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Reduced brain volume and impaired memory in betaine homocysteine *S*-methyltransferase knockout mice

Emily A.K. Prieur[a], Eneda Pjetri, MD[c], Steven H. Zeisel, MD[c], Nafisa M. Jadavji, PhD[a,b]

eprie015@uottawa.ca

eneda_pjetri@unc.edu

steven_zeisel@unc.edu

nafisa.jadavji@mail.mcgill.ca

[a] Department of Neuroscience, Carleton University, Ottawa, ON, Canada

[b] Department of Experimental Neurology, Center for Stroke Research Berlin, Charité University Medicine Berlin, Germany

[c] Department of Nutrition, Gillings School of Global Public Health & School of Medicine, University of North Carolina, Chapel Hill, NC Nutrition Research Institute, University of North Carolina, Chapel Hill, NC

Corresponding Author
Nafisa M. Jadavji, PhD
Department of Neuroscience
Carleton University
1125 Colonel By Drive
Ottawa, ON K1S 5B6
E-mail: nafisa.jadavji@mail.mcgill.ca

Abstract

Using a mouse model, this study examined the impact of lack of betaine homocysteine *S*-methyltransferase (BHMT) on neurological function. *Bhmt*$^{-/-}$ mice maintained on a control diet had elevated concentrations of homocysteine, reduced total brain MRI volume, as well as impaired reference and short-term memories. The results of this study indicate that the absence of BHMT may play a role in neurological function.

Keywords
Homocysteine, betaine homocysteine *S*-methyltransferase, MRI, Morris Water maze, Y-maze, memory

Introduction

Increased levels of the amino acid homocysteine (> 15μM) have previously been reported to be detrimental for brain development and function (Lawrance et al. 2011, Jadavji et al. 2014, 2015a). Previous reports have shown that high levels of homocysteine may increase vulnerability to neurological diseases, such as stroke (Castro et al. 2006) and neurodegeneration (Seshadri 2012). Changes in plasma concentrations of homocysteine reflect metabolic consequences of nutrient deficiency (Obeid 2013).

Choline is a metabolite of betaine and can also be derived from plant sources. Choline, betaine, and, betaine homocysteine *S*-methyltransferase (BHMT), reduce levels of homocysteine in the cell. Both humans and mice with elevated levels of homocysteine have reduced levels of betaine in the brain (Imbard et al. 2015). BHMT is expressed at low levels in human brain tissue (Gaulij et al. 1973, Delgado-Reyes et al. 2001) although, the role of BHMT in normal brain function is not well understood. However, epidemiological studies may have provided evidence for a link between reduced levels of BHMT and neurological function. For example, a common variant of BHMT, the 742G→A (R239Q), the QQ variant was associated with reduced risk of neural tube defects (NTD) in mothers as well as offspring (Morin et al., 2003). The QQ variant was also associated with reduced risk of vascular disease (Weisberg et al. 2003). Additionally, the 742 G→ A polymorphism is associated with reduced risk of developing Down Syndrome in the Brazilian population (Amorim et al. 2013). A single nucleotide polymorphism (rs3733890) has been reported to be associated with increased risk of NTDs in Caucasian American families especially when mothers that had high levels of dietary folic acid or had a polymorphism in methylenetetrahydrofolate reductase, an enzyme involved in folate metabolism (Boyles et al,

2006). These studies suggest that BHMT may have some implications for neurological function possibly through increased levels of homocysteine. This association is not clear since other polymorphisms in *Bhmt* have been reported that have no influence in neurological function (Heil et al. 2000) and to date, there have been no human reports of homozygous BHMT inactivation.

A knockout mouse model of BHMT was developed to study the *in vivo* effects (Teng et al. 2011). *Bhmt*$^{-/-}$ mice have increased levels of plasma homocysteine (~40μM) as well as increased susceptibility to fatty liver disease and hepatocellular cancer (Teng et al. 2011). Another study reported that supplementation with folic acid (20mg/kg) did not decrease plasma homocysteine levels in *Bhmt*$^{-/-}$ mice (Teng et al. 2012), suggesting that the role folic acid has in reducing plasma homocysteine levels is not as important as *Bhmt*. In the present study, we assessed the impact of a lack of BHMT on neurological function. We report that *Bhmt*$^{-/-}$ maintained on a control diet have decreased total brain volume, impaired reference memory on the Morris water maze and short-term memory on the y-maze.

Materials and Methods

All experiments were approved by the Landesamt für Gesundheit und Soziales Berlin, Germany. Experiments were performed in accordance with the German Animal Welfare Act and institutional guidelines. Generation of *Bhmt*-deficient mice, was previously described (Teng et al. 2011). The *Bhmt* null allele was backcrossed for more than 10 generations onto a C57BL/6 background. Mice were generated from multiple litters with heterozygous to heterozygous matings. Male wild-type, heterozygous, and knockout littermates were used and there were 10 mice per genotype group. Animals were assigned randomly into groups. At four-weeks-of-age *Bhmt*$^{+/+}$, *Bhmt*$^{+/-}$ and *Bhmt*$^{-/-}$ mice were fed control diet (CD) containing 2 mg folic acid/kg diet

(Envigo) for a period of 6 weeks prior to starting experiments. After which, at 10 weeks of age, behavioural experiments were conducted. Once experiments were completed brain tissue and blood was collected for analysis.

Learning and memory was assessed in BHMT deficient and wildtype mice using the Morris water maze (MWM). In brief, cues were present at fixed positions and a clear Plexiglas platform was submerged beneath the surface of the water in the center of one of the four quadrants. The latency to find the platform for each animal was tracked with a computer-based system (TSE Systems). Over 7 days mice were trained to find the hidden platform, with 3 trials per day and an inter-trial interval of 30 minutes. Each trial consisted of a maximum of 90 seconds and was randomly started from one the quadrants with the animal facing the wall. If an animal did not reach the platform after 90 seconds it was guided there. On day 8, a probe trial occurred, the platform was removed, mice could swim for 90 seconds and data was recorded.

The y-maze was used to measure spatial short-term working memory in mice. Briefly, each mouse was placed at the end of one arm and allowed to move freely through the maze during an 8-min session and the series of arm entries was recorded (Sarter et al. 1988). Alternation was defined as successive entries into 3 arms, on overlapping triplet sets. The percentage of alternation was calculated as the ratio of actual to possible alternations (defined as the total number of arms entered minus two), multiplied by 100 (Maurice et al. 1994).

The rotarod was used to measure balance and coordination, mice were placed on an accelerating rotarod (4 to 40 rpm). Animals were tested over 5 min and the latency to fall was recorded (Jadavji et al. 2015b).

All MRI experiments occurred on a 7T system (Bruker BioSpin) as previously described (Jadavji et al. 2015b). Whole brain and hippocampal volumes were calculated by manually drawing regions of interest on the T_2 scans using ImageJ freeware (National Institutes of Health).

At the conclusion of the study, mice were deeply anaesthetized with isoflurane, terminal blood samples were taken via cardiac puncture for total plasma homocysteine analysis as described in (Jadavji et al., 2015a). Brain tissue was also removed from all animals. The right hemisphere was fixed in 4% paraformaldehyde for 24 hours, processed, and then sectioned at 5 μm thick. Sections were stained using neuronal nuclei (NeuN; AbCam) and visualized with Alexa Fluor 488 (Cell Signalling) for assessment of neuronal density within the hippocampus. The cell counts were targeted to the dentate gyrus and cortical areas 1 and 3 (CA1, CA3) of the hippocampus in 4 mice per genotype group. For each mouse three sections were counted and an average was calculated.

Whole brain, cerebellum and hippocampus were dissected from *Bhmt*$^{+/+}$ mice and snap frozen for protein analysis of BHMT (gift from Timothy Garrow) using Western Blot. Protein extracts from brain (~60mg), cerebellum (~30mg) and hippocampus (~15mg) were prepared, separated and transferred to nitrocellulose members as previous described (Jadavji et al., 2015b). Primary antibodies used were BHMT and GAPDH (Cell Signalling Technology). Secondary antibody was a horseradish peroxidase (HRP)-conjugated donkey anti-rabbit IgG (GE Healthcare). Protein was detected by using ECL Chemiluminescence Plus (GE Healthcare).

Two investigators, blinded to group assignment, performed analysis of all behaviour and MRI data analysis. Statistical analysis was performed using Graph Pad 6.0. One-way analysis of variance (ANOVA) was used to compare genotype differences. Significant main effects in the ANOVAs were followed by the Tukey's post-hoc test to determine whether statistically significant differences existed between the groups. In all analyses, $p \leq 0.05$ was considered significant. All data are presented as mean $\pm$ standard error of the mean (SEM).

Results

Increased plasma homocysteine levels, BHMT expression in the brain and, reduced total brain volume

Plasma homocysteine levels were increased in *Bhmt*$^{-/-}$ mice while *Bhmt*$^{+/-}$ mice had levels similar to wild-type mice (Figure 1A; $F(_{2,23}) = 4.44$, $p = 0.03$).

There was no difference between genotype groups in body and brain weights at the time of tissue collection (*Bhmt*$^{+/+}$ 31.2 $\pm$ 1.3 *Bhmt*$^{+/-}$ 30.3 $\pm$ 0.8 *Bhmt*$^{-/-}$ 30.1 $\pm$ 1.2; $F(_{2,23})=0.24$, $p = 0.80$ and *Bhmt*$^{+/+}$ 0.59 $\pm$ 0.01 *Bhmt*$^{+/-}$ 0.53 $\pm$ 0.03 *Bhmt*$^{-/-}$ 0.59 $\pm$ 0.03; $F(_{2,23}) = 1.94$, $p = 0.17$).

Western Blot analysis showed expression of BHMT in cerebellum and hippocampal tissue in wildtype mice (Figure 1B).

To determine whether a lack of BHMT changed brain and hippocampal volume MRI analysis was conducted (representative images shown in Figure 1C-E). *Bhmt*$^{-/-}$ had reduced total brain volume (Figure 1D; ($F(_{2,23})= 6.82$,$p = 0.04$). There was no change in hippocampal volume between genotype groups, when it was corrected for total brain volume.

We assessed neuronal density within the hippocampus of BHMT mice using NeuN. We observed a slight decrease, but not significant ($p>0.05$), in the number of NeuN positive cells within the dentate gyrus (Figure 1F-I) and cortical areas 1 and 3 (CA1 and 3) (Figure 1J-N).

No changes in motor function in Bhmt$^{-/-}$ mice

Balance and coordination were measured using the accelerating rotarod, no difference was observed between *Bhmt*$^{+/+}$ (88.4 $\pm$ 24.2 seconds), *Bhmt*$^{+/-}$ (86.3 $\pm$ 15.0 seconds) and *Bhmt*$^{-/-}$ (104.1 $\pm$ 19.5 seconds) animals ($F_{(2,29)} = 0.219$, $p = 0.81$). This confirms that there was no motor function impairment that could have affected performance on MWM.

Reference memory altered in Bhmt$^{-/-}$ mice

Learning and memory was assessed using the MWM. There was no genotype difference and all the mice learned where the platform during the seven days of training on the MWM (Figure 2A). On day eight, reference memory was measured using the probe trial, the *Bhmt*$^{-/-}$ mice spent significantly less time in the platform quadrant (Figure 2B; $F_{(2,29)} = 4.1$, $p = 0.03$). Representative images of probe trial tracking are shown in Figure 2C.

Impaired spatial short-term working memory in Bhmt$^{-/-}$ mice

Spatial short-term working memory was measured using the y-maze task. *Bhmt*$^{-/-}$ mice made significantly less alternations indicating that these mice did not remember previous arm entries (Figure 2D; $F_{(2,25)} = 3.7$, $p = 0.03$). There was no difference in the number of entries between genotype groups *Bhmt*$^{+/+}$ (24.1 $\pm$ 1.7 number of entries), *Bhmt*$^{+/-}$ (22.7 $\pm$ 3.5 number of entries), *Bhmt*$^{-/-}$ (19.0 $\pm$ 2.0 number of entries).

Discussion

High levels of homocysteine are associated with negative health consequences (Castro et al. 2006). BHMT is an enzyme involved in reducing levels of homocysteine using the nutrient choline. Using BHMT knockout mouse model, we found elevated levels of homocysteine in the *Bhmt*$^{-/-}$ mice in concordance with previous findings (Teng et al. 2011). Furthermore, *Bhmt*$^{-/-}$ mice have reduced total brain volume, but no change in brain or body weight or neuronal density within the hippocampus. Additionally, reference and spatial memories were impaired in *Bhmt*$^{-/-}$ mice. We are the first to report these effects of BHMT deficiency in neurological function.

One potential mechanism through which an absence of BHMT may affect neurological function could be via epigenetics. BHMT uses betaine as a methyl donor and apart from liver, it has been reported to have a significant function also during early development by facilitating blastocyst development (Lee et al. 2012). Methylation is also critical for proper postnatal brain development. During early brain development methylation is involved in plasticity and synaptic transmission (Fagiolini et al. 2010, Szulwach et al. 2011). A lack of BHMT and subsequent methyl groups during development could be a contributor to the phenotype we observe in the present study. Another potential mechanism could be through the role of betaine as an osmolyte that can regulate cellular volume and fluid balance (Stange 1992, Lever and Slow 2010). Maintenance of the brain osmotic composition is critical for normal functioning of the brain because of its complex architecture (Stange 1992). Interestingly, *Bhmt*$^{-/-}$ mice have a 5-fold increase in levels of betaine within the brain (Teng et al., 2011). It is unknown how the osmotic composition is changed in these mice however, brain development was affected as shown in the

reduced volume and the role of the increased levels of betaine in *Bhmt*$^{-/-}$ mice might be some sort of compensation that needs to be further examined.

The present study provides some insights on the involvement BHMT may have in neurological function. Further studies are required to dissect how choline metabolism is affected in brain function.

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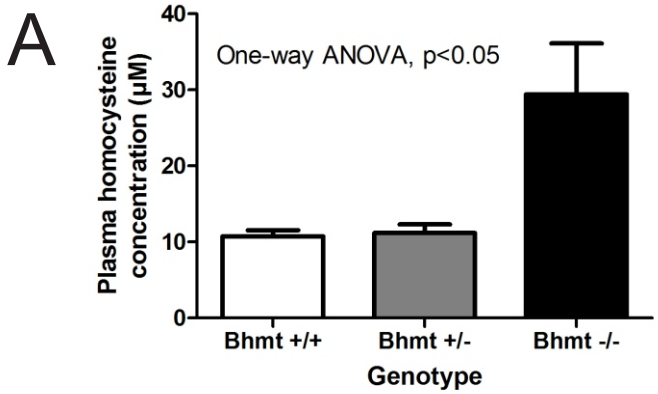
Figure captions

Figure 1. Impact of lack of BHMT on plasma homocysteine concentration (A). Expression of betaine homocysteine *S*-methyltransferase (BHMT) in total brain, cerebellum and hippocampus of wild-type animals (B). Representative T2 MRI images from *Bhmt* $^{+/+}$ (C), *Bhmt* $^{+/-}$ (D) and *Bhmt*$^{-/-}$ (E) mice and volumetric analysis of total brain (F). Representative NeuN staining in dentate gyrus of *Bhmt*$^{+/+}$ (G), *Bhmt*$^{+/-}$ (H), and *Bhmt*$^{-/-}$ (I) mice. Quantification of NeuN positive cells in dentate gyrus (J). Representative NeuN staining in cortical area (CA) within hippocampus of *Bhmt*$^{+/+}$ (K), *Bhmt*$^{+/-}$ (L), and *Bhmt*$^{-/-}$ (M) mice. Quantification of NeuN positive cells in CA (N). *p< 0.05 Tukey's post-hoc comparison between *Bhmt*$^{+/-}$ vs *Bhmt*$^{-/-}$.

Figure 2. The lack of betaine homocysteine *S*-methyltransferase (BHMT) on learning and memory. Morris water maze (MWM) escape latency (A) over 7-day testing period, and time spent in the platform quadrant (B) and representative tracking from probe trial of *Bhmt*$^{+/+}$, *Bhmt*$^{+/-}$ and *Bhmt*$^{-/-}$ mice (C). Percent alternations on y-maze (D). *p<0.05 Tukey's post-hoc comparison between *Bhmt*$^{+/+}$ vs *Bhmt*$^{-/-}$.


A
One-way ANOVA, p<0.05
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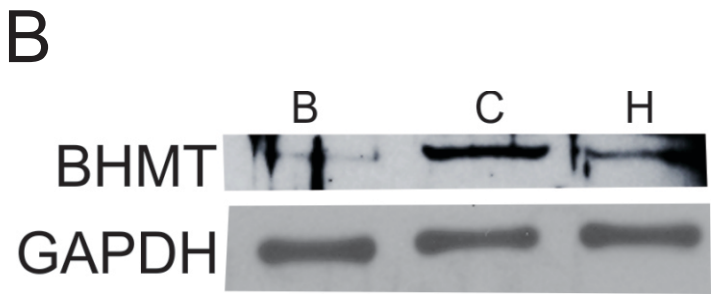
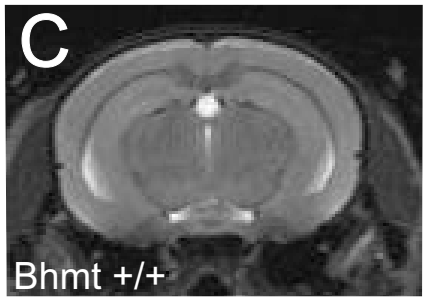
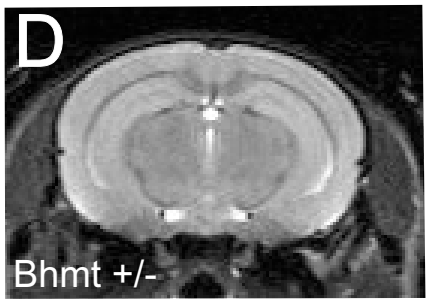
40
30
20
10
0
Bhmt +/+
Bhmt +/-
Bhmt -/-
Genotype


B
B
C
H
BHMT
GAPDH


C
Bhmt +/+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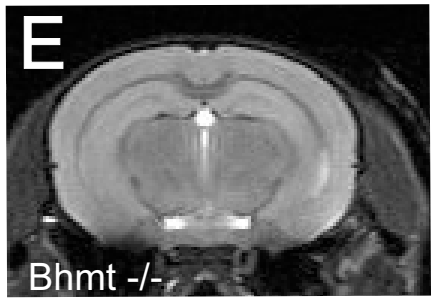
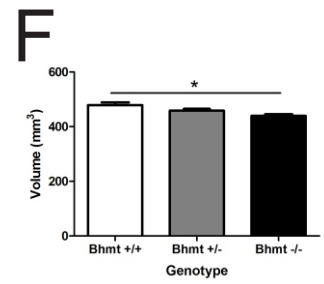
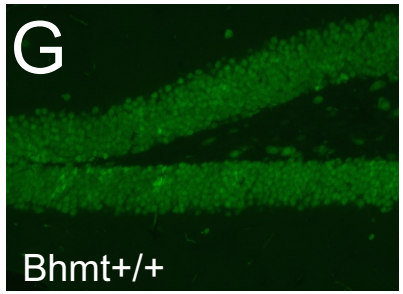
Bhmt +/-


E
Bhmt -/-


F
600
400
200
0
Volume (mm3)
*
Bhmt +/+
Bhmt +/-
Bhmt -/-
Genotype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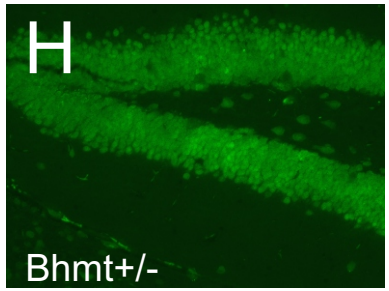
Bhmt+/+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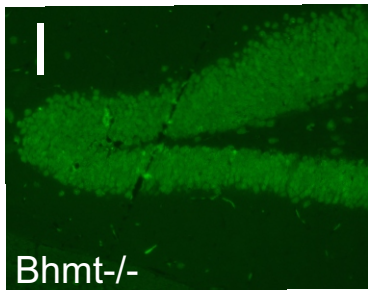
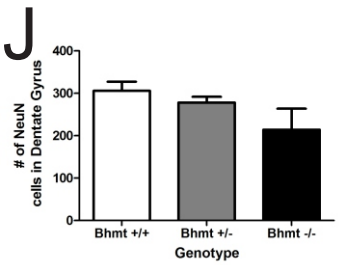
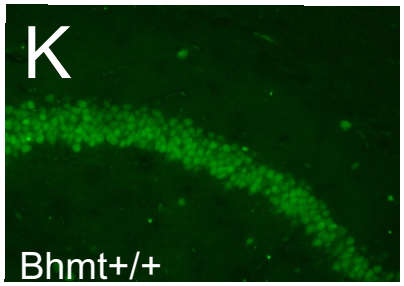
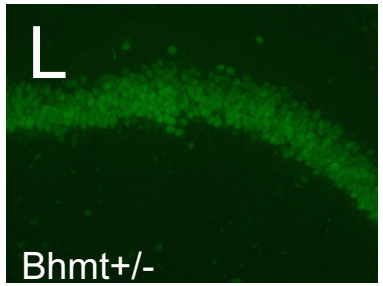
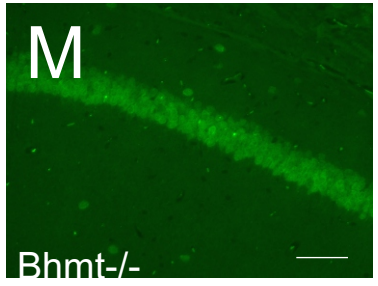
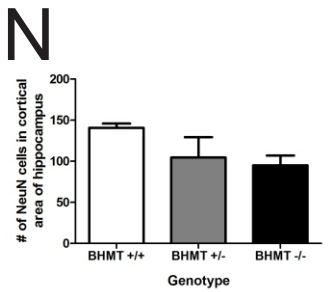

I
Bhmt-/-


J
400
300
200
100
0
# of NeuN cells in Dentate Gyrus
Bhmt +/+
Bhmt +/-
Bhmt -/-
Genotype


K
Bhmt+/+


L
Bhmt+/-


M
Bhmt-/-


N
200
150
100
50
0
# of NeuN cells in cortical area of hippocampus
BHMT +/+
BHMT +/-
BHMT -/-
Genotype

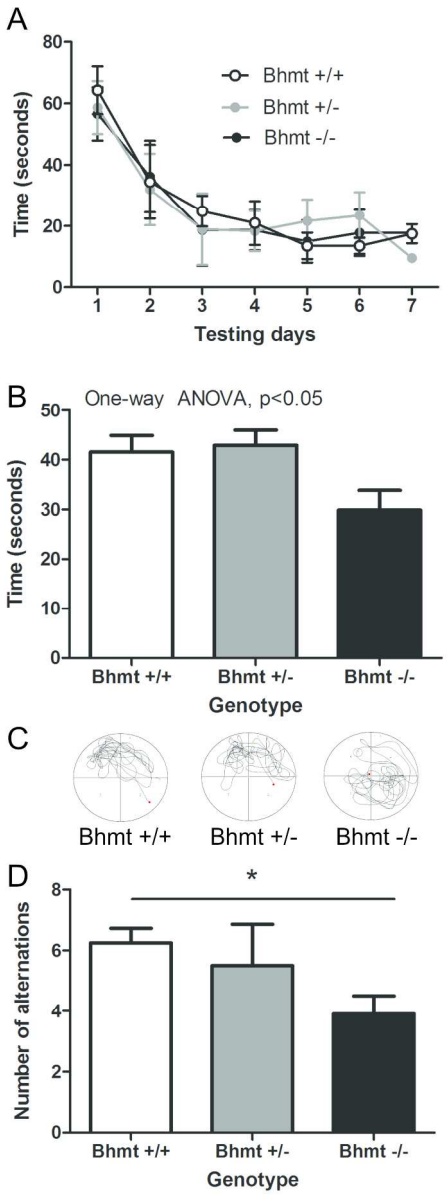


Figure 2. The lack of betaine homocysteine S-methyltransferase (BHMT) on learning and memory. Morris water maze (MWM) escape latency (A) over 7-day testing period, and time spent in the platform quadrant (B) and representative tracking from probe trial of Bhmt+/+, Bhmt+/- and Bhmt-/- mice (C). Percent alternations on y-maze (D). *p<0.05 Tukey's post-hoc comparison between Bhmt+/+ vs Bhmt-/-.

165x387mm (300 x 300 DPI)