Available online at www.sciencedirect.com
ScienceDirect
Reproductive Toxicology 22 (2006) 580–585
Reproductive Toxicology
www.elsevier.com/locate/reprotox

Manganese acts centrally to activate reproductive hormone secretion and pubertal development in male rats☆

Boyeon Lee, Michelle Pine, Larry Johnson, Valeria Rettori, Jill K. Hiney, W. Les Dees*

The Department of Veterinary Integrative Biosciences, Texas A&M University, College Station, TX 77843-4458, USA

Received 5 January 2006; received in revised form 6 March 2006; accepted 15 March 2006
Available online 12 May 2006

Abstract

Manganese (Mn) is an important element for normal growth and reproduction. Because Mn accumulates in the hypothalamus and is capable of stimulating puberty-related hormones in female rats, we assessed whether this metal could cause similar effects in male rats. We have demonstrated that $MnCl_2$, when administered acutely into the third ventricle of the brain, acts dose dependently to stimulate luteinizing hormone (LH) release. Furthermore, there was a dose dependent stimulation in the secretion of LH-releasing hormone (LHRH) from the medial basal hypothalamus in vitro, and administration of an LHRH receptor antagonist in vivo blocks Mn-induced LH release. To assess potential chronic effects of the metal, male pups were supplemented with 10 or 25 mg $MnCl_2$ per kg by gastric gavage from day 15 until days 48 or 55, at which times developmental signs of spermatogenesis were assessed. Results demonstrate that while significant effects were not observed with the 10 mg/kg dose, the animals receiving the 25 mg/kg dose showed increased LH ($p < 0.05$), FSH ($p < 0.01$) and testosterone ($p < 0.01$) levels at 55 days of age. Furthermore, there was a concomitant increase in both daily sperm production ($p < 0.05$) and efficiency of spermatogenesis ($p < 0.05$), demonstrating a Mn-induced acceleration in spermatogenesis. Our results suggest Mn is a stimulator of prepubertal LHRH/LH secretion and may facilitate the normal onset of male puberty. These data also suggest that the metal may contribute to male precocious pubertal development should an individual be exposed to low but elevated levels of Mn too early in life.

Keywords: Puberty; Manganese; Luteinizing hormone; Hypothalamus; Precocious puberty; Spermatogenesis

1. Introduction

Manganese (Mn) is an abundant, naturally occurring element that is found as a component of over 100 minerals. This metal is considered an essential nutrient that is necessary for many normal mammalian physiological events, including those related to normal growth and development of bone and cartilage [1], as well as connective tissue and the reproductive system [2,3]. Mn is abundant in the environment, found in water, food, soil and air, and its absorption is affected by the age of an individual, route of exposure, the chemical form, as well as other dietary factors [4]. Additionally, Mn is being used as an antiknock agent in gasoline [5] which also contributes to environmental sources of exposure.

Exposure to high levels of Mn is toxic, causing developmental and reproductive dysfunction [6,7]. Interestingly, a Mn deficiency leads to impaired growth and reproduction in both sexes [8,9], demonstrating that it must play an important role in mammalian reproduction. We recently questioned whether Mn could be a factor contributing to hypothalamic events leading to sexual maturation. In this regard, we determined that exposure to low, but moderately elevated levels of the metal acted within the hypothalamus to cause increased serum levels of puberty-related hormones and modestly, yet significantly, advanced the time of female puberty [10]. This is potentially important since it suggests that if Mn levels rise and accumulate in the hypothalamus too early in life, the seemingly beneficial effect to facilitate puberty may actually be harmful by causing or contributing to precocious pubertal development. In support of this, infants and children have been suspected as being more sensitive to elevations in Mn [4].

At this time, no studies have been conducted to determine whether Mn can influence the neuroendocrine control of puberty in males. Thus, the present study was conducted to assess the

☆ This work was supported by grants from the National Institutes of Health to W. Les Dees (ES013143) and to the Texas A&M University Center for Environmental and Rural Health (ES09106).
* Corresponding author. Tel.: +1 979 845 1430; fax: +1 979 847 8981.
E-mail address: ldees@cvm.tamu.edu (W.L. Dees).

0890-6238/$ – see front matter © 2006 Elsevier Inc. All rights reserved.
doi:10.1016/j.reprotox.2006.03.011

ability of Mn to stimulate LH secretion in prepubertal male rats and to discern whether the site of this action is at the hypothalamic or pituitary level. Furthermore, we assessed whether short-term, low dose exposure to the metal during juvenile development would accelerate peripubertal spermatogenesis, a reliable indicator of male pubertal maturation.

2. Materials and methods

Immature male Sprague-Dawley rats, raised in our colony at the Texas A&M University Department of Comparative Medicine were housed under controlled conditions of light (lights on, 06:00 h; lights off, 18:00 h) and temperature (23 °C), with ad libitum access to food and water. Harlan Teklad 2016 diet was provided, and contained 94.7 mg/kg Mn and 149.8 mg/kg iron, as assessed 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.

2.1. Effect of centrally administered $MnCl_2$ on LH secretion

Thirty-five-day-old male rats were anesthetized with 2.5% tribromoethanol (Aldrich, Milwaukee, WI) and stereotaxically implanted with a stainless steel cannula (23 gauge) in the third ventricle of the brain and allowed 5 days for recovery [11]. When the rats were 40 days old, a silastic cannula (Dow Corning, Midland, MI) was inserted into the right external jugular vein of each rat [12]. The next day at 09:00 h, an extension of tubing was attached to each cannula and flushed with heparinized saline (100 IU/ml). After the animals were acclimated for an hour, three basal blood samples (250 μl) were drawn from each animal at 15-min intervals. After the third basal sample, manganese chloride tetrahydrate ($MnCl_2$; Sigma Chemical Company, St. Louis, MO) at concentrations of 1, 2.5, 10, or 25 μg/3 μl, or an equal volume of saline was injected into the third ventricular cannula. Following the respective injection, four more samples were taken at 15 min intervals for a total of seven samples. After blood sampling, brains were examined for proper cannula placement. Blood samples were centrifuged at 4 °C, and serum was stored at −80 °C until assayed for LH.

2.2. Effect of $MnCl_2$ on LHRH released from hypothalami in vitro

Forty-day-old male rats were decapitated at 09:00 h, the medial basal hypothalamus (MBH) dissected under a stereomicroscope, rinsed, and incubated as we have described previously [10,13]. Briefly, the MBHs were placed in vials (one per vial) containing 0.35 ml incubation medium consisting of Locke' buffer (2 mM Hepes, 154 mM NaCl, 5.6 mM KCl, 1 mM $MgCl_2$, 6 mM $NaHCO_3$, 10 mM glucose, 1.25 mM $CaCl_2$, and 1 mg/ml BSA, pH 7.4). Tissues were preincubated inside a Dubnoff shaker at 37 °C with constant shaking in an atmosphere of 95% O_2 and 5% CO_2 for 15 min. The first incubation was discarded, and all MBHs were then incubated again in medium only for 30 min to establish basal LHRH release. After this incubation, the medium was collected, and replaced with medium only or medium containing 50, 250 or 500 μM $MnCl_2$. After the second 30-min incubation, the media were collected, boiled for 10 min to break down proteases, and then stored at −80 °C until assayed for LHRH. The MBHs were weighed to the nearest 0.01 mg.

2.3. Effect of LHRH receptor antagonism on Mn-induced LH release

Third ventricular and external jugular cannulae were implanted as described above. The next day at 09:00 h, an extension of tubing was attached to each cannula and flushed with heparinized saline (100 IU/ml). Half of the animals received a single subcutaneous injection of acyline at a dose of 10 μg/0.1 ml [10], a potent LHRH receptor antagonist (kindly provided by Dr. H.K. Kim, Contraception and Reproductive Health Branch, Center for Population Research, National Institute of Child Health and Human Development, Bethesda, MD). The other half of the animals was injected with an equal volume of saline. Acyline was allowed a 2-h absorption period and then three basal blood samples were drawn from each freely moving animal at 15-min intervals. Immediately following the third basal sample, a 3 V injection of $MnCl_2$ (10 μg/3 μl) was administered to all of the animals. Four more blood samples were taken at 15-min intervals for a total of seven samples. After the experiment, brains were examined for proper cannula placement. Blood samples were centrifuged at 4 °C and serum was stored at −80 °C until assayed for LH.

2.4. Effect of chronic oral Mn exposure on puberty-related hormones, steroidogenesis and spermatogenesis

Mature females were bred and delivered their pups normally. Litters were adjusted to 8–1 pups with at least six males per litter. At 15 days of age, the males from each litter were divided into three groups. Groups 1 and 2 received 10 and 25 mg/kg $MnCl_2$, respectively. Administration was such that 0.258 or 0.625 mg $MnCl_2$ was delivered in 0.2 ml/25 g rat daily by a gastric gavage injection. Group 3 served as controls and received an equal volume of saline. The rats were killed by decapitation between 09:00 and 10:00 h on day 48 or day 55. Trunk blood was collected and allowed to clot, then the serum stored frozen at −80 °C until used for RIA assessment of puberty-related hormones. At this time the testes were also collected and processed for analysis of spermatogenic development as described below.

Sperm production was measured by methods we have described previously [14]. Briefly, thawed testes were separated from epididymides before the testes were weighed. Both testes were decapsulated, the tunica albuginea weighed, and the testis homogenized for 2 min in 100 ml of homogenizing fluid containing 150 mM NaCl, 0.05% (v/v) Triton X-10, and 3.8 mM NaN_3 [14]. Testicular homogenates were stored at 5 °C and evaluated within 28 h. Only spermatid heads with a shape characteristic of steps 17–19 found in stages IV and VIII [15] are resistant to such homogenization; these were enumerated by phase-contrast cytometry. Duplicate evaluations were made by each of two evaluators for the right and left side of each rat [14]. Daily sperm production per testis (DSP/testis) is a measure of total daily sperm production by each testis, and was calculated by dividing the number of homogenization-resistant spermatids by the life span of these spermatids (6.3 days) [14,16]. Daily sperm production per gram testicular parenchyma (DSP/g), a measure of efficiency of spermatogenesis, was calculated by dividing the DSP/testis by the difference between testis and tunic weights. Both methods are reliable indicators of spermatogenic development [16].

2.5. Hormone analysis

Rat serum LH and FSH were measured using radioimmunoassay (RIA) procedures we have described previously [17]. Rat LH antiserum (NIDDK-anti-rLH-S-II), antigen (NIDDK-rLH-I-9), and reference preparation (NIDDK-rLH-RP-3); and rat FSH antiserum (NIDDK-rFSH-I-9), and reference preparation (NIDDK-rFSH-RP-2) were purchased from the NIH Pituitary Hormones & Antisera Center, Harbor-UCLA Medical Center, Torrance, CA. The LH assay had a sensitivity of 0.07 ng/ml and the FSH assay had a sensitivity of 0.4 ng/ml. The LHRH was measured as previously described [18] using antisera N0.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 sensitivity of the LHRH assay was 0.2 pg/tube. The serum levels of testosterone were measured by an RIA kit purchased from Diagnostic Products Corp. (Los Angeles, CA). The sensitivity of the testosterone assay was 0.1 pg/ml. All of the assays had inter- and intra-assay coefficients of variation of <10%.

2.6. Statistical analysis

All values are expressed as the mean (±S.E.M.). Gaussian distribution was determined by the method of Kolmogorov–Smirnov. Differences between treatment groups were analyzed either by Student's *t*-test or by Kruskal–Wallis nonparametric analysis of variance (ANOVA) followed by post hoc testing using Dunn's multiple comparisons test. *p*-values less than 0.05 were considered significant. The IBM PC programs INSTAT and PRISM (GraphPad, San Diego, CA) were used to calculate and graph the results.

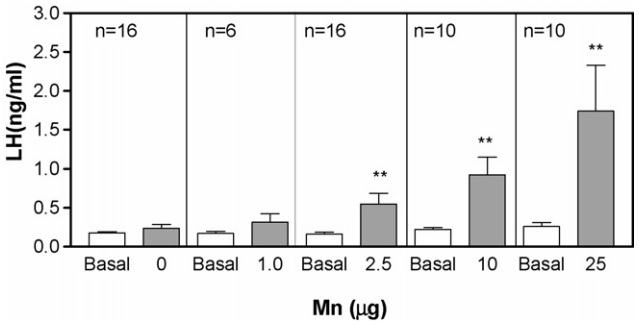

Fig. 1. The effect of 3 V administration of $MnCl_2$ on LH release in immature male rats during late juvenile phase of development. Bars depict basal vs. stimulated serum levels of LH. The animals received either saline (0) or the designated dose of $MnCl_2$. Animals which received the 2.5 μg dose of $MnCl_2$ or higher showed a marked increase in LH secretion compared to basal levels. Values represent mean ± S.E.M. The number of animals is depicted within each panel ($^{**}p < 0.01$).

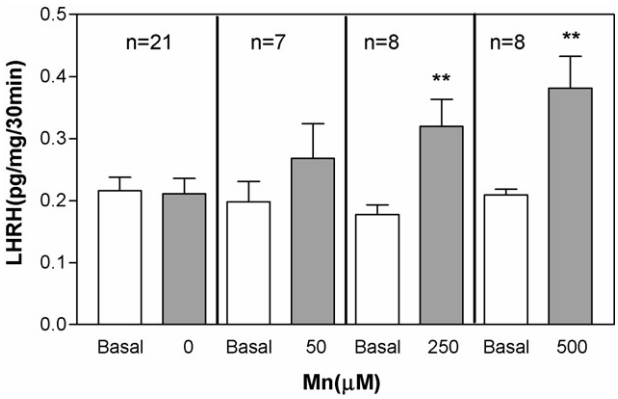

Fig. 2. The effect of $MnCl_2$ on LHRH release from MBHs of immature male rats in vitro. Bars depict basal vs. stimulated levels of LHRH. MBHs were first incubated in Locke's buffer alone to assess basal LHRH release, then again with either Locke's buffer alone (0) or with the buffer containing increasing concentrations of $MnCl_2$ to determine the stimulated response. The presence of Mn caused a dose dependent increase in LHRH release. Values represent mean ± S.E.M. The number of tissue samples is depicted within each panel ($^{**}p < 0.01$).

3. Results

3.1. Effect of centrally administered $MnCl_2$ on LH secretion

Central administration of $MnCl_2$ stimulated prepubertal LH release significantly and dose dependently over basal levels (Fig. 1). The rats that were injected with saline or the 1.0 μg dose of $MnCl_2$ exhibited no change in LH released as compared to their respective basal levels. However, the rats that were injected with the 2.5, 10 and 25 μg doses of $MnCl_2$ exhibited three-, six- and seven-fold increases in LH release, respectively, compared with their basal levels.

3.2. Effect of $MnCl_2$ on LHRH release from hypothalami in vitro

Hypothalami from prepubertal male rats were incubated in vitro with three concentrations of $MnCl_2$. The addition of $MnCl_2$ to the medium caused a dose dependent stimulation in the release of LHRH compared to basal levels (Fig. 2). Specifically, MBHs incubated in Locke's buffer without $MnCl_2$, or with the lowest dose of the metal (50 μM), showed no significant change in LHRH secreted between their respective basal and challenge incubation periods. However, marked increases in the amount of LHRH released were observed when the hypothalami were incubated with 250 μM ($p < 0.01$) and 500 μM ($p < 0.01$) concentrations of $MnCl_2$.

3.3. Effect of LHRH receptor antagonism on Mn-induced LH release

To further support that the Mn acted at the level of the hypothalamus and not the pituitary, rats were treated with the LHRH receptor antagonist, acyline, prior to the central administration of $MnCl_2$. Fig. 3 illustrates that $MnCl_2$ injected directly into the third ventricle markedly ($p < 0.01$) stimulated LH release over basal levels in control animals but not in the animals that were pre-treated with acyline.

3.4. Effect of chronic oral Mn exposure on puberty-related hormones, steroidogenesis and spermatogenesis

Compared to control animals, serum gonadotropin levels were not altered at 48 or 55 days in animals that received the 10 mg/kg supplemental dose of $MnCl_2$ (not shown). The 25 mg/kg dose caused non-significant elevations in LH, FSH, testosterone and spermatogenesis by 48 days (not shown). However, supplementation of animals with the 25 mg/kg dose of Mn until 55 days of age produced increased levels of mean serum LH (control: 0.99 ± 0.06 ng/ml versus Mn: 1.85 ± 0.36 ng/ml; $p < 0.02$) and FSH (control: 29.7 ± 1.5 ng/ml versus Mn: 36.9 ± 1.5 ng/ml; $p < 0.01$) and testosterone (control: 2 ± 0.19 ng/ml versus Mn: 2.85 ± 0.3 ng/ml; $p < 0.05$) compared with the control animals (Fig. 4A–C). Furthermore, com-

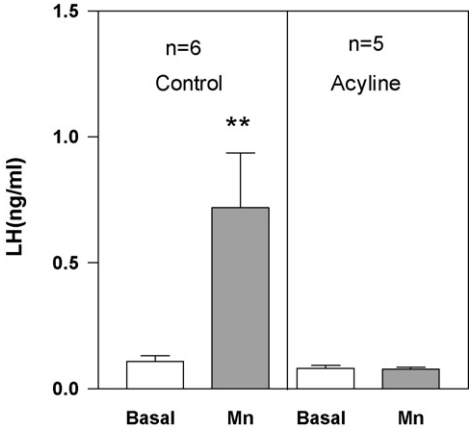

Fig. 3. The effect of pretreatment with the LHRH receptor antagonist, acyline, on $MnCl_2$-stimulated LH release in immature male rats. Control animals (no acyline) exhibited a marked Mn-induced increase in serum levels of LH compared to their basal levels. Conversely, the acyline-treated animals did not respond to the Mn stimulation. Values represent mean ± S.E.M. The number of animals is depicted within each panel ($^{**}p < 0.01$).

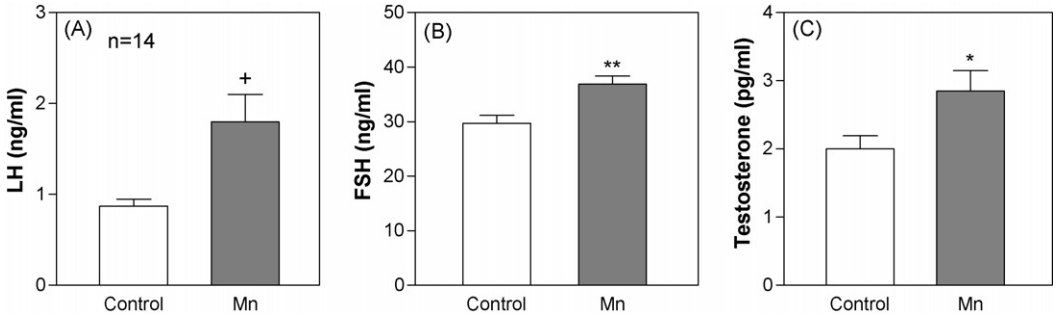

Fig. 4. Effect of chronic oral administration of $MnCl_2$ on serum LH, FSH and testosterone in 55-day-old male rats. Serum LH, FSH and testosterone levels from control animals and animals which received the 25 mg/kg dose of $MnCl_2$ from day 15 through day 55 of life are depicted (panels A–C, respectively). Values represent the mean ± S.E.M. The number of animals represented in each panel is depicted within panel A ($^{+}p < 0.02$; $^{*}p < 0.05$; $^{**}p < 0.01$, respectively, vs. control).

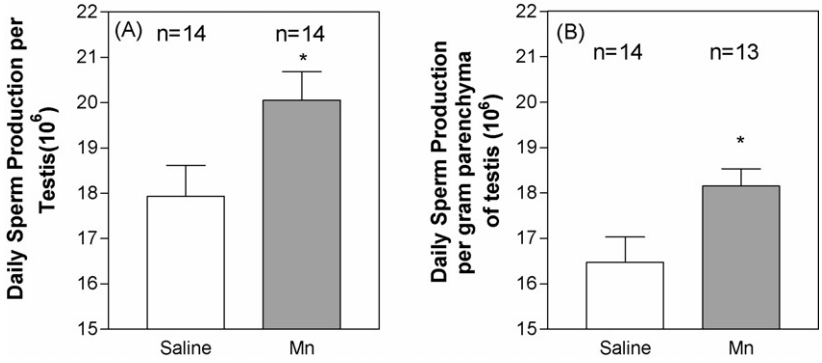

Fig. 5. Effect of chronic oral administration of $MnCl_2$ on spermatogenesis of immature male rats. Male rats received either $MnCl_2$ or saline by gastric gavage from day 15 through day 55 of life. These data depict the effect of $MnCl_2$ (25 mg/kg) supplementation on daily sperm production per testis (panel A) and efficiency of sperm production as assessed by daily sperm production per gram parenchyma (panel B). Values represent mean ± S.E.M. The number of animals is depicted within each bar ($^{*}p < 0.05$).

pared with levels in controls, daily sperm production per testis (DSP/testis) and daily sperm production per gram of testicular parenchyma (DSP/g), which are key indicators of male pubertal development, were both increased ($p < 0.05$) by 55 days of age in the animals supplemented with the 25 mg/kg dose of $MnCl_2$ (Fig. 5A and B).

4. Discussion

The present study demonstrates an action of Mn to stimulate prepubertal LHRH and gonadotropin secretion, and to significantly elevate the rate of daily mature sperm production, an indicator of pubertal development in males. Our results depict three lines of evidence demonstrating that the hypothalamus is the site of the Mn action on LH secretion in prepubertal male rats: (1) the third ventricular administration of $MnCl_2$ stimulated a dose dependent release in LH, (2) the metal stimulated LHRH release directly from the MBH in vitro, and (3) the LHRH receptor antagonist, acyline, effectively blocked LHRH receptors on the pituitary, thus inhibiting the Mn-induced release of LH following third ventricular administration. Collectively, these data indicate that Mn acts within the hypothalamus to facilitate the secretion of LH, a key hormone controlling Leydig cell production of testosterone, which subsequently, drives the increase in spermatogenesis during pubertal development.

The age at which the normal onset of puberty begins is variable and depends on a complex series of events within the hypothalamus that culminate in the increased secretion of LHRH. The current understanding of the initiation of puberty is that the pituitary and gonad are capable of function at any age after a short period of exposure to LHRH [19]. LHRH secretion is minimal during juvenile and childhood development and thus, this system is down-regulated; however, when the secretion pattern of the peptide is enhanced, the onset of puberty begins. The ability of low doses of Mn to act centrally to elicit prepubertal LHRH/LH secretion is important and suggests a facilitative role of the metal at the time of puberty by subsequently enhancing testosterone production and spermatogenic development.

Chronic administration of high levels of Mn is known to cause toxic effects to adult male reproduction by decreasing sperm count and motility, thus, resulting in decreased fertility [20,21]. The effects of low, yet slightly elevated levels of the metal during prepubertal development have not been investigated. The present study is the first to show that chronic low level $MnCl_2$ supplementation to immature male rats caused significant increases in the prepubertal levels of serum LH, FSH and testosterone, and that these elevations were associated with increased spermatogenesis compared to controls.

Regarding spermatogenesis, daily sperm production and efficiency, both key indicators of spermatogenesis used in the present study, showed significant increases in the Mn-treated

rats. The values obtained from our control animals were similar to those reported previously for rats at this age [22], but were less than those attained in adult rats [14,22,23]. The fact that the 25 mg/kg dose of Mn enhanced the efficiency of spermatogenesis to a DSP/g of 18 million is important. This increased efficiency, coupled with no change in testicular weight, yielded significantly enhanced daily sperm production per testis in the Mn-treated animals, results which were positively associated with puberty-related hormones. It is well known that LH acts on spermatogenesis via stimulating production of testosterone, and there is existing evidence that both FSH and testosterone are capable of stimulating all phases of spermatogenesis [24]. These authors presented evidence that a combination of FSH and testosterone supported spermatogenesis both qualitatively and quantitatively in a man hypophysectomized for a pituitary tumor. These observations are consistent with the results of our study showing that exposure to low levels of Mn increased puberty-related hormones, and enhanced spermatogenesis. Thus, our results suggest that the Mn-treated rats were maturing at an accelerated rate compared to the age matched control animals.

We have previously demonstrated similar effects in females [10]; however, by comparing results from the two studies we can now suggest a gender difference in responsiveness to this metal. In females, a 10 mg/kg dose of Mn was effective in significantly elevating serum gonadotropins and advancing the time of vaginal opening. In males, a 25 mg/kg dose of Mn was required to significantly elevate serum gonadotropins and accelerate spermatogenesis; hence, suggesting that the males are less sensitive to the hypothalamic influence of Mn than females. Even though a greater oral dose of Mn was required for males, it is important to note that it was still much lower than doses shown to produce neurotoxicological effects in adult rats and primates [25]. One explanation for the males requiring a higher level of Mn exposure could be metabolic variations due to gender differences in Mn metabolism. In this regard, male rats have been shown to clear Mn two times faster than female rats [26].

The onset of puberty in boys before the age of 9.5 years is usually considered precocious [19]. True precocious puberty is due to a premature activation of the LHRH pulse generator, and approximately 10% of these cases are idiopathic. Some underlying cause is inevitable and any substance that can act centrally to induce LHRH release could be involved. Results presented here suggest the potential for slightly elevated levels of Mn to contribute to such an effect. Importantly, we demonstrated conclusively that the site of action of Mn is within the hypothalamus, a brain region in close proximity to the third ventricle. Mn is able to enter the brain either through the cerebral vasculature or via the cerebral spinal fluid, and has been shown to accumulate in the hypothalamus of young animals [10,27]. The mechanism of crossing the blood brain barrier is not completely understood, but likely involves a function of Mn binding to transport systems such as transferrin [28,29]. Mn has been shown to cross the blood brain barrier over four times more efficiently in young animals, which do not have full capacity to eliminate this metal [30]. Although the minimum level of exposure is not well defined [2], infants and children have been classified as being potentially more sensitive to excess Mn [31]. These observations further support the potential for Mn to influence the timing of male puberty.

The fact that Mn has the potential to facilitate pubertal development places it in a unique category of a limited number of substances capable of advancing the timing of puberty. Other substances include insulin like growth factor-1 [17], leptin [32], kisspeptin [33], and excitatory amino acids that can activate the *N*-methyl-D-aspartic acid receptor [34]. Because Mn is a natural element, our results suggest it may be an environmental factor, which may work in concert with the above metabolic signals as well as genetic factors to influence pubertal development. At the present time the mechanism of Mn action to stimulate LHRH release has not been entirely discussed, but we have recent evidence that the stimulatory effects are mediated by the cGMP/protein kinase G pathway [35].

In conclusion, the present data clearly show that exposure to low levels of Mn during juvenile development can induce the prepubertal release of LHRH, causing elevated circulating levels of LH, FSH and testosterone; hence, resulting in increased spermatogenesis commensurate with accelerated pubertal development compared to age matched controls. These results suggest that Mn may play a role at the time of normal pubertal development, but also indicate a possible increased risk for precocious pubertal development if males are exposed to moderately elevated levels of Mn too early in life.

References

[1] Hurley L. Teratogenic aspects of manganese, zinc and copper nutrition. Physiol Rev 1981;61:249–95.
[2] Greger JL. Nutrition versus toxicology of manganese in humans: evaluation of potential biomarkers. Neurotoxicology 1999;20:205–12.
[3] Keen CL, Ensunsa JL, Watson MH, Baly DL, Donavan SM, Monaco MH, et al. Nutritional aspects of manganese from experimental studies. Neurotoxicology 1999;20:213–24.
[4] ATSDR (Agency for Toxic Substances and Disease Registry). Toxicological profile for manganese. US Department of Health And Human Services Public Health Service; 2000 [available at http://www.atsdr.cdc.gov/toxprofiles/tp151.html].
[5] Thibault C, Kennedy G, Gareau L, Zayed J. Preliminary assessment of atmospheric manganese tricarbonyl and particulate manganese in selected urban sites. J Toxicol Environ Health A 2002;65:503–11.
[6] Gray LE, Laskey JW. Multivariate analysis of the effects of manganese on the reproductive physiology and behavior of the male house mouse. J Toxicol Environ Health 1980;6:861–7.
[7] Laskey JW, Rehnberg JF, Hein. Effects of chronic manganese (Mn_3O_4) exposure on selected reproductive parameters in rats. J Toxicol Environ Health 1982;9:677–87.
[8] Boyer PH, Shaw JH, Phillips PH. Studies on manganese deficiency in the rat. J Biol Chem 1942;143:417–25.
[9] Smith SE, Medlicott M, Ellis GH. Manganese deficiency in the rabbit. Arch Biochem Biophys 1944;4:281–9.
[10] Pine M, Lee B, Dearth R, Hiney JK, Dees WL. Manganese acts centrally to stimulate luteinizing hormone secretion: a potential influence on female pubertal development. Toxicol Sci 2005;85:880–5.
[11] Antunes-Rodrigues J, McCann SM. Water, sodium chloride, and food intake induced by injections of cholinergic and adrenergic drugs into the third ventricle of the rat brain. Proc Soc Exp Biol Med 1970;133:1464–9.
[12] Harms PG, Ojeda SR. Method for cannulation of the rat jugular vein. J Appl Physiol 1974;309:261–3.
[13] Hiney JK, Srivastava V, Dearth RK, Dees WL. Influence of estradiol on insulin-like growth factor-1-induced luteinizing hormone secretion. Brain Res 2004;1013:91–7.

[14] Johnson L, Petty CS, Neaves WB. A comparative study of daily sperm production and testicular composition in humans and rats. Biol Reprod 1980;22:1233–43.
[15] Leblond CP, Clermont Y. Definition of the stages of the cycle of the seminiferous epithelium in the rat. Ann NY Acad Sci 1952;55:548–73.
[16] Amann RP, Johnson L, Thompson DL, Pickett BW. Daily spermatozoal production, epididymal spermatozoal reserves and transit time of spermatozoa through the epididymis of the Rhesus monkey. Biol Reprod 1976;15:586–92.
[17] Hiney JK, Srivastava V, Nyberg CL, Ojeda SR, Dees WL. Insulin-like growth factor I of peripheral origin acts centrally to accelerate the initiation of female puberty. Endocrinology 1996;137:3717–27.
[18] Hartter DE, Ramirez VD. Responsiveness of immature versus adult male rat hypothalami to dibutyryl cyclic AMP- and forskolin-induced LHRH release in vitro. Neurotoxicology 1985;40:476–82.
[19] Lee PA. Disorders of puberty. In: Lifshitz F, editor. Pediatric endocrinology. New York: Marcel Dekker, Inc.; 1996. p. 175–95.
[20] Ponnapakkam TP, Bailey KS, Graves KA, Iszard MB. Assessment of male reproductive system in the CD-1 mice following oral manganese exposure. Reprod Toxicol 2003;17:547–51.
[21] Elbetieha A, Bataineh H, Darmani H, Al-Hamood MH. Effects of long-term exposure to manganese chloride on fertility of male and female mice. Toxicol Lett 2001;119:193–201.
[22] Robb GW, Amann RP, Killian GJ. Daily sperm production and epididymal sperm reserves of pubertal and adult rats. J Reprod Tertil 1978;54:103–7.
[23] Johnson L, Lebovitz RM, Simson WK. Germ cell degeneration in normal and microwave-irradiated rats: potential sperm production rates at different developmental steps in spermatogenesis. Anat Record 1984; 209:501–7.
[24] Simoni M, Weinbauer GF, Gromoll J, Niesclag E. Role of FSH in male gonadal function. Ann Endocrinol (Paris) 1999;60:102–6.
[25] Newland MC. Animal models of manganese neurotoxicity. Neurotoxicology 1999;20:415–32.
[26] Zheng W, Kim H, Zhao Q. Comparative toxicokinetics of manganese chloride and methylcyclopentadienyl manganese tricarbonyl (MMT) in Sprague-Dawley rats. Toxicol Sci 2000;54:295–301.
[27] Deskin R, Bursain SJ, Edens FW. Neurochemical alterations induced by manganese chloride in neonatal rats. Neurotoxicology 1980;2:65–73.
[28] Aschner M, Aschner JL. Manganese transport across the blood brain barrier: relationship to iron homeostasis. Brain Res Bull 1990;24:857–60.
[29] Aschner M. Manganese: brain transport and emerging research needs. Environ Health Perspect 2000;108:429–32.
[30] Mena I. The role of manganese in human disease. Ann Clin Lab Sci 1974;4:487–91.
[31] USEPA. Health effects support document for manganese. EPA Report 02-029. US Environmental Protection Agency; 2002.
[32] Chehab FF, Mounzih K, Lu R, Lim ME. Early onset of reproductive function in normal female mice treated with leptin. Science 1997;275:88–90.
[33] Navarro VM, Fernandez-Fernandez R, Castellano JM, Roa J, Mayen A, Barreiro ML, et al. Advanced vaginal opening and precocious activation of the reproductive axis by KiSS-1 peptide, the endogenous ligand of GPR54. J Physiol 2004;561:379–86.
[34] Gay VL, Plant TM. *N*-methyl-D,L-aspartate elicits hypothalamic gonadotropin-releasing hormone release in prepubertal male rhesus monkeys. Endocrinology 1987;120:2289–96.
[35] Lee B, Hiney JK, Pine MD, Srivastava V, Dees WL. Manganese induces hypothalamic LHRH release by directly activating guanylyl cyclase. In: Proceedings of the Annual Meeting of the Endocrine Society. 2006.