Manganese Acts Centrally to Stimulate Luteinizing Hormone Secretion: A Potential Influence on Female Pubertal Development

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Manganese (Mn), an essential element considered important for normal growth and reproduction, has been shown in adults to be detrimental to reproductive function when elevated. Because Mn can cross the blood–brain barrier and accumulate in the hypothalamus, and because it has been suggested that infants and children are potentially more sensitive to Mn than adults, we wanted to determine the effects of Mn exposure on puberty-related hormones and the onset of female puberty. We demonstrated that MnCl₂ when administered acutely into the third ventricle of the brain acts dose-dependently to stimulate luteinizing hormone (LH) release in prepubertal female rats. Incubation of hypothalami in vitro showed that this effect was due to a Mn-induced stimulation of luteinizing hormone releasing hormone (LHRH). Further demonstration that this is a hypothalamic site of action was shown by in vivo blockade of LHRH receptors and lack of a direct pituitary action of Mn to stimulate LH in vitro. To assess potential short-term effects, animals were supplemented with MnCl₂ (10 mg/kg) by gastric gavage from day 12 until day 29, or, in other animals, until vaginal opening (VO). Mn caused elevated serum levels of LH, follicle stimulating hormone, and estradiol, and it initiated a moderate but significant advancement in age at VO. Our results are the first to show that Mn can stimulate specific puberty-related hormones and suggest that it may facilitate the normal onset of puberty. They also suggest that Mn may contribute to precocious puberty if an individual is exposed to elevated levels of Mn too early in development.

Key Words: puberty; manganese; reproduction; luteinizing hormone; hypothalamus; precocious puberty.

INTRODUCTION

Manganese (Mn) is a naturally occurring metal that is required for normal mammalian physiological functions. Both excesses and deficiencies of Mn affect brain function and cause serious health problems. Manganese is a known cofactor for a variety of brain enzymes such as glutamine synthetase and mitochondrial superoxide dismutase (Wedler and Denman, 1984), as well as transferases and hydrolases (Wedler, 1993). It is also necessary for the normal growth and development of bone and cartilage (Hurley, 1981), connective tissue, and the reproductive system (Greger, 1999; Keen et al., 1999).

With regard to reproduction, it has been known for many years that Mn deficiencies in laboratory animals are associated with impaired growth and reproduction in both sexes (Boyer et al., 1942; Smith et al., 1944); hence, suggesting a role in reproductive function. While it is known that exposure to high levels of Mn is toxic and causes developmental reproductive dysfunction (Gray and Laskey, 1980; Laskey et al., 1982), it is not known whether exposure to lower, but still elevated levels of the metal would facilitate or inhibit neuroendocrine development of reproductive function. This is important because infants and children have been identified as being potentially more sensitive to Mn and because the optimum level of oral Mn exposure is not well understood (EPA, 2002; Greger, 1999). Because Mn has been shown to cross the blood–brain barrier over four times more efficiently in young versus adult animals (Mena, 1974), and because it can accumulate in the hypothalamus (Deskin et al., 1980), we hypothesized that it may influence the neuroendocrine system prior to puberty. Thus, the present study assessed whether this natural environmental metal is involved in the hypothalamic control of prepubertal luteinizing hormone (LH) secretion, and whether short-term, low-dose exposure of the metal to prepubertal animals would affect levels of puberty-related hormones and alter the timing of female 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 that contained 94.7 mg/kg Mn and 149.8 mg/kg iron (Fe), as analyzed by the Heavy Metal Analysis Laboratory, Department of Veterinary Integrative Biosciences, College of Veterinary Medicine, Texas A&M University.
procedures used were approved by the University Animal Care and Use Committee and in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals.

**Effect of centrally administered Mn on LH secretion.** Twenty-three-day-old female rats were anesthetized with 2.5% trichloremethanol (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 (Antunes-Rodrigues and McCann, 1970). When the rats were 29 days old, they were anesthetized with 2.5% trichloremethanol, and istalactic cannulae were inserted into the right external jugular vein of each rat (Harms and Ojeda, 1974). The next day, an extension of tubing was attached to each cannula and flushed with heparinized saline (100 IU/ml). After a 1-h acclimation period, three basal blood samples (200 μl) were drawn from each freely moving animal at 15-min intervals. A third ventricular injection of 1.0, 2.5, 5.0, 10.0, or 25.0 μg/3.0 μM MnCl₂ (MnCl₂ tetrahydrate, Sigma Chemical Company, St. Louis, MO) or an equal volume of saline was administered immediately after the third basal sample. After the respective injection, four more samples were taken at 15-min intervals for a total of seven samples. After the experiment, animals were euthanized with an overdose of trichloremethanol, and brains were examined for proper cannula placement. Animals were confirmed to be in the juvenile phase of development by criteria we have previously used (Dees and Skelley, 1990). Blood samples were centrifuged at 4°C, and serum was stored at −80°C until assayed for LH.

**Effects of Mn on LHRH release from hypothalami in vitro.** The ability of Mn to induce luteinizing hormone releasing hormone (LHRH) release directly from the medial basal hypothalamus (MBH) incubated in *vitro* was evaluated. In this regard, 30-day-old female rats were decapitated, and the MBH was removed and incubated as described elsewhere (Hiney et al., 1999), with minor modifications. Briefly, each MBH was incubated in a vial containing 350 μl of Locke's buffer (2 mM Hepes, 154 mM NaCl, 5.6 mM KCl, 1 mM MgCl₂, 6 mM NaHCO₃, 10 mM glucose, 1.25 mM CaCl₂, and 1 mg/ml BSA, pH 7.4) inside a Dubnoff shaker (80 cycles/min) at 37°C in an atmosphere of 95% O₂ and 5% CO₂ for 60 min. This medium was discarded, and all APs were incubated in fresh medium for 30 min to establish basal LH release. The medium was removed, boiled for 10 min, and stored in microcentrifuge tubes, and replaced with medium containing 0, 50, 250, or 500 μM MnCl₂. The MBHs were incubated for an additional 30 min. This medium was collected, boiled for 10 min, and stored at −80°C until assayed for LHRH. MBHs were weighed to the nearest 0.01 mg.

**Effect of LHRH receptor antagonism on Mn-induced LH release.** Third ventricular and external jugular cannulae were implanted as described above. The next day, 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 (10 μg), a potent LHRH antagonist which was 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 remaining animals received an injection of equal volume saline. After a 2-h absorption period for the acyline, three basal blood samples were drawn from each freely moving animal at 15-min intervals. Immediately after the third basal sample, a third ventricular injection of MnCl₂ (10 μg) was administered to all of the animals. Four more samples were taken at 15-min intervals for a total of seven samples. After the experiment, the rats were euthanized with an overdose of trichloremethanol, and the brains were examined for proper cannula placement. Animals were confirmed to be in the juvenile phase of development by criteria we have previously used (Dees and Skelley, 1990). Blood samples were centrifuged at 4°C, and serum was stored at −80°C until assayed for LH.

**Effect of Mn on LH release from pituitary glands in vitro.** Thirty-day-old female rats were decapitated, and pituitaries were removed and incubated as described previously (Hiney et al., 1999). Briefly, the posterior lobe was removed and each anterior pituitary (AP) was incubated in a vial containing 1.0 ml of Krebs-Ringer bicarbonate (pH 7.4) containing glucose (4.5 mg/ml) inside a Dubnoff shaker (80 cycles/min) at 37°C in an atmosphere of 95% O₂ and 5% CO₂ for 60 min. This medium was discarded, and all APs were incubated in fresh medium for 30 min to establish basal LH release. The medium was removed and stored in microcentrifuge tubes, and replaced with medium containing 100 μM MnCl₂. The APs were incubated for an additional 30 min. This medium was collected and stored at −80°C until assayed for LH. Pituitaries were weighed to the nearest 0.01 mg.

**Effect of short-term Mn exposure on puberty-related hormones and the onset of female puberty.** Rats were bred and allowed to deliver their pups normally, at which time litters were adjusted to 8–11 pups with at least 5–6 females per litter. In a preliminary study, we delivered 5, 10, 25, and 100 mg/kg/day MnCl₂ by gastric gavage to female pups beginning on postnatal day 12. The minimum effective dose to advance puberty was 10 mg/kg, thus this dose was used in the following experiments. In the first experiment, MnCl₂ (10 mg/kg; 0.25 mg in 0.2 ml/25 g rat) or an equal volume of saline was administered daily by a single gastric gavage injection from day 12 until day 29, at which time the rats were killed by decapitation, the brain was removed, and the preoptic area (POA) and MBH were isolated and then stored at −80°C until Mn analysis. Trunk blood was collected for hormonal and Mn assessments. A second experiment was conducted exactly as above, except that the animals continued to be dosed with MnCl₂ until vaginal opening (VO) occurred.

**Hormone analysis.** Rat LH and follicle-stimulating hormone (FSH) were measured using radioimmunoassay (RIA) procedures as previously described (Hiney et al., 1996). The rat LH antiserum (NIDDK-anti-LH-S-II), antigen (NIDDK-anti-LH-I-9), reference preparation (NIDDK-rLH-RP-3), FSH antiserum (NIDDK-anti-FSH-S-I-9), and reference preparation (NIDDK-rFSH-RP-2) were purchased from the NIH Pituitary Hormones & Antiserum 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. Serum estradiol (E₂) was measured using an RIA kit purchased from Diagnostic Products Corp. (Los Angeles, CA) as previously described (Hiney et al., 1996). The E₂ assay sensitivity was 80 pg/ml. All assays had inter- and intra-assay coefficients of variation of <10%. The LH assay was measured as previously described (Harter and Ramirez, 1985) using Antiseria N0.R11B73 kindly provided by Dr. V. D. Ramirez. Synthetic LH used for the standards and inquisitions was purchased from Sigma Chemical Co. (St. Louis, MO). The sensitivity of the assay was 0.2 pg/tube, and the intraassay coefficient of variation was <10%.

**Metal analysis.** Manganese was measured by the Heavy Metal Analysis Laboratory, Department of Veterinary Integrative Biosciences, College of Veterinary Medicine, Texas A&M University. A PerkinElmer/Sciex DRC II inductively coupled plasma-mass spectrometer was used. Tissue digestion followed the method of da Silva et al. (1998) with the following modifications: 20–40 mg of wet tissue sample and QA/QC samples (SRM, spiked samples, sample duplicates, blank, and blank spike) were digested with 200 μl of H₂O₂ and 100 μl of HCl at 90°C for 6 h. The volume was then brought up to 10 ml. The method was considered in control when the SRMs, spike recoveries, and duplicates were ±15% of the expected value and blank values were <0.001 ppb.

**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 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.
Animals that received saline or the 1.0 μg dose of MnCl₂ exhibited no change in LH released as compared to their respective basal levels. However, animals which received 2.5 μg, 5.0 μg, and 25 μg doses of Mn showed marked increases in LH secretion when compared to their respective basal levels. We suggest that this is a hypothalamic site of action, and the effect is exerted by very low doses because the Mn is further diluted by cerebrospinal fluid in the third ventricle and because only some of the Mn diffuses into the basal hypothalamus to apparently induce LHRH secretion.

Effect of Mn on LHRH Release from Hypothalamus In Vitro

Because LHRH cannot be measured accurately in serum, in vitro studies using the MBH were conducted. The MBH was removed from 30-day-old female rats and incubated with three different concentrations of MnCl₂. The addition of MnCl₂ to the medium stimulated the release of LHRH in a dose-dependent fashion as compared to basal levels (Fig. 2). MBHs incubated in Locke’s Buffer without MnCl₂ showed no change in LHRH secretion over time, whereas those incubated in the buffer with 50 μM, 250 μM, and 500 μM concentrations of MnCl₂ showed significant increases in LHRH secretion when compared to their respective basal levels. Values represent mean ± S.E.M. The number of samples is depicted within each panel (*p < 0.05; **p < 0.02).

Effect of Mn on LH Release from Pituitary Glands In Vitro

In vitro incubation of hemipituitaries with 100 μM MnCl₂ indicated that MnCl₂ was incapable of stimulating LH release from the pituitary gland directly. In this regard, basal levels of secreted LH were 16.9 ± 2.4 ng/mg, and LH levels after a pretreated with acyline showed no significant change in LH levels after MnCl₂ administration (0.35 ± 0.04 ng/ml vs. 0.57 ± 0.02 ng/ml) (Fig. 3).

FIG. 1. The effect of third ventricular administration of MnCl₂ on LH release during the late juvenile phase of developing female rats. Each concentration point indicates basal LH levels vs. stimulated levels. The animals which received the saline and the 1.0 μg dose of MnCl₂ showed no significant changes in LH secretion when compared to basal levels. However, animals which received 2.5 μg, 5.0 μg, and 25 μg doses of Mn showed marked increases in LH secretion when compared to their respective basal levels. Values represent mean ± S.E.M., and the number of animals is depicted within each panel (*p < 0.05; **p < 0.01; ***p < 0.001).

FIG. 2. The effect of MnCl₂ on LHRH release from the medial basal hypothalamus in vitro. Each concentration point indicates basal LHRH levels versus stimulated levels. Tissue incubated in Locke’s buffer only showed no significant changes in LHRH secretion when compared to basal levels. Tissues incubated in buffer containing 50 μM, 250 μM, and 500 μM concentrations of MnCl₂ showed significant increases in LHRH secretion when compared to their respective basal levels. Values represent mean ± S.E.M. The number of samples is depicted within each panel (*p < 0.05; **p < 0.02).

FIG. 3. The effect of pretreatment with the LHRH receptor antagonist, acyline, on MnCl₂ stimulated LH release. Acyline-treated animals showed no significant change in LH release after third ventricular administration of MnCl₂ as compared to basal levels. Animals which were not pretreated with acyline exhibited a four-fold increase in LH as compared to basal levels (**p < 0.01). Values represent the mean ± S.E.M. The number of animals is depicted within each panel.
30-min incubation with MnCl₂ were 14.7 ± 6.8 ng/mg (n = 13), further suggesting a hypothalamic action.

**Effect of Short-Term Mn Exposure on Puberty-Related Hormones and the Onset of Female Puberty**

Short-term exposure to MnCl₂ during juvenile development caused increases in specific puberty-related hormones. In this regard, the serum levels of LH, FSH, and E₂ were all elevated (p < 0.05) by 29 days of age in Mn-treated versus control animals (Fig. 4). The dose and regimen of MnCl₂ administration produced an accumulation of Mn in the brain, resulting in elevated Mn levels in both the POA (p < 0.02) and the MBH (p < 0.05) when compared to controls (Fig. 5). As expected, serum Mn levels were not different from the controls at the time of blood collection (not shown) because a very low oral dose of MnCl₂ was used, and the rats did not receive the dosage on the final day of the experiment. When the dosing regimen was continued for several more days to assess the timing of puberty, we observed that short-term MnCl₂ exposure did not alter mean (± S.E.M.) daily weight gain (controls, 3.8 ± 0.07 g/day; Mn-treated, 3.7 ± 0.08 gm/day), but advanced (p < 0.001) the age at VO compared with saline controls. Specifically, mean (± S.E.M.) VO was 32.8 ± 0.21 days compared with 34.3 ± 0.22 days in the controls (Fig. 6), a 1.5-day advancement in the onset of puberty that appears to be moderate in terms of time, but a highly significant trend.

**DISCUSSION**

The results of this study are the first to show a stimulatory action of Mn on the hypothalamic control of prepubertal gonadotropin secretion, and the ability of short-term Mn exposure to moderately accelerate the onset of female puberty. The dose used for the short-term studies was low compared to previous studies using rats, and is comparable on a mg/kg basis to that which has been shown to have effects on movement-response tests in adult primates (Newland and Weiss, 1991). Although different end points were assessed, the low-level effect of Mn we described is important because many of the detrimental, neurotoxicological end point effects of the metal in adult rats and primates occurred after much higher doses (Newland, 1999). In the present study Mn facilitated specific puberty-related events, such as increased gonadotropins and estradiol. Because Mn appears to be involved in events leading to the onset of female puberty, we suggest the possibility that a Mn-induced activation of the LHRH releasing system too early in childhood may place an individual at risk for precocious pubertal development.
Obviously, more basic research is needed in this area, and epidemiological studies as well as experiments using primates would be helpful to more clearly assess the possible relationship between Mn levels and precocious puberty in children. Although the present study used rats, the results demonstrated a clear relationship between Mn and LHRH/LH secretion and thus raised several points worthy of discussion with regard to the potential influence of Mn on 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 culminates in the increased secretion of LHRH. This increase appears to require interactive participation of neuronal circuitries and glial cells within the hypothalamus (Ojeda and Urbanski, 1994), which are likely influenced by metabolic signals of peripheral origin, as well as genetic and environmental influences (Parent et al., 2003). Insulin-like growth factor-1 (IGF-1) is a peripheral metabolic signal that we have shown can stimulate prepubertal LHRH release (Hiney et al., 1996), an event that causes advanced onset of female puberty in rats (Hiney et al., 1996) and primates (Wilson, 1998). Influences that can stimulate excitatory amino acid receptors are also capable of inducing prepubertal LHRH release and advancing the timing of female puberty in both rats (Nyberg et al., 1993; Urbanski and Ojeda, 1990) and primates (Gay and Plant, 1987). Despite the normal variation in the timing of puberty, in recent years evidence has been presented that suggests puberty, especially in females, may be occurring at an earlier age (Herman-Giddens et al., 1997; Parent et al., 2003); although the mean age at menarche appears to have stabilized in both the United States and Europe (Apter and Hermanson, 2002). At the present time the cause of this possible trend is not known, but it has been suggested that assessing the onset of puberty may be a sensitive marker of interactions between environmental conditions and genetic susceptibility that may influence the pubertal process (Parent et al., 2003).

The onset of puberty before the age of 8 years in girls and the age of 9.5 years in boys is usually considered precocious (Lee, 1996). A “true” precocious puberty is due to central causes, whereas “pseudo” precocious puberty is due to peripheral causes. The central form can begin anytime after birth, is LHRH-dependent, and is characterized by hormonal changes similar to those that occur at the normal time of puberty. Such changes are increased pulsatile LH secretion, increased LH response to LHRH, and increased gonadal steroid secretion. Thus, in central precocious puberty there is a premature activation of the LHRH pulse generator. In boys, this activation is usually accounted for by hypothalamic hamartomas, other central nervous system lesions, or familial disease with less than 10% being idiopathic. In girls, however, over 65% of the cases are considered idiopathic. There must be some underlying cause(s), and any substance that can act centrally to stimulate LHRH release could possibly be involved.

The present study indicates that Mn may be a candidate for such an action. Manganese is able to enter the brain either through the cerebral vasculature or via the cerebral spinal fluid (CSF). 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 (Aschner, 2000; Aschner and Aschner, 1990). As blood levels rise, influx into the CSF rises and entry across the choroid plexus becomes more important (Murphy et al., 1991). The POA and hypothalamus are adjacent to the third ventricle and receive Mn from both capillaries and the CSF. Importantly, Mn has been shown to cross the blood–brain barrier over four times more efficiently in young animals compared with adults (Mena, 1974), and young animals do not have the full capacity to eliminate Mn (Fechter, 1999). Additionally, infants and children have been classified as being potentially more sensitive to excess Mn exposure (EPA, 2002), in large part because the optimum level of exposure is not well defined (Gregor, 1999). Gender differences in Mn metabolism have also been observed, with male rats clearing Mn two times faster than female rats (Zheng et al., 2000).

An early elevation of Mn, especially in females, could pose a risk for precocious sexual development. This may not necessarily mean high levels, but it may indicate that if Mn accumulates in specific brain regions too early in life and reaches levels not normally attained until later, then a potential problem could arise. Our results support this possibility because we noted increased Mn accumulation in both the POA and— as others have shown—in the hypothalamus (Deskin et al., 1980), which is directly responsible for synthesis and secretion of LHRH.

Environmental factors that may elevate Mn levels should also be considered as possible candidates that may alter the timing of puberty. Some regions have high levels of Mn in the drinking water, and certain foods are high in Mn. This could become an even greater concern in areas where diets are iron deficient. Both Mn and Fe are able to use a transferrin-dependent transport system (Malecki et al., 1999); thus Fe deficiency has been shown to potentiate the absorption and accumulation of Mn (Erikson et al., 2002).

In conclusion, our results demonstrate that Mn acts centrally to stimulate prepubertal LHRH/LH release, and can modestly advance the onset of puberty. 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 priming (Lee, 1996). Secretion of LHRH is minimal during juvenile and childhood development, and thus this system is downregulated; however, when the secretion pattern of the peptide is enhanced, puberty begins. The present results clearly show that Mn can induce LHRH secretion at this critical time of development. Whether this is a direct action on the LHRH neuronal system or an indirect action to activate or facilitate a known modulator of LHRH release will require further investigation. Also, the potential exists that early childhood exposure to elevated levels of Mn could affect the timing of puberty. Epidemiological...
research in children and experimental studies in primates could help address this important question with regard to child health.

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