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Manganese protects against the effects of alcohol on hypothalamic puberty-related hormones

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Abstract

Aims—Since manganese (Mn) is capable of stimulating the hypothalamic-pituitary unit and advancing female puberty, we assessed the possibility that this element might overcome some of the detrimental effects of prepubertal alcohol (ALC) exposure on the hypothalamic control of pituitary function.

Main Methods—Rats received either saline or Mn (10mg/kg) daily by gastric gavage from day 12 to day 31. After weaning, all rats were provided Lab Chow diet *ad libitum* until day 27 when they began receiving either the Bio Serv control or ALC diet regime. On day 31, the medial basal hypothalamus (MBH) was collected to assess luteinizing hormone-releasing hormone (LHRH) and cyclooxygenase 2 (COX2) protein levels. Release of prostaglandin-E₂ (PGE₂), LHRH and serum luteinizing hormone (LH) were also assessed. Other animals were not terminated on day 31, but remained in study to assess timing of puberty.

Key findings—Short-term ALC exposure caused elevated hypothalamic LHRH content, suggesting an inhibition in peptide release, resulting in a decrease in LH. Both actions of ALC were reversed by Mn supplementation. COX2 synthesis, as well as PGE₂ and LHRH release were suppressed by ALC exposure, but Mn supplementation caused an increase in COX2 synthesis and subsequent PGE₂ and LHRH release in the presence of ALC. Mn supplementation also ameliorated the action of ALC to delay puberty.

Significance—These results suggest that low level Mn supplementation acts to protect the hypothalamus from some of the detrimental effects of ALC on puberty-related hormones.

Keywords

Manganese; alcohol; glia; puberty; luteinizing hormone

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The authors declare there are no conflicts of interest

INTRODUCTION

Mammalian puberty results from complex interactions within the hypothalamus that involve the gradual decline of inhibitory neurotransmission along with the development of key excitatory neurotransmitters; hence, resulting in increased luteinizing hormone-releasing hormone (LHRH) secretion, the peptide controlling the release of pituitary luteinizing hormone (LH). Insulin-like growth factor-1 (IGF-1) [1-3], kisspeptin (Kp) [4-5], glutamate [6-7], norepinephrine [8] and transforming growth factor α (TGF α) [9], are examples of known stimulators of prepubertal LHRH secretion. The actions of all of these are dependent upon their specific receptor activation and the interactive participation of the neuronal circuits and glial cells responsible for their synthesis and secretion. Importantly, alcohol (ALC) suppresses prepubertal LHRH/LH secretion [10-14] and has also been shown to impair neuronal and glial cell developmental communications [15-16], as well as specific functions with regard to neuronal/glial interactions involved in LHRH release [17-22]. Defining methods to ameliorate the suppressive effects of ALC on prepubertal LHRH/LH secretion is of importance. In this regard, manganese (Mn) is an essential nutrient that for many years has been known to be necessary for reproductive development [23-24]. More recently, low level Mn supplementation in the diet of prepubertal rats was shown to cause elevated serum luteinizing hormone (LH) secretion and advanced puberty, through facilitating the release of LHRH [25, 26]. As a result of this timely action to stimulate LHRH release, we have suggested that Mn may provide a nutrient signal that contributes to the pubertal process along with the other above mentioned stimulators of this peptide. Therefore, the present study assessed the potential for Mn to override or protect against the hypothalamic effect of ALC to diminish the release of this neuropeptide necessary for the timely onset of puberty.

MATERIALS and METHODS

Animals

Immature female rats of the Sprague-Dawley line raised in our colony at the Texas A&M University Department of Comparative Medicine were used for these experiments. The animals were housed under controlled conditions of photoperiod (lights on, 0600 hr, lights off, 1800 hr) and temperature (23 °C), with ad libitum access to food and water. The diet was Harlan Teklad 2016 which contained 94.7 mg/kg Mn and 149.8 mg/kg iron as analyzed by the Heavy Metal Analysis Laboratory, Department of Veterinary Integrative Biosciences, College of Veterinary Medicine, Texas A&M University. All procedures used on animals were approved by the University Animal Care and Use Committee at Texas A&M University and in accordance with the NIH Guidelines for the Care and Use of Laboratory Animals.

Justification of Supplement dose

While the daily amount of food consumed depends on the age and size of the rat, for every 10 g of pellet diet consumed provides 0.95 mg of Mn. Regarding the supplement, the 10 mg/kg dose causes a cumulative intake of 9.7 mg of Mn over the 17 days. Because oral Mn intake is absorbed at about 3-10% and figuring 5% absorption [27], the Mn dose caused 0.49

mg of Mn absorption during the 17 day period meaning that they absorbed a total of approximately 4.9 mg Mn/kg animal. We did not, however; assess Mn obtained through the dam's milk because the dams were only fed the normal lab show diet and were not supplemented with Mn. Also, each dam nursed both SAL and Mn-treated pups. This low dose is important since much higher doses of Mn have been used for many neurotoxicological studies [27, 28].

Effects of Mn and ALC exposure on hypothalamic LHRH content and serum LH levels

Manganese was administered daily in the form of manganese chloride (0.25 mg in 0.2 ml/25 g rat) by a single gastric gavage injection from postnatal day 12 through postnatal day 31. Control (CON) animals received an equal volume of saline (SAL). Rats were weaned on postnatal day 21 and were fed ad libitum lab chow diet until 27 days of age. At 23 days of age, each animal was anesthetized with 2.5% Tribromoethanol (Sigma Aldrich, St. Louis MO) and surgically implanted with a permanent intragastric cannula. [29]. Animals were housed individually and when 27 days old, were weighed and divided into six groups, all closely matching in weight: Group 1 consisted of animals that received a 5% ALC liquid diet (Bioserve Inc., Frenchtown, NJ) in which ALC provided 36% of total calories, and also a daily dose of saline. Group 2 consisted of animals that received the 5% ALC liquid diet plus the daily dose of Mn. Group 3 received the companion Bioserve CON liquid diet in which dextramaltose was isocalorically substituted for the ALC, and also a daily dose of SAL. Group 4 received the liquid control diet plus the daily dose of Mn. Group 5 served as an additional set of controls that were cannulated and maintained on lab chow and water, ad libitum plus a daily dose of SAL. Group 6 was also maintained on lab chow and water and received the daily dose of Mn. Animals began receiving their respective diet regimens on 27 days old. These diets were administered with a total of 8 ml of diet (4 injections of 2 ml each) injected via the intragastric cannula, with injections equally dispersed over the lights-on period. Mn or SAL was given with the first injection of the liquid diet. On day 27, the ALC-treated animals received 1.5 ml of ALC diet and 0.5 ml of isocaloric CON diet in order to gradually condition the animals to the ALC. The animals in Groups 3 and 4 received 2 mls of isocaloric CON diet. On day 28, the animals began receiving 2.0 mls of ALC diet. From day 29 till day 30, the animals were receiving 2 and 3 mls of their respective diets at each injection.

On day 31, the animals received one injection of ALC or isocaloric CON diet and then were killed by decapitation 90 minutes after diet injections. This time period was chosen to allow for ALC absorption and for comparison of data with prior studies from this lab [10, 14, 21, 30]. The MBH was dissected from each brain by a method described previously (30) for LHRH assessment. Briefly, a rostral cut was made along the caudal border of the optic chiasm and then a caudal cut was made just behind the mammillary bodies. The block was formed by making cuts along the hypothalamic sulci laterally and along the border of the thalamus dorsally. Trunk blood was collected for serum LH and ALC levels.

Effects of Mn and ALC exposure on pubertal onset

Another experiment like the one described above was repeated twice with the following exceptions. The animals were not killed at 31 days of age but remained on their respective

diets and treatments until vaginal opening (VO) occurred. At that time, vaginal lavage was performed to detect estrus and diestrus by a previously described method [10].

Preparation of Hypothalamic Extracts

Each hypothalamus was lyophilized, placed in culture tubes and subjected to acetic acid extraction for LHRH by a procedure previously described [31]. Total extraction supernatant for each sample was lyophilized and reconstituted in 1ml of borate buffer (pH 8.6) containing 0.1% gelatin and 0.025 M EDTA, then measured for LHRH content by RIA.

Hormone analysis

LHRH was measured by radioimmunoassay (RIA) as previously described [25] using Antiserum R11B73 kindly provided by Dr. V.D. Ramirez. Synthetic LHRH used for the standards and iodinations was purchased from Sigma Chemical Co. (St. Louis, MO). The iodinations were performed using ^{125}I -Na (Perkin Elmer, Watham, MA) with the chloramine T method. The sensitivity of the assay was 0.2 pg tube^{-1} , and the intra and interassay coefficients of variation were $< 10\%$. Serum LH was measured by a kit purchased from the National Hormone and Pituitary Program, Harbor UCLA Medical Center, Torrance CA using the rLH-S-11 antiserum. The sensitivity of the assay was 0.07 ng/ml and the inter- and intra-assay variations were less than 5% .

Effects Mn and ALC exposure on hypothalamic PGE_2 release and COX2 protein levels

To assess PGE_2 release, rats were grouped and treated as above with the respective Mn and ALC dosing regimen. On day 31, rats representing all six groups were decapitated and the MBH was dissected out and the tissue incubated as described previously [25] with minor modifications. Briefly, each MBH was incubated in a vial containing $300 \mu\text{l}$ of Locke's Buffer inside a Dubnoff shaker (80 cycles/min) at 37°C in an atmosphere of $95\% \text{ O}_2$ and $5\% \text{ CO}_2$ for 60 minutes to determine basal PGE_2 and LHRH release. All of the samples were stored at -80°C until assayed for the respective hormones. MBHs were weighed to the nearest 0.1 mg .

To determine COX2 protein levels, rats were grouped and treated exactly as detailed above. On day 31, the rats representing all six groups were decapitated and the MBH was dissected out and frozen until analyzed for COX2 protein expression.

Enzyme-linked Immunosorbent Assay (ELISA)

PGE_2 levels were measured from the incubation media using an ELISA kit (26; Caymen Chemical Inc., Ann Arbor, MI) according to the manufacturer's instructions. The sensitivity of the assay was 5 pg/ml . Values are expressed as $\text{pg PGE}_2/\text{mg tissue}/60\text{minutes}$.

LHRH RIA

LHRH content was measured [12, 21] by RIA from the same *in vitro* media samples used above for PGE_2 [25]. Values are expressed as $\text{pg LHRH release}/\text{mg tissue}/30 \text{ minutes}$. The sensitivity of the assay was 0.07 ng/ml and the intra-assay variation was less than 5% .

Immunoblotting

MBH tissues were homogenized in 1% Igepal CA-630, 20 mM Tris-Cl, pH (8.0), 137 mM NaCl, 2 mM EDTA, 10% glycerol, 10 mM sodium pyruvate, 10 mM sodium fluoride, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 0.25% protease inhibitor cocktail (Sigma-Aldrich, Inc., St. Louis, MO) at 4C. The homogenates were incubated on ice for 30 minutes and centrifuged at 12,000X g for 15 minutes. The concentration of total protein in the resultant supernatants was determined by the Pierce 660nm Protein assay kit (Thermo Scientific, Rockford, IL) using bovine serum albumin as standard. Immunoblot analysis was performed by solubilizing the proteins (100 µg) in a sample buffer containing 25 mM Tris-Cl, pH 6.8, 1% SDS, 5% β-mercaptoethanol, 1 mM EDTA, 4% glycerol, and 0.01% bromophenol blue, and resolved on 10% SDS-PAGE for cyclooxygenase 2 (COX2) under reducing conditions. The fractionated proteins were electrophoretically transblotted onto polyvinylidene difluoride membranes. Following transfer, membranes were blocked with 5% nonfat dried milk/0.1% Tween 20 in PBS (pH 7.4) for 3 hours and incubated at 4C overnight with rabbit polyclonal antibody to COX2 (1: 300; Cayman Chemical, Ann Arbor, MI). After the incubation, membranes were washed in PBS/0.1% Tween 20 and subsequently incubated with goat anti-rabbit secondary antibody (1:50,000; Santa Cruz Biotech., Santa Cruz, CA) for 2 hours at room temperature. After washing, the specific signals were visualized using a chemiluminescence detection system (Western Lightning Plus-ECL; PerkinElmer, Shelton, CT) and the protein quantification was done using NIH Image J software version 1.43 (National Institute of Health, Bethesda, MD). Subsequently, membranes were stripped using Re-Blot Plus kit (EMD Millipore, Temecula, CA) and reprobed with mouse monoclonal antibody to β-actin (1:50,000; Sigma-Aldrich, Inc.) and goat anti- mouse secondary antibody (1:50,000; Santa Cruz Biotech., Santa Cruz, CA), to normalize for the amount of sample loading. After washing, the detection and quantitation of β-actin was conducted as described above.

Statistical Analysis

All values are expressed as the mean (± SEM). Differences between the ad libitum chow fed and the isocaloric liquid diet fed CON groups that also received a daily dose of saline were analyzed by Student's *t* test. Because no significant differences were detected in any of the parameters measured between the two CON diet-fed groups that received SAL, their data were combined for presentation in order to simplify comparative descriptions. The same statistical analysis was conducted for the two CON diet fed groups that also received Mn and this resulted in the combining of these two groups as well. Differences between the now four treatment groups (CON+SAL; CON+Mn; ALC+SAL; ALC+Mn) were then initially analyzed by using Kruskal-Wallis nonparametric analysis of variance (ANOVA) followed by post hoc testing using the Student Neuman Keul's multiple comparisons test. P-values less than 0.05 were considered significant.

Serum Alcohol Analysis

Blood alcohol concentrations (BACs) were assessed from trunk blood collected 1.5 hours after the last infusion of ALC on the final day of the experiment. Serum was transferred to

microcentrifuge tubes for the enzymatic analysis of serum ALC levels [32] using a diagnostic kit purchased from Sekisui Diagnostics (Lexington, MA).

RESULTS

The chow-fed and liquid diet-fed CON animals receiving the SAL showed no differences with regard to body weights or any of the protein measurements during the 5 days of the experiment. This is in agreement with previous reports using this feeding regimen and showing similar animal weights and hormone levels, as well as gene and protein expressions between these two CON groups of prepubertal, growing animals [10, 17, 20]. Similarly, no differences were detected between the two diet-fed CON groups supplemented with Mn. Because no differences were noted, data from the two diet CON groups receiving SAL were combined (CON+SAL), as were data from the two diet CON groups receiving Mn (CON+Mn), and are presented in the following figures as their respective single group in order to simplify comparative descriptions. The mean $\pm$ SEM of the BACs from all animals receiving ALC liquid diet was 222 ± 5 mg/dl.

Figure 1A shows that CON-fed animals supplemented daily with Mn (CON+Mn) have a lower content ($p < 0.05$) of hypothalamic LHRH compared to CON diet-fed animals and that were dosed daily with SAL (CON+SAL). Those animals receiving the ALC diet and dosed with SAL (ALC+SAL) exhibited an elevation ($p < 0.001$) in LHRH content when compared to both CON diet-fed groups. Animals receiving the ALC diet plus the Mn supplementation (ALC+Mn) showed lower ($p < 0.001$) hypothalamic levels of LHRH than those receiving ALC+SAL. Furthermore, these levels were similar to those of the CON+Mn group. Figure 1B shows that Mn supplementation elevated ($p < 0.01$) LH release in the CON+Mn group when compared to the CON+SAL group. The ALC+SAL animals had suppressed serum LH levels when compared to the CON+SAL ($p < 0.05$) and CON+Mn ($p < 0.01$) groups. The increased LH ($p < 0.05$) levels in the ALC+Mn group compared with the ALC+SAL group shows that Mn supplementation stimulated LH release in the presence of ALC.

The resulting increase in prepubertal LH secretion in the Mn+ALC-treated animals indicates Mn supplementation may ameliorate the delay in puberty caused by ALC. Thus, in a separate experiment, we allowed animals to continue on their specific diet and treatment regimen until VO was observed. Figure 2 demonstrates that the CON+Mn group of animals showed advanced ($p < 0.01$) VO when compared with the CON+ SAL group. Although both the ALC-treated groups showed delayed VO ($p < 0.001$) compared to the CON groups, there was a critical difference between the two ALC groups. Specifically, the mean ($\pm$ SEM) age of VO in the ALC+Mn group was 41 ± 0.7 days, demonstrating they attained VO while still under the influence of ALC. Conversely, on day 42, since none of the ALC+SAL animals had shown VO, the ALC diet was removed and replaced with pellet food. Only after another 5-6 days did the VO occur in the ALC-SAL group at a mean ($\pm$ SEM) of 47 ± 0.6 days, approximately 6 days later ($p < 0.01$) than the ALC+Mn group.

Since Mn supplementation overrode the ALC effect on LHRH/LH release and improved pubertal onset, we assessed whether this action was due, at least in part, to the element protecting the hypothalamus from the detrimental effects of ALC on the PGE₂ control of

LHRH. PGE₂ is a key component to the communication between glia and LHRH neurons with regard to controlling LHRH release [33]. Mn has been shown to induce PGE₂ and LHRH release [26] and ALC exposure is known to block PGE₂ and LHRH release [12]; thus, we determined whether Mn supplementation could alter the ability of ALC to block PGE₂ release and thus, restore LHRH release. Figure 3 shows that the animals in the CON +Mn group exhibited increased *in vitro* release of both basal PGE₂ (p<0.05) and LHRH (p<0.01) compared with the CON+SAL animals. Animals in the ALC+SAL group showed reduced (p<0.05) basal PGE₂ and LHRH release versus CON+SAL group. Conversely, the animals in the ALC+Mn group showed an increase (p<0.05) in both PGE₂ and LHRH release compared to the ALC+SAL group, with levels equal to those observed in the CON +SAL group.

Since ALC blocked basal PGE₂ release *in vitro*, we investigated the effect of Mn supplementation on COX2 protein synthesis, the inducible rate limiting enzyme that is necessary for the synthesis of prostaglandins. Using western blot analysis, figure 4 demonstrates that COX2 protein levels were elevated in the CON+Mn group (p <0.01) when compared to the CON+SAL group. The ALC+SAL group showed reduced COX2 protein levels when compared to the CON+SAL (p<0.05) and CON+Mn (p<0.01) groups. Importantly, ALC-treated rats that were supplemented with Mn (ALC+Mn) showed that this element was able to override the suppressive effect of ALC, causing an increase (p<0.05) in COX2 protein levels over the ALC+SAL group, with levels similar to the CON+SAL group.

DISCUSSION

The present results reveal that diet supplementation with a low dose of Mn is capable of overriding some of the detrimental effects of ALC on key puberty-related hormones. Initially, we showed that Mn supplementation alone, beginning on day 12, resulted in increased serum LH levels by prepubertal day 31 compared with controls. Interestingly, these levels were inversely correlated with a decrease in the content of LHRH within the MBH, suggesting the hypothalamic peptide was released. This supports previous work showing that Mn stimulates LHRH secretion through an action at the hypothalamic level [25, 34]. Conversely, animals that were administered an ALC-liquid diet resulted in suppressed LH levels, an effect that was inversely correlated with the increased content of LHRH within the MBH and therefore, suggesting the release of the hypothalamic peptide was inhibited; hence, supporting the well-documented effect of ALC to act at the hypothalamic level to suppress LHRH/LH secretion [10, 12, 13, 17, 31, 35]. Importantly, other ALC-treated rats that also received Mn supplementation showed increased serum LH and decreased LHRH peptide content in the MBH, similar to the Mn supplemented animals that did not receive ALC. This indicates that the Mn was capable of protecting against the negative effect of ALC within the hypothalamus and thus, allowed for the increased release of LH.

This study also provides evidence that Mn can antagonize the well-known action of ALC to delay the timing of puberty. Mn alone has been shown to stimulate LHRH release and advance the onset of puberty [25]. While this could be detrimental if an individual is exposed to low but elevated levels of Mn too early in life, the ability of Mn to stimulate

LHRH release presents the possibility that supplementation with this element could ameliorate the delay in puberty caused by ALC. The present study showed that ALC-treated rats supplemented with Mn eventually attained VO, whereas ALC-treated animals without Mn supplementation did not show VO until 5-6 days after the ALC was removed from their diet. This further suggests that Mn can antagonize the hypothalamic action of ALC to delay the pubertal process. Interestingly, Mn supplementation has also been shown to antagonize other actions of ALC, enabling the element to overcome some aspects of ALC-induced alterations in glial cell development [15, 36]. Taken together, these results suggests that gaining a better understanding as to the actions and interactions of ALC and Mn on glial-neuronal relationships could be important for determining how Mn can override the hypothalamic effect of ALC to suppress prepubertal LHRH/LH secretion.

It is known that glial cells can regulate LHRH neuronal activity during puberty [37-39] through their close association with LHRH nerve terminals in the median eminence (ME) region of the MBH. Growth factors of glial origin are recognized as being important for the functional control of puberty through hypothalamic glial-neuronal communications [37, 40, 41]. TGF α and epidermal growth factor are glial-derived peptides that signal through the erbB1 tyrosine kinase receptor. Specifically, once the growth factor is released it binds to and activates the erbB1 receptor on adjacent glial cells [42] and thus, initiates an intracellular signaling pathway [43] resulting in the in synthesis and release of glial-derived PGE₂ [44]. PGE₂ is a critical component in the glial regulation of LHRH secretion, since once it is released it binds to its receptor on nearby LHRH nerve terminals in the ME, causing the release of this peptide [33, 41, 45]. Because ALC can suppress both TGF α and PGE₂ secretions [20], we also investigated in this study whether Mn supplementation could antagonize the actions of ALC on PGE₂ synthesis since it represents a critical pathway to LHRH neuronal secretion [33, 41, 45]. In support of this rational, previous studies have indicated Mn supplementation can up-regulate COX₂ expression *in vivo* and can stimulate PGE₂ and LHRH release from MEs incubated *in vitro* [26]. The present results showed that the administration of ALC between days 27 and 31 caused suppressed release of PGE₂ and LHRH from hypothalami incubated *in vitro* on the final day of the study; however, the animals that were supplemented with Mn showed continued release at levels similar to the control groups. These results clearly indicate that Mn supplementation can antagonize the suppressive effects of short-term (5 days) ALC administration on the secretion of PGE₂ and subsequently LHRH.

The influence of Mn to protect against the inhibitory effect of ALC on prepubertal PGE₂ secretion is of potential importance, and while the mechanism of the effect is yet to be discerned, earlier work supports the possibility that Mn can protect against the adverse effects of ALC on glial-derived substances [36]. Because Mn facilitates PGE₂ secretion, even in the presence of ALC, the resulting upregulation of PGE₂ receptors on glial cells and LHRH nerve terminals promotes glial-neuronal communications. PGE₂ is known to enhance blood flow within the ME and to promote plasticity of specialized glial cells known as tanycytes. Specifically, PGE₂ induces production of TGF β 1 which induces retraction of the tanycyte end feet from the endothelial wall [45, 46], hence creating a better access of the LHRH nerve endings to the blood vessel and thus, enhancing the release of the peptide into

the portal vasculature [47]. Additional research will be needed to conclusively determine whether the Mn effects on PGE₂ are due specifically, or in part, to glial involvement.

Finally, it is well known that Mn is beneficial unless an individual is exposed to toxic amounts of the element; thus, it is important to note that the supplemental dose of Mn resulted in a total of 0.49 mg of this essential element absorbed during the 17 day course of this study. Importantly this dose is much lower than doses used in toxicological studies [27, 28]. Previous studies show that Mn supports numerous physiological functions such as being a co-factor for a variety of brain enzymes [48] and is a beneficial factor for normal growth and development of the reproductive system [49, 50], including puberty-related hormone secretions [25, 34]. Our present results are in support of these actions by indicating diet supplementation with a low dose of Mn can antagonize the inhibitory effects of ALC on prepubertal PGE₂ and LHRH secretion, as well as the timing of puberty.

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Figure 1.

Effect of daily supplementation of manganese (Mn) on hypothalamic luteinizing hormone-releasing hormone (LHRH) content and serum levels of luteinizing hormone (LH) after short-term ALC exposure.

Control diet-fed animals were supplemented with Mn (CON+Mn) or received saline (ALC+SAL). Animals fed the ALC diet either received saline (ALC+SAL) or were supplemented with Mn (ALC+Mn). Taken together these results suggest that Mn protects against the negative effects of ALC. Panel A: a vs. b and d, $p < 0.05$; c vs a, b and d, $p < 0.001$. Panel B: a vs b, $p < 0.01$; a vs c, $p < 0.05$; b vs c, $p < 0.01$; d vs. c, $p < 0.05$. Bars with the same letters are not different. N=10-12 animals per group.

Figure 2.

Effect of daily manganese (Mn) supplementation on the age at vaginal opening (VO) following short term ALC exposure.

The same four treatment groups of animals as described in Figure 1 were used except the animals were maintained on their diet and treatments until VO occurred. The only exception was the ALC+SAL group, as these animals did not show VO until after the ALC was removed from their diet on day 42. Note that animals in the ALC+Mn group showed a marked amelioration of the delay in VO. a vs b, $p < 0.01$; a vs c and d, $p < 0.001$; b vs c and d, $p < 0.001$; d vs c, $p < 0.01$. N=8-10 rats per group.

Figure 3.

Effect of daily manganese (Mn) supplementation on basal prostaglandin E₂ (PGE₂) release from prepubertal female rats after short term ALC exposure.

The same four treatment groups of animals as described in Figure 1 were used. On day 31 the medial basal hypothalamus (MBH) of animal was excised and incubated *in vitro*. Note that Mn stimulates PGE₂ and subsequently LHRH release, even in the presence of ALC. Panel A: a vs b and c, $p < 0.05$; b vs c, $p < 0.01$; b vs d, $p < 0.05$; c vs d, $p < 0.05$; Bars with same letters are not different. Panel B: a vs b, $p < 0.01$; a vs c, $p < 0.05$; b vs c, $p < 0.01$; b vs d, $p < 0.05$; c vs d, $p < 0.05$. N=10-12 animals per group.

Figure 4.

Effect of daily manganese (Mn) supplementation on hypothalamic cyclooxygenase 2 (COX2) protein levels in prepubertal female rats after short term ALC exposure.

The same four treatment groups of animals as described in Figure 1 were used. Panel A) Representative immunoblot of the 72 kDa COX2 protein isolated from the medial basal hypothalamus of prepubertal female rats are shown from the CON+SAL group (lanes 1-3); the CON+Mn group (lanes 4-6); the ALC+SAL group (lanes 7-9); and the ALC+Mn group (lanes 10-12). Panel B) Composite graph represents the densitometric quantitation of all the bands from 2 blots that correspond to the COX2 protein. Note that animals in the ALC+Mn group showed restored COX2 protein synthesis. a vs b, $p < 0.01$; a vs c, $p < 0.05$; b vs. c, $p < 0.01$; b vs d, $p < 0.05$; c vs d, $p < 0.05$; Bars with same letters are not different. N=6 animals/group.