Manganese Induces IGF-1 and Cyclooxygenase-2 Gene Expressions in the Basal Hypothalamus during Prepubertal Female Development

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Precocious puberty is a significant child health problem, especially in girls, because 95% of cases are idiopathic. Our earlier studies demonstrated that low-dose levels of manganese (Mn) caused precocious puberty via stimulating the secretion of luteinizing hormone–releasing hormone (LHRH). Because glial-neuronal communications are important for the activation of LHRH secretion at puberty, we investigated the effects of prepubertal Mn exposure on specific glial-derived puberty-related genes known to affect neuronal LHRH release. Animals were supplemented with MnCl₂ (10 mg/kg) or saline by gastric gavage from day 12 until day 22 or day 29, then decapitated, and brains removed. The site of LHRH release is the medial basal hypothalamus (MBH), and tissues from this area were analyzed by real-time PCR for transforming growth factor α (TGF α), insulin-like growth factor-1 (IGF-1), and cyclooxygenase-2 (COX-2) messenger RNA levels. Protein levels for IGF-1 receptor (IGF-1R) were measured by Western blot analysis. LHRH gene expression was measured in the preoptic area/anteroventral periventricular (POA/AVPV) region. In the MBH, at 22 days, IGF-1 gene expression was increased ($p < 0.05$) with a concomitant increase ($p < 0.05$) in IGF-1R protein expression. Mn also increased ($p < 0.01$) COX-2 gene expression. At 29 days, the upregulation of IGF-1 ($p < 0.05$) and COX-2 ($p < 0.05$) continued in the MBH. At this time, we observed increased ($p < 0.05$) LHRH gene expression in the POA/AVPV. Additionally, Mn stimulated prostaglandin E₂ and LHRH release from 29-day-old median eminences incubated *in vitro*. These results demonstrate that Mn, through the upregulation of IGF-1 and COX-2, may promote maturational events and glial-neuronal communications facilitating the increased neurosecretory activity, including that of LHRH, resulting in precocious pubertal development.

Key Words: manganese; IGF-1; COX-2; puberty.

Manganese (Mn) is a natural element that is important for numerous physiological functions in mammals. It has been shown that Mn is involved in the pubertal process and that prepubertal exposure to low but elevated levels of the element can cause precocious pubertal development in female rats (Pine *et al.*, 2005). This action of Mn to induce early puberty is

due to its ability to stimulate the prepubertal secretion of luteinizing hormone–releasing hormone (LHRH) from the hypothalamus (Lee *et al.*, 2007; Pine *et al.*, 2005). This increase in LHRH secretion, resulting in the enhanced pulsatile release of luteinizing hormone, is responsible for initiating puberty in all mammals, including humans (Rosenfield, 2002). The fact that Mn stimulates this peptide indicates that it acts through normal channels, perhaps as an important environmental component in the pubertal process; however, this action to induce LHRH secretion can be detrimental if it occurs too early during development.

Mn-induced precocious puberty is potentially important and supported by several facts. The element accumulates in the hypothalamus (Deskin *et al.*, 1980; Pine *et al.*, 2005) and is taken up by both neurons and glial cells (Tholey *et al.*, 1990), suggesting a role for Mn in hypothalamic neuronal-glial communications. In recent years, it has been determined that puberty is occurring at an earlier age, especially in females (Herman-Giddings *et al.*, 1997; Parent *et al.*, 2003). Although the cause of this trend to earlier puberty is not known, it is important to note that central, or true, precocious puberty is LHRH dependent and characterized by changes like those at the normal time of puberty, although prematurely (Lee, 1996). The cause of central precocious puberty in boys can usually be accounted for by hypothalamic hamartomas, central nervous system lesions, or familial disease; however, the cause of precocious puberty in girls is not known in about 95% of the cases (Lee, 1996; Rosenfield, 2002). Our animal research to date indicates a possible risk for developing precocious puberty if exposed to low but elevated levels of Mn during juvenile or early adolescent development. We have shown that the Mn-induced secretion of LHRH causes elevated gonadotropin and gonadal steroid levels, resulting in advanced puberty in both sexes, with the females being much more sensitive (Lee *et al.*, 2006; Pine *et al.*, 2005). Environmental sources of Mn are abundant, with infants and children being classified as more sensitive to the element (EPA, 2002), mainly due to the fact that the minimum levels of exposure have not been well

defined (Greger, 1999). Because Mn accumulates in the hypothalamus, we suggest that should moderate levels of this element accumulate in this brain region too early in life, reaching levels not normally obtained until later, this could result in an increased risk for precocious development to occur. Thus, when taken together, this information suggests that gaining a better understanding of the role of Mn in stimulating prepubertal LHRH release is important with regard to factors controlling or altering the timing of puberty.

In recent years, it has been shown that the development of upstream connections governing LHRH synthesis, as well as downstream glial to glial and glial to neuronal communications involved in controlling LHRH release, are important for the onset of puberty. Insulin-like growth factor-1 (IGF-1), transforming growth factor α (TGF α), and prostaglandin E₂ (PGE₂) are three such downstream glial substances that are involved in glial-neuronal communications that are critically important for prepubertal LHRH secretion (Dhandapani *et al.*, 2003; Hiney *et al.*, 1991, 1996; Ojeda *et al.*, 2008). The effects of Mn on these substances have not been studied, and because Mn can induce precocious puberty, we assessed whether this element could cause an early upregulation of the expression of any of these puberty-related genes following exposure during early juvenile development. Thus, the intent of this study is to determine, prior to the onset of puberty, the potential involvement of Mn in the maturation or control of glial-neuronal communication networks in the basal hypothalamus that subsequently facilitate LHRH release at 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 h; lights off, 1800 h) 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 were approved by the University Animal Care and Use Committee and in accordance with the NIH Guidelines for the Care and Use of Laboratory Animals.

Experimental Design

Effects of Mn on puberty-related genes. Adult female rats were bred and then allowed to deliver their pups normally, at which time litters were adjusted to 8–11 pups with at least five to six females per litter. In a previous study (Pine *et al.*, 2005), we delivered 5, 10, 25, and 100 mg/kg/day MnCl₂ to female pups beginning on postnatal day 12. The minimum effective dose to advance puberty was 10 mg/kg; thus, this dose was utilized in the following experiments. In the first experiment, four litters were used that contained six female pups. Half of the females in each litter received MnCl₂ (10 mg/kg; 0.25 mg in 0.2 ml/25 g rat) and the other half an equal volume of saline (control group) administered daily by a single gastric gavage injection from day 12 until day 22, at which time the rats were killed by decapitation and the brains removed. The preoptic-hypothalamic region was dissected out into two blocks, one containing the

preoptic area (POA)/anteroventral periventricular (AVPV) nucleus and the other the medial basal hypothalamus (MBH) as previously described (Hiney *et al.*, 2009). Tissues were frozen on dry ice and stored for real-time PCR and Western blot analysis. The second experiment used the same protocol except animals were dosed until day 29, then killed by decapitation, and the same two blocks of tissue were removed and frozen for real-time PCR analysis.

Effects of Mn on PGE₂ and LHRH release from median eminence *in vitro*. The ability of Mn to induce PGE₂ and LHRH release directly from the median eminence (ME) incubated *in vitro* was evaluated. In this regard, 29-day-old female rats were decapitated, the ME removed, and incubated as described previously (Hiney *et al.*, 1998) with minor modifications. Briefly, each ME was incubated in a vial containing 150 μ l of Lockes Buffer (2mM Hepes, 154mM NaCl, 5.6mM KCl, 1mM MgCl₂, 6mM NaHCO₃, 10mM glucose, 1.25mM CaCl₂, and 1 mg/ml bovine serum albumin, pH 7.4) inside a Dubnoff shaker (80 cycles per minute) at 37°C in an atmosphere of 95% O₂ and 5% CO₂ for 30 min. This medium was discarded, and all MEs were incubated in fresh medium for 30 min to establish basal PGE₂ and LHRH release. The medium was removed, boiled for 10 min, stored in microfuge tubes, and replaced with medium containing 0 or 50 μ M MnCl₂. The MEs were incubated for an additional 30 min. This dose of MnCl₂ was selected from our previous dose-response studies in which we determined this metal to be an effective stimulator of LHRH release *in vitro*. This medium was collected, boiled for 10 min, and stored at –80°C until assayed for PGE₂ and LHRH. MEs were weighed to the nearest 0.01 mg.

Isolation of Total RNA

Total cellular RNA was initially isolated from the tissue blocks by homogenizing in TRIZOL reagent (Invitrogen Lifetech, CA). Each homogenate was further extracted for RNA using the QIAGEN RNeasy kit and treated with RNase-free DNase I to remove genomic DNA according to the manufacturer's instructions (Qiagen Inc., Valencia, CA). The integrity of the RNA was checked by the visualization of the ethidium bromide-stained 28S and 18S ribosomal RNA bands. The concentration of RNA was measured spectrophotometrically by absorbance at 260 nm in a Model Smartspec 3000 spectrophotometer (Bio-Rad Laboratories, Hercules, CA).

Reverse Transcription and Real-Time Quantitative PCR

One microgram total RNA was reverse transcribed into complementary DNA (cDNA) using oligo (dT) and SuperScript III First-strand Synthesis System (Invitrogen Lifetech). Real-time PCR was performed in 25 μ l reactions containing 2 μ l cDNA, 500nM primer pairs, and 1 $\times$ SYBR green PCR master mix in 96-well plates on an ABI PRISM 7500 sequence detection system (Applied Biosystems, Foster City, CA). PCR primers for the analysis were designed according to the guidelines of Applied Biosystems with help of the Primer Express 3.0 software (Applied Biosystems), and each primer was checked by BLAST search for the absence of any cross-reactivity. To normalize for the quantity of RNA in the initial reverse transcription reaction, the housekeeping gene, β -actin, was included in all reactions separately under the same experimental conditions. A reaction without reverse transcriptase was also performed to rule out the possibility of any contamination of genomic DNA. The primers for the PCR reactions are IGF-1 (GenBank accession NM_178866): forward, 5'-GCACCACAGACGGGCATT-3', reverse, 5'-ACATCTCCAGCCTCCTCAGATC-3' (product size 67 bp); cyclooxygenase-2 (COX-2) (GenBank accession S67722): forward, 5'-GGCACAATATGATGTTTCGCATT-3', reverse, 5'-CAGGTCCTCGCTTCTGATCTGT-3' (product size 79 bp); TGF α (GenBank accession M31076): forward, 5'-GCCAGATTCCACACTCA-3', reverse, 5'-TCTCTTCTGCACCAAAAACCT-3' (product size 63 bp); LHRH (GenBank accession S50870): forward, 5'-GGGCAAGGAGGAGGATCAAA-3', reverse, 5'-GGCCAGTGGACAGTGCATT-3' (product size 60 bp); and rat β -actin (GenBank accession NM_031144): forward, 5'-ATGCCCCGAGGCTCTCTT-3', reverse, 5'-TGGATGCCACAGGATTCCA-3' (product size 57 bp). The PCR cycling conditions were as follows: initial denaturation and enzyme activation at 95°C for 10 min, followed by 40 cycles at 95°C for 15 s and 60°C for 1 min.

Dissociation curve analysis was also done for each gene at the end of the PCR reaction. In this regard, each amplicon generated a single peak and did not show any peak when the template was not included in the PCR reaction. Additionally, each PCR-generated DNA product was electrophoresed onto a 2% agarose gel containing 0.5 µg/ml ethidium bromide, which showed a single band of the expected size. The raw data from each experiment were used to determine the relative levels of expression for each gene by the delta-delta C_T method as described by *Hettinger et al.* (2001).

Immunoblotting

Brain tissues were homogenized in 1× PBS, 1% Igepal CA 630, 0.5% sodium deoxycholate, 0.1% SDS, 1mM phenylmethylsulfonyl fluoride, 0.25% protease inhibitor cocktail (Sigma Aldrich, St Louis, MO), and 1mM sodium orthovanadate at 4°C. The homogenates were incubated on ice for 30 min and centrifuged at $12,000 \times g$ for 15 min. Protein concentration in the supernatant was measured by the DC Protein Assay kit (Bio-Rad Laboratories, Richmond, CA) using bovine serum albumin as standard. Immunoblot analysis was performed by solubilizing the equal amounts of proteins (100 µg) in a sample buffer containing 25mM Tris-HCl, pH 6.8, 1% SDS, 5% β-mercaptoethanol, 1mM EDTA, 4% glycerol, and 0.01% bromophenol blue and electrophoresed through a 10% SDS-polyacrylamide gel electrophoresis under reducing conditions. Following electrophoresis, proteins were transferred electrophoretically onto polyvinylidene difluoride membranes. Membranes were blocked with 5% nonfat dried milk/0.05% Tween 20 in PBS (pH 7.4) for 3 h and subsequently incubated at 4°C overnight with rabbit anti-IGF-1R (1:300; Abcam Inc., Cambridge, MA). Following incubation, membranes were washed in PBS buffer containing 0.05% Tween 20 and then incubated with horseradish peroxidase-labeled goat anti-rabbit immunoglobulin G (1:12,000; Abcam Inc.) for IGF-1 receptor (IGF-1R) for 2 h at room temperature. After washing, the protein signals were visualized by chemiluminescence (Western Lightning Plus-ECL, PerkinElmer, Shelton, CT) according to the manufacturer's instruction and quantified with NIH Image J software version 1.43 (National Institutes of Health, MD). To confirm equal loading, membranes were washed and blocked at room temperature in the presence of 5% nonfat dried milk/0.05% Tween 20 in PBS (pH 7.4) for 2 h. Following washing, membranes were reprobed with a mouse monoclonal antibody to the β-actin and goat anti-mouse secondary antibody. After washing, the detection and quantitation of β-actin was done as described above.

Measurement of PGE₂ and LHRH

PGE₂ ELISA. PGE₂ was measured from the same samples by an ELISA kit purchased from Cayman Chemical Inc. (Ann Arbor, MI) with a sensitivity of 7.8 pg/ml. Values are expressed as picograms PGE₂ per milligram tissue per 30 minutes.

LHRH radioimmunoassay. LHRH was measured as previously described (*Nyberg et al.*, 1993) using Antiserum R11B73 kindly provided by Dr V.D. Ramirez. Synthetic LHRH used for the standards and iodinations was purchased from Sigma Chemical Co. The sensitivity of the assay was 0.2 picograms per tube, and the intra and interassay coefficients of variation were < 10%.

Statistical Analysis

All values are expressed as the mean (± SE). Differences between control and Mn-treated groups were analyzed by the Mann-Whitney *U*-test. Probability values less than 0.05 were considered significant. The IBM PC programs INSTAT and PRISM 3.0 (GraphPad, San Diego, CA) were used to calculate and graph the results.

RESULTS

Effect of Mn during Juvenile Development

Mn exposure caused differential effects on TGFα and IGF-1 gene expression in the MBH at 22 days of age. TGFα was not affected by Mn treatment in this region (control, 1.58 ± 0.23 vs.

Mn, 1.53 ± 0.08 ; Fig. 1A); however, exposure to this element did cause an increase ($p < 0.05$) in IGF-1 gene expression (Fig. 1B) when compared with the control animals that received saline (control, 1.26 ± 0.07 vs. Mn, 1.73 ± 0.1). We also observed that the level of IGF-1R protein expression in the MBH was increased ($p < 0.05$) by 22 days in the Mn-treated compared with control animals (control, 0.42 ± 0.03 vs. Mn, 0.58 ± 0.04 ; Fig. 2). Because IGF-1 can stimulate PGE₂ release from the ME (*Hiney et al.*, 1998), we assessed the effect of Mn on COX-2 gene expression in these 22-day-old animals. Figure 3 shows that Mn exposure induced ($p < 0.01$) the COX-2 gene in the MBH compared with the control animals (control, 1.38 ± 0.13 vs. Mn, 2.30 ± 0.07).

Effect of Mn during Precocious Peripubertal Development

Figures 4A and 4B demonstrate that the Mn-treated animals continued to show elevated gene expressions for both IGF-1 (control, 1.16 ± 0.05 vs. Mn, 2.02 ± 0.25 ; $p < 0.05$) and COX-2 (control, 1.39 ± 0.1 vs. Mn, 1.83 ± 0.16 ; $p < 0.05$) in the MBH at 29 days of age. At this age, we also incubated ME tissues *in vitro* and observed that Mn can stimulate ($p < 0.01$) the release of both PGE₂ (control, 95.4 ± 6.5 vs. Mn, 186 ± 26 ;

FIG. 1. Effect of low-dose Mn exposure on TGFα (A) and IGF-1 (B) gene expression in the MBH of 22-day-old female rats as determined by real-time PCR. Panel (A) shows the gene expression of TGFα in 22-day-old rats was not affected by Mn exposure (solid bar) versus those that received saline (open bar). Panel (B) shows that in these same animals, Mn exposure markedly increased expression of the IGF-1 gene (solid bar) compared with the controls (open bar). The respective bars illustrate the mean (± SE) of an *N* of 5 per group. * $p < 0.05$ versus control.

FIG. 2. Effect of low-dose Mn exposure on IGF-1R protein expression in the MBH of 22-day-old female rats. (A) Representative Western immunoblot of IGF-1R and β -actin proteins in the MBH isolated from control (lanes 1–3) and Mn-treated (lanes 4–6) animals. (B) Densitometric quantitation of all the bands from two blots assessing IGF-1R protein expression in the MBH. These data were normalized to the internal control β -actin protein, and the densitometric units represent the IGF-1R/ β -actin ratio. Note that IGF-1R protein expression was increased following Mn exposure (solid bar) of these 22-day-old animals when compared with the control animals treated with saline (open bar). The respective bars illustrate the mean ($\pm$ SE) of an N of 8 per group. $*p < 0.05$ versus control.

Fig. 5) and LHRH (not shown). Because of the effect of Mn to stimulate the secretion of LHRH, it was also important to determine if Mn could induce synthesis of LHRH in order to keep pace with release of the peptide at this stage of development. Because LHRH is not synthesized in the MBH of the rat, we assessed the effect of Mn on LHRH gene expression in the POA/AVPV nuclei, the principal area for LHRH synthesis. Assessments were made at both 22 and 29 days to determine if enhanced expression occurred as pubertal maturation progressed. Compared with the controls, LHRH

FIG. 3. Effect of low-dose Mn exposure on COX-2 gene expression in the MBH of 22-day-old female rats as determined by real-time PCR. Note that Mn exposure significantly induced COX-2 gene expression (solid bar) in the MBH of 22-day-old animals when compared with the control animals that received saline (open bar). The respective bars illustrate the mean ($\pm$ SE) of an N of 5 per group. $**p < 0.01$ versus control.

FIG. 4. Effect of low-dose Mn on IGF-1 (A) and COX-2 (B) gene expression in the MBH of 29-day-old animals as determined by real-time PCR. Panel (A) shows that IGF-1 gene expression was increased following Mn exposure (solid bar) when compared with control animals exposed to saline (open bar). Panel (B) shows that in these same animals, Mn exposure also increased COX-2 gene expression (solid bar) compared with the controls (open bar). The respective bars illustrate the mean ($\pm$ SE) of an N of 9 per group. $*p < 0.05$ versus control.

gene expression was not affected by Mn at 22 days (not shown); however, there was an increase ($p < 0.05$) in its expression at 29 days (control, 1.47 ± 0.21 vs. Mn, 2.18 ± 0.18 ; Fig. 6).

DISCUSSION

The onset of mammalian puberty is the result of complex interactions within the hypothalamus leading to increased secretion of LHRH. Key excitatory neurotransmitters controlling LHRH at puberty are IGF-1 (Hiney *et al.*, 1996; Longo *et al.*, 1998; Wilson, 1998; Zhen *et al.*, 1997), kisspeptin (Navarro *et al.*, 2004; Shahab *et al.*, 2005), and neuronal glutamate (Gay and Plant 1987; Urbanski and Ojeda, 1987). The actions of these neurotransmitters at puberty are dependent upon the maturation and interactive participation of neuronal circuits and glial cells. Whereas the arcuate nucleus of the basal hypothalamus contains neuronal perikarya, nerve fibers, and glial cells, the ME portion is mainly made up of glial cells and nerve terminals, but not perikarya. Glial cells within the ME

FIG. 5. Effect of Mn on the *in vitro* release of PGE₂ from the MEs obtained from 29-day-old female rats. MEs were incubated in medium only or medium containing 50 µM MnCl₂. Note that the presence of Mn in the medium caused a marked increase in the release of PGE₂ (hatched bar) compared with the basal release of PGE₂ from the medium-only controls (open bar). The respective bars illustrate the mean ($\pm$ SE) of an *N* of 8 per group. ***p* < 0.01 versus control.

are in close association with LHRH nerve terminals and facilitate prepubertal LHRH release by producing cell adhesion molecules and growth factors. Glial-derived TGF α and IGF-1 are growth factors recognized as key components in the control of mammalian puberty via downstream hypothalamic glial to neuronal signaling (Duenas *et al.*, 1994; Garcia-Segura *et al.*, 2010; Ma *et al.*, 1994; Ojeda *et al.*, 2008). Recently, we showed that environmental Mn can also stimulate LHRH secretion and that an early, chronic exposure to low levels of the element can initiate precocious puberty (Lee *et al.*, 2007; Pine *et al.*, 2005). These actions, along with the fact that Mn crosses the blood-brain barrier (Aschner and Aschner, 1990) and accumulates in the hypothalamus (Deskin *et al.*, 1980; Pine *et al.*, 2005), support the notion that this element may be an environmental factor that normally contributes to the pubertal process. As a result of these actions, we suggest that exposure

FIG. 6. Effect of low-dose Mn exposure on LHRH gene expression in the POA/AVPV region of 29-day-old female rats as determined by real-time PCR. Note that at 29 days of age, the LHRH gene expression is increased in those animals exposed to Mn (solid bar) compared with control animals that received saline (open bar). The respective bars illustrate the mean ($\pm$ SE) of an *N* of 5 per group. **p* < 0.05 versus control.

to low but elevated levels of Mn too early in life may cause a risk for precocious pubertal development, a serious endocrine disorder. The present study focused on discerning specific actions of Mn on downstream glial components during juvenile and peripubertal maturation that contribute to prepubertal LHRH release.

Effects of Mn during Juvenile Development

Chronic exposure to elevated levels of Mn caused differential effects on TGF α and IGF-1, peptides that are important for the pubertal process (Hiney *et al.*, 1991, 1996; Ma *et al.*, 1992; Ojeda *et al.*, 2008). TGF α gene expression was not altered in the MBH; however, IGF-1 expression was markedly enhanced by the element. This timely Mn-induced effect on the IGF-1 system in the MBH is of potential importance because it occurred at 22 days of age, whereas it has been shown that IGF-1 gene expression normally increases at 30 days of age (Daftary and Gore, 2003), just prior to entering the peripubertal period. Furthermore, our observation indicates an early effect of Mn in this brain region to upregulate the IGF-1R, which is present in large numbers on glia and nerve terminals in the ME (Lesniak *et al.*, 1988; Marks *et al.*, 1991). This Mn-induced activation of the IGF-1R is important because it has been shown that during juvenile development, IGF-1R synthesis is necessary for progression through pubertal development (DiVall *et al.*, 2010; Hiney *et al.*, 2004). Importantly, IGF-1R transgenic mice, which lack the receptor on LHRH neurons, showed delayed puberty (DiVall *et al.*, 2010). These authors suggested that IGF-1R signaling in LHRH neurons facilitates the establishment of synaptic structures necessary for receiving excitatory inputs for the induction of the pulsatile pattern of LHRH release at puberty. Thus, we suggest that this action of Mn to induce IGF-1 and its receptor initiates trophic actions of the peptide to support cellular survival, maturation, and differentiation of neural cells (Duenas *et al.*, 1996; Ye and D'Ercole, 2006), as well as facilitates glial and neuronal plasticity (Cardona-Gomez *et al.*, 2000; Fernandez-Galaz *et al.*, 1999), functions dependent on adequate levels of E₂, and its receptor (Garcia-Segura *et al.*, 2010; Hiney *et al.*, 2004). In this regard, we showed previously that Mn exposure causes the precocious elevation in serum levels of this steroid (Pine *et al.*, 2005), indicating that the early induction of IGF-1 may contribute to development of glial-neuronal communication networks needed to support the increased LHRH secretion in the coming days.

In this study, we also showed that Mn induced the expression of the COX-2 gene in the MBH at 22 days of age. COX-2 is the rate-limiting inducible form of the enzyme necessary for PGE₂ synthesis. PGE₂ is not stored in any cellular compartment but is rapidly synthesized and released (Hamberg and Samuelsson, 1971). Our observation that Mn exposure induces COX-2 expression in the MBH is the first to

show an *in vivo* increase in brain, supporting *in vitro* studies showing Mn-induced activation of microglia (Bae *et al.*, 2006) and astrocytes via the COX-2 pathway (Liao *et al.*, 2006). Whether Mn activates COX-2 in the MBH directly or first acts on another substance that mediates this action remains to be determined. Previous studies showed that IGF-1 (Hiney *et al.*, 1998), which is produced in both glia and neurons (Daftary and Gore, 2005; Duenas *et al.*, 1994), as well as glial-derived epidermal growth factor (EGF)-related peptides (Hiney *et al.*, 2003; Ma *et al.*, 1997; Ojeda *et al.*, 1990), bind to specific receptors in the ME, activating intracellular signaling events leading to increased COX-2/PGE₂ secretion. Because we demonstrated here that Mn can induce IGF-1 in the MBH, we suggest that this peptide may, in part, mediate the effect of Mn on COX-2/PGE₂ production. With regard to EGF-related peptides, our results indicate that Mn does not affect TGF α expression; however, we cannot rule out that other erbB receptor ligands, like EGF or epiregulin, may also mediate Mn effects on COX-2/PGE₂ production. Regardless of the exact mechanism, the fact that Mn induces COX-2 by 22 days of age indicates it may contribute to maturation of the MBH prior to the onset of puberty. In support of this, stimulation of PGE₂ synthesis/release would result in upregulation of its receptors located on adjacent glial cells and on the nearby LHRH neuron terminals (Rage *et al.*, 1997), further contributing to glial-neuronal interactions. Furthermore, PGE₂ is involved in angiogenesis by promoting endothelial cell sprouting (Namkoong *et al.*, 2005) and increases blood flow via vasodilation (Gordon *et al.*, 2007), hence indicating enhanced vascular development and function in preparation for the onset of puberty when increased secretion of puberty-related neurohormones, including LHRH, are released directly into the hypophyseal portal vessels.

Effects of Mn during Precocious Peripubertal Development

We observed that LHRH gene expression in the POA/AVPV region was similar between Mn-treated and control animals at 22 days; however, Mn caused increased LHRH expression in this region at 29 days. This is important because this is the principal brain region responsible for LHRH synthesis. Furthermore, this increased synthesis coincides with our earlier report showing that Mn stimulated serum gonadotropin and estradiol (E₂) levels at 29 days, followed by an earlier age at puberty than normal (Pine *et al.*, 2005). Taken together, these results further demonstrate that Mn acts during the peripubertal phase of development, in addition to the earlier action during juvenile development. The mechanism by which Mn influences LHRH gene expression is not known. It is possible that Mn acts directly on the LHRH neuron in the POA/AVPV region, but more likely, it is involved in activating a specific gene controlling LHRH neuronal activity. In this regard, IGF-1 has been shown to act on LHRH neurons in the POA to enhance LHRH gene expression (Daftary and Gore, 2003). However, Mn does not act through IGF-1 in the POA/AVPV

nucleus because Mn-induced LHRH gene expression at 29 days was not associated with a change in IGF-1 gene expression in this region (Hiney, Srivastava, and Dees, unpublished observation). Hence, we suggest that Mn upregulates the LHRH gene expression by activating some as yet unknown upstream gene. Identification of this affected gene will be important in identifying a potential cause of precocious puberty.

The influence of Mn on PGE₂ in relation to LHRH secretion from the MBH at 29 days of age is important. We demonstrated that chronic exposure to Mn induces gene expressions of both IGF-1 and COX-2 in MBH and furthermore showed that Mn induced the secretion of both PGE₂ and LHRH from ME tissues incubated *in vitro*. The latter effect of Mn on LHRH release confirms our earlier report (Lee *et al.*, 2007). When glial PGE₂ is released, it binds to its receptors on LHRH terminals in the ME, causing secretion of the LHRH peptide (Ojeda and Negro-Vilar, 1985). As with juvenile development, whether the Mn action to stimulate PGE₂ during peripubertal development is due to a direct effect or is mediated via another substance remains to be determined. IGF-1 can induce PGE₂ (Hiney *et al.*, 1998), but whether it mediates the Mn effect to stimulate PGE₂ has not been studied. Regardless of the mechanism, the ability of Mn to facilitate PGE₂ secretion is important for several reasons. Upregulation of PGE₂ receptors on glial cells and LHRH nerve terminals will promote glial-neuronal communications. PGE₂ will continue enhancing blood flow in the ME and was shown to promote the plasticity of specialized glial cells known as tanycytes. In this regard, as E₂ levels rise, PGE₂ stimulates production of TGF β 1, causing the tanocyte end feet to retract from the endothelial wall (Prevot *et al.*, 1999, 2003). This action allows for a better access of the LHRH nerve endings to the vessel and therefore enhances peptide release into the portal vasculature (King and Letourneau, 1994; King and Rubin, 1996).

It is important to further discuss the relationship between Mn, IGF-1, and LHRH release from the MBH. Both Mn (Lee *et al.*, 2007) and IGF-1 (Hiney *et al.*, 1996) can stimulate LHRH secretion. Although IGF-1 is certainly an important stimulator of prepubertal LHRH, it does not play a major role in mediating Mn-induced LHRH release directly (Dees *et al.*, 2009); however, induction of IGF-1 by chronic Mn exposure likely contributes to the above-mentioned effects on tanycytes and other glia within this region because IGF-1 and its receptor also respond to increasing E₂ at puberty and together play a role in synaptic plasticity (Cardona-Gomez *et al.*, 2000; Fernandez-Galaz *et al.*, 1999). Furthermore, upregulation of IGF-1R would further promote glial-neuronal interactions and be receptive to the circulating IGF-1 that contributes to peripubertal LHRH secretion (Hiney *et al.*, 1996). Taken together, the induction of IGF-1 and PGE₂ by Mn is important and suggests that in addition to stimulating LHRH release, the element may facilitate synaptogenesis and entry of the released peptide into hypophyseal portal vessels for transport to the anterior pituitary gland.

The actions of Mn described above with regard to PGE₂ and IGF-1 are relevant because they represent downstream effects within the MBH, at the level of glial-neuronal interactions, to enhance LHRH secretion and drive the pubertal process. Our study, however, does not investigate other potential mechanisms of Mn action such as assessment of effects on specific puberty-related genes or on additional neuroactive substances that may act upstream to influence LHRH neuronal activity. This potential is supported by the fact that in addition to the MBH, Mn accumulates in the POA/AVPV region (Deskin *et al.*, 1980; Pine *et al.*, 2005). Therefore, we cannot rule out the possibility that Mn exposure during prepubertal development may have multiple effects within the hypothalamus, and thus, additional research in this regard is warranted.

The present study clearly demonstrates that chronic Mn exposure during juvenile and peripubertal development causes precocious increases in hypothalamic IGF-1 and COX-2/PGE₂. Our results indicate that Mn, through upregulation of these two puberty-related substances, may promote maturational events and glial-neuronal communications facilitating the increased neurosecretory activity, including that of LHRH, occurring at puberty. It has been known for many years that Mn can be both beneficial and harmful. Although accumulation of Mn in the hypothalamus may play a beneficial, normal role in the timing of puberty, we suggest that exposure to elevated levels too early in development, causing an earlier than normal accumulation of the element, contributes to precocious pubertal development. Precocious puberty, especially in females, is a child health concern, and more research to determine the effects and mechanisms of Mn actions in this regard will be important.

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