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Galectin-1 suppresses Methamphetamine induced neuroinflammation in human brain microvascular endothelial cells: Neuroprotective role in maintaining Blood Brain Barrier integrity

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Abstract

Methamphetamine (Meth) abuse can lead to the breakdown of the blood-brain-barrier (BBB) integrity leading to compromised CNS function. The role of Galectins in the angiogenesis process in tumor-associated endothelial cells (EC) is well established; however no data are available on the expression of Galectins in normal human brain microvascular endothelial cells and their potential role in maintaining BBB integrity. We evaluated the basal gene/protein expression levels of Galectin-1, -3 and -9 in normal primary human brain microvascular endothelial cells (BMVEC) that constitute the BBB and examined whether Meth altered Galectin expression in these cells, and if Galectin-1 treatment impacted the integrity of an in-vitro BBB. Our results showed that BMVEC expressed significantly higher levels of Galectin-1 as compared to Galectin-3 and -9. Meth treatment increased Galectin-1 expression in BMVEC. Meth induced decrease in TJ proteins ZO-1, Claudin-3 and adhesion molecule ICAM-1 was reversed by Galectin-1. Our data suggests that Galectin-1 is involved in BBB remodeling and can increase levels of TJ proteins ZO-1 and Claudin-3 and adhesion molecule ICAM-1 which helps maintain BBB tightness thus playing a neuroprotective role. Galectin-1 is thus an important regulator of immune balance from neurodegeneration to neuroprotection, which makes it an important therapeutic agent/target in the treatment of drug addiction and other neurological conditions.

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8.0 Conflict of Interest.

The authors do not have any conflict of interests.

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Keywords

Methamphetamine; Galectin-1; Human Brain Microvascular endothelial cells; Blood Brain Barrier; Tight Junctions; Oxidative stress; Proinflammatory cytokines; Transendothelial electrical resistance

1.0 Introduction

Galectins are a family of β -galactoside-binding lectins that regulate a variety of biological functions and modulate immune response under various pathophysiological conditions. Expression of Galectins is significantly increased under neuroinflammatory conditions and neuroinflammation contributes to the pathogenesis of several neurological diseases. 3 members of the Galectin family namely Galectin-1, -3 and -9 in particular are believed to be involved in the neuromodulation via cytokine production contributing to CNS pathology and/or neuro-preservation.

Galectin-1 regulates microglial activation that targets the activation of p38MAPK-, CREB-, and NF- κ B-dependent signaling pathways and suppresses downstream proinflammatory mediators, such as iNOS, TNF- α , thereby deactivating microglia and restrict brain inflammation and neurodegeneration and therefore has important therapeutic implications in neuroinflammation (Starosom SC et al 2012; Nonaka M, and Fukuda M. 2012). Similar, neuroprotective properties of Galectin-1 were demonstrated in astrocytes via the production of brain-derived neurotrophic factor (BDNF) which is known to promote neuronal survival and hence brain function and the inhibition of glutamate toxicity via modulation of NMDA receptor expression (Endo T 2005; Sasaki T et al 2004; Lekishvili T et al 2006).

Galectin-3 expression is significantly enhanced in IFN- γ -stimulated glia and produced high levels of proinflammatory mediators via the activation of the JAK-STAT pathway. Galectin-3 presence in the brain could thus be indicative of neuropathology in the CNS. (Jeon SB et al 2010)

Under basal conditions astrocytes did not express Galectin-9 however IL-1 β , IFN- γ , and TNF- α via the activation of Galectin-9 modulate the neuroinflammatory processes and contribute to CNS pathology. Galectin-9 functions as an astrocyte-microglia communication signal and promote cytokine production from microglia. (Steelman AJ et al 2013). Galectin-9 is induced in astrocytes by TNF- α via the JNK/c-Jun pathway and astrocyte-derived Galectin-9 functions as an immunoregulatory protein in response to ongoing neuroinflammation. (Steelman AJ and Li J. 2014). Astrocytes produce Galectin-9 in response to the stimulation with IL-1 β , which contributes to neuroinflammation in the CNS. (Yoshida H et al 2001).

Previously, we have shown that addictive drugs such as opiates like morphine and Meth increased the expression of Galectin-1 in human monocytes-derived macrophages (MDM) and exogenous Galectin-1 and morphine potentiated HIV-1 infection of MDM (Reynolds JL, et al 2012^a; Reynolds JL, et al 2012^b). These studies demonstrated that Galectin-1 was an important mediator involved in immune-mediated responses observed in HIV-1 infected

drug abusing subjects and that Galectin-1 could be included as a therapeutic target in combination therapy for HIV-1 infected drug abusers. Targeting Galectin-1 could decrease cell adhesion between viral proteins and host macrophages thereby decreasing HIV-1 infectivity of the macrophages in HIV-1 infected drug abusers.

Several studies report the expression of Galectins in tumor-associated endothelial cells (EC) and have implicated Galectins in angiogenesis process; however no data are available on the expression of Galectins in normal human brain microvascular endothelial cells. (D'Haene N, et al 2014). Based on previous reports Galectin-1, -3 and -9 play an important role in the CNS, however their expression levels, cellular localization and role in human central nervous system tissue is not well defined. Galectins may modulate cell adhesion by inhibiting or enhancing adhesive potential between cells or between cells and the extracellular matrix. (Wada, J. and Makino H. 2001) The brain microvascular endothelial cells and the astrocytes are the two major cells that constitute the blood brain barrier. Given the important role of Galectins on cell adhesion, migration, polarity, and chemotaxis, it is likely that modulation of Galectin levels in the brain microvascular endothelial cells that constitute the blood brain barrier (BBB) could affect the integrity of the blood brain barrier and consequently contribute to neuroinflammation. Further, we have shown that the Galectin expression levels may be modulated by viral infections and inflammatory agents such as drugs of abuse but whether they directly or indirectly alter BBB permeability is not known. (Reynolds JL, et al 2012^a; Reynolds JL, et al 2012^b).

We have previously shown that Meth treatment alter BBB permeability via the release of pro-inflammatory cytokines (Mahajan SD, et al 2008; Reynolds, J. L et al 2011), however a detailed investigation of the effect of Meth on Galectin expression and its role in modulating BBB permeability has not been done. In the current study we first evaluated the basal gene expression levels of Galectin-1, -3 and -9 in normal human brain microvascular endothelial cells (BMVEC). Our results showed that Galectin-1 was highly expressed in BMVEC as compared to Galectin- 3 and -9, therefore subsequent studies examined if Meth treatment altered Galectin-1 expression in these cells. A previous study using vascular endothelial cells demonstrated that increased Galectin expression contributed to increased leukocyte traffic through the vascular endothelium (Ishikawa A, et al 2004; Imaizumi T et al. 2002). Therefore, we examined whether treatment of an in-vitro BBB with Galectins impacted the integrity of the BBB and evaluated if galectin-1 treatment modulated the expression of tight junction proteins ZO-1 and Claudin-5. Since Galectins play a major role in cell-cell and cell-ECM adhesion, we further examined if Galectin-1 treatment modulated levels of intercellular adhesion molecule-1 (ICAM-1) that could enhance the responsiveness of endothelium thereby regulating BBB integrity. Additionally, we examined the mechanisms that may underlie the neuroprotective effects of Galectin-1 in human brain microvascular endothelial cells.

2.0 Results

2.1 Basal expression of Galectin-1, 3 and 9 in BMVEC

We evaluated basal expression levels of Galectin-1, 3 and 9 in Human Brain microvascular endothelial cells using real time QPCR. Figure 1 shows the basal gene expression levels of

Galectin-1, 3 and 9 levels in BMVEC. Our results show significantly higher gene expression of Galectin-1 under basal conditions in BMVEC as compared to Galectin-3 or Galectin-9 (Figure 1). Galectin-1 basal gene expression levels ($114\% \pm 2.82$) were 30 % ($87.5\% \pm 7.77$; $p < 0.04$) higher than Galectin-3 and 60% higher than Galectin-9 ($71\% \pm 7.07$; $p < 0.001$) gene expression levels in BMVEC. Since basal expression of Galectin-1 was high in BMVEC, our further investigations have focused on Galectin-1. The basal gene expression levels were calculated using the delta delta CT or Livak Method (Livak KJ and TD Schmittgen, 2001), where Reference Gene vs Target Gene expression was computed using the formula $CT = (CT(\text{ref}, \text{untreated}) - CT(\text{ref}, \text{treated})) - (CT(\text{target}, \text{untreated}) - CT(\text{target}, \text{treated}))$, which assumes approximately equal PCR efficiencies of the reference control gene and the target gene of interest and also that the PCR amplification efficiency is a 100%.

2.2 Effect of Meth treatment on Galectin-1 expression in BMVEC

To evaluate the dose and time kinetics of Meth treatment on Galectin-1 expression, BMVEC cells were treated with Meth (50- and 100nM) concentrations for a time period of 24–96 hr. Prior to all the gene and protein expression studies the cell viability was checked using trypan blue dye exclusion and we observed greater than 90% cell viability for all treatment conditions. After the incubation period was complete, BMVEC were harvested, RNA extracted and reverse transcribed to DNA followed by real time PCR using Galectin-1 specific primers. Our results (Figure 2A) showed Meth treatment of BMVEC at both 50 and 100nM concentrations showed no significant increase in Galectin-1 gene expression 24 hrs post treatment, however an 39% increase ($p < 0.05$) when treated with 50nM Meth and a 17% increase ($p = \text{NS}$) in Galectin-1 gene expression when treated with Meth (100nM) was observed at 96 hr post treatment, as compared to the untreated control. Thus, our gene expression data indicates that Meth treatment increases Galectin-1 expression. The Meth concentrations found in blood, urine or tissue samples of Meth users range from $0.001\mu\text{M}$ to $1000\mu\text{M}$ (Takayasu et al. 1995; Kalasinsky et al. 2001; Schepers et al. 2003; Klette et al. 2005; Morefield et al. 2011 Abdul Muneer PM et al, 2011; Fisher D et al 2015). The concentration of Meth used in vitro studies, depends on the type of target cell. For example, previous studies from our group have used Meth concentration between $1\mu\text{M}$ – $100\mu\text{M}$ when targeting monocyte derived macrophages (MDM). Meth doses in that concentration range produced maximum biological response without causing toxicity to the MDM and were based also on published in vitro studies (Reynolds et al. 2007; Tallóczy et al. 2008; Tipton et al. 2009). Typically for studies involving the BBB permeability we have previously (Mahajan SD Et al 2008) used Meth concentrations in the range of 10–50 nM which are well within the physiological range and have been used by other investigators in prior studies (NIDA info Facts <http://www.drugabuse.gov/publications/drugfacts/methamphetamine>). In the current study, we used 50nM–100nM (i.e. 0.05 – $0.1\mu\text{M}$) concentration of Meth which were relatively lower and our rationale for a lower dose was to eliminate any inflammatory response by Meth. Meth treatment typically stimulates the production of the inflammatory cytokines, such as tumor necrosis factor α TNF- α , IL-1 β etc which facilitates the activation of the redox-responsive transcription factors associated with inflammation.

We also measured Galectin-1 protein levels using an ELISA kit, 24 and 96 hr post Meth treatment (50–100nM). Our results (Figure 2B) a slight but not significant increase in Galectin-1 protein expression at 24 hr post when treated with 50nM Meth (309.73 ± 21.09 pg/ml) as compared to the untreated control (297.72 ± 46.34 pg/ml), however a significant increase (588.49 ± 57.01 pg/ml, $p < 0.01$) in Galectin-1 protein levels at 96hr with 50nM Meth as compared to the untreated control (348.29 ± 32.3 pg/ml) was observed. At a higher dose of 100nM Meth, a ~28% decrease ($p < 0.05$) in Galectin-1 levels was observed at both 24hr (214.91 ± 19.01 pg/ml) and 96 hr (249.05 ± 32.03 pg/ml) post Meth treatment when compared to the respective untreated controls (297.72 ± 46.34 pg/ml and 348.29 ± 32.3 pg/ml respectively).

Figure 3 (a–c) shows the representative immunofluorescence staining images for Galectin-1 in BMVEC cells treated with Meth. Our results show that treatment of BMVEC cells with Meth at both 50 and 100nM concentrations increased Galectin-1 expression. The intensity of the fluorescence staining was measured using the Image J software software (National Institutes of Health, Bethesda, MA, USA) and the results were expressed as mean pixel units (Figure 3d) of a representative image from each treatment.

2.3 Effect of Galectin-1 alone and in combination with Meth on BBB permeability

To evaluate the role of Galectin-1 in neuroinflammation we examined the effect of Galectin-1 alone and in combination with Meth on BBB permeability using an in-vitro BBB model. We used a well validated, in-vitro BBB model to evaluate integrity of the BBB as measured by transendothelial electrical resistance (TEER). An intact BBB was treated with Meth (50–100 nM) alone and in combination with Galectin-1 (10 μ M). Our data (Figure 4) shows that treatment of the intact BBB with Meth resulted in a 52% ($p < 0.01$) and 63% ($p < 0.001$) decrease in TEER at both 50nM and 100 nM concentrations. Treatment with Galectin-1 (10 μ M) did not alter BBB integrity, however when Galectin-1 (10 μ M) was added in combination with Meth (50–100 nM) a reversal of Meth induced effect on the BBB were observed indicating that Galectin-1 plays a neuroprotective role and helps maintain the integrity of the BBB.

2.4 Effect of Galectin-1 and/or Meth on expression of Tight junction proteins and adhesion molecule ICAM-1 gene expression

Disruption of tight junctions (TJ) alters BBB permeability, increases paracellular permeability and contributes to neuroinflammation. We have previously shown that Meth treatment alters TJ protein expression levels (Mahajan SD et al 2008). In the current study, our TEER experiments showed that Galectin-1 helps maintain the integrity of the BBB, we therefore wanted to evaluate if Galectin-1 treatment modulates the expression levels of adhesion molecules such as ICAM and TJ proteins ZO-1 and Claudin-5 that are key to BBB integrity. Our results (Figure 5) show that 50nM Meth treatment significantly decreased Claudin-5 gene expression by 37% ($TAI = 0.63 \pm 0.14$; $p < 0.05$) and ZO-1 gene expression by 74% ($TAI = 0.27 \pm 0.06$; $p < 0.01$) and 100nM Meth treatment significantly decreased Claudin-5 gene expression by 19% ($TAI = 0.81 \pm 0.11$; $p = NS$) and ZO-1 gene expression by 80% ($TAI = 0.20 \pm 0.04$; $p < 0.001$). Treatment of BMVEC with Galectin-1 (10 μ M) alone did not change TJ expression, however, the Meth induced decrease in Claudin and ZO-1

expression was reversed when cells were treated with a combination of Meth and Galectin-1. Our results show that a combination of Meth (50nM) + Galectin-1 (10 μ M) significantly increased Claudin-5 gene expression by 47% (TAI=1.47 $\pm$ 0.23; p <0.05) and ZO-1 gene expression by 29% (TAI=1.29 $\pm$ 0.10; p <0.05) and Meth (100nM) + Galectin-1 (10 μ M) treatment significantly increased Claudin-5 gene expression by 86% (TAI=1.869 $\pm$ 0.24; p <0.001) and ZO-1 gene expression by 58% (TAI=1.58 $\pm$ 0.18; p <0.01). All statistical comparisons were made to the untreated controls.

Upregulation of ICAM-1 expression plays an important role in regulating BBB function and structure (Huber JD et al 2005). Our results (Figure 6) show that 50nM Meth treatment decreased ICAM-1 gene expression by 50% (TAI=0.507 $\pm$ 0.11; p <0.01) and 100nM Meth treatment decreased ICAM-1 gene expression by around 55% (TAI=0.45 $\pm$ 0.09; p <0.01). Treatment of BMVEC with Galectin-1 (10 μ M) alone did not change ICAM-1 expression (TAI=0.95 $\pm$ 0.11) however, the Meth induced decrease in ICAM-1 expression was reversed when cells were treated with a combination of Meth and Galectin-1. Our results show that a combination of Meth (50nM) + Galectin-1 (10 μ M) significantly increased ICAM-1 gene expression by 46% (TAI=1.45 $\pm$ 0.13; p <0.001) and Meth (100nM) + Galectin-1 (10 μ M) treatment significantly increased ICAM-1 gene expression by 25% (TAI=1.25 $\pm$ 0.19; p <0.05). All statistical comparisons were made to the untreated controls.

2.5 Mechanisms that underlie the Galectin-1 induced neuroprotective effects

We measured the effect of Meth (50nM) alone and in combination with Galectin-1 (10 μ M) on the gene expression levels of some proinflammatory cytokines IL-1 β , and TNF- α . Our results (Figure 8) show that 50nM Meth treatment increased IL-1 β , gene expression by 140% (TAI=2.4 $\pm$ 0.19; p <0.001), IL-8 gene expression by 62% (TAI=1.62 $\pm$ 0.13; p <0.01), and TNF- α gene expression by around 31% (TAI=1.31 $\pm$ 0.15; p <0.05). The Meth induced increase in the gene expression of these proinflammatory cytokines was reversed when cells were treated with a combination of Meth and Galectin-1. Our results show that a combination of Meth (50nM) + Galectin-1 (10 μ M) significantly decreased IL-1 β , 1 gene expression by 43% (TAI=1.37 $\pm$ 0.21; p <0.01), IL-8 gene expression by 38% (TAI=1.02 $\pm$ 0.07; p <0.05), and TNF- α gene expression by 72% (TAI=0.27 $\pm$ 0.05; p <0.001). All statistical comparisons were made between Meth (50nM) treated vs Meth (50nM) + Galectin-1 (10 μ M) BMVEC cells. These data confirm that Meth treatment significantly increased pro-inflammatory cytokine levels and that the neuro-protective properties of Galectin-1 (10 μ M) are mediated by decreased neuroinflammation due to reduction in the levels of proinflammatory cytokines. The MAPK and CREB signaling pathways may be involved in the neuroprotective effect of Galectin-1 (Starossam SA et al, 2012). We therefore investigated the ERK and CREB protein levels in cell lysates of BMVEC treated with Meth and/or Galectin-1. Our results (Figure 9) show that 50nM Meth treatment did not significantly change ERK and CREB protein expression, further treatment of BMVEC with Galectin-1 (10 μ M) alone did not change significantly change ERK and CREB expression levels, however a combination of Meth (50nM) + Galectin-1 (10 μ M) increased CREB (31% increase; p <0.05) expression levels as compared to Meth (50nM) alone, bringing them back to baseline levels similar to the untreated controls. These data suggest that Galectin-1

mediates its neuroprotective effects via the activation of the CREB signaling pathways whereas no significant modulation of the ERK signaling pathway was observed.

Treatment of BMVEC with Meth results in increase in oxidative stress. Meth induces reactive oxygen species (ROS) production in concentration- and time dependent manner. ROS include superoxide (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radical ($OH^\bullet$), which under physiological conditions are generated at low levels and play important roles in signaling and metabolic pathways, however increased oxidative stress on treatment with Meth, causes the generation of ROS which are potentially toxic for cells further contributing to neuroinflammation. We measured the total free radical presence using a commercially available in-vitro ROS Assay Kit and our results (Figure 7) show that treatment with Meth 50nM and 100nM resulted in a 1.8 fold ($p<0.05$) and 6.7 fold ($p<0.01$) increase in oxidative stress respectively as compared to the untreated control. Treatment of BMVEC cells with Galectin-1 alone (10 μ M) did not induce oxidative stress, however when cells were treated with a combination of Meth (50nM) + Galectin-1 (10 μ M) or Meth (100nM) + Galectin-1 (10 μ M) the oxidative stress was significantly reduced by 30% ($p<0.01$) and 86% ($p<0.0001$) respectively when compared to the respective Meth treatment dose alone (i.e. Meth 50 nM and 100nM respectively), thus bringing the ROS levels back to baseline levels similar to those observed in untreated controls. These data suggest that Galectin-1 can significantly reduce oxidative stress and was able to eliminate the oxidative stress induced by Meth treatment.

3.0 Discussion

Several Galectin family members have been associated with the control of CNS macrophages and microglia (Starossom et al, 2012; Nonaka & Fukuda, 2012). Galectins are endogenous glycan-binding proteins that regulate microglial activation through modulation of signaling pathways such as p38-MAPK and CREB, to prevent neurodegeneration and promote neuroprotection. Astrocytes are known to be a major source of Galectin-1 in the CNS; however no data is available on the Galectin expression by brain microvascular endothelial cells that constitute the blood-brain barrier (BBB). The BBB is formed by capillary endothelial cells, surrounded by basal lamina and astrocytic perivascular endfeet and the astrocytes provide the cellular link to the neurons. The major Galectins expressed in the CNS are Galectins 1, 3, 4, 8, and 9 and are important modulators participating in homeostasis of the CNS and neuroinflammation (Chen HL et al, 2014). The integrity of the BBB is compromised in neuroinflammation and the Galectins may serve as extracellular mediators or intracellular regulators in controlling the inflammatory response or conferring the remodeling capacity in a compromised BBB. In this study, we wanted to evaluate expression of key Galectins by primary human brain microvascular cells and examine if Galectins can modulate TJ expression and thereby BBB integrity.

We evaluated basal expression levels of Galectin-1, 3 and 9 in Human Brain microvascular endothelial cells. Our results showed significant expression of Galectin-1 under basal conditions in BMVEC as compared to Galectin-3 or Galectin-9 (Figure 1). Overexpression of Galectin-1 in activated tumor endothelium has been well documented (Thijssen VL et al, 2006, 2008, 2010; Salomonsson E et al 2011; Astorgues-Xerri L et al 2014). Tumor

endothelial cells express several members of the Galectin family and that their expression and distribution changes on cell activation, resulting in different profiles in the tumor vasculature. Thijssen VL et al (2008) showed significant expression of Galectin-1 in the endothelial cells of normal tissues, as compared to Galectin-3, -8, and -9 whose expression levels were more variable and depended on the environment/tissue surrounding the endothelial cell. Since basal expression of Galectin-1 was highest in BMVEC and given the ability of Galectin-1 to modulate cell-cell and cell-matrix interactions, it can be speculated that Galectin-1 may play a role in maintaining BBB integrity, therefore our further investigations have focused on Galectin-1 alone.

We and several others have shown that Meth abuse can lead to the breakdown of the blood-brain-barrier (BBB) integrity leading to compromised CNS function (Mahajan SD et al 2008; Reynolds JL et al 2012; Fernandes S et al 2014). Reynolds JL et al (2012) showed that treatment of monocyte derived macrophages with Meth (10 μ M) resulted in an increase in Galectin-1 expression. Our results (Figure 2A–2B & 3) showed that treatment with Meth resulted in increased gene and protein levels of Galectin-1. However at a higher dose of 100nM Meth, at 96 hr post treatment, a ~30% decrease ($p < 0.05$) in Galectin-1 protein levels were observed (Figure 2B). The difference in the expression pattern of Galectin-1 when treated with different concentrations of Meth, may be due to several reasons: a) the fact that Galectin-1 is present both intracellularly and extracellularly and has distinct physiological roles in those cellular compartments. Galectin-1 exists as a noncovalent homodimer and its physiological effects are mainly mediated by the monomeric form of this protein which is present both within the cytoplasm and the extracellular environment (Barrionuevo P et al 2007; Cho M & RD Cummings, 1996). The extracellular functions require the carbohydrate-binding properties of Galectin-1 while the intracellular ones are associated with carbohydrate-independent interactions between Galectin-1 and other proteins. In our in-vitro experiments, depending on the concentration used Meth treatment may potentiate release of Galectin-1 from either the intracellular or extracellular compartment. b) transcriptional changes are not always translated into changes in protein expression, a recent integrative biology study showed that altered transcription levels appeared responsible for 26% of the protein changes (Taylor, R. et al. 2013). Change in mRNA levels and protein levels do not always correlate, mainly due to the transcriptional regulatory control at different levels. mRNA levels of some genes strongly correlate with change in their protein levels, this occurs when regulation of gene expression of these genes are tightly controlled at levels upstream of translation, when this regulation control is not so tight it results in weak correlation between mRNA and protein levels. Thus, changes in gene expression level are frequently not reflected at the protein level. c) yet another explanation for this discrepancy could be the fact that the half life of Meth is about 10–12 hrs and the acute effects of Meth which persist at 24 hr may steadily wane by 96 hr in culture. Meth induced Galectin-1 release can induce increase in RhoA expression and alter the polymerization of the actin cytoskeleton (Camby et al, 2002) which would then modulate BBB integrity.

Meth-induced toxicity results in the degradation of the neurovascular matrix components and tight junctions (TJs) disruption (Fernandes S et al 2014). Disruption of tight junctions drastically alters paracellular permeability and is a hallmark of many pathologic states. Meth alters BBB integrity and modifies the expression of tight junction proteins and adhesion

molecules resulting in increased transendothelial monocyte migration. (Eugenin EA et al 2013). In the current study we evaluated if Galectin-1 could play a neuroprotective role by maintaining BBB integrity and reversing the neuroinflammatory effects induced by Meth treatment in BMVEC.

We and others have shown that Meth increases BBB permeability in vitro and vivo, and that exposure of BMVEC to Meth diminishes the tightness of BMVEC monolayers in a dose- and time-dependent manner (Rosas-Hernandez et al 2013; Northrop NA & BK Yamamoto 2015; Ramirez SH et al 2009, Mahajan SD et al 2008). We observed similar effects of Meth on BBB permeability (Figure 4). Meth treatment significantly decreased the trans endothelial electric resistance (TEER), thereby increasing the permeability of the BBB. This change in BBB permeability is mediated by decrease in the expression of cell membrane-associated tight junction (TJ) proteins ZO-1 and Claudin-5 and adhesion molecules ICAM-1 (Figure 5 & 6) and further these changes were accompanied by the enhanced production of reactive oxygen species (Figure 7). ROS induces rapid tyrosine phosphorylation and redistribution of TJ proteins leading to a decrease in TEER in endothelium and subsequent disruption of BBB integrity. ZO-1 is a TJ protein that acts as scaffold to organize occludin at cell junction sites and/or links occludin to actin cytoskeleton thereby playing a regulatory role in cellular permeability. ICAM-1 plays an important role in immune-mediated cell-cell adhesive interactions and upregulation in ICAM-1 expression may play a primary role in regulating BBB function and structure. When treated with a stimulant such as Meth, the endothelial cell may undergo morphological changes that include changes in the surface expression of adhesion molecules, cytoskeletal reorganization by TJ modulation, and activation of signaling pathways. Meth treatment results in a decrease in ICAM-1 gene expression, however Galectin-1 treatment causes an induction of ICAM-1. Meth treatment also involved rapid activation of endothelial nitric oxide synthase, indicating that nitric oxide is a mediator of BBB disruption in response to Meth (Figure 7). The Meth induced decrease in TJ proteins ZO-1, Claudin-5 and adhesion molecule ICAM-1 was reversed by Galectin-1. Galectin-1 is an important regulator of immune balance from neurodegeneration to neuroprotection, which makes it an important therapeutic molecule that may play a key role in several neurological diseases. Our data suggests that Galectin-1 is involved in BBB remodeling and can increase levels of TJ proteins ZO-1 and Claudin-5 and adhesion molecule ICAM-1 which helps maintain BBB tightness thus playing a neuroprotective role. Based on the data from this study we believe that Galectin-1 via the modulation of TJ proteins and adhesion molecules maintains BBB integrity as evident by the increased TEER value in the BBB treated with a combination of Meth and Galectin-1.

Meth increases levels of proinflammatory cytokines like IL-1 β , IL-6, IL-8 and TNF- α (O'Callaghan JP et al 2008) and induce IL-6 and TNF- α in the striatum and hippocampus of mice (Sriram K et al, 2006; Goncalves J et al 2010) and IL-1 β in the hypothalamus of rats (Yamaguchi T, et al 1991). Meth significantly increased inducible nitric oxide synthase (iNOS) expression in a concentration-dependent manner and significantly increased the levels of tumor necrosis factor (TNF)- α mRNA (Permpoonputtana K & Govitrapong P; 2013). We observed an increase in the gene expression levels of levels of IL-1 β , IL-6, IL-8 and TNF- α on Meth treatment and that Galectin-1 treatment decreased the levels of the Meth induced proinflammatory cytokines (Figure 8). It is speculated that Meth induces

oxidative stress and mitochondrial dysfunction which can increase proinflammatory cytokines by increasing the activities of transcription factors such as nuclear factor-Kappa B (NF- κ B), and the cAMP-response element-binding protein (CREB) (Lee YW et al 2002). The neuroinflammatory effects of Meth are also mediated by mitogen-activated protein kinase (MAPK) pathway followed by the activation of caspases and the induction of apoptosis (Lee YW et al 2001). Our results show that the Meth induced neuroinflammatory effects may be mediated via the CREB (Figure 9). Changes in BBB integrity are related to Meth-induced activation of the mitogen-activated protein kinase (MAPK) JNK1/2 (Oshea E et al 2014). It was recently shown that Galectin-1 can modulate (MAPK) JNK1/2 and CREB signaling pathways to suppress downstream proinflammatory mediators, such as iNOS, TNF- α (Starossam SA et al, 2012). Our data showed that the CREB signaling pathway is involved in the Galectin-1 mediated suppression of proinflammatory cytokines and reversal of oxidative stress induced by Meth.

This is the first report showing the Galectin expression on human brain microvascular endothelial cells that constitute the BBB. The difference in BMVEC expression levels of Galectin family members studied may be attributed to differences in tissue distribution and subcellular localization rather than differences in their carbohydrate-binding specificities. Galectins are important modulators participating in homeostasis of the CNS and neuroinflammation. In light of the neuroprotective properties of Galectin-1, its targeted overexpression would be a novel approach for the treatment of neuroinflammation, as evident in several neurological diseases such as Multiple sclerosis, HIV-1 associated neurological disorders, drug addiction etc. and other neurodegenerative pathologies.

The current investigation confirms that Galectin-1 plays a role in maintaining BBB integrity via the modulation of TJ proteins and significantly reduces Meth induced neuroinflammation.

4.0 Materials and Methods

4.1 Cell culture

Primary cultures of human brain microvascular endothelial cells (BMVEC) and normal human astrocytes (NHA) are obtained from Applied Cell Biology Research Institute (ACBRI) Kirkland, WA. Characterization of these cells demonstrated that >95% of BMVECs were positive for cytoplasmic VWF/Factor VIII and >99% of the NHA were positive for glial fibrillary acidic protein (GFAP). BMVEC are cultured in CS-C complete medium (ACBRI) with attachment factors (ACBRI) and Passage Reagent GroupTM (ACBRI). NHA are cultured in CS-C complete serum-free medium (ACBRI), supplemented with 10 μ g/ml human epidermal growth factor (EGF), 10 mg/ml insulin, 25 μ g/ml progesterone, 50 mg/ml transferrin, 50 mg/ml gentamicin, 50 μ g/ml amphotericin, and 10% fetal bovine serum (FBS). Both BMVEC and NHA are obtained at passage 2 for each experiment and are used for all experimental paradigms between 2–8 passages, within 6 to 27 cumulative population doublings.

4.2 Cell culture conditions

BMVECs (1×10^6 /ml) were plated in 6 well and allowed to adhere overnight and grown to 100% confluence following which cells were treated with Meth (50–100nM) and/or Galectin-1 (10 μ M) for a time period of 24–96 hr, after which cells were harvested and used for gene/protein expression studies. BMVEC cells that were untreated were used as media controls.

4.3 In-vitro BBB model

The in-vitro BBB model used in this study utilizes 2-compartment, 12 well culture plates with the upper compartment separated from the lower by a 3 μ pore-size polyethylene terephthalate (PET) insert (Transwell plates, Costar). BMVEC are cultured to confluence on the upper side of the PET insert, while a confluent layer of normal human astrocytes (NHAs) are grown on the underside (Persidsky, 1997). The transendothelial electrical resistance (TEER) across the in vitro BBB is used to evaluate its functional integrity. TEER is measured with an ohm meter (Millicell ERS system, Millipore, Bedford, MA). Electrodes are sterilized using 95% alcohol and rinsed in sterile distilled water prior to measurement. Probes are positioned such that one end is immersed in media inside the upper filter chamber and the other is placed through the basolateral access hole into the media below. A constant distance of 0.6 cm is maintained between the electrodes at all times during TEER measurement. Formation of a functionally intact BBB with untreated BMVEC occurs by day 4–5 of culture and a value of $\sim 110 \Omega/\text{cm}^2$ indicates an intact BBB.

4.4 Cell viability assay

BMVECs (1×10^4 /ml) are treated with Meth (50–100nM) and/or Galectin-1 (10 μ M) or untreated media controls for 24hr. At the end of the incubation period, cell viability is determined using the Trypan blue dye exclusion method.

4.5 RNA extraction

Cytoplasmic RNA was extracted by an acid guanidinium-thiocyanate-phenol-chloroform method as described using Trizol reagent (Invitrogen-Life Technologies, Carlsbad, CA). The amount of RNA was quantitated using a Nano-Drop ND-1000 spectrophotometer (Nano-DropTM, Wilmington, DE) and isolated RNA is stored at -80°C until used.

4.6 Real time, quantitative PCR (Q-PCR)

Q-PCR is used to quantitate Galectin1,-3, 9, ZO-1, Claudin-5, IL-1 β , TNF- α , IL-8 and ICAM-1 gene expression in BMVEC cultures. Approximately 1×10^6 cells/ml BMVEC were treated with Meth (10–100nM) for 24–96hr and RNA is extracted as described above. The RNA is then reverse transcribed to cDNA using a reverse transcriptase kit (Promega Inc, Madison, WI). Relative abundance of each mRNA species is quantitated by Q-PCR using specific primers and the Brilliant® SYBR® green Q-PCR master mix (Stratagene Inc, La Jolla, CA). The following were the sequences of the primers for real time PCR. Galectin-1 (forward primer 5'-GGTCTGGTCGCCAGCAACCTGAAT-3', reverse primer 5'-TGAGGCGGTTGGGGAACCTTG-3'); Galectin-3 (forward primer 5'-CCAAAGAGGGAATGATGTTGCC-3', reverse primer 5'-

TGATTGTACTGCAACAAGTGAGC-3'); Galectin-3 (forward primer 5'-CAGTGCTCAGAGGTTCCACA-3', reverse primer 5'-TGAGGCAGTGAGCTTCACAC-3'); ZO-1 (forward primer 5'-GGGGCCTACACTGATCAAGA-3', reverse primer 5'-GGTCTCTGCTGGCTTGTTTC-3'); Claudin-5 (forward primer 5'-AAGGTGTACGACTCGCTGCT-3', reverse primer 5'-AGTCCCGGATAATGGTGTTG-3'); ICAM-1 (forward primer 5'-CGCAAGTCCAATTCACACTGA-3', reverse primer 5'-CAGAGCGGCAGAGCAAAAG-3'); IL-8 (forward primer 5'-TGC AGC TCT GTG TGA AGG TG -3', reverse primer 5'-TCT GCA CCC AGT TTT CCT TG -3'); TNF- α (forward primer 5'-GCTCTGGAGAGCAAACACGG -3', reverse primer 5'-GCCTTCATAAATAGTCCCCTCCC -3'); and IL-1 β (forward primer 5'-CAAAGGCGGCCAGGATATAA -3', reverse primer 5'-AAGTGAGTAGGAGAGGTGAGAG-3'). The housekeeping gene β -actin (forward primer 5'-TGACGGGGTCACCCACACTGTGCCCATCTA -3', reverse primer 5'-AGTCATAGTCCGCCTAGAAGCATTGTGCGGT -3'); was used as the internal control. To provide precise quantification of the initial target in each PCR reaction, the amplification plot is examined and the data are calculated as described. Relative expression of mRNA species was calculated using the comparative threshold cycle number (C_T) method (Bustin, 2002; Radoni et al., 2004). Briefly, for each sample, a difference in C_T values (ΔC_T) is calculated for each mRNA by taking the mean C_T of duplicate tubes and subtracting the mean C_T of the duplicate tubes for the reference RNA (β -actin) measured on an aliquot from the same RT reaction. The ΔC_T for the treated sample is then subtracted from the ΔC_T for the untreated control sample to generate a $\Delta\Delta C_T$. The mean of these $\Delta\Delta C_T$ measurements is then used to calculate the levels in the targeted cytoplasmic RNA relative to the reference gene and normalized to the control as follows: Relative levels or Transcript Accumulation Index = $2^{-\Delta\Delta C_T}$. This calculation assumes that all PCR reactions are working with 100% efficiency. All PCR efficiencies were found to be >95%; therefore, this assumption introduces minimal error into the calculations. All data were controlled for quantity of RNA input and by performing measurements on an endogenous reference gene, β -actin.

4.7 Immunofluorescent staining of BMVEC

BMVEC are grown to 70% confluence in a petri dish with a glass bottom and treated with Meth (10–100nM) for 24hr. Untreated BMVEC cells are fixed for 10 min at 37°C in 4% formaldehyde, followed by permeabilization with ice-cold 90% methanol. Cells are then washed in 1X phosphate buffered saline (PBS) and treated with anti Galectin-1 antibody to determine Galectin expression. Immunofluorescent staining of Galectin-1 was done using anti human primary antibodies raised in goat obtained from Santacruz Biotech, CA (Cat # sc-19276 (N-16) goat polyclonal IgG. The secondary antibody was a fluorescence labeled (Alexa Fluor 647 rabbit anti-goat IgG (H+L) Cat # A-21446, Molecular Probes Inc-Life Technologies, Grand Island, NY). DAPI (Cat #D1306 Molecular Probes Inc-Life Technologies, Grand Island, NY) was nuclear to stain the nuclei. The protein expression levels of Galectin-1 were quantitated based on the intensity of the fluorescent signal analyzed using the computer image analysis image J software (National Institutes of Health, Bethesda, MA, USA). Standard immunofluorescent staining procedures were followed.

Imaging was performed with the EVOS® FL Cell Imaging System (Life Technologies, Grand Island, NY).

4.8 Galectin-1 ELISA

We measured Galectin-1 levels using a commercially available ELISA kit. Approximately 1×10^6 (cells/ml) BMVEC were treated for 96 hr with Meth (50–100nM) alone and/or Galectin-1 (10 μ M). Cell supernatants were harvested at the end of the incubation period and the levels of Galectin-1 were measured in the supernatants using a commercially available the ELISA kit (Cat # BG-HUM10999; from Novovite Biosciences, Woburn, MA). This assay has high sensitivity and excellent specificity for detection of Galectin-1 with an assay range of 0.01–1000 ng/ml and sensitivity of ~25pg/ml. No significant cross reactivity between analyte and analogues was observed.

4.9 Total CREB and ERK ELISA

We used the commercially available InstantOne™ ELISA kits (eBioscience, San Diego, CA) Cat #85-86151 to measure total CREB and Cat # #85-86011 to measure total ERK in cell lysates as per manufacturers instruction.

4.10 Measurement of Reactive oxygen species

We measured the total free radical present in the Meth and/or Galectin-1 treated BMVEC cell lysates using a commercially available in-vitro ROS/RNS Assay Kit (Cell Biolabs Inc, San Diego, CA, Cat # STA-347). The assay employs a proprietary quenched fluorogenic probe, dichlorodihydrofluorescein DiOxyQ (DCFH-DiOxyQ), which is a specific ROS/RNS probe that is based on similar chemistry to 2', 7'-dichlorodihydrofluorescein diacetate. Briefly, the DCFH-DiOxyQ probe is first primed with a quench removal reagent, and subsequently stabilized in the highly reactive DCFH form. In this reactive state, ROS and RNS species react with DCFH, which is rapidly oxidized to the highly fluorescent 2', 7'-dichlorodihydrofluorescein (DCF). The amount of DCF in the sample is determined based on the Relative fluorescence units (RFU) obtained using a a DCF standard curve and the fluorescence intensity obtained is proportional to the total ROS/RNS levels within the sample.

4.11 Statistical Analysis

All data are expressed as means $\pm$ SD and analyzed using Graphpad Prism software. Statistical analysis was done using an analysis of variance (One-way ANOVA). A post hoc analysis using Bonferroni's test was done. A statistical significant difference was accepted when $p < 0.05$.

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Highlights

- Galectins are important modulators of CNS homeostasis and neuroinflammation.
- Galectin-1 is highly expressed in Brain Microvascular endothelial cells.
- Galectin-1 helps maintain BBB integrity via modulation of Tight Junction proteins.
- Galectin-1 is neuroprotective significantly reduces Meth induced neuroinflammation.
- CREB signaling is involved in mediating the neuroprotective effects of Galectin-1.

Figure 1.

Basal galectin expression in BMVEC. Figure shows the basal Galectin-1, 3 and 9 gene expression levels. The basal gene expression levels were calculated using the delta delta CT method where Reference Gene vs Target Gene expression was computed using the formula $CT = (CT(\text{ref, untreated}) - CT(\text{ref, treated})) - (CT(\text{target, untreated}) - CT(\text{target, treated}))$. Results are expressed as the mean $\pm$ SD from $n=3$ separate experiments. A p value of <0.05 is considered a statistically significant difference.

Figure 2.

Dose and time kinetics of the effect of Meth on Galectin-1 gene/protein expression. Data in Figure 2A shows Galectin-1 gene expression at 24 and 96 hrs post treatment with Meth. At 24 hr, marginal increase in Galectin-1 (12% increase, p=NS) gene expression was observed on treatment with 50 nM Meth and an 20% increase, (p<0.067 (NS) increase in Galectin-1 gene expression was observed when treated with 100nM Meth, as compared to the untreated control. At 96 hr post Meth treatment, we observed significant increase 39% (p<0.05) in Galectin -1 gene expression when treated with 50 nM Meth and a 17% increase (p=NS) in Galectin-1 gene expression when treated with 100nM Meth, as compared to the untreated controls. Results are expressed as the mean $\pm$ SD from n=3 separate experiments. A p value

of <0.05 is considered a statistically significant difference. Data in Figure 2B shows Galectin-1 levels in culture supernatants of BMVEC cells treated with Meth (50–100nM) over a 24–96hr time period using a commercially available ELISA kit. Our results showed that Meth (50nM) treatment resulted in a significant increase ($p<0.01$) in Galectin-1 protein levels at 96hr as compared to the untreated control, however at a higher dose of 100nM Meth, a ~30% decrease ($p<0.05$) in Galectin-1 levels were observed both 24 and 96 hr post Meth treatment. Results are expressed as the mean $\pm$ SD from $n=3$ separate experiments. A p value of <0.05 is considered a statistically significant difference.

Figure 3.

Effect of Meth on Galectin-1 expression as quantitated by Immunofluorescence.

Immunofluorescent staining of Galectin-1 was done using anti human Galectin-1 antibody, the secondary antibody used was Alexa Fluor 647 (yellow color) and the nucleus was stained with DAPI (blue). The expression levels of Galectin-1 were quantitated based on the intensity of the fluorescent signal analyzed using the computer image analysis image J software (National Institutes of Health, Bethesda, MA, USA). Standard immunofluorescent staining procedures were followed. Imaging was performed with the EVOS® FL Cell Imaging System and images shown are representative images of BMVEC a: untreated control; b: Meth (50 nM) treated and c: Meth (100nM) treated. d: Histogram showing the intensity of the fluorescent signal in mean pixel units for the 3 representative images. n= 3 separate immunostaining experiments were done.

Figure 4.

Effect of Meth and/or Galectin on BBB integrity using an in-vitro BBB model. An intact in-vitro BBB was treated with Meth (50–100 nM) alone and in combination with Galectin-1 (10µM). Treatment of the intact BBB with Meth resulted in a 52% ($p < 0.01$) and 63% ($p < 0.001$) decrease in TEER at both 50nM and 100nM concentrations, while treatment with Galectin-1 (10µM) did not alter TEER however when treated with a combination of Galectin-1 (10µM) and Meth (50–100nM); a significant increase in TEER was observed indicating a reversal of Meth induced effect on the BBB. Results are expressed as the mean $\pm$ SD from $n=3$ separate experiments. A p value of <0.05 is considered a statistically significant difference.

Figure 5.

Effect of Galectin-1 and/or Meth on Tight junction protein gene expression. BMVEC cells were treated with Meth (50–100 nM) alone and in combination with Galectin-1 (10 μ M) for 24 hr, RNA was extracted, reverse transcribed and the gene expression levels of Claudin-5 and ZO-1 were measured using real time Q-PCR. Our data shows that 50nM Meth treatment significantly decreased Claudin-5 gene expression by 37% ($p<0.05$) and ZO-1 gene expression by 74% ($p<0.01$) while 100nM Meth treatment significantly decreased Claudin-5 gene expression by 19% ($p=NS$) and ZO-1 gene expression by 80% ($p<0.001$). Treatment of BMVEC with Galectin-1 (10 μ M) alone did not change TJ expression. A combination of Meth (50nM) + Galectin-1 (10 μ M) significantly increased Claudin-5 gene expression by 47% ($p<0.05$) and ZO-1 gene expression by 29% ($p<0.05$) and Meth (100nM) + Galectin-1 (10 μ M) treatment significantly increased Claudin-5 gene expression by 86% ($p<0.001$) and ZO-1 gene expression by 58% ($p<0.01$). All statistical comparisons were made to the untreated controls. Results are expressed as the mean $\pm$ SD from $n=3$ separate experiments. A p value of <0.05 is considered a statistically significant difference.

Figure 6.

Effect of Galectin-1 and/or Meth on ICAM-1 gene expression. BMVEC cells were treated with Meth (50–100 nM) alone and in combination with Galectin-1 (10 μ M) for 24 hr, RNA was extracted, reverse transcribed and the gene expression levels of ICAM-1 were measured using real time Q-PCR. Treatment of BMVEC with 50nM and 100 nM Meth treatment decreased ICAM-1 gene expression by 50% ($p<0.01$) and 55% ($p<0.01$) respectively. Treatment of BMVEC with Galectin-1 (10 μ M) alone did not change ICAM-1 expression. Meth (50nM) + Galectin-1 (10 μ M) significantly increased ICAM-1 gene expression by 46% ($p<0.001$) while Meth (100nM) + Galectin-1 (10 μ M) treatment significantly increased ICAM-1 gene expression by 25% ($p<0.05$). All statistical comparisons were made to the untreated controls. Results are expressed as the mean $\pm$ SD from $n=3$ separate experiments. A p value of <0.05 is considered a statistically significant difference.

Figure 7.

Effect of Galectin-1 and/or Meth on ROS production in BMVEC. BMVEC cells were treated with Meth (100 nM) alone and in combination with Galectin-1 (10 μ M) for 24 hr, we measured the total free radical presence in cell lysates using a commercially available in-vitro ROS Assay Kit. Our data shows treatment with Meth 50nM and 100nM resulted in a 1.8 fold ($p<0.05$) and 6.7 fold ($p<0.01$) increase in oxidative stress respectively as compared to the untreated control. Treatment of BMVEC cells with Galectin-1 alone (10 μ M) did not induce oxidative stress, however when cells were treated with a combination of Meth (50nM) + Galectin-1 (10 μ M) or Meth (100nM) + Galectin-1 (10 μ M) the oxidative stress was significantly reduced by 30% ($p<0.01$) and (86% ($p<0.0001$) respectively when compared to the respective Meth treatment dose alone (i.e. Meth 50 nM and 100nM respectively), thus bringing the ROS levels back to baseline levels similar to those observed in untreated controls. Results are expressed as the mean $\pm$ SD from $n=3$ separate experiments. A p value of <0.05 is considered a statistically significant difference.

Figure 8.

Effect of Galectin-1 and/or Meth on gene expression levels of pro-inflammatory cytokines, IL-1 β , IL-8 and TNF- α in BMVEC. BMVEC cells were treated with Meth (50nM) alone and in combination with Galectin-1 (10 μ M) for 24 hr, RNA was extracted, reverse transcribed and the gene expression levels of IL-1 β , IL-8 and TNF- α were measured using real time Q-PCR. Our data show that 50nM Meth treatment increased IL-1 β , gene expression by 140% ($p < 0.001$), IL-8 gene expression by 62% ($p < 0.01$), and TNF- α gene expression by 31% ($p < 0.05$). Treatment with a combination of Meth (50nM) + Galectin-1 (10 μ M) significantly decreased IL-1 β , gene expression by 43% ($p < 0.01$), IL-8 by 38% ($p < 0.05$), and TNF- α gene expression by 72% ($p < 0.001$). All statistical comparisons were made between Meth (50nM) treated vs Meth (50nM) + Galectin-1 (10 μ M) BMVEC cells. Results are expressed as the mean $\pm$ SD from $n=3$ separate experiments. A p value of < 0.05 is considered a statistically significant difference.

Figure 9.

Effect of Galectin-1 and/or Meth on ERK and CREB protein expression. BMVEC cells were treated with Meth (50nM) alone and in combination with Galectin-1 (10 μ M) for 96 hr followed by measurement of Total ERK and CREB in the cell lysates using a commercially available kit. Our results show that 50nM Meth treatment did not significantly change ERK and CREB protein expression. Galectin-1 (10 μ M) alone did not change significantly change ERK and CREB expression levels, however treatment with a combination of Meth (50nM) + Galectin-1 (10 μ M) resulted in an 31% increase ($p<0.05$) in CREB expression levels as compared to Meth (50nM) alone. Results are expressed as the mean $\pm$ SD from $n=3$ separate experiments. A p value of <0.05 is considered a statistically significant difference.