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Early-Life Manganese Exposure Up-regulates Tumor Associated Genes in the Hypothalamus of Female Rats: Relationship to Manganese-Induced Precocious Puberty

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Running Title: Manganese induced tumor associated genes
Abstract
Prepubertal exposure to low, but elevated levels of manganese (Mn) can induce increased secretions of puberty-related hormones resulting in precocious pubertal development in female rats. These events are due to an action of the element within the hypothalamus to induce the secretion of gonadotropin-releasing hormone (GnRH). Because of these prepubertal effects of Mn, and because precocious puberty is a serious neuroendocrine disorder, we have assessed whether early life exposure to this environmental element is capable of precociously up-regulating the expression of a select group of genes previously associated with tumor growth or suppression, and that have more recently been shown to increase at the normal time of puberty. Female rat pups received a daily dose of either 10 mg/kg MnCl$_2$ or an equal volume of saline by gastric gavage from postnatal day 12 through day 22 or 29. At this time blood was collected for E$_2$ analysis and hypothalamic brain tissue frozen on dry ice until assessed for gene expressions. Rats exposed to the elevated Mn showed a precocious increase in GnRH gene expression in the preoptic area and rostral hypothalamus on day 29, an action associated with precociously increased expressions of specific tumor associated, puberty-related genes. These results demonstrate for the first time that prepubertal Mn exposure is capable of activating specific upstream genes regulating hypothalamic GnRH and suggest that these actions are involved in the mechanism by which this element can induce precocious puberty.

Key words: Manganese, puberty, KISS-1, Ras homologue enriched in brain (Rheb), mammalian target of rapamycin (mTOR)
Introduction

The age at which normal puberty occurs depends on a complex series of events within the hypothalamus that culminate in the increased synthesis and release of gonadotropin-releasing hormone (GnRH). It is accepted that GnRH neuronal functions associated with the onset of puberty are regulated by metabolic signals, genetic and environmental influences. Thus, any endogenous or exogenous substance capable of facilitating or inhibiting prepubertal GnRH release could impact the central activation of the peptide and therefore, potentially alter normal developmental processes leading to puberty.

Several neuronal products within the hypothalamus, including insulin like growth factor -1 (IGF-1), excitatory amino acids, and kisspeptin (Kp) have all been shown to activate the prepubertal hypothalamic-pituitary axis and drive the pubertal process in rats (Hiney et al., 1996; Navarro et al., 2004a, 2004b; Urbanski and Ojeda, 1987;) and monkeys (Gay and Plant, 1987; Shahab et al., 2005; Wilson, 1998). In recent years, we have also shown that environmental manganese (Mn) acts within the hypothalamus to induce prepubertal GnRH release in the rat (Pine et al., 2005). Furthermore, the time of puberty was advanced when prepubertal animals were exposed to a very low dose of the element, an effect associated with females being more sensitive (Pine et al., 2005) than males (Lee et al. 2006). This hypothalamic action of Mn indicates that the normal beneficial effect of Mn at puberty could be harmful if an individual is exposed to low but elevated levels of this heavy metal too early in life. Precocious puberty is a serious endocrine disorder; hence, this raises a concern regarding environmental Mn exposure in the young, especially females, since their age at puberty onset appears to be occurring earlier (Parent et al., 2003), and most cases have no identifiable cause (Rosenfield, 2002). Thus understanding more about the mechanisms of Mn involvement at puberty is important since the
young are considered more sensitive to Mn (EPA, 2002), their optimum level of oral exposure is not well understood (Gregor, 1999), Mn crosses the blood brain barrier over four times more efficiently in young vs. adults (Mena, 1974) and Mn accumulates in the hypothalamus (Deskin et al., 1980; Pine et al., 2005).

An emerging concept regarding the onset of puberty is the participation of specific genes that, once activated, may coordinate early events controlling the neuroendocrine timing of puberty. In recent years, specific genes associated with tumor growth or suppression, which we refer to as tumor associated genes (TAGs), have been linked to events leading to puberty. The most important of these identified to date is the KiSS-1 gene. This gene encodes the Kp family, which were first identified as tumor suppressors (Lee et al., 1996; Ohtaki et al., 2001), but since have also been shown to be potent stimulators of prepubertal GnRH secretion (Keen et al., 2008; Navarro et al., 2004a). KiSS-1 gene expression increases in the hypothalamus as puberty approaches in both primates (Shahab et al, 2005) and rats (Navarro et al., 2004a), and is critical for the pubertal process in all species studied, including humans (de Roux et al., 2003; Navarro et al., 2004b; Seminara et al., 2003; Smith et al., 2007). While KiSS-1 is essential for the onset of puberty, upstream hypothalamic regulators of its expression at the time of puberty are only now beginning to emerge (Hiney et al., 2009; Mueller et al., 2011).

There have been numerous other TAGs recently shown to have increased expressions in the hypothalamus at puberty (Roth et al., 2007), although for most of them, their definitive functions related to the pubertal process are not known. Evidence has been provided that these genes appear to be organized in an intricate network, with a select few being designated as potential upper level genes with the highest degree of interconnectivity with other genes within the network, including KiSS-1 (Roth et al., 2007). Determining factors influencing the activation
of these and other TAGs at puberty, as well as assessing any associations/relationships between their expression and the expression of KiSS-1, will be important. Since Mn is an environmental substance with the ability to stimulate prepubertal GnRH release from the hypothalamus and cause precocious pubertal development, we have assessed whether prepubertal exposure to this element is capable of up-regulating the expression of a select group of puberty-related TAGs.

**Materials and Methods**

**Animals.** Immature female Sprague-Dawley rats raised in our colony at the Texas A&M University Laboratory Animal Research and Resources Facility were used for this study. All animals were housed under controlled conditions of light (lights on, 0600h, lights off, 1800 h) and temperature (23°C). The diet used was Harlin Teklad 2016 and the contents of Mn and iron in every batch purchased were confirmed by our departmental trace element lab so that supplemental doses would be precise. All procedures were approved by the University Care and Use Committee and were in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals.

**Experimental Procedure.** Rats were bred and delivered their pups normally. All litters were adjusted to 10-12 pups, with each having 4-6 females. Food and water was available to the mothers *ad libitum*, which was also available to the pups once they began eating solid food. When the animals were 12 days-old, the females were numbered by tail markings and approximately half of the female pups in each litter were supplemented with a daily gastric gavage dose of 10 mg/kg MnCl₂ (0.25 mg in 0.2 ml/25 g of rat). The other female pups in each litter received an equal volume of saline. We have previously reported that this dose of Mn was the minimum effective dose to increase puberty-related reproductive hormones and advance female puberty (Pine et al. 2005). When the pups were 21 days old, the mothers and the male
pups were removed from the cage. The dosing continued for the female pups until they were
terminated by decapitation when either 22 or 29 days old. At both times, one saline and one Mn-
treated female pup was selected from each litter; hence, the final groupings of animals from
which the tissues were assessed contained pups from different litters. Because Mn-treated rats
attain puberty early, beginning about day 30, it was important to take the tissues prior to this time
so that some of the rats would not be in first proestrus or estrus. This identical procedure was
repeated 4 times for each time point to complete tissue collections for the various assessments
(see below). Trunk blood was collected from some of the animals for estradiol (E\textsubscript{2}) and Mn
measurements. The brains were removed for gene and Mn assessments. They were divided into
two blocks, frozen on dry ice and stored at -80 °C until subsequent gene analysis. Briefly, the
rostral tissue block contained the preoptic area and the rostral hypothalamic area (POA/RHA),
which includes the anteroventral periventricular (AVPV) nucleus, for which the KiSS-1
expressing neurons in this region are localized. For this block, the first cut was made
approximately 1 mm rostral to the optic chiasm (OC), then a second cut made at the caudal
border of the OC. Finally, the block was formed by making cuts along the borders of the OC
laterally and along the border of the anterior commissure dorsally. The caudal tissue block
contained the medial basal hypothalamus (MBH), which includes the arcuate (ARC) nucleus, for
which the KiSS-1 expressing neurons in this region are localized. This block extended from the
previous cut to the mammillary bodies caudally. This block was formed by making cuts along
the hypothalamic sulci laterally and along the border of the thalamus dorsally. These two
regional tissue blocks were used to assess site specific effects of Mn on the expression of specific
puberty-related TAGs in the reproductive hypothalamus. Using well-established criteria for
assessing stages of puberty (Dees and Skelley, 1990), all of the animals were confirmed to be immature when their tissues were collected.

**Gene Selection.** The first group of genes consisted of GnRH, KiSS-1 and the Kp receptor, GPR54. These puberty-related genes were selected for analysis because of their essential roles in puberty and to determine if low, but elevated Mn exposure could induce their expression. Importantly, once the Mn-induced simultaneous up-regulations of KiSS-1 and GnRH were observed, then this project was expanded over the next coming months by repeating the above experimental procedure several times in order to collect enough tissues for discerning potential upstream regulators of the critically important KiSS-1 gene. As specific puberty-related genes were identified and selected the frozen tissues were retrieved and subjected to PCR analysis. Most of the other TAGs in our study were chosen from a network of genes revealed to have been increased at the time of puberty in non-human primates and/or rats (Roth et al. 2007). In this regard, we first selected the genes, cutlike-1 (Cutl-1) and ying-yang 1 (YY1), which show the strongest interconnectivity with KiSS-1 (Mueller et al., 2011), and upstream stimulatory factor-2 (USF2) based on its role in reproductive function (Hermann and Heckert, 2007). We also examined tuberous sclerosis complex 1 (TSC1), musculoaponeurotic fibrosarcoma (Maf), p53 and cyclin D1 which are known cell regulators of differentiation, growth and development (Huang and Manning, 2008; Kataoka, 2007; Roth et al., 2007; Vogelstein et al., 2000; Xiong et al., 1997) and are also important at the time of puberty. Additionally, we assessed ras homologue enriched in brain (Rheb) and mammalian target of rapamycin (mTOR), based on their associations with KiSS-1 (Roa et al., 2009), and their respective ability to mediate nutrient signaling (Huang and Manning, 2008; Tee et al, 2003; Wullschleger et al, 2006) and divalent metal transporter 1 (DMT1) which is associated with Mn transport (Jiang et al., 2010).
Isolation of total RNA. Total RNA was initially extracted from the brain tissues by homogenizing in TRIzol Reagent (Invitrogen, CA). The homogenates were further extracted for RNA using QIAGEN RNeasy kit and treated with RNase-free DNase I to eliminate genomic DNA contamination according to the manufacturer’s instructions (Qiagen Inc., Valencia, CA). The concentration of total RNA was quantitated spectrophotometrically by measuring its absorbance at 260nm.

Reverse Transcription and quantitative Real-time Polymerase Chain Reaction (PCR).

Total RNA (1µg) from each sample was reverse transcribed into cDNA in a total volume of 20 µl according to the instruction manual using oligo (dT) and SuperScript III First-strand Synthesis System (Invitrogen Life Tech., CA). Real-time PCR was performed on an ABI PRISM 7500 sequence detection system as described previously (Hiney et al., 2009). Briefly, PCR reactions were done in 25 µl reactions containing 2 µl cDNA, 500nM primer pairs and 1X SYBR green PCR master mix in 96-well plates. Primers for each gene were designed according to the guidelines of Applied Biosystems (Foster City, CA) with the help of Primer Express 3.0 software. Each primer was checked for the absence of cross-reactivity by BLAST search. Primers specific for the housekeeping gene, β-actin, were also included in all reactions separately under the same experimental conditions to normalize for the amount of RNA in the initial reverse transcription reaction. Reactions without reverse transcriptase were also carried out as negative controls. The primer sequences for each gene amplification are in Table 1S (Supplemental Material). The PCR cycling conditions were 95°C for 10 min, followed by 40 cycles at 95°C for 15 seconds and 60°C for 1 minute. PCR product purity was confirmed by dissociation curve analysis for each gene at the end of the PCR reaction. Each PCR generated DNA product was also electrophoresed onto 2% agarose gel containing ethidium bromide, which
showed a single band of the expected size. The relative levels of expression for each gene were calculated using the comparative threshold cycle method described previously (Hettinger et al., 2001). The $\Delta - C_T$ for each gene was determined by subtracting the corresponding $\beta$-actin $C_T$ value from the target gene $C_T$ of each sample. Calculation of $\Delta - \Delta C_T$ in each gene involves using the highest sample $\Delta - C_T$ value as an arbitrary constant to subtract from all other $\Delta - C_T$ sample values. The changes in the relative gene expression were then determined by the formula $2^{\Delta - \Delta C_T}$.

**Hormone analysis.** Serum E$_2$ levels were measured as previously described (Hiney et al. 2009). The E$_2$ assay kit was purchased from Diagnostic Products Corp. (Los Angeles, CA). The sensitivity for the E$_2$ assay was 5 pg/ml. The intra-assay variation was less than 5%.

**Mn Analysis.** Serum and brain Mn measurements were measured by a PerkinElmer/Sciex DRC 11 inductively coupled mass spectrometer by a method we have described previously (Pine et al., 2005). All assessments were conducted by the Heavy Metal Analysis Laboratory, Department of Integrative Biosciences, College of Veterinary Medicine, Texas A&M University.

**Statistical Analysis.** The differences between control and Mn-treated animals were analyzed by Student’s t test. Thirty-four control and 34 Mn-treated animals, respectively, were used at 22 days of age and 41 control and 41 Mn-treated animals, respectively, used at 29 days of age. The total number of animal tissues used for each gene analyzed is listed within the respective legend. Serum levels of E$_2$ and Mn were conducted on 8 control and 8 Mn-treated rats at 22 days of age and 13 control and 13 Mn-treated 29 days of age. The Mn content in brain POA/RHA and MBH was measured in these same animals. All tests were conducted using INSTAT and Prism software for the IBM PC (GraphPad, Inc., San Diego, CA). All data are reported as mean±SEM and p values of < 0.05 were considered to be statistically different.

**Results**
The Mn-treated animals showed an increase in the accumulation of the element in both the AVPV/POA and MBH brain regions at both time points (Table 1). Serum E$_2$ levels were similar between saline and Mn-treated animals at 22 days of age (Saline: 14.9±0.83 pg/ml, N=8 vs Mn: 14.4 ± 1.50 pg/ml; N=8). Subsequently, a marked increase (p<0.01) in E$_2$ was observed by 29 days in the Mn-treated animals (Saline: 12.9 ± 0.085 pg/ml; N=13; vs Mn: 16.4 ± 0.59 pg/ml; N=13). Figure 1 shows that Mn did not alter GnRH gene expression in the POA/RHA brain region at 22 days of age (A), but caused an increase (p<0.05) in its expression by 29 days (B), an action associated with the increased levels of serum E$_2$. Since GnRH neurons are not present in the MBH of the rat (Kozlowski and Dees, 1984), this region was not assessed.

Because KiSS-1 is a TAG directly linked to GnRH and is essential for puberty, we first assessed it and the Kp receptor, GPR54, to determine if any specific increases in expression were associated with the Mn-induced increase in GnRH expression at 29 days of age. We also determined if these genes may have been induced earlier, at 22 days old. Figure 2 demonstrates that increases over saline (basal) expression levels were observed within the POA/RHA for both KiSS-1 (p<0.05, B) and GPR54 (p<0.05, D) at 29 days, and only GPR54 (p<0.01, C) at 22 days of age. In the MBH, Mn did not affect the expression of either KiSS-1 or GPR54 when compared to the basal levels expressed by the saline-treated tissue at either time point (not shown).

Figure 3 demonstrates differential effects of Mn on the activation of other TAGs associated with reproductive functions, including ones that mediate nutrient signaling. Panels A - C show that Mn did not affect expression of USF2, Cutl-1 and YY1 in the POA/RHA region at 29 days of age nor were they affected at 22 days of age (not shown). Figure 4 demonstrates that at 29 days, Mn induced the expressions of both Rheb (p<0.05; A) and mTOR (p<0.01; B).

Likewise, Mn also induced both Rheb (SAL: 1.23±.08 vs Mn: 1.5±.08; N=9/group; p<0.05) and
mTOR (SAL: 1.76±.19 vs Mn: 2.42±.13; N=6/group; p<0.05) in the MBH at 29 days. While this latter gene has not been designated a TAG, it was assessed because it can be activated by Rheb (Avruch J. et al. 2009). At 22 days of age, Mn did not affect the expression of any of these genes either in the POA/RHA or the MBH (not shown).

Figure 5 shows differential effects of Mn on specific genes that are capable of mediating important cellular events such as cell differentiation, growth and development. Panel A shows that Mn supplementation did not alter the expression of TSC1 in the POA/RHA region at 29 days of age. Conversely, panels B and C demonstrate that Mn exposure resulted in the increased (p<0.01) expression of both cyclin D1 and p53, respectively, at 29 days of age. Mn did not alter the expression of any of these genes within the POA/RHA on day 22 of age (not shown). Figure 6 shows that Maf exhibited increased expressions within the POA/RHA at both 22 (A; p<0.01) and 29 (B; p<0.05) days of age in the Mn-treated animals. In the MBH, only cyclin D1 was increased at 29 days of age (SAL: 1.31±.13 vs Mn: 1.87±.14; N=6/group; p<0.05) and no changes were observed in the expression of any of these genes at 22 days of age (not shown).

DMT1 has been shown to be a TAG that synthesizes an integral membrane protein that transports metal ions, including Mn, across the cell membrane (Gunshin et al., 1997). Figure 7A demonstrates that while Mn did not alter DMT1 gene expression in the POA/RHA at 22 days of age, it caused an increase (p<0.01) in gene expression at 29 days of age (B). No changes were observed in DMT1 expression within the MBH at either 22 or 29 days of age (not shown).

Discussion

The animals supplemented with a low dose of Mn showed an increase in the accumulation of the element in the prepubertal hypothalamus compared to the controls. While serum Mn levels were consistently higher they were not significantly different; however, this is
explained by the fact that the animals were not dosed on the morning of days 22 or 29 when the tissues were collected, coupled with continued excretion over the last 24 hours. These results are similar to those reported previously (Pine et al., 2005). This dosing regimen has now revealed for the first time that low level Mn exposure resulted in increased prepubertal hypothalamic GnRH gene expression in the POA/RHA at 29 days of age, an action that was associated with increased expressions of both KiSS-1 and GPR54 in that same region (which includes the AVPV nucleus), but not in the MBH. The fact that KiSS-1 gene expression was increased over basal levels in one area but not the other is explained by the regional differences in responsiveness of this gene to circulating levels of E$_2$. Several studies have shown that E$_2$ differentially regulates Kp expression in the AVPV and ARC nuclei of rodents (Mayer et al. 2010; Smith et al., 2005; 2007). Mayer et al. (2010) showed that using a conditional knockout of the estrogen receptor alpha (ER$\alpha$) gene in Kp neurons revealed that these neurons in the AVPV region were stimulated by the positive feedback of circulating E$_2$ in early puberty, which resulted in GnRH synthesis, but that during this same time the Kp neurons in the ARC region were inhibited by ER$\alpha$ signaling. Our studies are in line with these studies in that Mn exposure caused precociously elevated levels of serum E$_2$, resulting in differential regional response of the KiSS-1 gene to this steroid. Differential expression was also seen with regard to GPR54 expression in the POA/RHA region being up-regulated at 22 days but not in MBH, whereas the KiSS-1 gene was not up-regulated until 29 days in the POA/RHA. While the GPR54 gene is responsive to Mn at 22 days, it is important to note that even though the transcriptional regulation of this event is not yet known, it is not due to rising levels of E$_2$ since this steroid is still low in serum at 22 days. This Mn-induced response is an important event since these kisspeptin receptors are located on GnRH neurons in this brain region. As stated above, KiSS-1 expression is under the positive influence of E$_2$, which had
increased precociously by 29 days in Mn-treated compared to control animals. Thus, the timely increase in GPR54 expression, followed by increased KiSS-1 expression, is supportive of the enhanced prepubertal GnRH secretion observed after low-level Mn exposure. Given that KiSS-1 activation is essential for the pubertal process, we began determining if a select group of transcription factors identified as TAGs exhibiting increased expression at the time of puberty, and are associated with transcriptional control of the KiSS-1 gene, were also influenced by Mn.

Although the transcription factors USF2, Cutl-1 and YY1 were not affected by Mn, we have shown that Mn induced the gene expressions of both Rheb and mTOR in the POA/RHA and the MBH at 29 days. These two genes were assessed because Rheb is a TAG (Jiang et al., 2010) that acts as a mediator of the nutrient signaling input to mTOR (Tee et al., 2003), and because central mTOR signaling is a key modulator of puberty and reproduction via its regulation of KiSS-1 (Roa et al., 2009). Thus, our results are the first to demonstrate that Mn can induce Rheb, an upstream modulator of mTOR. More work will be needed to define whether Mn is acting as a nutrient signal or by some other biochemical mechanism to activate Rheb. Once activated, however, Rheb binds directly to mTOR (Avruch et al., 2009), an interaction that is essential for activation of the mTOR complex 1. This complex is the nutrient-responsive mediator for cell growth regulation (Avruch et al., 2009; Wullschleger et al., 2006), and involved in the central activation of puberty (Roa et al., 2009). It is important to note that the increased Rheb and mTOR expressions at 29 days were associated with increased KiSS-1 expression in the POA/RHA, but not within the MBH. The fact that Rheb and mTOR were elevated in the MBH may suggest another role for them, perhaps nutrient signaling, in this region.

We have clearly demonstrated that Rheb, mTOR and KiSS-1 gene expressions were increased precociously by 29 days in the POA/RHA of Mn-treated animals, and importantly, that
these increases correlated with the increased expression of the GnRH gene. This, as well as the other gene responses at 29 days, is likely due to the combination of the longer exposure to the Mn that had accumulated in brain, as well as the rising prepubertal levels of E\textsubscript{2}. A previous study showed that the central blockade of mTOR resulted in decreased gene expressions of both KiSS-1 and GnRH (Roa et al., 2009). These two studies are complimentary in that they show that either the activation or inactivation of this pathway results in opposite actions with regard to KiSS-1 and GnRH gene expressions. Additionally, they further indicate a role for KiSS-1/Kp in the regulation of the GnRH gene, an action supported by the fact that GnRH neurons express the Kp receptor, GPR54 (Messager et al., 2005). Thus, our present results clearly suggest that the activation of this pathway by Mn is associated with increased prepubertal GnRH neuronal function and precocious pubertal development.

TSC1 has been shown to negatively regulate Rheb and the mTOR pathway (Tee et al., 2003; Zhang et al., 2003). Importantly, we showed that Mn did not alter TSC1 expression in either brain region studied; hence, further suggesting the importance of Mn to selectively induce Rheb. Mn did, however, precociously induce the expressions of cyclin D1 and p53 in the POA/RHA at 29 days, with Maf being responsive both at 22 and 29 days in this brain region. Only cyclin D1 showed a Mn-induced increase in the MBH, which was earlier than its normal pubertal increase (Roth et al., 2007). Importantly, while Mn stimulated p53, it did not affect YY1, an important negative regulator of p53 (Wu et al., 2012). Because cyclin D1, p53 and Maf all have multiple functions regarding cellular differentiation and proliferation, and are associated with increased gene expressions during the normal onset of puberty, it is possible that these genes may play roles during pubertal development, since Mn induces precocious increases in their respective gene expressions.
In order for Mn to be involved in the pubertal process, and for it to induce precocious development, it must enter and accumulate in the hypothalamus at an early prepubertal age. The optimal levels and timing of Mn entry into the brain is important, since deficiencies and excesses can result in altered brain functions. DMT1 is a TAG protein that transports metals, including Mn, across the cell membrane (Gunshin et al., 1997). In brain, DMT1 is localized in neurons, capillary endothelial cells associated with the blood brain barrier (BBB) and choroid plexus epithelial cells associated with the blood and cerebral spinal fluid (CSF) barrier (Burdo et al., 2001). Mn enters the brain either through the blood vasculature or the CSF. The POA/RHA and the MBH are closely associated with the third ventricle. This element readily accumulates in the choroid plexus (Michotte et al., 1977), a structure involved in brain maturation, homeostasis and neuroendocrine functions (Strazielle and Ghersi-Egea, 2000; Zheng, 2001). As blood Mn levels increase, influx into the CSF rises and entry across the choroid plexus becomes more important (Murphy et al., 1991). Our results are the first to demonstrate that Mn induces an increase DMT1 gene expression at 29 days in the POA/RHA. Whether Mn transport is the only apparent role for DMT1 at puberty, or whether it, like some of the other TAGs, may also influence more specific aspects of the pubertal process, such as neuronal KiSS-1 expression, will be important to determine.

Our results provide the first evidence that Mn may be a peripherally derived metabolic signal playing a facilitative role in the timing of puberty, at least in part, by activating the following proposed critical pathway leading to GnRH secretion:

\[
\text{Mn} \rightarrow \text{Rheb mRNA} \rightarrow \text{mTOR mRNA} \rightarrow \text{KiSS-1 mRNA} \rightarrow \text{GnRH mRNA} \rightarrow \text{GnRH peptide release}
\]
We suggest that because of this capability, as well as other potential influences, Mn may contribute to precocious puberty if exposure to low but elevated levels of this element occur too early in life. This is supported by numerous lines of evidence, in that Mn more efficiently crosses the BBB in the young (Mena, 1974), which do not yet have full capacity to eliminate the element (Fechter, 1999). Prepubertal females accumulate Mn in the POA/RHA, as well as the MBH (Pine et al., 2005). Furthermore, infants and children have been classified as being more sensitive to excess Mn (EPA, 2002), largely because the optimum level of exposure is not well defined (Greger, 1999). Gender differences have also been observed with regard to Mn metabolism, with male rats clearing the element two times faster than females (Zheng et al., 2000).

Current evidence indicates that the age of pubertal onset is decreasing and the incidence of precocious puberty is increasing, especially in girls (Mouritsen, 2010). While the cause of this trend has not been determined, assessing the timing of pubertal onset may be an early marker for potential interactions between environmental and genetic influences over the pubertal process (Parent et al., 2003, Mouritsen et al., 2010). Central precocious puberty is GnRH-dependent and caused by the premature activation of the GnRH releasing system. Interestingly, in boys hypothalamic harmatomas are usually the cause of this precocious activation, along with other CNS lesions or familial disease, with only 10% of the cases being idiopathic. In females, however, over 95% of precocious puberty cases have no identifiable cause, and puberty appears normal other than occurring too early (Rosenfield, 2002). While Mn can cause early signs of puberty in both sexes (Lee et al., 2006; Pine et al., 2005), the dose was 2.5 times greater in males; thus, suggesting that females were more sensitive to the element. This sensitivity issue in females may be important with regard to identifying factors, such as environmental Mn, that may
contribute to the onset of puberty, and in the case of a potential early exposure, cause precocious development.

We demonstrated that low level Mn exposure activates specific upstream genes regulating prepubertal hypothalamic GnRH. These actions, at least in part, appear to be involved in the mechanism by which Mn induces precocious puberty in research animals; hence, suggesting a potential child health concern following early life exposure.

Supplementary Material
Contains the primer sequences for each gene amplification.

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References


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**Legends**

**Figure 1.** Effect of Mn exposure on GnRH gene expression in the POA/RHA. Note that Mn did not alter GnRH mRNA at 22 days (A), but induced an increased expression at 29 days (B) of age. Bars represent an N of 5 at 22 days and 7 at 29 days of age in each group. *p*<0.05 vs saline.
Figure 2. Effects of Mn exposure on KiSS-1 and GPR54 gene expressions in the POA/RHA.

Note that Mn did not alter KiSS-1 mRNA at 22 days (A), but induced an increased expression at 29 days (B) of age. GPR54 expression was increased at both 22 and 29 days of age (C and D, respectively). Bars represent an N of 5 at 22 days and 7 at 29 days in each group. *p<0.05 vs saline; **p<0.01 vs saline.

Figure 3. Assessment of Mn exposure on USF2, Cutl-1 and YY1 gene expressions in the POA/RHA. Panels A-C show that Mn exposure did not affect the mRNA expression level of any of these genes at 29 days of age. Bars represent an N of 5 in each group.

Figure 4. Effects of Mn exposure on Rheb and mTOR gene expressions in the POA/RHA.

Panels A and B show that Mn exposure caused increased mRNA expression levels of both genes at 29 days of age. Bars represent an N of 5 for Rheb and 11 for mTOR in each group. *p<0.05 vs saline; **p<0.01 vs saline.

Figure 5. Effects of Mn exposure on TSC1, cyclin D1 and p53 gene expressions in the POA/RHA. Note that Mn exposure did not affect the mRNA expression of TSC1 (A), but induced increases in both cyclin D1 and p53 (B and C, respectively) at 29 days of age. Bars represent and N of 5 for TSC1 and and N of 6 for cyclinD1 and p53 in each group. **p<0.01 vs saline.

Figure 6. Effect of Mn exposure on Maf gene expression in the POA/RHA. Panels A and B, respectively, show that Mn induced an increase in Maf mRNA at both 22 and 29 days of age. Bars represent an N of 5 at 22 days and 6 at 29 days. *p<0.05 vs saline; **p<0.01 vs saline.

Figure 7. Effect of Mn exposure on DMT1 gene expression in the POA/RHA. Note that Mn did not alter DMT1 mRNA at 22 days (A), but induced an increased expression at 29 days (B) of age. Bars represent an N of 5 at 22 days and 6 at 29 days. **p<0.01 vs saline.
Table 1.

Mn content in serum and brain tissues of 22 and 29 days old female rats after oral administration.

<table>
<thead>
<tr>
<th></th>
<th>SAL 22 day</th>
<th>Mn 22 day</th>
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<tbody>
<tr>
<td>Serum (ppm)</td>
<td>.028±.006</td>
<td>.037±.006</td>
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<tr>
<td>MBH (ppm)</td>
<td>.678±.03</td>
<td>.879±.07 *</td>
</tr>
<tr>
<td>POA/RHA (ppm)</td>
<td>.645±.02</td>
<td>.922±.09 **</td>
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<table>
<thead>
<tr>
<th></th>
<th>SAL 29 day</th>
<th>Mn 29 day</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum (ppm)</td>
<td>.025±.004</td>
<td>.037±.01</td>
</tr>
<tr>
<td>MBH (ppm)</td>
<td>.651±.03</td>
<td>.968±.08 **</td>
</tr>
<tr>
<td>POA/RHA (ppm)</td>
<td>.680±.04</td>
<td>.898±.06 **</td>
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Note: Daily MnCl₂ exposure caused a significant increase in the Mn content in both the MBH (medial basal hypothalamus) and the POA/RHA (preoptic area/rostral hypothalamic area) at both ages. Values represent the mean±SEM. N=8 for 22 day old values; N=13 for 29 day old values. *p<0.05; **p<0.01; ppm (parts per million)
71x35mm (300 x 300 DPI)