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# Bardoxolone methyl prevents high-fat diet-induced alterations in prefrontal cortex signalling molecules involved in recognition memory

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## Abstract

High fat (HF) diets are known to induce changes in synaptic plasticity in the forebrain leading to learning and memory impairments. Previous studies of oleanolic acid derivatives have found that these compounds can cross the blood-brain barrier to prevent neuronal cell death. We examined the hypothesis that the oleanolic acid derivative, bardoxolone methyl (BM) would prevent diet-induced cognitive deficits in mice fed a HF diet. C57BL/6J male mice were fed a lab chow (LC) (5% of energy as fat), a HF (40% of energy as fat), or a HF diet supplemented with 10 mg/kg/day BM orally for 21 weeks. Recognition memory was assessed by performing a novel object recognition test on the treated mice. Downstream brain-derived neurotrophic factor (BDNF) signalling molecules were examined in the prefrontal cortex (PFC) and hippocampus of mice via Western blotting and N-methyl-d-aspartate (NMDA) receptor binding. BM treatment prevented HF diet-induced impairment in recognition memory ( $p < 0.001$ ). In HF diet fed mice, BM administration attenuated alterations in the NMDA receptor binding density in the PFC ( $p < 0.05$ ), however, no changes were seen in the hippocampus ( $p > 0.05$ ). In the PFC and hippocampus of the HF diet fed mice, BM administration improved downstream BDNF signalling as indicated by increased protein levels of BDNF, phosphorylated tropomyosin related kinase B (pTrkB) and phosphorylated protein kinase B (pAkt), and increased phosphorylated AMP-activated protein kinase (pAMPK) ( $p < 0.05$ ). BM administration also prevented the HF diet-induced increase in the protein levels of inflammatory molecules, phosphorylated c-Jun N-terminal kinase (pJNK) in the PFC, and protein tyrosine phosphatase 1B (PTP1B) in both the PFC and hippocampus. In summary, these findings suggest that BM prevents HF diet-induced impairments in recognition memory by improving downstream BDNF signal transduction, increasing pAMPK, and reducing inflammation in the PFC and hippocampus.

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**Bardoxolone methyl prevents high-fat diet-induced alterations in prefrontal cortex signalling  
molecules involved in recognition memory**

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## Abstract

High-fat (HF) diets are known to induce changes in synaptic plasticity in the forebrain leading to learning and memory impairments. Previous studies of oleanolic acid derivatives have found that these compounds can cross the blood brain barrier to prevent neuronal cell death. We examined the hypothesis that the oleanolic acid derivative, bardoxolone methyl (BM) would prevent diet-induced cognitive deficits in mice fed a HF diet. C57BL/6J male mice were fed a lab chow (LC) (5% of energy as fat), HF (40% of energy as fat), or HF diet supplemented with 10 mg/kg/day BM orally for 21 weeks. Recognition memory was assessed by performing a novel object recognition test on the treated mice. Downstream brain derived neurotrophic factor (BDNF) signalling molecules were examined in the prefrontal cortex (PFC) and hippocampus of mice via western blotting and *N*-methyl-D-aspartate (NMDA) receptor binding. BM treatment prevented HF diet induced impairment in recognition memory ( $p < 0.001$ ). In HF diet fed mice, BM administration attenuated alterations in NMDA receptor binding density in the PFC ( $p < 0.05$ ), however, no changes were seen in the hippocampus ( $p > 0.05$ ). In the PFC and hippocampus of HF diet fed mice, BM administration improved downstream BDNF signalling as indicated by increased protein levels of BDNF, phosphorylated tropomyosin related kinase B (pTrkB) and phosphorylated protein kinase B (pAkt), and increased phosphorylated AMP-activated protein kinase (pAMPK) ( $p < 0.05$ ). BM administration also prevented the HF diet induced increase in the protein levels of inflammatory molecules, phosphorylated c-Jun N-terminal kinase (pJNK) in the PFC, and protein tyrosine phosphatase 1B (PTP1B) in both the PFC and hippocampus. In summary, these findings suggest that BM prevents HF diet induced impairments in recognition memory by improving downstream BDNF signal transduction, increasing pAMPK, and reducing inflammation in the PFC and hippocampus.

## Introduction

Obesity is a major risk factor for the development of cognitive decline in neurodegenerative disease such as vascular dementia (Hassing et al. , 2002). A number of studies provide direct evidence demonstrating a link between high-fat (HF) diet-induced obesity and impairments in learning and memory performance, including a decline in recognition memory (Greenwood and Winocur, 1990, 1996, Heyward et al. , 2012). Furthermore, preclinical animal studies have demonstrated that a HF diet reduces synaptic plasticity in the prefrontal cortex (PFC) (Val-Laillet et al. , 2011) and hippocampus (Molteni et al. , 2002, Wu et al. , 2003), which leads to learning and memory impairments (Laroche et al. , 2000). A HF diet can further induce cognitive decline by promoting neuroinflammation in the forebrain (Miller and Spencer, 2014). Despite this, therapeutic interventions targeting HF diet induced cognitive impairment are lacking.

The oleanolic acid synthetic derivative, bardoxolone methyl (BM) has attracted attention due to its potential application in a wide variety of diseases (Camer and Huang, 2014, Camer et al. , 2014, Liby and Sporn, 2012, Reisman et al. , 2012, Wang et al. , 2011). A recent study found that BM can promote dopaminergic neuroprotection via attenuation of the inflammatory mediator, tumour necrosis factor alpha (TNF $\alpha$ ), and reactive oxygen species (ROS) production *in vitro* (Tran et al. , 2008). Despite this finding no study has subsequently investigated the effects of BM on the brain *in vivo*. However, a derivative of BM, CDDO-MA, improved spatial memory and reduced hippocampal amyloid plaques in a mouse model of Alzheimer's disease (Dumont et al. , 2009). It has been demonstrated that administration of oleanolic acid has been found to reverse recognition memory impairments in mice (Park et al. , 2014). Furthermore, synthetically modifying side chains on oleanolic acid to a derivative form, such as BM, significantly increases its potency (Zhang et al. , 2008). Therefore, this suggests that BM has the potential to significantly prevent recognition memory decline, which was examined in our study.

Obesity induced cognitive impairment is attributed to a reduction of synaptic plasticity (Molteni, Barnard, 2002). Recent evidence has indicated that HF diet-induced impairment in neuronal plasticity may be caused notably by the reduction of brain derived neurotrophic factor (BDNF) protein expression in the PFC and hippocampus, which are key brain areas in learning and memory (Kanoski et al. , 2007). BDNF signalling is a critical pathway for promoting long term potentiation (LTP), a form of synaptic plasticity responsible for long term memory (LTM) formation, and neurogenesis in the forebrain (Noble et al. , 2011). Tropomyosin related kinase B (pTrkB) receptor phosphorylation and activation by BDNF leads to a downstream intracellular cascade resulting in activation of protein kinase B (pAkt) signalling (Cunha et al. , 2010). Akt signalling regulates the translation and transport of synaptic proteins in order to promote synaptic plasticity in learning and memory (Yoshii and Constantine-Paton, 2007). Along with the activation of TrkB, BDNF also triggers the opening of Na<sup>+</sup> gated ion channels, resulting in an influx of Ca<sup>2+</sup> and the enhancement of glutamate activation of *N*-methyl-D-aspartate (NMDA) receptors (Rose et al. , 2004). NMDA receptors also play a crucial role in synaptic plasticity with their activation by glutamate leading to the induction of LTP (Bliss and Collingridge, 1993, Cooke and Bliss, 2006). A previous study has reported that a HF diet desensitises NMDA receptors in the hippocampus in mice causing impairment in NMDA-induced long term depression (LTD), suggesting that its alteration may also account for cognitive defects (Valladolid-Acebes et al. , 2012). Another important signalling protein that is linked to BDNF is phosphorylated AMP-activated protein kinase (pAMPK). Studies have demonstrated that pAMPK activation increases BDNF expression in the brain (Gomez-Pinilla et al. , 2008, Yoon et al. , 2008, Zhao et al. , 2008), suggesting that its activation plays a crucial role in promoting synaptic plasticity. Furthermore, it has been reported that a HF diet reduces phosphorylation of AMPK in the hippocampus in rats (Wu et al. , 2006). However, the effect of chronically administered BM in preventing HF diet-induced alterations in BDNF signalling, pAMPK, and NMDA receptor neurotransmission in the PFC and hippocampus of mice remains unexplored, and was investigated in this study.

It is widely accepted that consumption of a HF diet and obesity leads to obesity-induced chronic inflammation in a number of tissues, including the brain (Weisberg et al. , 2003, Xu et al. , 2003). Several rodent studies have demonstrated that chronic inflammation in the brain induced by a HF diet is also associated with a decline in cognitive performance (Morrison et al. , 2010, Pistell et al. , 2010, Singh et al. , 2012). In the forebrain, synaptic plasticity is disrupted by increased expression of the inflammatory mediators, protein tyrosine phosphatase 1B (PTP1B) (Fuentes et al. , 2012) and phosphorylated c-Jun N-terminal kinase (pJNK) (Jiang et al. , 2013). However, whether BM administration can prevent HF diet- induced increases in expression of these inflammatory mediators is unknown and therefore was examined in this study.

Although beneficial effects of BM have been demonstrated in animal models and human clinical trials in a variety of tissues (Pergola et al. , 2011, Pitha-Rowe et al. , 2009), the effect of BM in the central nervous system during HF diet-induced obesity has not been examined previously. Furthermore, no study has yet investigated whether chronic BM treatment can prevent HF diet-induced decline in recognition memory and synaptic plasticity. Therefore, the purpose of the current study was to determine whether chronic oral BM administration in mice fed a HF diet for 21 weeks could prevent impairments to recognition memory. Our findings suggest that chronic BM supplementation may be useful in reducing impairments in recognition memory by improving BDNF downstream signal transduction, increasing phosphorylation of AMPK, and decreasing PTP1B in the PFC and hippocampus. In addition to these effects BM supplementation in HF diet fed mice prevented alterations in NMDA receptors and the inflammatory mediator pJNK in the PFC, but not the hippocampus. Therefore, the present study suggests that BM prevents HF diet-induced alterations in signalling molecules involved in recognition memory, with a stronger effect on the PFC compared to the hippocampus.

## Materials and Methods

### Animals and HF diet-induced obesity model

Male C57BL/6J mice (12 weeks old) were purchased from the Animal Resource Centre (Perth, Western Australia) and maintained in the animal facility at the University of Wollongong. The experiments were performed in accordance with the *Australian Code of Practice for the Care and Use of Animals for Scientific Purposes*. All procedures were approved by the Animal Ethics Committee, University of Wollongong, Wollongong, Australia (AE 12/15). Mice were housed in environmentally controlled conditions (temperature 22 °C, 12hr light/dark cycle) and 1 week after acclimatisation were randomly divided into 3 groups (n=7 per group). For the next 21 weeks one group of mice were fed a lab chow (LC) diet (5% of energy as fat; Vella Stock Feeds, Doonside, New South Wales, Australia), and the other two groups a HF diet (40% of energy as fat; SF11-095, Specialty Feeds, Glen Forrest, Western Australia). The fat present in the HF diet consisted of half lard and half sunflower oil. Mice in the treatment group were one of the groups fed a HF diet for 21 weeks, which also received an oral daily dose of BM (10 mg/kg) in their drinking water. We chose the BM dose according to a previous study (Wu et al. , 2014). Body weight was measured weekly for the duration of the experiment (Final average body weight after 21 weeks: LC, 27.15g; HF, 40.84g; HF+BM, 28.13g). Area under the curve (AUC) for glucose following a glucose tolerance test was measured (AUC glucose: LC, 969.14 mmol/l; HF, 1102.83 mmol/l; HF+BM, 942.75mmol/l).

### Novel Object Recognition Test

Recognition memory was assessed by performing a novel object recognition test based on a previously described protocol (Fernandez et al. , 2012). Briefly, a white open-field square box measuring 55cm in length, 55cm in width, and 35cm in height was used as the experimental apparatus. The open-field box was located in a sound proof room, and lit at approximately 14 lux. The experimental procedure consisted of habituation, training and retention sessions, which were recorded using a video camera placed above the open-field box. All objects and the open-field box were cleaned with 70% ethanol



between each mouse. For habituation, mice were individually placed in the box for 10 minutes to explore the environment in the absence of objects. During the training session, two identical objects (A) were placed at opposing corners of the box, 5cm from the adjacent wall. Each mouse was then placed in the middle of the open-field box individually and left to explore the objects for 10 minutes. A mouse was considered to be exploring the object if it was sniffing, touching or facing the object within 2cm or less, and measurements were recorded in seconds. For the retention session, one familiar object (A) was replaced with one novel object (B) and measurements were taken according to how much time each mouse spent at each object as per the training session. The retention session commenced upon placing the mouse individually in the middle of the open-field box ninety minutes after its training session, and leaving it to explore for another 10 minutes. A recognition index was calculated using the formula: Recognition Index = Object B/ (Object A + Object B).

#### **Tissue collection**

For tissue analysis (n=7 per group), mice were euthanised at week 21 of the experiment. Brains were dissected from the mice, snap frozen in liquid nitrogen and stored at -80 °C until use.

#### **Microdissection**

Frozen brain sections containing the PFC and hippocampus regions were cut into 14 µm coronal sections with a cryostat at -18°C before being mounted on Polylysine™ microscope slides for receptor autoradiography. Further coronal brain sections were cut at 500µm before the PFC and hippocampus regions were dissected for western blotting. Sections were collected at levels ranging from Bregma 3.70mm to -5.20mm based on a standard mouse brain atlas (Paxinos, 2002). Brain sections were stored in -20°C until use.

#### **Receptor Autoradiography**

The procedure for receptor autoradiography to assess NMDA receptor density was based on the protocol described by Newell et al. (2005) (Newell et al. , 2005). Briefly, brain sections were incubated in 30nM N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid (HEPES) buffer, containing 100mM

glycine and 100mM glutamate, 1 mM ethylenediaminetetraacetic acid (EDTA) and 20nM of the radioligand, [<sup>3</sup>H]MK-801 (specific activity 17.1 Ci/mmol, Perkin Elmer, Boston, Massachusetts, USA) for 2.5 hours at room temperature. All solutions for this procedure had a pH of 7.5. Non-specific binding was determined by incubating adjacent brain sections with [<sup>3</sup>H] MK-801 in 20 mM MK-801. Following incubation, each section was washed twice for 20 minutes at 0 °C in a solution of 30 mM HEPES and 1 mM EDTA. NMDA receptor binding autoradiographic images were taken using a Beta-Imager™ camera (BioSpace, Paris, France). Sections were scanned at a high-resolution setting for 3.5 hours. A series of sections used as standards with known amount of radioactivity were included in all scans. Quantitative analysis of these images was performed using the β-Image Plus software (version 4, BioSpace).

### Western Blot analysis

For protein extraction the frozen PFC and hippocampus tissue samples were homogenised in Nonidet P-40 lysis buffer. The following antibodies were used to quantify specific proteins: BDNF (sc-546), pTrkB (sc-135645), TrkB (sc-377218), pAkt (sc-135650), Akt (sc-1618), pAMPK (sc-33524), AMPK (sc-25792) and pJNK (sc-6254) (Santa Cruz Biotechnology, Dallas, TX); PTP1B (#5311) (Cell Signalling Technology, Beverly, MA). The bands corresponding to the proteins of interest were scanned and the band density analysed using the automatic imaging analysis system, Quantity One (Bio-Rad Laboratories, Hercules, California). All quantitative analyses were normalised to β-actin. Western blots were performed in triplicate for each sample; however, in some cases only two values for each sample were collected. The average of the duplicate/triplicate numbers for each sample were calculated and this number was used for statistical analysis

### Statistics

Data were analysed using the statistical package SPSS 20 (SPSS, Chicago, IL). Data was first tested for normality before differences between mice fed a LC, HF, and HF supplemented with BM diet were determined by one-way analysis of variance (ANOVA). This was followed by the post hoc Tukey-Kramer honestly significant difference (HSD) test for multiple comparisons among the groups. A p value

of  $<0.05$  was considered statistically significant. Values are expressed as mean  $\pm$  SEM. Pearson's correlations were used to examine the relationship between recognition index and BDNF levels, recognition index and NMDA receptor density, and AMPK phosphorylation and BDNF levels in the PFC and hippocampus.

## Results

### Bardoxolone Methyl prevented a decline in novel object recognition in mice fed a high-fat diet

To assess whether BM treatment can prevent HF diet-induced long term memory deficits, we performed a novel object recognition test in mice fed a HF diet for 21 weeks. During the training session of the test, the percentage of time spent exploring the identical objects in the open-field was not significantly different among mice fed a LC diet (18.65%), HF diet (22.83%), and HF diet treated with BM (17.29%). However, HF diet fed mice were found to have a significantly reduced recognition index compared to mice fed a LC diet, determined from the novel object recognition test (LC= 50.07%, HF= 26.28%,  $p = <0.001$ , Figure 1). This impairment in memory was prevented through BM administration, indicated by a significantly higher recognition index (56.82%) in BM treated mice fed a HF diet compared to untreated HF diet fed mice ( $p = <0.001$ , Figure 1A). These results show that recognition memory deficits caused by a HF diet may be prevented with BM treatment. In addition, the total sum of exploration time between the familiar and novel object is presented in Figure 1B (Difference in time between familiar and novel object (LC= +19.5%, HF= -62.0%, HF+BM= -4.7%)

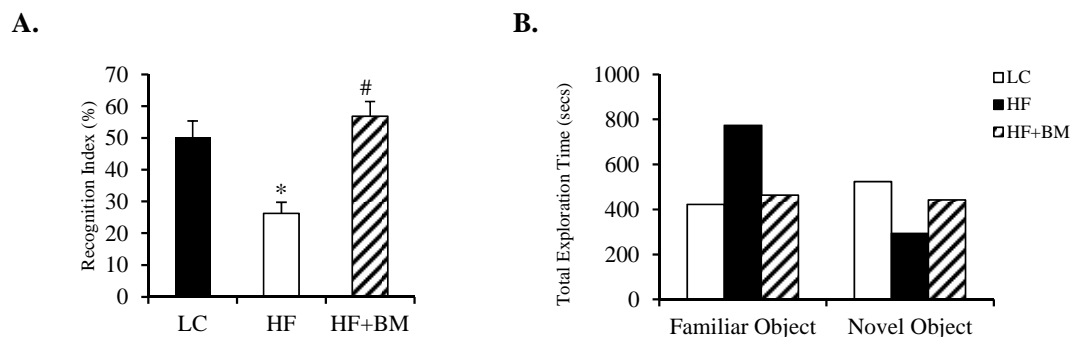


Figure 1. Effect of chronic bardoxolone methyl (BM) treatment on recognition memory in mice fed a high-fat diet for 21 weeks ( $n = 7$  per group). Chronic treatment of BM significantly prevented high-fat (HF) diet-induced decline in recognition index in mice (A). Total exploration time between familiar and novel object (B). \*,  $p < 0.05$  vs. lab chow (LC) group, #,  $p < 0.05$  vs. HF group, values are means  $\pm$  SEM.

## Bardoxolone Methyl prevented an increase in NMDA receptor density in the prefrontal cortex of mice fed a high-fat diet

[<sup>3</sup>H]MK-801 binding density was measured in mouse PFC and hippocampus in order to determine the density of NMDA receptors. NMDA receptor density in the PFC of HF diet fed mice was significantly increased compared with LC fed mice ([<sup>3</sup>H] MK-801 receptor binding density difference: 26.81%,  $p < 0.05$ , Figure 2 and Table 1). However, the level of [<sup>3</sup>H]MK-801 binding to NMDA receptors in BM treated mice fed a HF diet was significantly reduced compared to the HF diet controls in the PFC ([<sup>3</sup>H]MK-801 binding density difference: 27.76%,  $p < 0.05$ , Figure 2 and Table 1). In mouse hippocampus there were no significant differences in NMDA receptor density among LC, HF and BM groups (Table 1). These results suggest that BM administration in mice fed a HF diet prevents the obesity induced alteration of NMDA receptor binding density in the PFC, but not in the hippocampus.

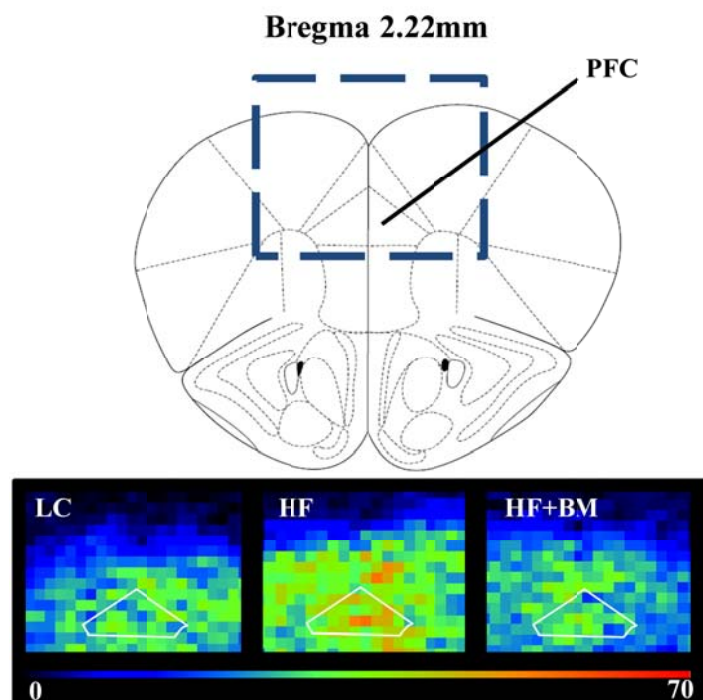


Figure 2. Effect of chronic bardoxolone methyl (BM) treatment on N-methyl-D-aspartate (NMDA) receptor binding density in the prefrontal cortex (PFC) in mice fed a high-fat diet for 21 weeks ( $n = 7$  per group). Chronic treatment of BM significantly prevented high-fat (HF) diet-induced alterations in NMDA receptor binding density in the PFC in mice. \*,  $p < 0.05$  vs. lab chow (LC) group, values are means  $\pm$  SEM. Scale bar: 0-70 fmol/mg tissue equivalent.

Table 1 NMDAR binding density in mouse forebrain following 21 weeks of LC, HF or HF + BM diet

Brain Area	LC	HF	HF+BM	F value	P value
Prefrontal Cortex	14.5±1.7 <sup>b</sup>	19.8±0.9 <sup>a</sup>	14.3±1.9 <sup>b</sup>	2.798	0.038
Hippocampus	16.7±1.9	19.1±2.1	19.5±1.5	0.785	0.473

Values are means ±SEM. LC, lab chow diet, HF, high-fat diet, HF+BM, high-fat diet and bardoxolone methyl treatment. <sup>a</sup> $p < 0.05$  vs LC, <sup>b</sup> $p < 0.05$  vs HF.

### Bardoxolone methyl prevented HF diet induced decline in downstream BDNF signalling and phosphorylation of AMPK in the forebrain of mice fed a HF diet

We evaluated the effect of BM on the expression of BDNF and its associated signalling molecules in the PFC and hippocampus of HF diet fed mice using western blotting analysis (n = 6-7 per group for each protein). In both the PFC and hippocampus, western blot analysis showed that a HF diet reduced BDNF levels and the phosphorylation of TrkB, which was significantly reversed by BM treatment ( $p = < 0.05$ , Figure 3A and B). Furthermore, HF diet induced decreases in phosphorylation of the proteins Akt and AMPK was prevented by BM administration ( $p = < 0.05$ , Figure 3A and B). In addition, BDNF levels were positively correlated to the phosphorylation of AMPK in the PFC ( $r = 0.674$ ,  $p = < 0.01$ ), and hippocampus ( $r = 0.798$ ,  $p = < 0.001$ ) (Figure 4A and B). In examining total protein expression, BM prevented HF diet-induced decreases in TrkB levels in the hippocampus, but not the PFC. There were no significant differences between the total protein expressions of Akt and AMPK in the PFC or hippocampus. These results suggest that BM prevents HF diet induced decreases in BDNF, phosphorylation of TrkB and the phosphorylation of associated signalling molecules, Akt and AMPK in the PFC and hippocampus.

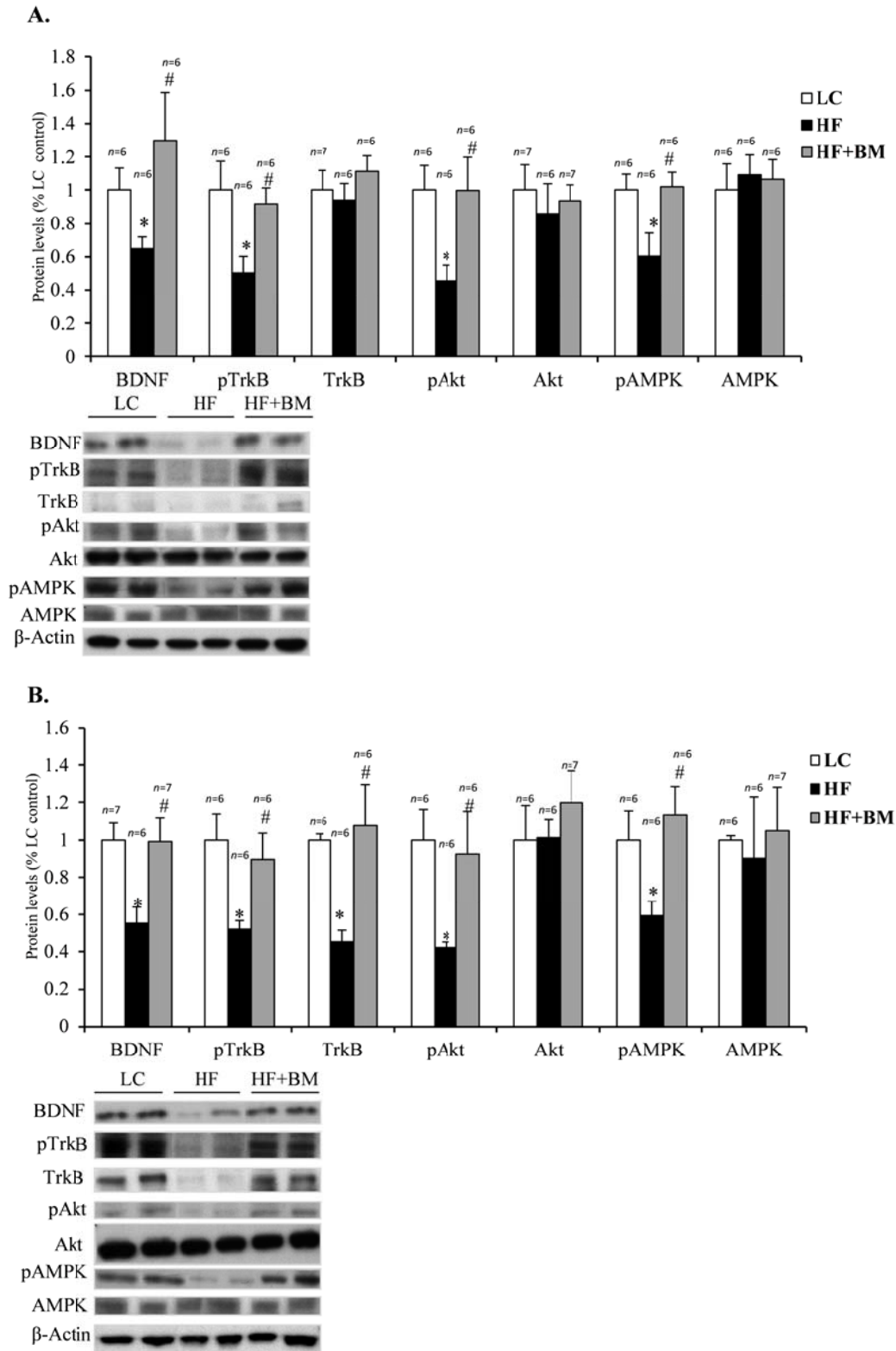


Figure 3. Effect of chronic administration of bardoxolone methyl (BM) treatment on protein levels of key signalling molecules involved in BDNF signalling of mice ( $n = 6-7$  per group) fed a HF diet for 21 weeks. Chronic treatment of BM significantly prevented high-fat (HF) diet-induced decreases in BDNF, and protein phosphorylation of TrkB, Akt, and AMPK in the prefrontal cortex (PFC) (A) and hippocampus (B). \*,  $p < 0.05$  vs. lab chow (LC) group, #,  $p < 0.05$  vs. HF group, values are means  $\pm$  SEM.

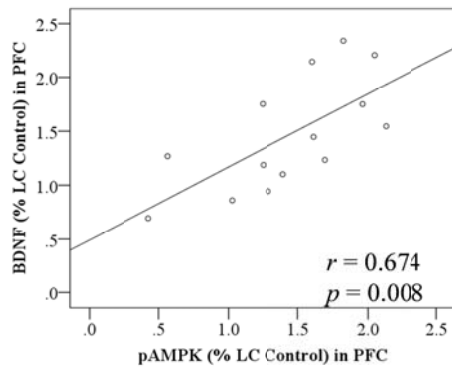
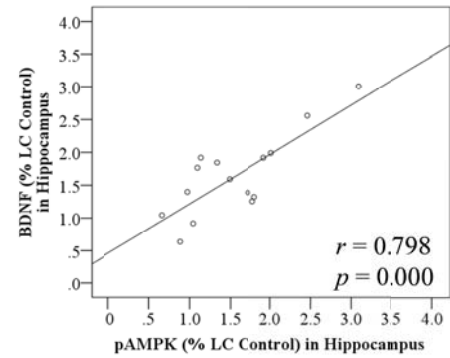
**A.****B.**

Figure 4. Correlation between BDNF and AMPK protein phosphorylation in the prefrontal cortex (PFC) (A) and hippocampus (B) in mice fed a high-fat (HF) diet and treated with bardoxolone methyl (BM) for 21 weeks ( $n = 6-7$  per group).  $n$  numbers used for quantification are indicated above each column; they are different because of variations in western blotting between membranes that did not allow us to quantify several lanes for some samples. \*,  $p = <0.05$  vs. lab chow (LC) group, values are means  $\pm$ SEM.

Bardoxolone methyl prevented HF diet induced elevations in the inflammatory mediators, PTP1B and phosphorylation of JNK in mouse prefrontal cortex

The downstream inflammatory mediators, PTP1B and pJNK, were measured in the prefrontal cortex and hippocampus of mice fed a HF diet using western blotting in order to assess if BM treatment could prevent neuroinflammation ( $n = 6-7$  per group for each protein). PTP1B levels were significantly increased in both the PFC and hippocampus, and protein phosphorylation of JNK were significantly increased in the PFC in mice fed a HF diet, which was significantly reduced by BM administration ( $p = <0.05$ , Figure 5A and B). However, no differences were seen in the protein phosphorylation of JNK between any of the groups in the hippocampus ( $p = >0.05$ ). These results suggest that BM prevents HF diet induced elevations in inflammatory mediators in the PFC and hippocampus.

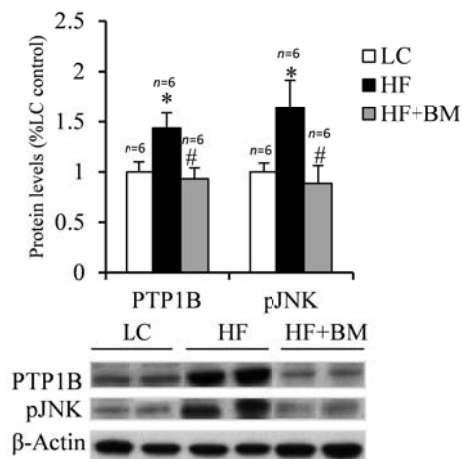
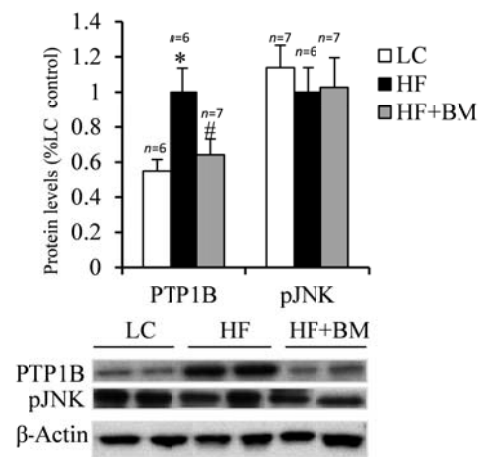
**A.****B.**

Figure 5. Effect of chronic administration of bardoxolone methyl (BM) treatment on protein levels of inflammatory mediators of mice ( $n=6-7$  per group) fed a high-fat (HF) diet for 21 weeks. Chronic treatment of BM significantly prevented a HF diet-induced increase in PTP1B and JNK protein phosphorylation in the prefrontal cortex (PFC) (A) and PTP1B in the hippocampus (B). No significant differences were found in the phosphorylation of JNK between any groups in the hippocampus.  $n$  numbers used for quantification are indicated above each column; they are different because of variations in western blotting between membranes that did not allow us to quantify several lanes for some samples. \*,  $p < 0.05$  vs. lab chow (LC) group, #,  $p < 0.05$  vs. HF group, values are means  $\pm$  SEM.

The relationship between recognition index, BDNF levels, and NMDA receptor density in brain regions examined

In the PFC there was a significant positive correlation between recognition index and BDNF levels ( $r = 0.547$ ,  $p < 0.05$ , Figure 6A and B), and a significant negative correlation between recognition index and NMDA receptor density ( $r = -0.532$ ,  $p < 0.05$ ) in the PFC. However, in the hippocampus, there were no significant correlations between recognition index and BDNF levels ( $r = 0.382$ ,  $p = 0.118$ ) or between recognition index and NMDA receptor binding density ( $r = -0.174$ ,  $p = 0.473$ ) in the hippocampus. This suggests that both NMDA and BDNF expression in the PFC may be related to the changes of recognition memory reported in the different tested groups.



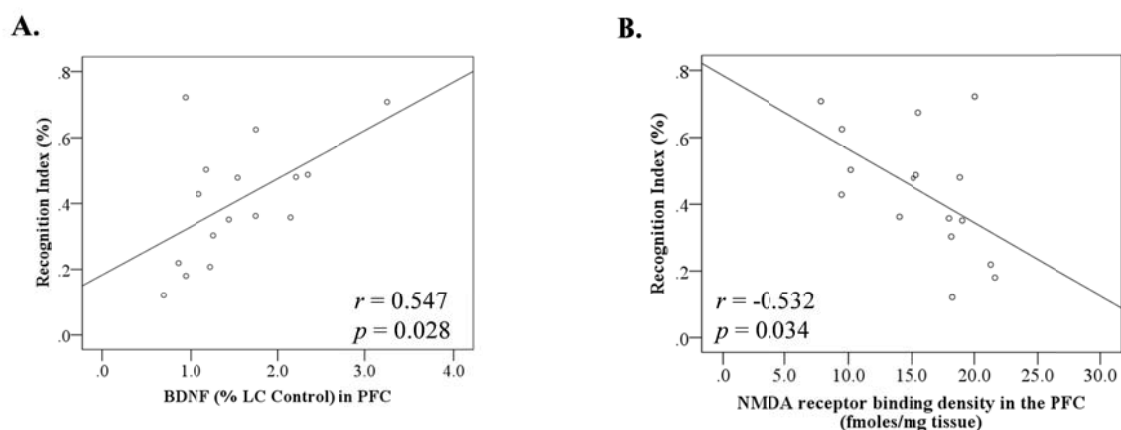


Figure 6. Correlation between recognition index and BDNF (A), and recognition index and N-methyl-D-aspartate (NMDA) receptor binding density (B) in the prefrontal cortex (PFC) in mice fed a high-fat (HF) diet and treated with bardoxolone methyl (BM) for 21 weeks ( $n = 6-7$  per group). \*,  $p < 0.05$  vs. lab chow (LC) group, values are means  $\pm$  SEM.

## Discussion

Rodents fed a HF diet show elevated body weight gain, along with cognitive decline, including impairments in recognition memory (Carey et al. , 2014, Heyward, Walton, 2012, Valladolid-Acebes et al. , 2011). In the current study, a chronic HF diet decreased recognition index in the novel object recognition test, which reflects that recognition memory was impaired in HF diet-induced obesity. Previously, a derivative of BM, CDDO-MA, improved spatial memory and reduced inflammation in the hippocampus in a mouse model of Alzheimer's disease (Dumont, Wille, 2009). In the present study, we found that BM prevented deficits in recognition memory in mice fed chronic HF diet, suggesting that BM has the potential to improve cognition in obesity and associated neurodegenerative disorders.

In the PFC and hippocampus, signalling through the BDNF pathway promotes neuronal plasticity and neurogenesis, which are both important for learning and memory (Kanoski, Meisel, 2007, Sakata et al. , 2013). Rats fed an unrestricted HF diet display decreased BDNF levels in both hippocampus and PFC, which is coupled with impaired discrimination learning (Kanoski, Meisel, 2007). In the brain, BDNF binds to TrkB receptors causing its phosphorylation and subsequent activation of the Akt intracellular signalling cascade to promote synaptic plasticity (Cunha, Brambilla, 2010). Our study demonstrated that BM treatment in mice fed a HF diet for 21 weeks promoted the downstream BDNF signalling cascade by increasing BDNF levels, and protein phosphorylation of TrkB, and Akt in the hippocampus

and PFC. This suggests that the actions of BM on the downstream BDNF signalling cascade contributed to improved neuronal plasticity in the hippocampus and PFC of mice fed a HF diet, which further contributed to an improvement in recognition memory. Furthermore, BDNF levels in the PFC were positively correlated to recognition index suggesting that BDNF signalling in the PFC is important for recognition memory.

Several studies have recently suggested an association between BDNF and the phosphorylation of AMPK (Gomez-Pinilla, Vaynman, 2008, Yoon, Oh, 2008, Zhao, Shen, 2008). Rats who performed a week of exercise demonstrated increases in AMPK protein phosphorylation and BDNF mRNA levels, which was coupled with an enhancement to spatial memory (Gomez-Pinilla, Vaynman, 2008). Furthermore, the activation of AMPK has been found to increase the expression of BDNF in mouse hippocampus (Zhao, Shen, 2008). Along with increased BDNF levels, our results demonstrated that BM administration increased AMPK protein phosphorylation in the PFC and hippocampus of mice fed a HF diet for 21 weeks. Furthermore, our results found a positive correlation between the phosphorylation of AMPK and BDNF levels in both the PFC and hippocampus. These results suggest that the BM-induced elevation of AMPK protein phosphorylation may modulate BDNF expression, which enhanced synaptic plasticity in the PFC and hippocampus, leading to improved recognition memory.

NMDA receptor activation is important for glutamatergic neurotransmission in learning and memory processes, including recognition memory (Bliss and Collingridge, 1993, Cooke and Bliss, 2006, Warburton et al. , 2013). It has been reported that a HF diet induces a desensitisation of NMDA receptors in the brains of mice, resulting in cognitive deficits (Valladolid-Acebes, Merino, 2012). Therefore, the increased NMDA receptor density we observed in the PFC after a chronic HF diet may reflect compensation for reduced glutamatergic NMDA receptor function. Importantly, our results found that BM prevented alteration of NMDA receptor expression in the PFC during a HF diet, which may be involved in its improvement of recognition memory in these mice. Furthermore, NMDA receptor binding density was negatively correlated to recognition index, suggesting that HF diet induced alterations of

NMDA receptors in the PFC leads to impairments in recognition memory that can be prevented by BM administration. The PFC and hippocampus are known important brain regions in learning and memory that both utilise NMDA receptor dependent synaptic plasticity (Banks et al. , 2012). Previous studies have demonstrated that recognition memory can be impaired by lesions on the PFC in monkeys and rats (Bachevalier and Mishkin, 1986, Kolb et al. , 1994). On the other hand, studies in rodents showed contradictory results regarding the implication the hippocampus in recognition memory, reporting that hippocampal lesions may have no effect in object recognition memory in rats (Forwood et al. , 2005, Langston and Wood, 2010, Mumby et al. , 2002). This suggests that the PFC may have a more influential role in recognition memory compared to the hippocampus in rodents. Our results were in line with these studies, since there were no significant changes in NMDA receptor binding density in the hippocampus between the groups.

PTP1B and phosphorylation of JNK in the hippocampus and PFC also influences synaptic plasticity, as their activation has been shown to impair learning and memory retention in mice (Fuentes, Zimmer, 2012, Wang et al. , 2013). Both pJNK and PTP1B are known to cause memory impairments by negatively regulating Akt signalling (Lu et al. , 2011, Sunayama et al. , 2005). In addition to preventing HF diet induced decline in Akt protein phosphorylation, our results demonstrated that BM prevented HF diet induced increases in PTP1B and phosphorylation of JNK in the PFC, and PTP1B in the hippocampus. This suggests that HF diet induced impairments of learning and memory via these inflammatory mediators were attenuated by BM administration, leading to the promotion of Akt signalling and subsequent improvement of recognition memory.

In conclusion, the findings of this study demonstrate that chronic administration of BM prevents HF diet induced impairment to recognition memory in mice. Furthermore our results suggest that BM targets signalling molecules in both the PFC and hippocampus that contribute to an improvement in recognition memory. However, it appears that the PFC has a more influential role in this effect. A proposed model of molecular targets of BM in the PFC in promoting recognition memory is summarised in Figure 7. Our

data suggests that a decline in neuronal plasticity in the hippocampus and PFC was attenuated by an increase in BDNF signalling, indicated by increased protein levels of BDNF, and phosphorylation of TrkB and Akt, an increase in AMPK protein phosphorylation, and through a decrease in PTP1B. This was further supported by BM preventing HF diet-induced PFC alterations in NMDA receptor density and prevents protein phosphorylation of the inflammatory mediator, JNK. Since HF diet-induced obesity has been implicated in the progression of neurodegenerative diseases such as Alzheimer's disease, BM may have beneficial effects in attenuating the progression of cognitive decline. In the future, female mice also need to be studied to rule out gender differences. With further research and eventual human clinical trials, the possibility of using BM for the prevention of HF diet-induced cognitive deficits, including recognition memory impairments, and associated neurodegeneration, appears promising.

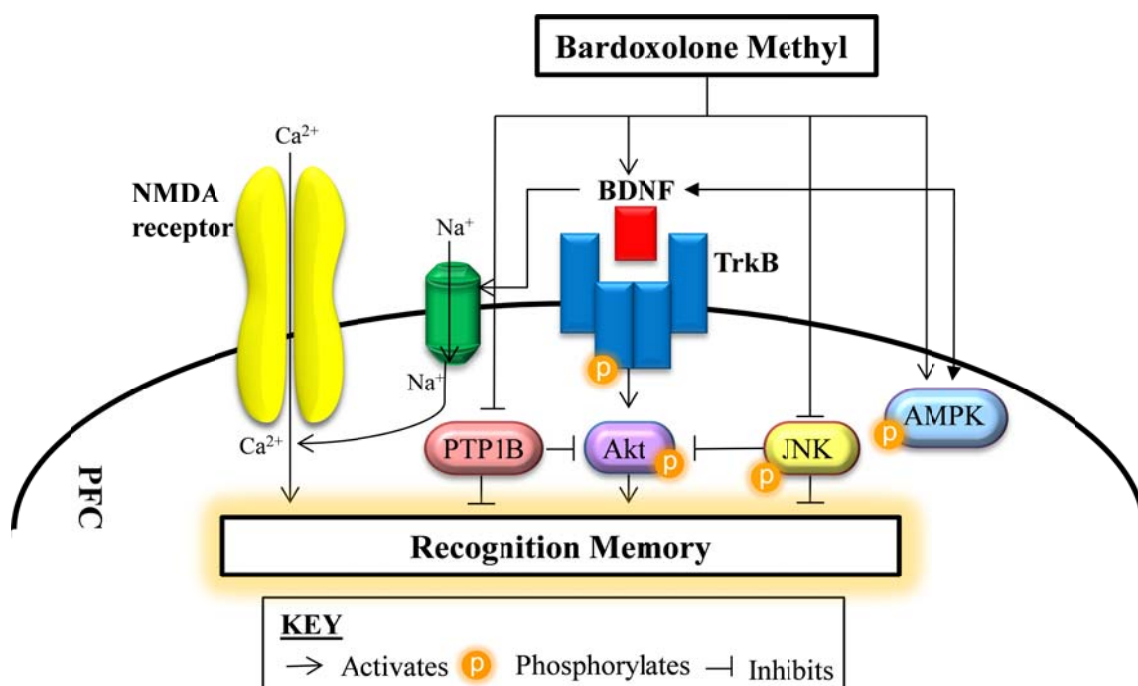


Figure 7: A proposed model of molecular targets of bardoxolone methyl (BM) in the prefrontal cortex (PFC) in preventing high-fat (HF) diet-induced decline in recognition memory. Our study found that BM prevents HF diet induced decreases in BDNF, protein phosphorylation of TrkB, Akt, and AMPK, and increases in PTP1B and JNK protein phosphorylation in mouse PFC. Furthermore, our study showed that BM prevented HF diet induced alterations in NMDA receptor density. This suggests that BM can activate BDNF resulting in activation of TrkB receptors, AMPK protein phosphorylation, and NMDA receptor activation. The activation of these targets by BDNF promotes downstream signalling pathways that promote recognition memory.

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