BL/6NCrl mice (strain 027) were purchased from Charles River Laboratories (Wilmington, MA) at 7 weeks of age and housed in an Association for Assessment and

Accreditation of Laboratory Animal Care (AAALAC) accredited animal care facility, with a 12-hour light/dark cycle, temperature, and humidity-controlled setting. Studies were approved by and performed in accordance with the Rutgers University Institutional Animal Care and Use Committee (Protocol 09–037). Mice were acclimated for up to two weeks before the experiment, and then administered one of the following treatments via intraperitoneal injection (10 ml/kg) once daily for 7 days (n=4–7): vehicle (sterile saline or corn oil), VPA (400mg/kg) dissolved in 0.9% sterile saline, or apicidin (5mg/kg) dissolved in corn oil. Mice had access to standard chow diet and water *ad libitum*. Brains were excised 6 h after the last dose, and sections of cortex, striatum, midbrain, and hippocampus were collected, each divided in half and snap frozen in liquid nitrogen. Samples were stored in −80°C until further analyses. *RNA Extraction, Reverse Transcription, and Real-Time Quantitative Polymerase Chain Reaction (qPCR)*

An entire half of each frozen brain section (10 to 20mg) was homogenized in 1% β-mercaptoethanol in Buffer RLT using a TissueLyser LT with stainless steel beads (QIAGEN, Valencia, CA). The homogenates were centrifuged, and the supernatant was collected and added with the same volume of 70% ethanol. Subsequent total RNA isolation was performed using QIAGEN RNeasy® Mini Kit (QIAGEN) following the manufacturer's protocol. The purity and concentration of isolated RNA samples were measured using a Nanodrop 1000 spectrophotometer (Thermo Scientific, Rockford, IL). Complimentary DNA (cDNA) was obtained from total RNA using the High-Capacity cDNA Reverse Transcription Kit with RNase Inhibitor (Applied Biosystems, Foster City, CA). Then, the expression of *Mdr1a (Abcb1a), Mdr1b* (*Abcb1b)*, and *Bcrp (Abcg2)* was analyzed by qPCR. Specific forward and reverse primers for each gene (Integrated DNA Technologies, Coralville, IA) were added to one microgram of cDNA from each sample, and then amplified products were detected using SYBR Green (Applied Biosystems). Sequences of the primers are listed in Supplemental Table 1. qPCR was performed in a 384-well plate format using the ViiA™7 real-time PCR instrument (Applied Biosystems). Ct values were converted to delta delta Ct values by comparing to ribosomal protein L13a (*Rpl13a*) which was used as a reference gene.

Western Blot Analysis

An entire half of each frozen brain section was homogenized in 250mM sucrose-10mM Tris-base buffer (pH 7.5) containing 1% protease inhibitor cocktail (Sigma-Aldrich). Protein concentrations were determined using the Pierce™ bicinchonicic acid (BCA) protein assay kit (Thermo Scientific, Rockford, IL). Tissue lysates (10 μg protein/well) were loaded on NuPAGE™ 4–12% Bis-Tris Midi Gel (Life Technologies, Carlsbad, CA) and then separated by SDS-PAGE electrophoresis. Proteins were then transferred overnight at 4ºC to Immobilon®-FL polyvinylidene fluoride transfer membranes (Millipore, Billerica, MA). Membranes were blocked for one hour in 5% non-fat dry milk in phosphate-buffer saline (PBS) with 0.5% Tween-20 (PBS-T). Blocking was followed by incubation at room temperature for three hours with the following primary antibodies diluted in 2% non-fat dry milk at a 1:1000 concentration: Mdr1 (E1Y7S, 13978S, Cell Signaling Technology, Danvers, MA), Bcrp (BXP-53, ALX-801–036, Enzo Life Sciences, Inc., Farmingdale, NY), acetylated histone H3K9/K14 (9677S, Cell Signaling Technology), and alpha-tubulin

(T6199, Sigma-Aldrich). After washing three times in PBS-T, membranes were incubated with species-appropriate secondary antibodies conjugated to horseradish peroxidase (Sigma-Aldrich). Proteins were detected using SuperSignal® West Dura Extended Duration Substrate (Thermo Scientific) and a FluorChem E imager (Protein Simple, Santa Clara, CA). Protein expression was semi-quantified using AlphaView SA Ver. 3.4.0 (Protein Simple) and normalized to alpha-tubulin.

Statistical Analysis

GraphPad Prism v5© was used for statistical analysis (GraphPad Software, La Jolla, CA). Differences between groups were compared using a two-tailed student's t-test with statistical significance set at $p < 0.05$.

RESULTS

Regional Expression of Mdr1 and Bcrp Proteins in Mouse Brains

Assessment of baseline expression of transporters revealed largely similar mRNA and protein expression of Mdr1 and Bcrp in homogenates from different mouse brain regions (Supplemental Table 2, Fig. 1). There was a trend for lower protein expression of both transporters in striatum; however, this was not statistically significant.

HDAC Inhibitors Up-Regulate Brain Transporter Proteins in a Region-Specific Manner

To determine whether HDACi alter the expression of brain transporters *in vivo*, adult, male C57BL/6 mice were administered VPA or apicidin intraperitoneally once daily for 7 days. Selected doses of VPA and apicidin were largely equivalent to human dose ranges based on body surface area scaling [35]. Interestingly, the expression of transporter proteins was altered in different brain regions where increases in acetylated histone H3 K9/14 proteins were detected. While cortical transporter protein expression was largely unaffected by HDACi (Fig. 2), changes were prominently detected in the striatum. Notably, Mdr1 protein expression was elevated by 30 to 70% along with acetylated histone H3 protein in the striatum upon treatment with HDACi (Fig. 3). These two HDAC inhibitors also caused modest up-regulation of the Bcrp transporter in other regions. VPA increased acetylated H3 and Bcrp protein levels in the midbrain by about 30% (Fig. 4). By comparison, apicidin led to a similar increase in Bcrp protein in the hippocampus, although no change in histone H3 acetylation was observed. (Fig. 5).

Treatment of Mice with HDAC Inhibitors Does Not Alter Regional Transporter mRNA

Similar to protein expression, different regions of the brain were assessed for the changes in Mdr1a, Mdr1b, and Bcrp mRNA expression. Results are summarized in Tables 1 through 4. Some modest changes were observed in cortex and hippocampus in mice treated with VPA, a more brain penetrable HDAC inhibitor. There was an approximately 35% reduction in Mdr1a mRNA in cortex and 30% induction in Bcrp mRNA in hippocampus of VPA-treated mice. However, both HDACi generally did not cause significant changes in the mRNA levels of Mdr1a, Mdr1b, or Bcrp across different brain regions as was observed with transporter protein expression.

DISCUSSION

MDR1 and BCRP efflux transporters are expressed in various cell types within the brain, where they regulate chemical disposition and provide protection of neural cells. Previous studies revealed the ability of HDACi to up-regulate MDR1 and BCRP in cancer cells. To determine whether similar regulation is relevant *in vivo*, we tested the effects of two HDACi, VPA and apicidin, on Mdr1 and Bcrp expression in the brains of adult, male mice. We demonstrated that: (1) treatment with HDACi significantly up-regulated the protein expression of Mdr1 or Bcrp in specific regions of mouse brains; and (2) transporter up-regulation largely occurred in regions with enhanced histone H3K9/K14 acetylation.

The constitutive mRNA and protein expression of Mdr1 and Bcrp were relatively similar across the four analyzed regions of the mouse brain. In response to HDACi treatment, we observed modest changes in Mdr1 or Bcrp mRNA in VPA-treated cortex and hippocampus. However, in general, HDACi did not significantly change transporter mRNA levels in mouse brains. It is possible that mRNA induction may have peaked earlier than 7 days and normalized with the repeated daily exposure to HDACi. Further studies assessing different time points and treatment duration may explain the apparent lack of mRNA changes in response to HDACi.

In contrast, transporter protein expression was significantly altered in a region-specific manner. In the striatum, both HDACi significantly up-regulated Mdr1 protein. The sensitivity of the striatum to Mdr1 up-regulation may be related to the region's relatively lower baseline Mdr1 expression. Bcrp, which was more uniformly expressed across brain regions at baseline, was moderately up-regulated in VPA-treated midbrains and apicidin-treated hippocampus. There was good correlation between transporter up-regulation and increases in acetylated histone H3 protein. Therefore, HDACi-mediated regulation of Mdr1 and Bcrp transporters in mouse brains may involve transcriptional activation following the histone acetylation at *Mdr1* and *Bcrp* gene promoters. Interestingly, the hippocampus from apicidin-treated mice revealed an up-regulation of Bcrp protein in the absence of histone H3 acetylation. We hypothesize that other histone modifications such as histone H4 acetylation may have been involved[16, 19].

Moderate changes in transporter mRNA in VPA-treated cortex and hippocampus were not reflected at the protein level. This was distinguished from other regions with significant changes in transporter protein levels, but not in mRNA. This suggests time-dependent changes distinctive to each brain region. Alternatively, decreases in transporter mRNA may be compensatory responses to maintain overall transport across the brain. It is also possible that localized changes in transporters within specific brain cell types may have been diluted and not detectable in brain lysates. Further studies assessing the transporter regulation at each cellular component of the brain using immunohistochemistry or laser capture microdissection can better elucidate the effects of HDACi in specific cell populations within each brain region.

In summary, we have demonstrated that treatment with HDACi elicit brain region-specific enhancement of histone H3 acetylation and up-regulation of Mdr1 and Bcrp transporters in

mice. Modulating MDR1 and BCRP transporters via HDAC inhibition may affect brain levels of important transporter substrates. Several HDACi, including those in our study, are being investigated as treatments for brain disorders including glioblastoma, Alzheimer's, and Parkinson's disease where transporter function is important in optimal drug delivery. Understanding the ability of HDACi to act as epigenetic regulators of MDR1 and BCRP transporters will aid in assessing the pharmacokinetics and pharmacodynamics of these compounds as treatments for brain disorders.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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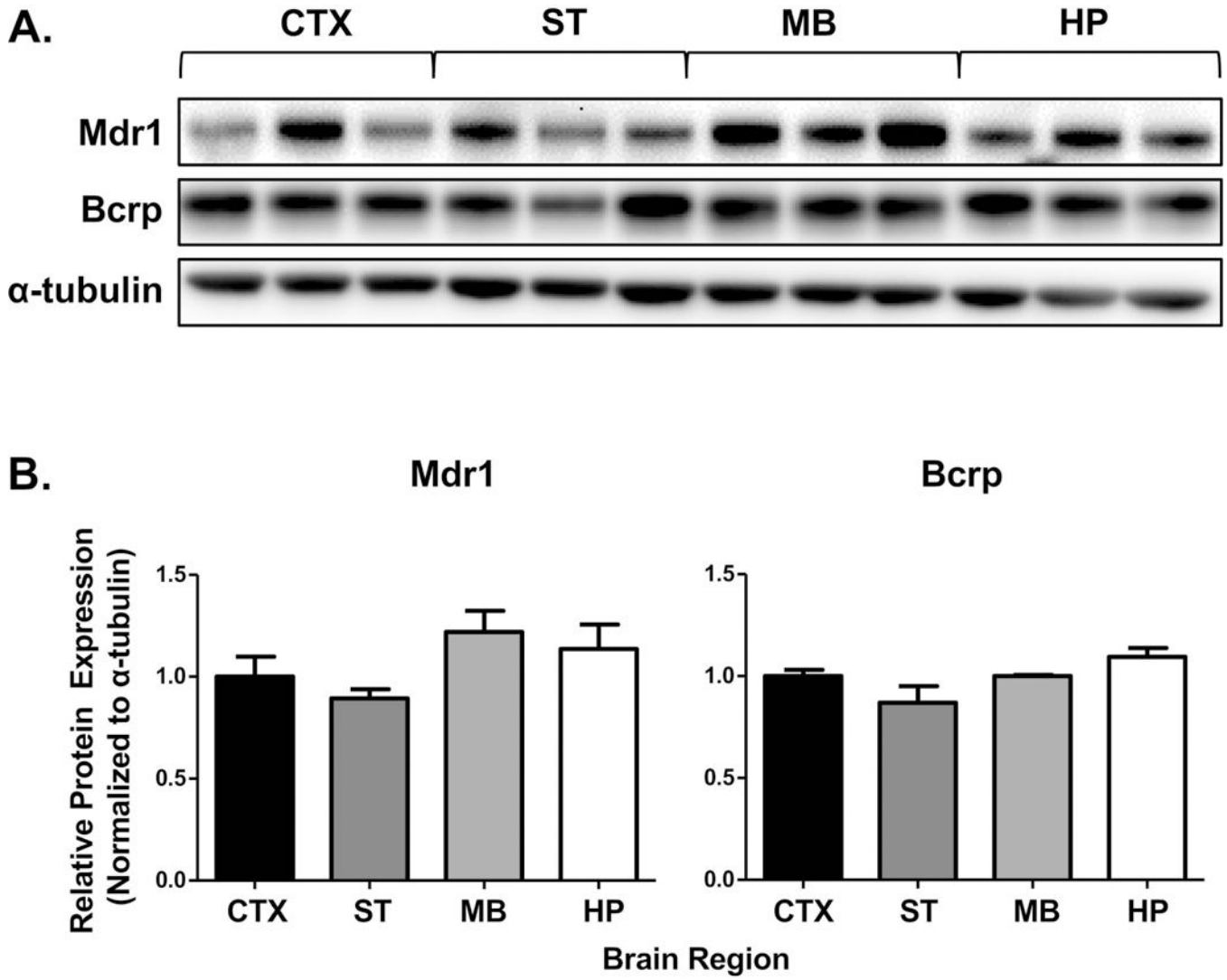

Fig. 1. Baseline transporter protein expression in different brain regions of mice.

Basal levels of transporter proteins in different brain regions of saline-treated mice were analyzed by (A) western blot and (B) semi-quantified by densitometry. Alpha-tubulin (α-tubulin) was used as a loading control. Data are presented as mean ± SEM. CTX: Cortex; ST: Striatum; MB: Midbrain; HP: Hippocampus.

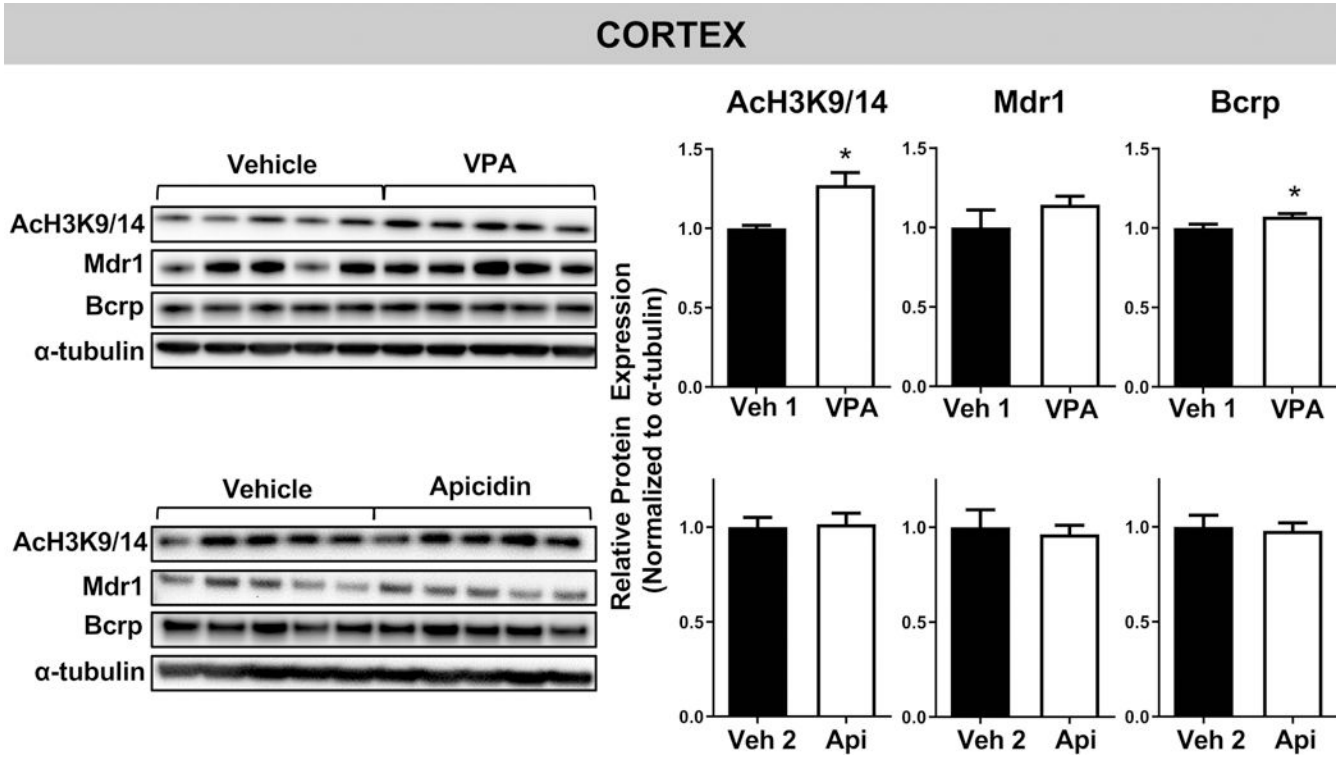

Fig. 2. Efflux transporter protein expression in the cortex of mice treated with HDAC inhibitors.

Protein expression of acetylated H3 histone K9/K14 and efflux transporters in cortex samples of mice treated with vehicle or a HDAC inhibitor (VPA 400mg/kg or apicidin 5mg/kg IP) (n=5) daily for 7 days was analyzed by western blot and semi-quantified by densitometry. Alpha-tubulin (α-tubulin) was used as a loading control. Data are presented as mean ± SEM and analyzed by two-tailed Student's t-test compared to the respective vehicle control (*) with statistical significance set at $p < 0.05$. Vehicle 1 (Veh 1): Normal saline; Vehicle 2 (Veh 2): Corn oil.

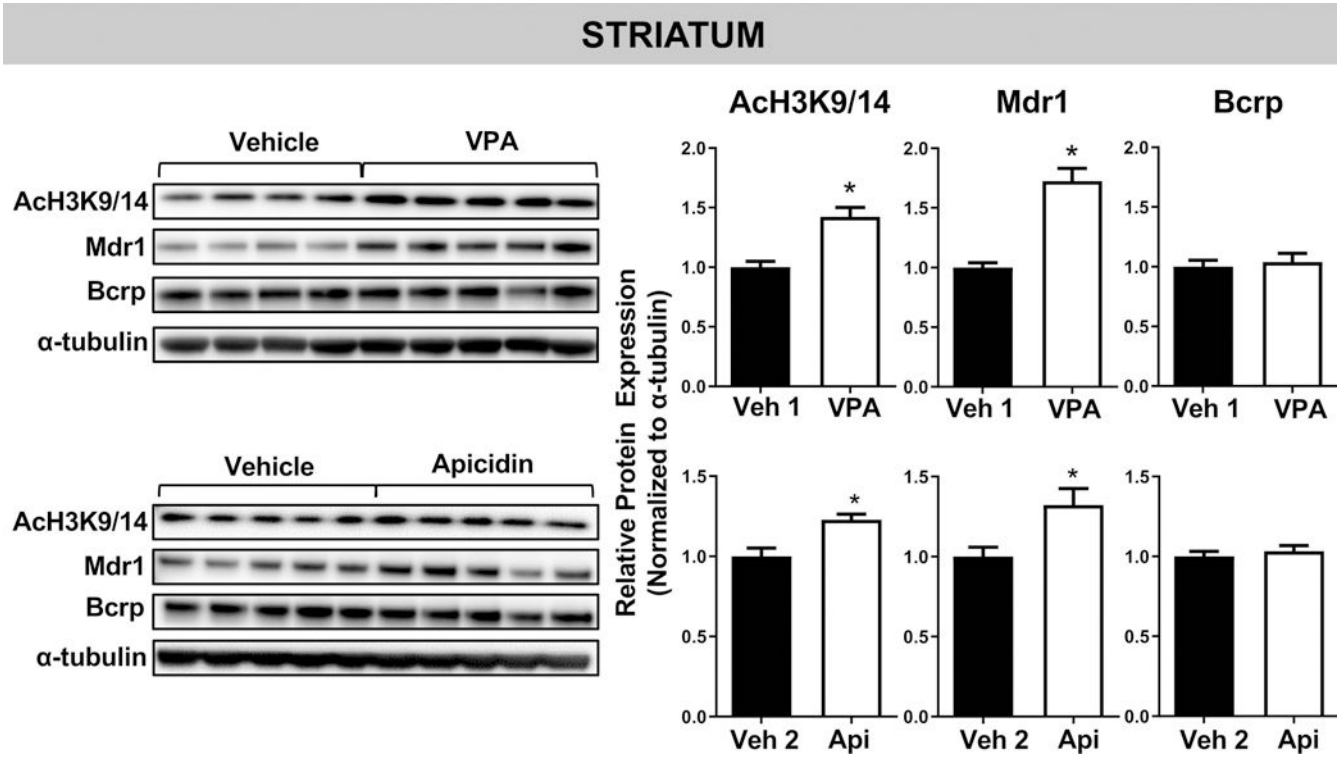

Fig. 3. Efflux transporter protein expression in the striatum of mice treated with HDAC inhibitors.

Protein expression of acetylated H3 histone K9/K14 and efflux transporters in striatum samples of mice treated with vehicle or a HDAC inhibitors (VPA 400mg/kg or apicidin 5mg/kg IP) (n=4–5) daily for 7 days was analyzed by western blot and semi-quantified by densitometry. Alpha-tubulin (α-tubulin) was used as a loading control. Data are presented as mean ± SEM and analyzed by two-tailed Student's t-test compared to the respective vehicle control (*) with statistical significance set at $p < 0.05$. Vehicle 1 (Veh 1): Normal saline; Vehicle 2 (Veh 2): Corn oil.

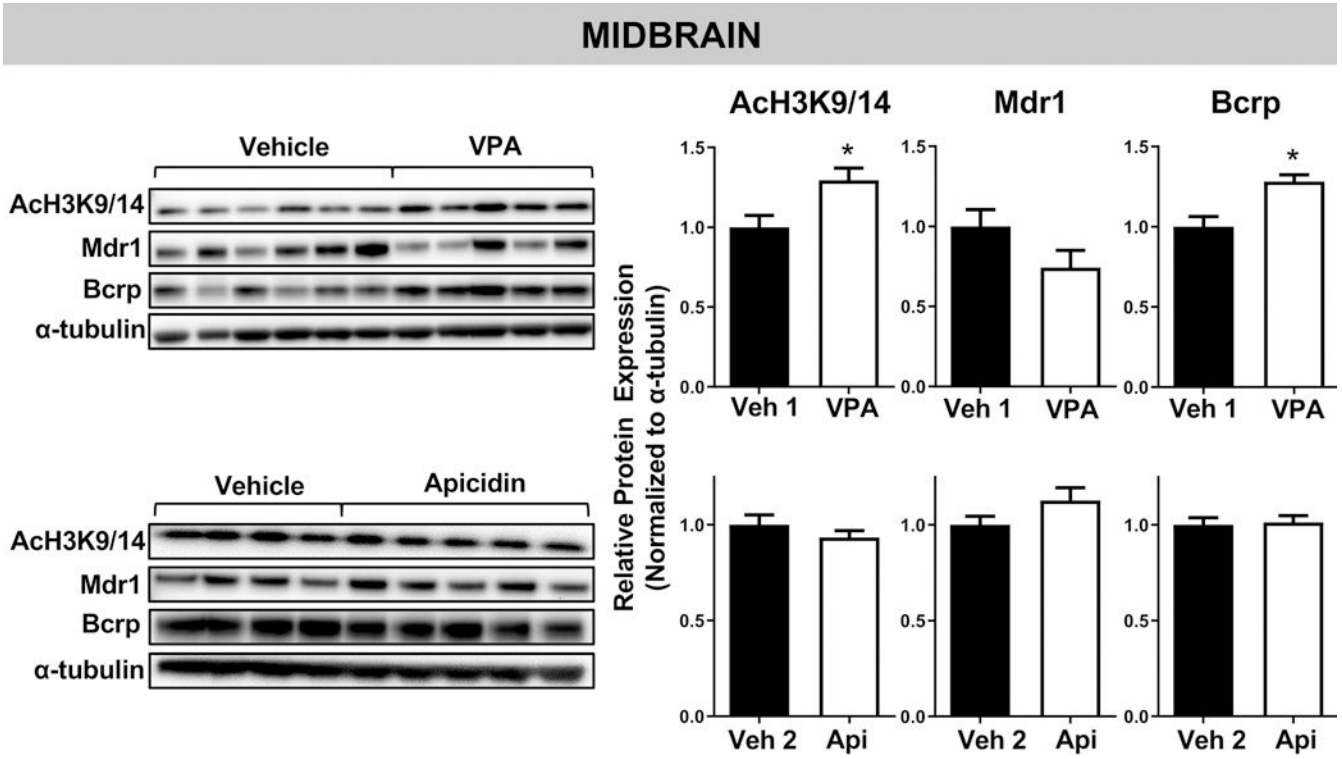

Fig. 4. Efflux transporter protein expression in the midbrains of mice treated with HDAC inhibitors.

Protein expression of acetylated H3 histone K9/K14 and efflux transporters in midbrain samples of mice treated with vehicle or a HDAC inhibitors (VPA 400mg/kg or apicidin 5mg/kg IP) (n=4–6) daily for 7 days was analyzed by western blot and semi-quantified by densitometry. Alpha-tubulin (α-tubulin) was used as a loading control. Data are presented as mean ± SEM and analyzed by two-tailed Student's t-test compared to the respective vehicle control (*) with statistical significance set at $p < 0.05$. Vehicle 1 (Veh 1): Normal saline; Vehicle 2 (Veh 2): Corn oil.

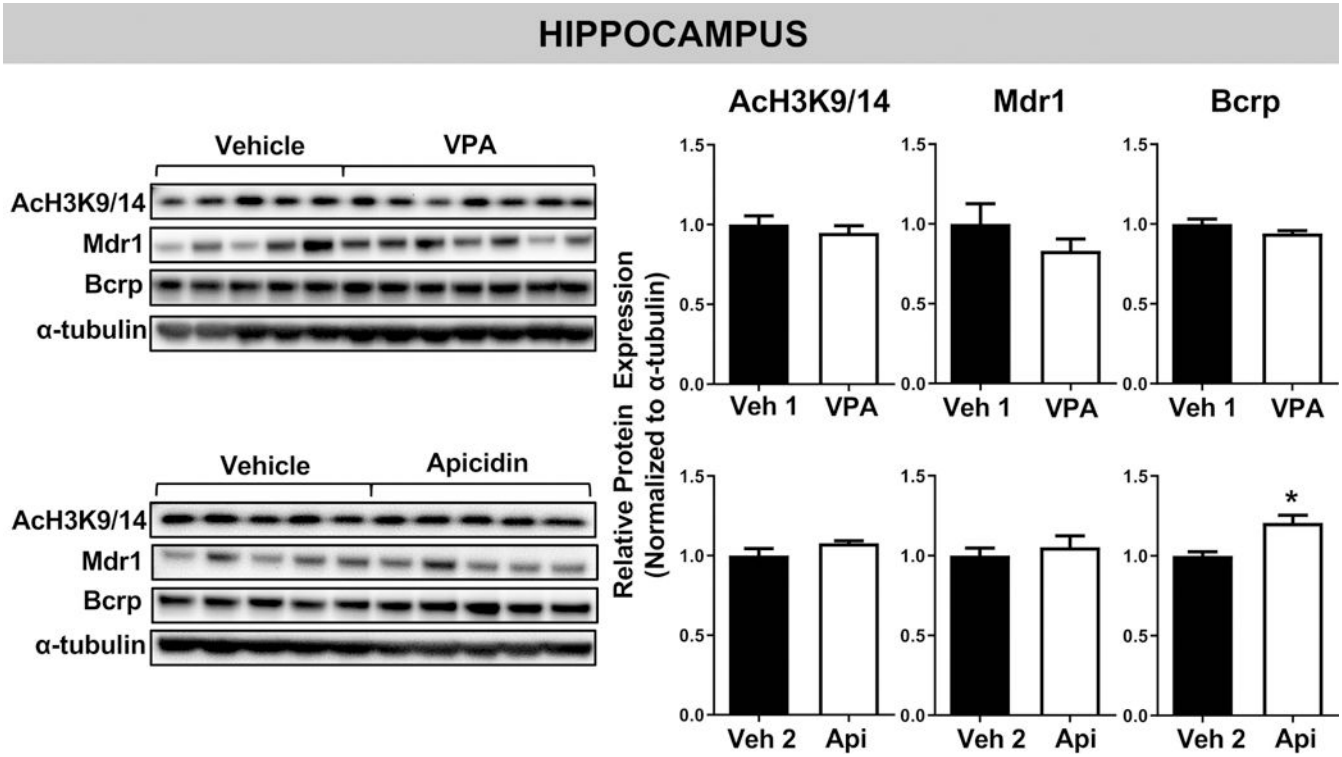

Fig. 5. Efflux transporter protein expression in the hippocampus of mice treated with HDAC inhibitors.

Protein expression of acetylated H3 histone K9/K14 and efflux transporters in hippocampus samples of mice treated with vehicle or a HDAC inhibitors (VPA 400mg/kg or apicidin 5mg/kg IP) (n=5–7) daily for 7 days was analyzed by western blot and semi-quantified by densitometry. Alpha-tubulin (α-tubulin) was used as a loading control. Data are presented as mean ± SEM and analyzed by two-tailed Student's t-test compared to the respective vehicle control (*) with statistical significance set at $p < 0.05$. Vehicle 1 (Veh 1): Normal saline; Vehicle 2 (Veh 2): Corn oil.

Table 1.

Efflux transporter mRNA expression in the cortex of mice treated with HDAC inhibitors

Region	CORTEX													
TX	VEHICLE 1			VPA			p-value	VEHICLE 2			APICIDIN			p-value
Gene	Mean[a]	SEM	N	Mean	SEM	N		Mean	SEM	N	Mean	SEM	N	
Mdr1a	**1**	**0.13**	**5**	**0.657**	**0.036**	**6**	**0.020**	1	0.053	5	0.91	0.044	5	0.208
Mdr1b	1	0.16	5	0.790	0.059	6	0.225	1	0.074	5	0.88	0.021	5	0.172
Bcrp	1	0.16	5	0.795	0.034	6	0.190	1	0.106	5	0.87	0.054	5	0.343

[a]Mean values are relative to the corresponding vehicle-treated controls.

Table 2.

Efflux transporter mRNA expression in the striatum of mice treated with HDAC inhibitors

Region	STRIATUM												
TX	VEHICLE 1			VPA			p-value	VEHICLE 2			APICIDIN			p-value
Gene	Mean[a]	SEM	N	Mean	SEM	N		Mean	SEM	N	Mean	SEM	N	
Mdr1a	1	0.062	4	1.067	0.140	6	0.722	1	0.041	5	1.01	0.027	5	0.784
Mdr1b	1	0.090	4	1.049	0.091	6	0.723	1	0.027	5	1.04	0.044	5	0.438
Bcrp	1	0.017	4	1.121	0.084	6	0.285	1	0.024	5	0.99	0.038	5	0.777

[a]Mean values are relative to the corresponding vehicle-treated controls.

Table 3.

Efflux transporter mRNA expression in the midbrains of mice treated with HDAC inhibitors

Region	MIDBRAIN												
TX	VEHICLE 1			VPA			p-value	VEHICLE 2			APICIDIN			p-value
Gene	Mean[a]	SEM	N	Mean	SEM	N		Mean	SEM	N	Mean	SEM	N	
Mdr1a	1	0.077	6	1.529	0.298	6	0.117	1	0.030	4	0.92	0.035	5	0.151
Mdr1b	1	0.130	6	1.582	0.261	6	0.074	1	0.166	4	1.06	0.098	5	0.769
Bcrp	1	0.128	6	1.379	0.121	6	0.057	1	0.144	4	1.04	0.154	4	0.861

[a]Mean values are relative to the corresponding vehicle-treated controls.

Table 4.

Efflux transporter mRNA expression in the hippocampus of mice treated with HDAC inhibitors

Region	HIPPOCAMPUS												
TX	VEHICLE 1			VPA			p-value	VEHICLE 2			APICIDIN			p-value
Gene	Mean[a]	SEM	N	Mean	SEM	N		Mean	SEM	N	Mean	SEM	N	
Mdr1a	1	0.054	6	0.910	0.055	7	0.274	1	0.039	5	1.07	0.059	5	0.337
Mdr1b	1	0.057	6	1.066	0.031	7	0.310	1	0.032	5	0.95	0.086	5	0.634
Bcrp	**1**	**0.054**	**6**	**1.326**	**0.060**	**7**	**0.002**	1	0.115	5	1.19	0.056	5	0.178

[a]Mean values are relative to the corresponding vehicle-treated controls.