ACTION OF MANGANESE ON PUBERTY

A Dissertation

by

BO YEON LEE

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

May 2007

Major Subject: Toxicology

ACTION OF MANGANESE ON PUBERTY

A Dissertation

by

BO YEON LEE

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

Approved by:

Chair of Committee,	W. Les Dees
Committee Members,	Gerald Bratton
	Weston Porter
	Shashi Ramaiah
Chair of Toxicology Faculty,	Robert Burghardt

May 2007

Major Subject: Toxicology

ABSTRACT

Action of Manganese on Puberty. (May 2007)

Bo Yeon Lee, B.S., Chonnam National University

Chair of Advisory Committee: Dr. W. Les Dees

Manganese (Mn) is considered important for normal growth and reproduction. 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 puberty, and discern the site and mechanism of Mn action.

We demonstrated that the central administration of manganese chloride ($MnCl_2$) stimulated luteinizing hormone (LH) release in prepubertal rats. Incubation of medial basal hypothalamus (MBH) *in vitro* showed 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 the lack of a direct pituitary action of Mn to stimulate LH release *in vitro*. Chronic supplementation of low dose of $MnCl_2$ caused elevated serum levels of LH, follicle stimulating hormone (FSH) and estradiol or testosterone. Importantly, Mn supplementation advanced the timing of puberty in both sexes.

We investigated the mechanism by which Mn induces LHRH/LH release from the hypothalamus. Blocking the NMDA receptor, IGF1 receptor, or inhibiting nitric oxide synthase *in vivo* was ineffective in altering Mn-induced LH release. Dose-

response, pharmacological blockade and nitrite assessments indicated that the lowest doses of Mn used stimulated LHRH release, but did not induce nitric oxide (NO) production, while only the highest dose of Mn stimulated NO. Conversely, a dose-dependent inhibition of Mn-induced LHRH release was observed in the presence of ODQ, a specific blocker of soluble guanylyl cyclase. Furthermore, Mn stimulated the release of cyclic GMP (cGMP) and LHRH from the same MBH, and a protein kinase G (PKG) inhibitor, KT5823, blocked Mn-induced LHRH release.

Collectively, these data demonstrate that Mn can stimulate specific puberty-related hormones both acutely and chronically, and furthermore, suggest that low levels of Mn facilitate the normal onset of puberty. The principal action of Mn within the hypothalamus is to facilitate the activation of guanylyl cyclase, which subsequently stimulates the cGMP/PKG pathway resulting in the stimulation of prepubertal LHRH secretion.

DEDICATION

To my husband, Hyeong-il Kwak,

my parents, Jaewoo Lee and Sunghun Park,

and my pastors, Chong Kim and Youngsik Ahn.

ACKNOWLEDGEMENTS

I wish to thank my major advisor, Dr. W. Les Dees, for his patience and support throughout my graduate career. I am also grateful to the members of my dissertation committee, Dr. Gerald Bratton, Dr. Weston Porter, and Dr. Shashi Ramaiah.

I would also like to thank the following colleagues for their help and friendship: Dr. Jill Hiney, Dr. Michell Pine, and Dr. Vinod Srivastava. I would also like to acknowledge Kim Daniel for her administrative assistance.

TABLE OF CONTENTS

CHAPTER Page

LIST OF FIGURES

FIGURE Page

CHAPTER I

INTRODUCTION

Manganese (Mn) is a naturally occurring elemental metal that is required for normal mammalian physiological processes, including growth and development of bones and cartilage (Hurley, 1981), as well as connective tissue and the reproductive system (Greger, 1999; Keen *et al*.,1999). Furthermore, Mn plays roles in metabolic regulation, as well as protein and energy metabolism by participating as a catalytic or regulatory factor for enzymes. The classes of enzymes that have Mn as a cofactors are very broad and include oxidoreductases, transferases, hydrolases, lyases, isomerases, ligases, lectins, and integrins (Wedler and Denman, 1984; Keen *et al*., 2000; Takeda A., 2003).

Everyday, people are exposed to Mn through food, air, soil, and water. Mn can enter the environment from iron, steel, and power plants into air, and from mining operations into dust, and natural deposits, disposal of wastes, or deposits from airborne sources into the water and soil. This metal is used for various purposes and products including batteries, fertilizers, pesticides, ceramics, and dietary supplements. Individuals who work in occupations that mine Mn or improperly use pesticides such as maneb and mancozeb are likely to be exposed to excess levels through their work environment (Gerber *et al*., 2002). Exposure to methylcyclopentadienyl manganese tricarbonyl (MMT), a fuel additive in some gasolines, is another source of exposure (EPA, 2001).

This dissertation follows the style of *Toxicological Sciences*.

Absorption of Mn is affected by an individual's age, route of exposure, the chemical form and to other dietary factors (USEPA, 2002). Intake varies greatly, depending mainly on the consumption of rich sources, such as unrefined grains, cereals, green leafy vegetables, and tea. The usual oral intake of this mineral is 2 to 5 mg/day, and absorption is 5 to 10% (Greger, 1998).

Both excesses and deficiencies of Mn are known to cause serious health problems. Exposure to high levels of airborne manganese can cause Parkinsonism-like symptoms (Jankovic, 2005; Calne *et al*., 1994). Industries associated with manganese emissions include ferroalloy production, iron and steel foundries, metal fumes from welding, battery production, and power plant and coke oven combustion emissions (Lioy, 1983). The mechanisms of Mn neurotoxicity have become an important area of concern because excesses of this element can cause numerous central nervous system (CNS) disorders (Beuter *et al*., 1994; Pal *et al*., 1999). Exposure to high levels of the metal also can cause respiratory problems and sexual dysfunction (Aschner *et al*., 2005). Conversely, low levels of manganese in the body have been associated with infertility, bone malformation, weakness, and seizures (Freeland-Graves and Llanes, 1994; Keen *et al*., 1999). Mn deficiencies are considered rare, since it is relatively easy to obtain adequate amounts of manganese through the diet. However, it has been known for a long time that Mn deficiencies are associated with impaired growth and reproduction in females and males (Boyer, 1942; Smith, 1944), suggesting important roles in these processes.

Because of its potential role in reproductive function, we questioned whether Mn plays a role in adolescent development and the pubertal process. There have been only a few studies assessing Mn exposure in immature animals. These studies were not done specifically to discern puberty related events and the onset of puberty and used high doses of different forms of Mn, and the Mn insult was either removed before the peripubertal period or hormones were assessed after castration. Also, the studies were conducted before accurate, sensitive luteinizing hormone (LH) assays were available for rodents and we could find no attempt to critically discern the actual timing of puberty (USEPA, 2002). Preliminary data for this study suggested that low but elevated levels of manganese chloride ($MnCl_2$**)** advanced the onset of puberty, and that this was associated with elevated puberty-related hormones. Should Mn levels in brain be elevated even moderately at an early age, this may activate luteinizing hormone releasing hormone (LHRH) secretion or facilitate an endogenous modulator of LHRH release; therefore, placing an individual at risk for a precocious pubertal development.

Precocious puberty is defined as the onset of puberty before the age of 8 in girls and 9 in boys (Lee, 1980). Recently, it has been reported that there has been a trend for earlier onset of the puberty. A “true” precocious puberty is due to central causes and reflects increased gonadotropins, where as “Pseudo” precocious puberty is due to peripheral causes. Central precocious puberty can begin anytime after birth, and is hypothalamic LHRH-dependent, and characterized by hormonal changes similar to those that occur at the normal time of puberty. The classic changes noted are an increased afternoon pulsatile secretion of LH, an increased LH responsiveness to LHRH, and

increased gonadal steroid secretion. Thus, in central precocious puberty there is a premature activation of the LHRH pulse generator. In boys, such activation is usually accounted for by hypothalamic hamartomas, other CNS lesions or familial disease, with less than 10% being idiopathic. In girls, however, it is much different with over 65% being idiopathic with no identifiable cause. There must be some underlying pathophysiological causes and any substance that can act centrally to stimulate LHRH release could be involved in the initiation of precocious pubertal development.

Precocious puberty also places children at risk for not achieving their genetic height potential. The rapid maturation of the growth plate will often result in temporary acceleration of linear growth, but the accompanying early closure of growth plates results in early cessation of growth and ultimately shorter than what would be expected for their adult height (Wheeler and Styne, 1990). Precocious puberty can be an early sign of metabolic syndrome (syndrome X), obesity, and insulin resistance (Ibanez, 1997; Pathomvanich, 2000).

The possibility exists that Mn is beneficial as an environmental factor that works in concert with other substances to facilitate LHRH release at the normal time of puberty. Interestingly, if this is the case then it is also possible that an early elevation, especially in females, could activate the LHRH releasing system prematurely. This may not necessarily mean very high levels, but if Mn accumulates in the brain too early in life and reaches levels that normally are not attained until later, then this may actually be harmful. Other indicators that support the possibility that Mn may accumulate in brain are the observations of high levels of Mn in the drinking water in some regions, concern

for high levels in some baby formulas (Lonnerdal *et al*., 1987), and increased amounts of MMT in the air from the gasoline. It may mean that any one of these in excess could be harmful, or it could take a combination of low level insults. Infants and children have been identified as being potentially more sensitive for excess Mn exposure (USEPA, 2002). This is largely because the optimum level of oral exposure is not well defined (Greger, 1999), some evidence in animals that the young do not yet have full capacity to eliminate Mn (Fechter, 1999), and because the tissue accumulation and health effects in humans, especially children, are not well understood.

Mn enters the brain either through cerebral vasculature or via the cerebrospinal fluid (CSF). The mechanism of crossing the blood brain barrier (BBB) is not completely understood, but likely involves binding of Mn to transport mechanisms such as transferrin (Aschner and Aschner, 1990; Aschner, 2000). As blood levels rise, influx into CSF increases and entry across the choroid plexus becomes more important (Murphy *et al*., 1991). Brain regions, like the preoptic area (POA) and the hypothalamus, which are adjacent to the third ventricle and responsible for LHRH production and secretion, respectively receive Mn from both capillaries and CSF. Mn has been shown to cross the blood-brain barrier over 4 times more efficiently in young vs. adult animals (Mena, 1974) and can accumulate in the hypothalamus (Deskin, 1980). This suggests the need to further investigate the actions of Mn on the hypothalamic control of the onset of puberty.

The CNS plays the critical role in bringing together events that lead to the onset of puberty by controlling both anterior pituitary function via hypothalamic hormones and ovarian function via pituitary hormones and direct neural inputs. The physiological

pattern of LH and follicle-stimulating hormone (FSH) secretion is periodic and intermittent, although more pronounced for LH. Before the onset of puberty the amplitude of release is low and after onset, the release becomes more prominent in the afternoon. This basic pattern is similar in both rats and humans (Urbanski and Ojeda, 1990; Delemarre-Van De Waal *et al*., 1991) and is a centrally driven event reflective of the pulsatile release of hypothalamic LHRH (Knobil, 1980*).* The current understanding is that the pituitary and gonad are capable of function at any age after a short period of priming (Lee, 1996). LHRH secretion is minimal during juvenile and childhood development and thus, this system is down-regulated; however, when the pulsatile frequency and amplitude is enhanced, the onset of puberty begins. The exact signal initiating this change in LHRH release is not known, but may be due to removal of an inhibitory tone or more likely, to completion of the development or activation of excitatory inputs.

Recently, in rats and primates, activation of a specific class of brain excitatory amino acid receptors, namely N-methyl-DL- aspartic acid receptors (NMDA-R; Gay and Plant, 1987; Urbanski and Ojeda, 1987), and activation of insulin-like growth factor-1 receptors (IGF1-R; Hiney *et al*., 1996; Wilson, 1998) have been shown to stimulate LHRH release and advance female puberty. Normally, as puberty approaches the LHRH/ LH levels rise, estradiol (E_2) increases and the central component of the E_2 positive feedback on the hypothalamus is enhanced. During this phase there is an increased hypothalamic capacity to synthesize prostaglandin E_2 (PGE_2), a compound known to mediate norepinepherine (NE)-induced LHRH release (Ojeda *et al*., 1979). NE is an

important neurotransmitter downstream in the pathway of both IGF-1 receptors and NMDA receptors, and appears to participate in the first preovulatory surge of LH (Sarkar *et al*., 1981). E_2 not only increases PGE_2 production prior to the first surge of gonadotropins (Claypool *et.al*., 2000), but also aids in neuronal growth and maturation of synaptic connections (Matsumaoto and Arai, 1977). As E_2 levels gradually rise, they eventually reach levels high enough to produce the preovulatory LHRH and gonadotropin surges and subsequently, first ovulation and reproductive maturity. Importantly, the complex events within the hypothalamus that lead to LHRH release and puberty appear to require interactive participation of neuronal circuitries and glial networks of the hypothalamus (Ojeda, 1994) (Fig. 1.1).

It has been shown that Mn is capable of permeating the NMDA-R channel (Mayer and Westbrook, 1987). Mechanisms of LHRH release after these receptors have been activated are not completely worked out; however, downstream from these receptors the pathways do appear to share some common functional sites such as the presence of nitric oxide synthase/ nitric oxide (NOS/NO), and when the system is activated it causes increased PGE_2, and finally, LHRH release (Ojeda *et al*., 1979; Rettori *et al*., 1993; Hiney *et al*., 1998). When NO diffuses into LHRH terminals it may activate guanylyl cyclase which activates the cyclic GMP (cGMP)/protein kinase G (PKG) pathway, triggering LHRH secretion. NMDA-R activated LHRH release may also utilize the cyclic GMP (cGMP)/protein kinase G (PKG) pathway (Karanth *et al*., 2003).

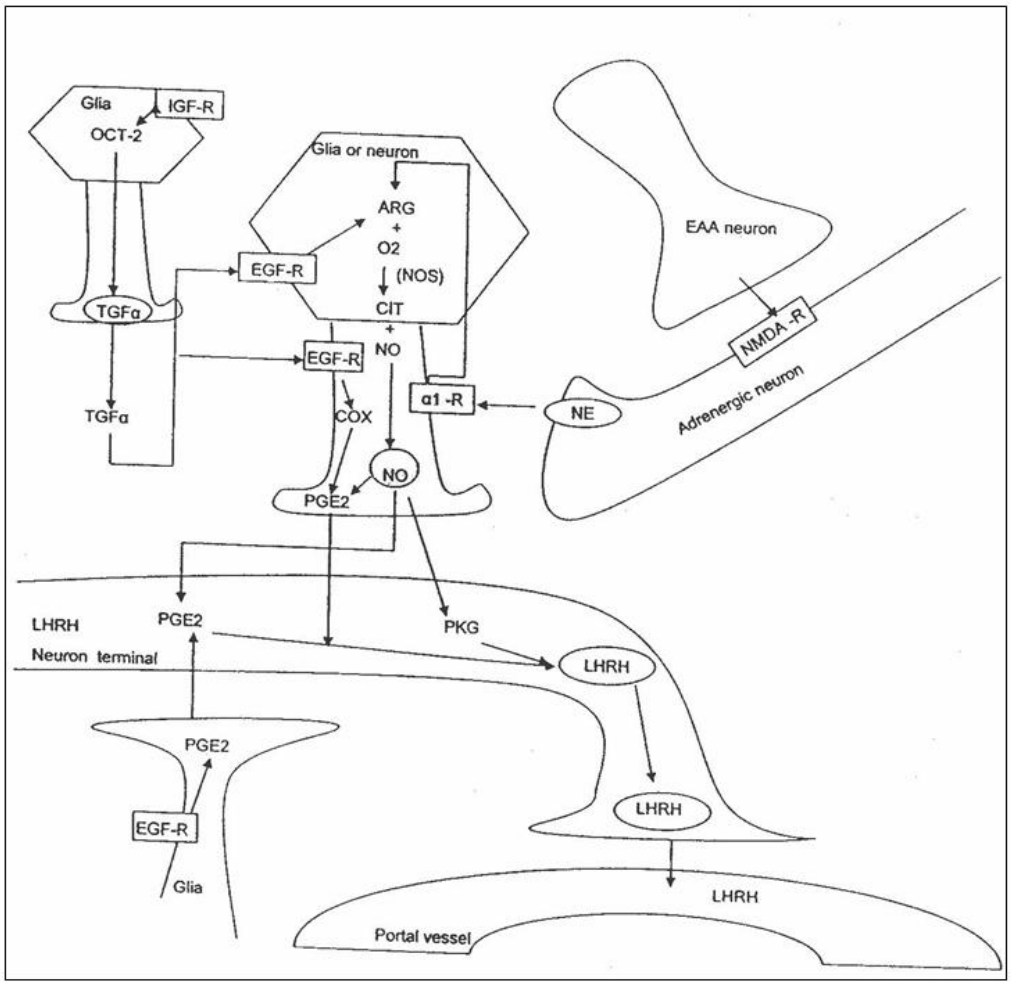

Figure 1.1. Schematic drawing of the hypothalamic excitatory inputs controlling LHRH secretion.

All arrows represent stimulatory actions. IGF-R, insulin-like growth factor receptor; OCT 2, POU homeodomain gene; TGFα, transforming growth factor α; EGF-R, epidermal growth factor receptor; ARG, arginine; CIT, citruline; NOS, nitric oxide synthase; NO, nitric oxide; EAA, glutamate and aspartate neuron; NMDA-R, N-methyl-D, L-aspartic acid receptor; NE, norepinephrine; α1R, α 1 adrenergic receptor; PKG, protein kinase G; COX, cyclooxygenase; PGE_2, prostaglandin-E_2

It is also possible that Mn could stimulate other components that are beginning to emerge as being potentially important for the onset of puberty. In this regard, Mn could induce or facilitate expression of the POU homeodomain gene which increases in the

female hypothalamus as puberty approaches. This is associated with increased hypothalamic content of transforming growth factor-α (TGFα) (Ojeda *et al*., 1999). TGFα binds to the epidermal growth factor-receptor (EGF-R) in the medial basal hypothalamus (MBH) and the activation of this receptor results in LHRH release (Ojeda *et al*., 1990; Hiney *et al*., 2002). Another possibility is for Mn to enhance hypothalamic IGF-1 and glutamate, suggesting increased availability of these naturally occurring ligands for IGF-1 and NMDA receptors within the POA and medial basal hypothalamus (MBH); the sites responsible for synthesis and secretion of LHRH.

The present study will assess the ability of Mn to contribute to pubertal development and will begin to discern mechanisms of Mn action. We hypothesize that Mn may precociously activate the reproductive control center in the hypothalamus to stimulate the secretion of LHRH; hence initiating the onset of puberty. We will use both in vivo and in vitro approaches to identify the hypothalamic site and mechanism of this action.

CHAPTER II

MANGANESE ACTS CENTRALLY TO STIMULATE LUTEINIZING HORMONE SECRETION: A POTENTIAL INFLUENCE ON FEMALE PUBERTAL DEVELOPMENT

INTRODUCTION

Manganese (Mn) is a naturally occurring elemental metal that is required for normal mammalian physiological functions. Both excesses and deficiencies of Mn affect brain function and cause serious health problems. Mn 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). Mn 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 are associated with impaired growth and reproduction in females and males (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 since 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 (USEPA, 2002; Greger, 1999). Since Mn has been shown to cross the blood-brain barrier over four times more efficiently in young vs. adult animals (Mena, 1974), and 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 LH secretion, and whether chronic, 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

Immature female rats of the Sprague-Dawley line that were raised in our colony at the Texas A&M University Laboratory Animal Facility were used for these experiments. The animals were housed under controlled conditions of photoperiod (lights on, 0600 hr, lights off, 1800 hr) and temperature (23 ºC), with ad libitum access to food and water. The diet was Harlan Teklad 2016 which contained 94.7 mg/kg Mn and 149.8 mg/kg iron (Fe) as analyzed by the Heavy Metal Analysis Laboratory, Department of 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.

Effect of centrally administered Mn on LH secretion

Twenty-three-day-old female rats were stereotaxically implanted with a stainless steel cannula (23 gauge) in the third ventricle (3V) of the brain and allowed five days for recovery (Antunes-Rodrigues and McCann 1970). When the rats were 29 days old, silastic 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). Following a one hour acclimation period, three basal blood samples were drawn from each freely moving animal at 15-minute intervals. A 3V injection of manganese chloride ($MnCl_2$; 1.0, 2.5, 5.0, 10.0, or 25.0 μg/ 3.0 μl; Sigma Chemical Company, St. Louis, Mo) or an equal volume of saline immediately followed the third basal sample. After the respective injection, four more samples were taken for a total of seven samples. After the experiment, brains were examined for proper cannula placement, and 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 –70 °C until assayed for LH.

Effects of Mn on LHRH release from hypothalami in vitro

The ability of Mn to induce LHRH release directly from the median basal hypothalamus (MBH) incubated in vitro was evaluated. In this regard, 30 day old female rats were decapitated, the MBH removed, and incubated as described previously (Hiney *et al*., 1999) with minor modifications. Briefly, each MBH was incubated in a vial containing 350 μl of Lockes Buffer (2mM 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 (50 cycles/min) at 37 ºC in an atmosphere of 95% O_2 and 5% CO_2 for 30 minutes. This medium was discarded, and all MBH's were incubated in fresh medium for 30 minutes to establish basal LHRH release. The medium was removed, boiled for 10 minutes, and stored in microcentrifuge tubes, and replaced with medium containing 0, 50, 250, or 500 μM $MnCl_2$. The MBHs were incubated for an additional 30 minutes. This medium was collected, boiled for 10 minutes, and stored at – 80 ºC 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. Following a two hour absorption period, three basal blood samples were drawn from each freely moving animal at 15-minute intervals. Immediately following the third basal sample, a 3V injection of $MnCl_2$ (10 μg) was administered to all of the animals. Then four more samples were taken for a total of seven samples. After the experiment, brains were examined for proper cannula placement, and 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 –70 °C until assayed for LH.

Effect of Mn on LH release from pituitary glands in vitro

Thirty-day-old female rats were decapitated, pituitaries 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 (50 cycles/ min) at 37 ºC in an atmosphere of 95% O_2 and 5% CO_2 for 60 minutes. This medium was discarded, and all APs were incubated in fresh medium for 30 minutes to establish basal LH release. The medium was removed and stored in microcentrifuge tubes, and replaced with medium containing 100 μM $MnCl_2$. The APs were incubated for an additional 30 minutes. This medium was collected and stored at – 80 ºC until assayed for LH. Pituitaries were weighed to the nearest 0.01 mg.

Effect of chronic Mn exposure on puberty-related hormones and the onset of female puberty

Litters were adjusted to 8-11 pups with at least 5-6 females per litter. In the first experiment, $MnCl_2$ (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 and blood collected for hormonal and Mn assessments. A second experiment was conducted exactly as above except that the animals continued to be dosed with $MnCl_2$ until VO occurred. This Mn dose caused a cumulative intake of 9.7 mg of Mn over the entire dosing period. Oral Mn intake is

absorbed at about 5% and therefore, this dose and method resulted in approximately 0.49 mg of Mn absorbed during the 17 day period. Since the Mn-treated rats were about 100 grams body weight at this time (~ 32 days of age) this means they absorbed a total of approximately 4.9 mg Mn/kg animal.

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-rLH-S-II), antigen (NIDDK-rLH-I-9), reference preparation (NIDDK-rLH-RP-3), 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, California. 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 (E_2) was measured by an RIA kit purchased from Diagnostic Products Corp. (Los Angeles, California) as we have previously described (Hiney *et al*., 1996). The E_2 assay sensitivity was 8.0 pg/ml. All assays had inter- and intra-assay coefficients of variation of < 10%. The LHRH was measured as previously described (Hartter and Ramirez, 1985) 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 assay was 0.2 pg/ tube, and the intraassay coefficient of variation was < 10%.

Metal analysis

Mn was measured by the heavy metal analysis lab, Department of Integrative Biosciences, College of Veterinary Medicine, Texas A&M University. A Perkin-Elmer/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 H_2O_2 and 100 µl of HCl at 90˚C for 6 hours. 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 (± SEM). 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, California) were used to calculate and graph the results.

RESULTS

Effect of centrally administered Mn on LH secretion

Central administration of Mn stimulated LH release significantly and dose dependently over basal levels (Fig. 2.1). Animals that received saline or the 1.0 µg dose

of Mn exhibited no change in LH released as compared to their respective basal levels. However, animals that received 2.5, 5 and 25 μg doses of $MnCl_2$ exhibited 3- 18- and 33-fold increases in LH release, respectively, compared with basal levels. We suggest that this is a hypothalamic site of action, and the effect is exerted by very low doses since the Mn is further diluted by cerebrospinal fluid in the 3V and because only some of the Mn diffuses into the basal hypothalamus to apparently induce LHRH secretion.

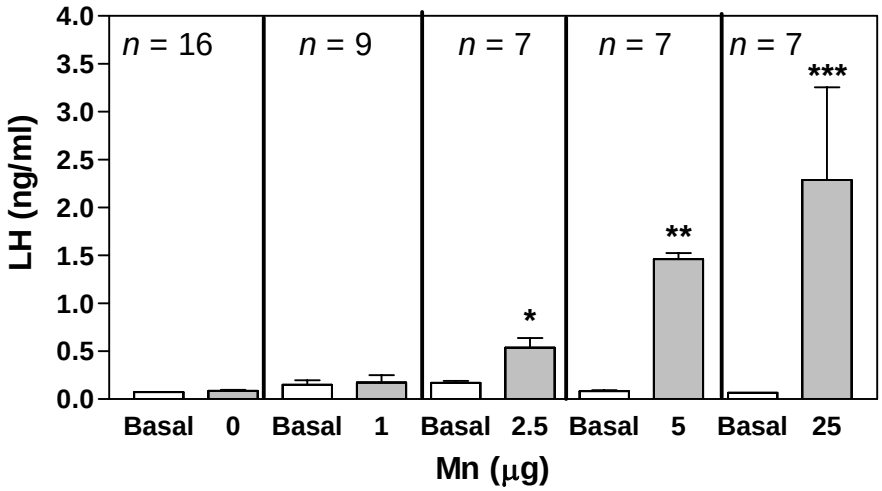

Figure 2.1. The effect of 3V administration of Mn on LH release during the late juvenile phase of developing female rats.
Each concentration point indicates basal LH levels vs. stimulated levels. The animals which received the saline and the 1.0 μg dose of Mn showed no significant changes in LH secretion 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 ± SEM, and the number of animals is depicted within each panel (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

Effect of Mn on LHRH release from hypothalami in vitro

Because LHRH cannot be measured accurately in serum, in vitro studies using the MBH were conducted. The MBH was removed from 29 day old female rats and incubated with three different concentrations of Mn. The addition of Mn to the challenge medium stimulated the release of LHRH in a dose dependent fashion as compared to basal levels (Fig. 2.2). MBHs incubated in Locke's Buffer without Mn showed no change between incubation and challenge levels while those incubated in the 50 ($P < 0.05$), 250 ($P < 0.02$), and 500 uM ($P < 0.05$). Mn demonstrated a marked increase in the amount of LHRH released.

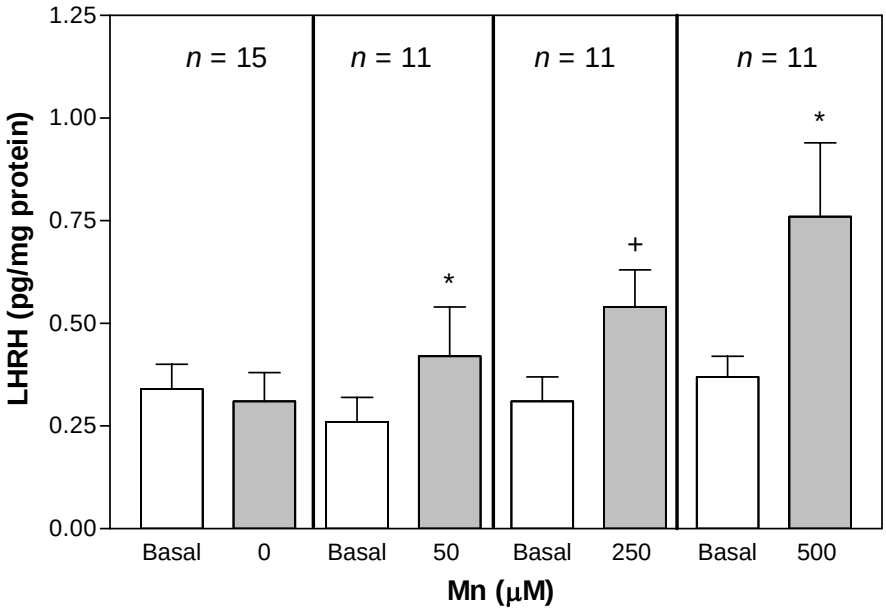

Figure 2.2. The effect of $MnCl_2$ on LHRH release from the medial basal hypothalamus *in vitro*. Each concentration point indicates basal LHRH levels vs. stimulated levels. Tissue incubated in Lockes buffer only showed no significant changes in LHRH secretion when compared to basal levels. Tissues incubated in 50 μM, 250 μM, and 500 μM concentrations of Mn showed significant increases in LHRH secretion when compared to their respective basal levels. Values represent mean ± SEM. The number of samples is depicted within each panel (*, $P < 0.05$; +, $P < 0.02$).

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 animals were treated with the LHRH receptor antagonist, acyline, prior to Mn administration. The 10 ug dose of Mn injected directly into the third ventricle of the non-acyline treated animals markedly stimulated LH release over basal levels (.37± .03 vs. 1.7 ± .5). However, animals pre-treated with acyline showed no significant change in LH levels after the Mn was administered (.35 ± .04 vs. .57 ± .02*)* (Fig. 2.3).

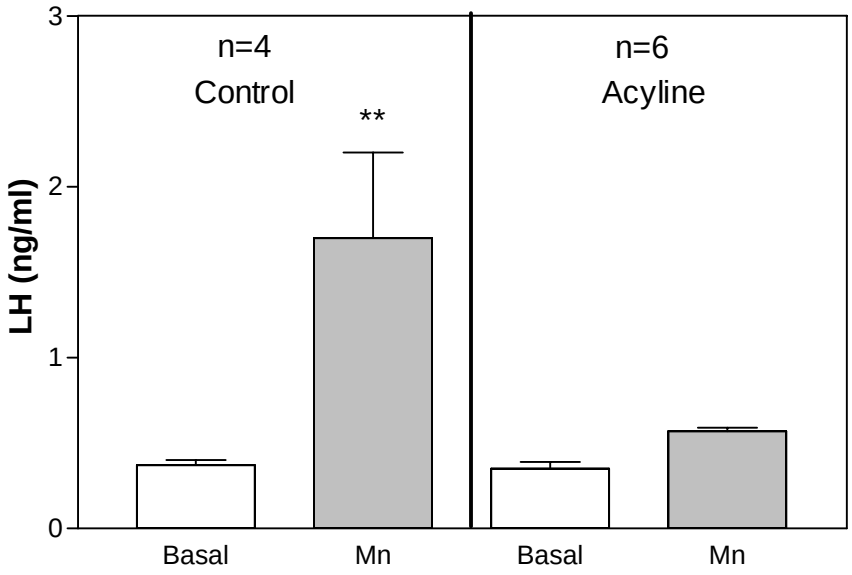

Figure 2.3. The effect of pretreatment with the LHRH receptor antagonist, acyline, on $MnCl_2$ stimulated LH release.
Acyline treated animals showed no significant change in LH release after 3V administration of $MnCl_2$ as compared to basal levels. Animals which were not pretreated with acyline exhibited a four-fold increase in LH as compared to basal levels (**, $P < 0.01$). Values represent the mean ± SEM. The number of animals is depicted within each panel.

Effect of Mn on LH release from pituitary glands in vitro

In vitro incubation of hemipituitaries with 100 μM Mn indicated that Mn was incapable of stimulating LH 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 30 minute incubation with manganese were 14.7 ± 6.8 ng/mg (n = 13), further suggesting a hypothalamic action.

Effect of chronic Mn exposure on puberty related hormones and the onset of female puberty

Chronic exposure to a low dose of Mn during juvenile development caused increases in specific puberty-related hormones. In this regard, the serum levels of LH, FSH and E_2 were all elevated ($P < 0.05$) by 29 days of age in Mn-treated vs. control animals (Fig. 2.4). The dose and regimen of Mn administration produced an accumulation of Mn in the brain, resulting in elevated Mn levels in both the preoptic area ($P < 0.02$) and the MBH ($P < 0.05$) when compared to controls (Fig. 2.5). As expected, serum Mn levels were not different from the controls at the time of blood collection (not shown) since a very low oral dose of Mn 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 chronic Mn exposure did not alter mean (± SEM) daily weight gain (controls, 3.8 ± 0.07 gm/day; Mn-treated, 3.7 ± 0.08 gm/day), but advanced ($P < 0.001$) the age at VO compared with saline controls. Specifically, mean (± SEM) VO was 32.8 ± 0.21 days compared with 34.3 ± 0.22 days in the controls (Fig. 2.6), a 1.5 day advancement in the

onset of puberty that appears to be moderate in terms of time, but a highly significant trend.

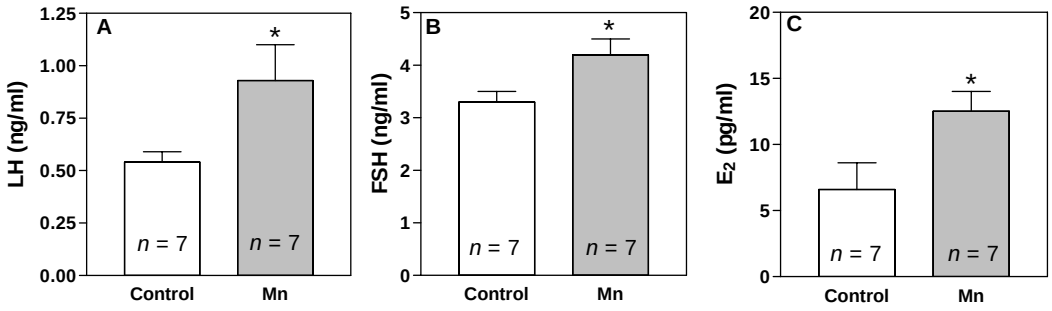

Figure 2.4. Effect of chronic oral administration of $MnCl_2$ on puberty related hormones in 29 day old female rats.
(A-C) serum levels of LH (A), FSH (B), and E_2 (C). Values represent the mean ± SEM, and the number of animals is depicted within each bar (*, $P < 0.05$ vs. control).

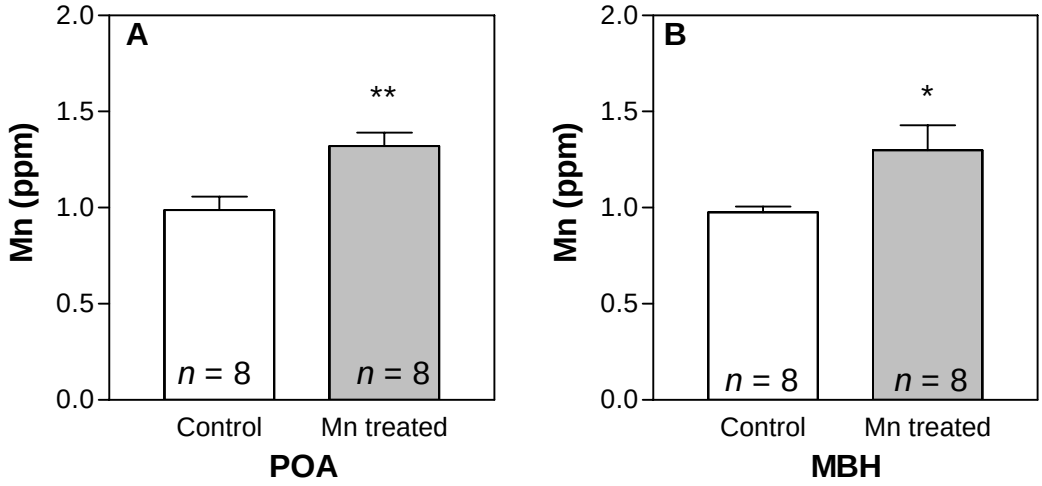

Figure 2.5. Accumulation of Mn in brain after chronic oral administration.
Daily administration of $MnCl_2$ at 10mg/kg increased Mn levels in both the MBH and the POA by 33% (*, $P < 0.05$; **, $P < 0.01$). Values represent the mean (n = 8) ± SEM.

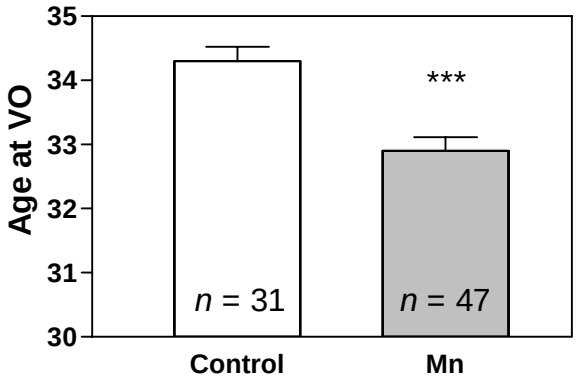

Figure 2.6. Effect of chronic oral administration of $MnCl_2$ on the age at vaginal opening (VO). Note that low level Mn exposure during the juvenile period moderately advanced the age at VO in terms of days (1.5 days), yet this was a highly significant trend. Values represent the mean ± SEM, and the number of animals is depicted within each bar (***, $P < 0.001$).

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 chronic, low dose Mn exposure to moderately accelerate the onset of female puberty. The dose used for the chronic 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 effortful response tests in adult primates (Newland and Weiss, 1991). Although different endpoints were assessed, the low level effect of Mn we described is important since many of the detrimental, neurotoxicological endpoint effects of the metal in adult rats and primates occurred after much higher doses (Newland, 1999). The fact that the low dose of Mn used in the present study facilitated specific puberty-related events, such as increased gonadotropins and estradiol, may be of importance for understanding the control of the onset of female puberty and furthermore, under certain circumstances it

may be possible that this seemingly beneficial effect could be harmful. In this regard, we suggest that a Mn-induced activation of the LHRH releasing system early in childhood may place an individual at risk for precocious 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). Despite the normal variation in the timing of puberty, in recent years there has been evidence presented that suggests puberty, especially in females, may be occurring at an earlier age (Herman-Giddens *et al*., 1997; Parent *et al*., 2003). At the present time the cause of this 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 age 8 in girls and 9.5 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 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 causes 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 and that an early elevation of Mn, especially in females, could pose a risk for precocious sexual development. This may not necessarily mean high levels, but 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 since 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.

Mn enters 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 and Aschner, 1990; Aschner, 2000). 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 gender differences in Mn metabolism have been observed with male rats clearing Mn two times faster than female rats (Zheng *et al*., 2000).

Other facts also suggest we consider the possibility that elevated Mn levels may affect the timing of puberty. Some regions have high levels of Mn in the drinking water, and there is concern for high levels in some baby formulas (Lonnerdal *et al*., 1987). Also, there are now increased amounts in the air from addition of methylcyclopentadienyl Mn tricarbonyl (MMT) to gasoline (EPA, 2001), and certain foods are high in Mn and this could relate to consumption habits of families and certain cultures. This could become an even greater concern in areas where diets are iron deficient. Mn and Fe are both able to use a transferrin-dependent transport system (Malecki and Devenyi, 1999), thus Fe deficiency has been shown to potentiate the absorption and accumulation of Mn.

It may be that any one of these excesses could be harmful, or it could take a combination of low level insults. Infants and children have been classified as being potentially more sensitive to excess Mn exposure (EPA, 2002) because the optimum level of exposure is not well defined (Greger, 1999), some evidence in animals that the young do not yet have full capacity to eliminate Mn (Fechter, 1999), and because tissue accumulation and health effects in humans, especially children, are not well understood.

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). LHRH secretion is minimal during juvenile and childhood development and thus, this system is down-regulated; however, as stated above when the secretion pattern of the peptide is

enhanced, the onset of puberty begins. The present results clearly show that Mn can act centrally to elicit the release of LHRH 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.

CHAPTER III

MANGANESE ACTS CENTRALLY TO ACTIVATE REPRODUCTIVE HORMONE SECRETION AND PUBERTAL DEVELOPMENT IN MALE RATS

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 (Hurley, 1981), as well as connective tissue and the reproductive system (Greger, 1999; Keen *et al*., 1999). 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 (ATSDR, 2000). Additionally, Mn is being used as an antiknock agent in gasoline (Thibault *et al*., 2002) which also contributes to environmental sources of exposure.

Exposure to high levels of Mn is toxic, causing developmental and reproductive dysfunction (Gray and Laskey, 1980; Laskey *et al*., 1982). Interestingly, a Mn deficiency leads to impaired growth and reproduction in both sexes (Boyer *et al*., 1942; Smith *et al*., 1944), 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 (Pine *et al*., 2005). 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 (ATSDR, 2000).

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 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.

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, 0600hr, lights off, 1800hr) and temperature (23 ºC), 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.

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 five days for recovery (Antunes-Rodrigues *et al*., 1970). 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 (Harms, 1974). The next day at 0900 hours, 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-minute intervals. After the third basal sample, manganese chloride ($MnCl_2$; 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 minute 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.

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

Forty-day-old male rats were decapitated at 0900 hours, the medial basal hypothalamus (MBH) dissected under a stereomicroscope, rinsed, and incubated as we

have described previously (Pine *et al*., 2005). Briefly, the MBHs were placed in vials (one per vial) containing 0.35 ml incubation medium consisting of Locke's 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 minutes. The first incubation was discarded, and all MBHs were then incubated again in medium only for 30 minutes 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-minute incubation, the media were collected, boiled for 10 minutes to break down proteases, and then stored at -80˚C until assayed for LHRH. The MBHs were weighed to the nearest 0.01mg.

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 0900 hours, 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/0.1ml (Pine *et al*., 2005), 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 two hour absorption period and then three basal blood samples were drawn from each freely moving animal at 15-minute intervals. Immediately following the third basal sample, a 3V injection of

$MnCl_2$ (10 µg/3µl) was administered to all of the animals. Four more blood samples were taken at 15-minute 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.

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 8-11 pups with at least 6 males per litter. At 15 days of age, the males from each litter were divided into 3 groups. Groups 1 and 2 received 10 and 25 mg/kg $MnCl_2$, respectively. Administration was such that 0.258 mg 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 0900-1000 hours on day 48 or day 55. Trunk blood was collected and allowed to clot, then the serum stored frozen at -80ºC 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 (Johnson *et al*., 1980). 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 two minutes in 100 ml of homogenizing fluid containing 150 mM NaCl, 0.05% (v/v) Triton X-10, and 3.8 mM NaN_3 (Johnson *et al*., 1980). Testicular homogenates were stored at 5 °C and evaluated within 28h. Only spermatid

heads with a shape characteristic of steps 17 through 19 found in stages IV and VIII (Leblond *et al*., 1952) 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 (Johnson *et al*., 1984). 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) (Johnson *et al*., 1980; Amann *et al*., 1976). 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 (Amann *et al*., 1976).

Hormone analysis

Rat serum LH and FSH were measured using radioimmunoassay (RIA) procedures we have described previously (Hiney *et al*., 1996). 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, California. 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 (Hartter *et al*., 1985) 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%.

Statistical analysis

All values are expressed as the mean (±SEM). 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, California) were used to calculate and graph the results.

RESULTS

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. 3.1). 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 3-, 6- and 7-fold increases in LH release, respectively, compared with their basal levels.

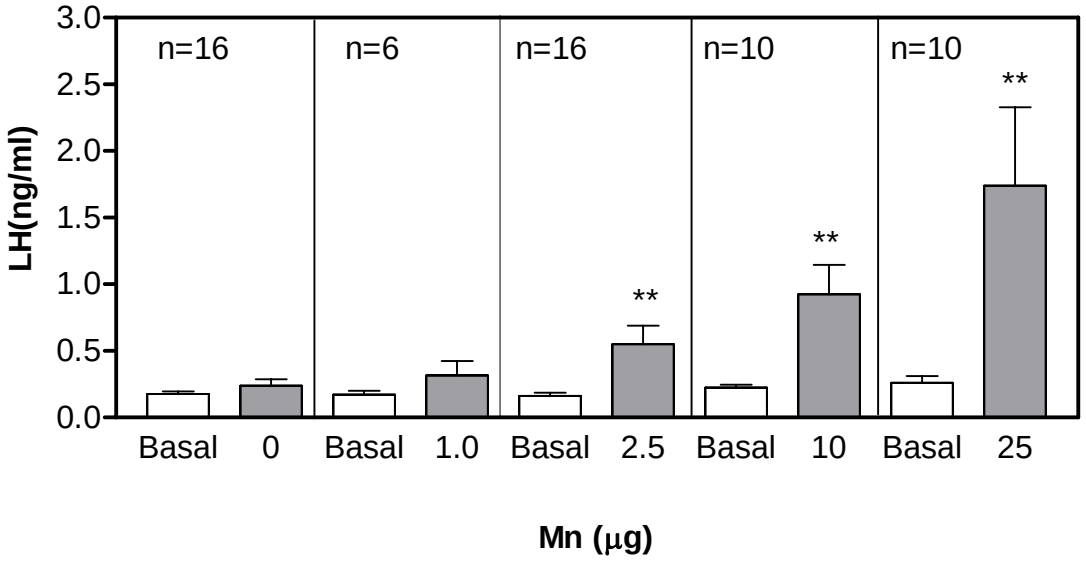

Figure 3.1. The effect of 3V administration of $MnCl_2$ on LH release in immature male rats during late juvenile phase of development.
Bars depict basal vs. stimulated serum levels of LH. The animals received either saline (0) or the designated dose of $MnCl_2$. Animals which received the 2.5 μg dose of $MnCl_2$ or higher showed a marked increase in LH secretion compared to basal levels. Values represent mean ± SEM. The number of animals is depicted within each panel (**, $p < 0.01$).

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. 3.2). 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 $MnCl_2$.

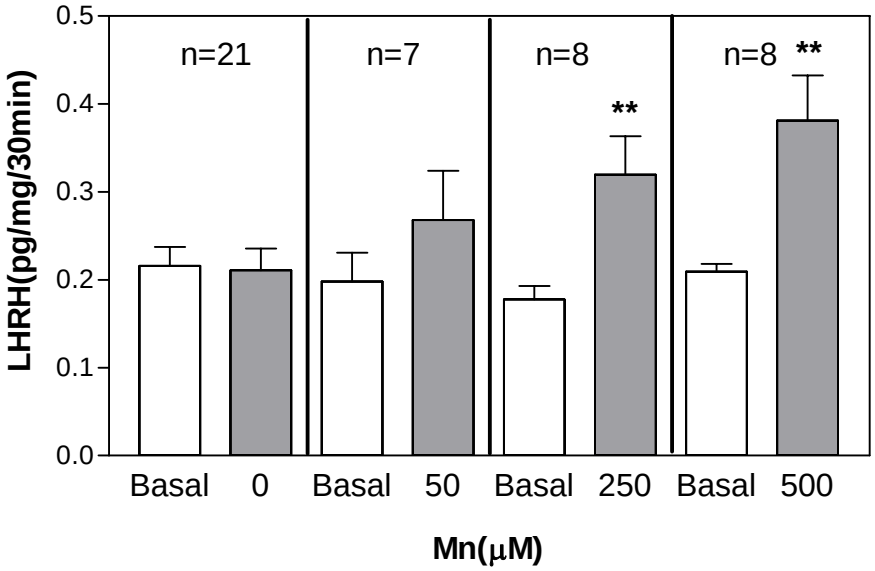

Figure 3.2. The effect of $MnCl_2$ on LHRH release from MBHs of immature male rats *in vitro*. Bars depict basal vs. stimulated levels of LHRH. MBHs were first incubated in Locke's Buffer alone to assess basal LHRH release, then again with either Locke's buffer alone (0) or with the buffer containing 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 ± SEM. The number of tissue samples is depicted within each panel (**, $p < 0.01$).

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 $MnCl_2$. Figure 3.3 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.

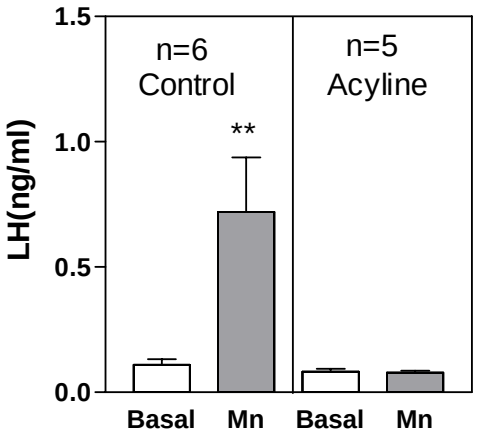

Figure 3.3. The effect of pretreatment with the LHRH receptor antagonist, Acyline, on $MnCl_2$-stimulated LH release in immature male rats.
Control animals (no acyline) exhibited a marked Mn-induced increase in serum levels of LH compared to their basal levels. Conversely, the acyline-treated animals did not respond to the Mn stimulation. Values represent mean ± SEM. The number of animals is depicted within each panel (**, $p< 0.01$).

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 vs. Mn: 1.85 ± 0.36 ng/ml; $P<0.02$) and FSH (Control: 29.7 ± 1.5 ng/ml vs. Mn: 36.9 ± 1.5 ng/ml; $P<0.01$) and testosterone (Control: 2 ± 0.19 ng/ml vs. Mn: 2.85 ± 0.3 ng/ml; $P<0.05$) compared with the control animals (fig. 3.4 a, b & c). Furthermore, compared 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. 3.5 a & b).

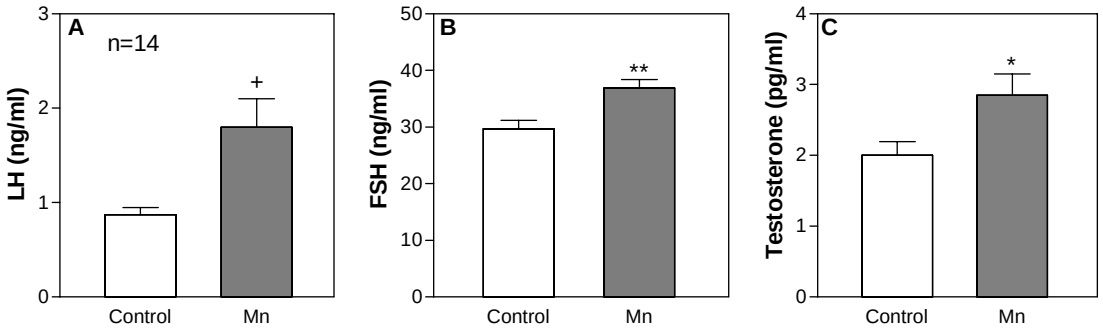

Figure 3.4. Effect of chronic oral administration of $MnCl_2$ on serum LH, FSH and testosterone in 55 day old male rats.
Serum LH, FSH and testosterone levels from control animals and animals which received the 25 mg/kg dose of $MnCl_2$ from day 15 through day 55 of life are depicted (panels A-C, respectively). Values represent the mean ± SEM. 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).

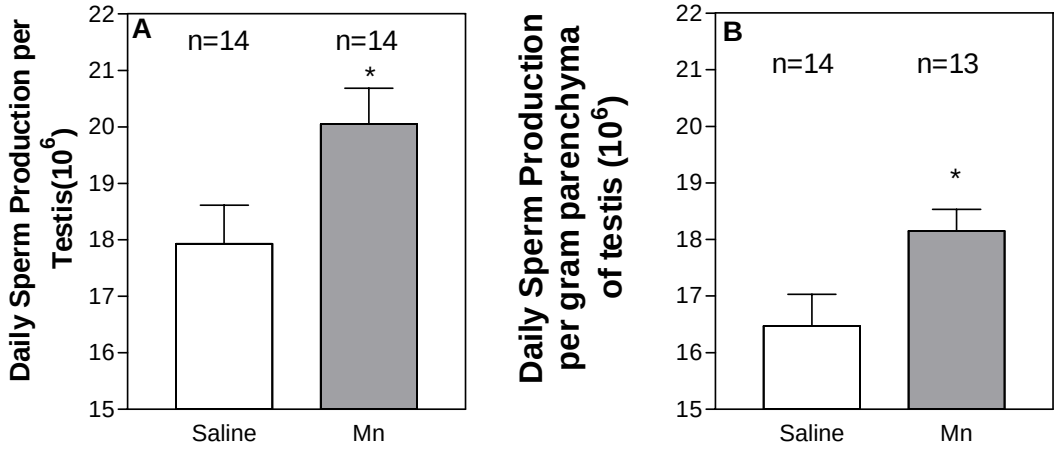

Figure 3.5. Effect of chronic oral administration of $MnCl_2$ on spermatogenesis of immature male rats.
Male rats received either $MnCl_2$ or saline by gastric gavage from day 15 through day 55 of life. These data depict the effect of $MnCl_2$ (25mg/kg) supplementation on daily sperm production per testis (panel A) and efficiency of sperm production as assessed by daily sperm production per gram parenchyma (panel B). Values represent mean ± SEM. The number of animals is depicted within each bar (*, $p < 0.05$).

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 (Lee, 1996). 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 (Ponnapakkam *et al*., 2003; Elbetieha *et al*., 2001). 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 (Robb *et al*., 1978), but were less than those attained in adult rats (Johnson *et al*., 1980; Robb *et al*., 1978; Johnson *et al*., 1984). 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 (Simoni *et al*., 1999). 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 homones, 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 (Pine *et al*., 2005); 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 (Newland, 1999). 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 (Zheng *et al*., 2000).

The onset of puberty in boys before the age of 9.5 years is usually considered precocious (Lee, 1996). 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 (Pine *et al*., 2005; Deskin *et al*., 1980). 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 and Aschner, 1990; Aschner, 2000). 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 (Mena, 1974). Although the minimum level of exposure is not well defined (Greger, 1999), infants and children have been classified as being potentially more sensitive to excess Mn (USEPA, 2002). 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 (Hiney *et al*., 1996), leptin (Chehab *et al*., 1997), kisspeptin (Navarro *et al*., 2004), and excitatory amino acids that can activate the N-methyl-D-aspartic acid receptor (Gay and Plant, 1987). 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 (Lee *et al*., 2006).

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.

CHAPTER IV

MANGANESE INDUCES HYPOTHALAMIC LUTEINIZING HORMONE RELEASING HORMONE SECRETION BY ACTIVATING GUANYLYL CYCLASE IN THE FEMALE RAT

INTRODUCTION

Manganese (Mn) is an abundant, naturally occurring essential element that is required for normal mammalian physiological events, including those related to normal growth and development of bone and cartilage (Hurley, 1981), as well as connective tissue and the reproductive system (Greger, 1999; Keen *et al*., 1999). 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); thus, suggesting a role in the reproductive process. Because Mn crosses the blood brain barrier over four times more efficiently in young versus adult animals (Deskin *et al*., 1980), and because it can accumulate in the hypothalamus (Deskin, *et al*., 1980; Pine, *et al*., 2005), we hypothesized that it may influence the neuroendocrine system just prior to puberty. Therefore, we have recently conducted studies to assess specifically, whether Mn contributes to the secretion of puberty-related hormones and the timing of puberty. In this regard, we used both in vitro and in vivo studies to demonstrate that Mn can stimulate the secretion of luteinizing hormone releasing hormone (LHRH) and luteinizing hormone (LH), respectively, in immature female rats (Pine *et al*., 2005). In that same study, we also assessed the effects of supplementing the diet of immature

female rats with a low dose of Mn from day 12 until day 29, or, in some animals until vaginal opening (VO). This diet regimen resulted in a significant elevation in the serum levels of LH, follicle stimulating hormone (FSH) and estradiol (E_2), and a moderate but significant advancement in the age at VO. More recently, we have observed similar elevations in puberty-related hormones in immature males, as well as increases in both daily sperm production and efficiency of spermatogenesis in age matched male controls. While this indicates that Mn can accelerate maturation in males (Lee *et al*., 2006), it is important to note that the Mn dose required for this was 2.5 fold greater than that which we showed to accelerate female puberty (Pine *et al*., 2005); hence, suggesting that the female is more sensitive in this regard. Taken together, the above studies indicate clearly that Mn is capable of acting within the hypothalamus to influence LHRH release during prepubertal development.

At the present time the hypothalamic mechanism by which Mn acts to facilitate prepubertal LHRH secretion is not known. Because nitric oxide (NO) is stimulatory on LHRH release (Rettori *et al*., 1993), and because of the ability of NO to activate soluble guanylyl cyclase (sGC) and the fact that the Mn cation is the preferred cofactor for activation of this enzyme (Murad, 1994), we questioned whether this pathway was the site of action for Mn-induced LHRH secretion. Since it is not known whether the Mn can effectively stimulate hypothalamic NO directly, or whether the principal action may reside in regulating activation of sGC, we investigated whether Mn stimulation of LHRH secretion involves an action within the NO/ guanylyl cyclase (GC)/ cyclic GMP (cGMP)/ protein kinase G (PKG) signaling pathway.

MATERIALS AND METHODS

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

In vivo *Experiments*

Inhibition of N-methyl-DL- aspartic acid receptors (NMDA-R)

Twenty-three-day-old female rats were anesthetized with 2.5% tribromoethanol (Aldrich, Milwaukee, WI) and stereotaxically implanted with a stainless steel cannula (23 gauge) in the third ventricle (3V) of the brain and allowed five days for recovery (Antunes-Rodrigues and McCann, 1970). When the rats were 29 days old, they were anesthetized with 2.5% tribromoethanol and silastic 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). Following a one hour acclimation period, two basal blood samples (250 µl) were drawn from each freely moving animal at 15-minute intervals. Half of the animals were

given an intravenous injection of a specific antagonist of NMDA-R, MK801 (0.15 mg/kg; Macdonald and Wilkinson, 1990; Brann and Mahesh, 1991), and the other half were given saline. Two additional samples were taken at 15 minute intervals and then a third ventricular injection of 10 μg/3 μl of manganese chloride ($MnCl_2$; Sigma Chemical Company, St. Louis, MO) was administered into the 3V. Following this injection, four additional samples were taken for a total of eight samples. This dose of $MnCl_2$ was determined in a previous study (Pine *et al.,* 2005). After the experiment, animals were euthanized with an overdose of tribromoethanol, 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.

Inhibition of insulin-like growth factor-1 receptors (IGF1-R)

Twenty-three-day-old female rats were treated in the same manner as described above except for the following modifications. After the two basal blood samples were taken, a IGF1-R inhibitor, JB1 (8 mg; Quesada and Etgen, 2002) or an equal volume of saline was administered into the 3V, then two blood samples were taken at 15 minute intervals. $MnCl_2$ was then administered into the 3V and three additional samples were taken for a total of seven samples. Euthanasia of animals, confirmation of cannula placement and stage of cycle, as well as blood sample storage were as described above.

Inhibition of nitric oxide synthase (NOS)

Twenty-three-day-old female rats were treated as above except for the following modifications. After the two basal blood samples were taken, the NOS inhibitor, N-

monomethyl-L-arginine (NMMA) (1 mg; Rettori *et al*., 1993) or an equal volume of saline was administered into the 3V. After 60 minutes, a third blood sample was taken and then a second dose of NMMA or saline was administered as above. Fifteen minutes later, a fourth blood sample was taken then $MnCl_2$ (10 µg/3µl) was administered into the 3V and three additional samples were taken for a total of seven samples. Euthanasia of animals, confirmation of cannula placement and stage of cycle, as well as blood sample storage were as described above.

In vitro *Experiments*

Thirty day-old female rats were decapitated, and the brain removed. The medial basal hypothalamus (MBH) was dissected by vertical cuts along the posterior side of the optic chiasm and the anterior edge of the mammillary bodies and laterally at the tuberoinfundibular sulci of the hypothalamus. The tissue was incubated as described previously (Nyberg *et al*., 1993) with minor modifications. Briefly, each MBH was incubated in a vial containing 350 µl of Locke's Buffer (2mM 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^{-1} BSA, pH 7.4) inside a Dubnoff shaker (80 cycles/min) at 37 ºC in an atmosphere of 95% O_2 and 5% CO_2. After a 30 min equilibration period, the initial incubation medium was discarded, and all MBH's were incubated in fresh medium for 30 minutes to establish basal LHRH release. The medium was replaced with medium only or medium plus NMMA (300 or 500 µM; Karanth *et al*., 2004), 1H-[1,2,4], oxadiazolo [4,3-a] quinoxalin-1-one (ODQ) (100 or 250 µM; Karanth *et al*., 2004), or KT5823 (10 µM), for 30 minutes then removed and stored in microcentrifuge tubes. The medium was then

replaced with medium containing 50 or 250 μM $MnCl_2$ (Pine *et al*., 2005) or medium with $MnCl_2$ plus NMMA, ODQ, or KT5823. The MBHs were incubated for an additional 30 minutes before these medium samples were collected. All of the samples were boiled for 10 minutes, and then stored at – 80 ºC until assayed for LHRH. MBHs were weighed to the nearest 0.1 mg.

In two other experiments, the medium was removed after equilibration and replaced with fresh medium for a 30 min incubation in order to establish basal secretion of total nitrite (NO_2^-) and LHRH, or cGMP and LHRH. In each case the medium was saved, then replaced with medium containing 50 or 250 μM $MnCl_2$ and the tissues incubated for a final 30 minutes. The medium samples were collected, aliquoted, boiled for 10 minutes and then stored frozen until assayed for NO_2^- and LHRH, or cGMP and LHRH, respectively. As above, the tissues were weighed to the nearest 0.1 mg.

Hormone analysis

Rat LH was measured using radioimmunoassay (RIA) procedures as previously described (Hiney *et al.,* 1996). The rat LH antiserum (NIDDK-anti-rLH-S-II), antigen (NIDDK-rLH-I-9), and reference preparation (NIDDK-rLH-RP-3) 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 inter- and intra-assay coefficients of variation was < 10%. The LHRH was measured as previously described (Nyberg *et al*., 1993) 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 assay was 0.2 pg/ tube, and

the intraassay coefficient of variation was < 10%. Total NO_2^- and cGMP were measured by kits purchased from Caymen Chemical, Ann Arbor, MI. The sensitivity of the NO_2^- and nonacetylated cGMP assays were 1 μM and 0.1 pmol ml^{-1}, respectively.

Statistical analysis

All values are expressed as the mean (± SEM). Differences between treatment groups were analyzed by Kruskal-Wallis nonparametric analysis of variance (ANOVA) followed by post hoc testing using Student-Neuman-Keuls multiple range test and where appropriate, the Student *t* 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 result.

RESULTS

Effects of NMDA-R inhibition on $MnCl_2$-induced LH release

Figure 4.1 shows that the intravenous administration of saline or MK801 alone did not alter basal LH levels. The central administration of $MnCl_2$ (10 μg/ 3 μl) stimulated LH release in both control (panel A; **p<0.01) and compared with basal levels in animals that received either saline or MK801-treated (panel B; ***p<0001) animals.

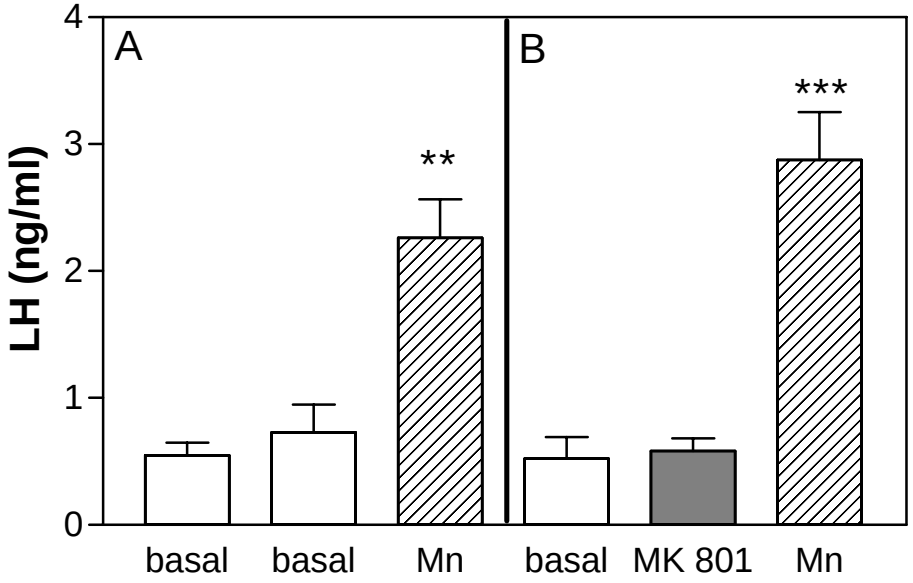

Figure 4.1. Effect of MK801 on Mn-induced LH release *in vivo*.
Open bars represent basal LH release. Grey bar represents LH release after MK801. Hatched bars represent LH release following central administration of $MnCl_2$ (10 ug/3ul) in the absence (panel A) or presence (panel B) of the inhibitor. Note that Mn stimulated LH release (panel A) and this action was not inhibited by the presence of the NMDA-R inhibitor (panel B). **p< 0.01 vs basals. ***p< 0.001 vs basal and MK801. Each bar represents the mean (±SEM). N= 5 for panels A and B respectively.

Effect of the IGF1-R inhibition on $MnCl_2$-induced LH release

Figure 4.2 shows that basal LH release was not altered by a 3V injection of either saline or JB1. The central administration of $MnCl_2$ (10 µg/ 3 µl) stimulated (**p<0.01) LH release in both groups similarly compared to their basal levels.

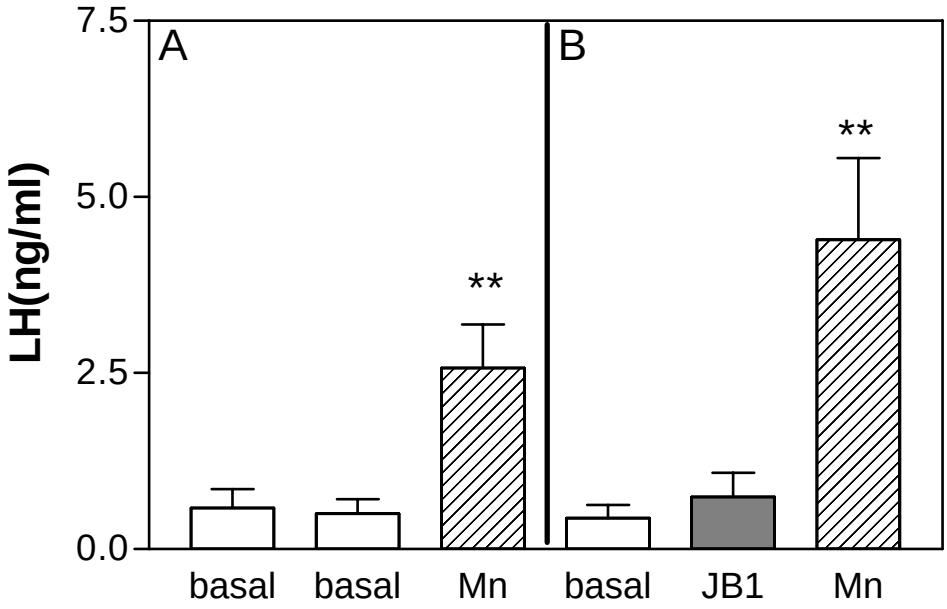

Figure 4.2. Effect of JB1 on Mn induced-LH release *in vivo*.
Open bars represent basal LH release. Grey bar represents LH release after JB1. Hatched bars represent LH release following central administration of $MnCl_2$ (10 ug/3ul) in the absence (panel A) or presence (panel B) of the inhibitor. Note that Mn stimulated LH release (panel A) and this action was not inhibited by the presence of the IGF1-R inhibitor (panel B). **p< 0.01 vs basals and JB1. Each bar represents the mean (±SEM). N= 13 for panels A and B respectively.

Effect of NOS inhibition on $MnCl_2$-induced LH release

Figure 4.3 shows that the central administration of saline or NMMA (1 mg), did not alter basal LH levels. The central administration of $MnCl_2$ (10 μg/ 3 μl) stimulated LH release in both control (panel A; ***p<0.001) or NMMA-treated (panel B; *p<0.05) animals compared with basal levels.

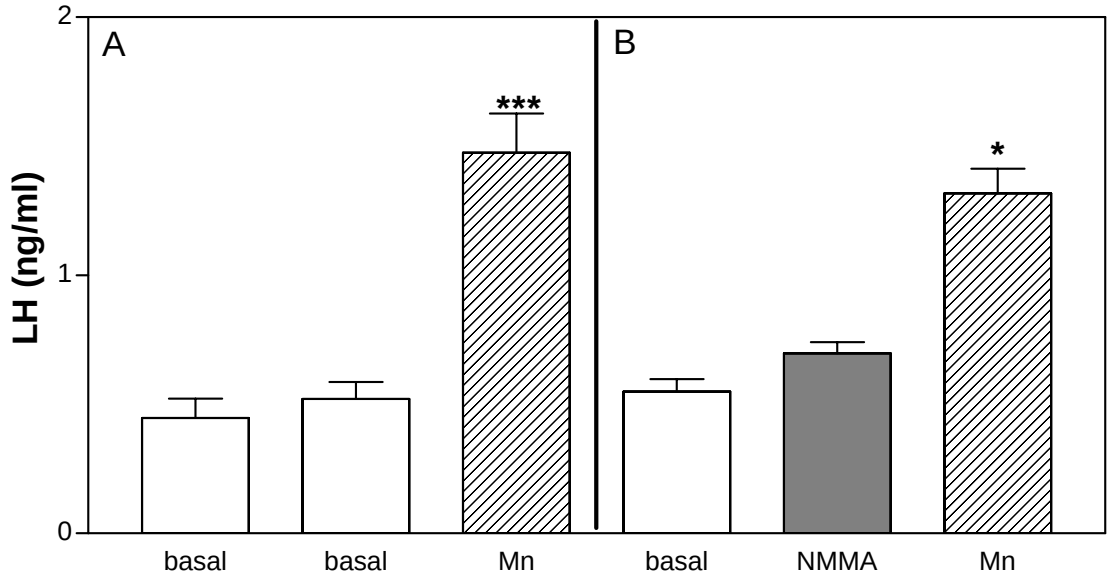

Figure 4.3. Effect of NMMA on Mn-induced LH release *in vivo*.
Open bars represent basal LH release. Grey bar represents LH release after NMMA. Hatched bars represent LH release following central administration of $MnCl_2$ (10 ug/3ul) in the absence (panel A) or presence (panel B) of the inhibitor. Note that Mn stimulated LH release (panel A) and this action was not inhibited by the presence of the NOS inhibitor (panel B). *** $p< 0.01$ vs basals. * $p<0.05$ vs basal and NMMA. Each bar represents the mean (±SEM). N= 9 and 8 for panels A and B respectively.

Effect of NOS inhibition on $MnCl_2$-induced LHRH release

Figure 4.4 demonstrates that the basal release of LHRH from the MBH was not altered by medium alone (Fig 4.4 A and C) or the medium containing 300µM NMMA (Fig 4.4 B and D). The addition of 50µM $MnCl_2$ to the medium caused an increase in the amount of LHRH released from both control tissues (Fig 4.4 A; $p<0.05$) and tissues exposed to 300µM NMMA (Fig 4.4 B; $p<0.05$). Furthermore, the addition of 250µM $MnCl_2$ likewise caused increased LHRH release from control tissues (Fig 4.4.C; $p<0.01$), as well as in tissues exposed to both 300µM NMMA (Fig 4.4 D; $p<0.05$) and 500µM NMMA ($p<0.05$, not shown).

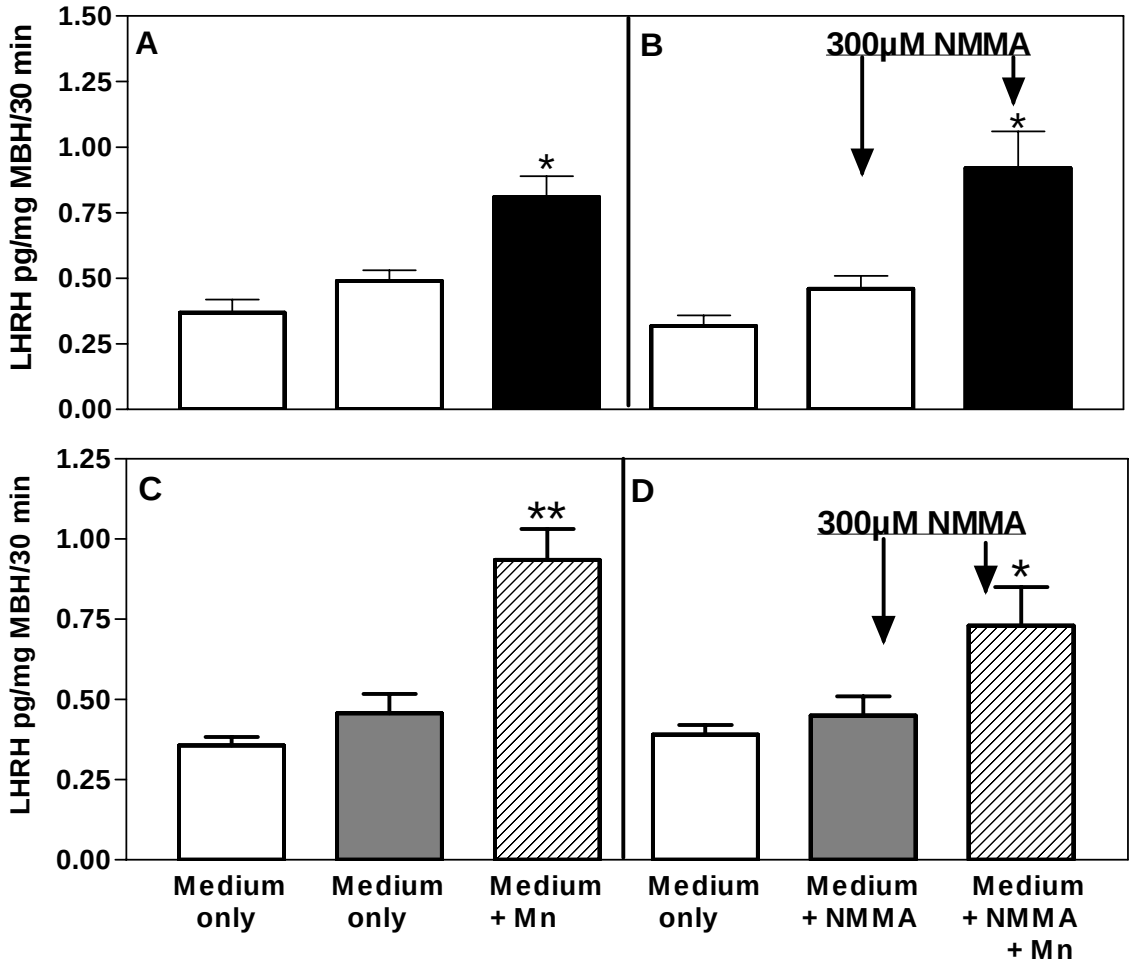

Figure 4.4. Effect of NOS inhibition using NMMA on $MnCl_2$-induced LHRH release.
Open bars represent basal LHRH release. Grey bar represents LHRH release in the presence of NMMA only. Black bars represent LHRH released following 50µM $MnCl_2$ stimulation in the absence (panel A) or presence (panel B) of NMMA. Hatched bars represent LHRH released following 250µM $MnCl_2$ stimulation in the absence (panel C) or presence (panel D) of the inhibitor. Note that Mn stimulated LHRH release in control tissues after both doses (panels A and C), and that this action was not altered by the presence of the NOS blocker (panels B and D). *$p<0.05$ vs medium only or medium plus NMMA; ** $p<0.01$ vs respective medium only. Each bar represents the mean (±SEM). N= 8, 9, 11 and 10 for panels A, B, C and D respectively.

Effect of $MnCl_2$ on NO_2^- release

In order to assess more directly a potential effect of Mn on tissue NO formation, we measured the amount of total NO_2^-, an indicator of NO generation, and LHRH

release from the same tissue incubates. The 50μM $MnCl_2$ failed to induce NO_2^- released into the medium (Fig 4.5 A), but did stimulate a marked increase in LHRH released (Fig 4.5 B; $p<0.001$). Identical results were noted following a 100μM dose of $MnCl_2$ (not shown). The 250μM dose of $MnCl_2$ did, however, induce the release of both NO_2^- (Fig 4.5 C; $p<0.001$) and LHRH (Fig 4.5 D; $p<0.001$).

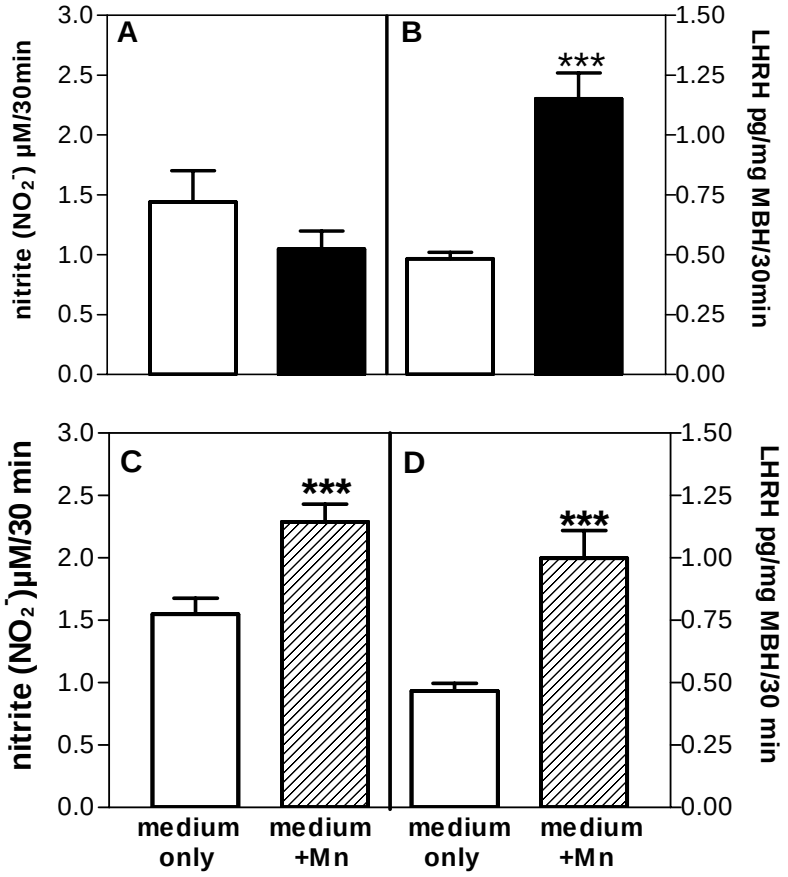

Figure 4.5. Effect of $MnCl_2$ on NO_2^- (as an assessment of NO production) and LHRH release from the same tissues.

Open bars represent basal NO_2^- and LHRH released into the medium. Black or hatched bars represent NO_2^- and LHRH released following MnCl2 stimulation at doses of 50μM and 250 μM, respectively. Note that the 50μM Mn dose did not stimulate NO_2^- (panel A), but markedly induced LHRH release (panel B). The 250μM Mn dose caused stimulations in both NO_2^- (panel C) and LHRH (panel D). Together, this suggests that Mn can only stimulate NO production at higher concentrations. Each bar represents the mean (±SEM). The number of tissues/group is depicted within each panel. ***$p < 0.001$ vs medium only. N=9 for panels A and B; N=14 for panels C and D.

Effect of GC inhibition on $MnCl_2$-induced LHRH release

Figure 4.6.A-C shows that the basal secretion of LHRH was not affected by medium alone or medium containing doses of 100 and 250µM ODQ. The subsequent addition of $MnCl_2$ to the medium elicited a marked increase ($p<0.001$) in LHRH released from control tissues (Fig 4.6 A), but this action was blunted markedly by the 100µM dose of ODQ and blocked by the 250µM dose of the inhibitor (Figs 4.6 B and C, respectively).

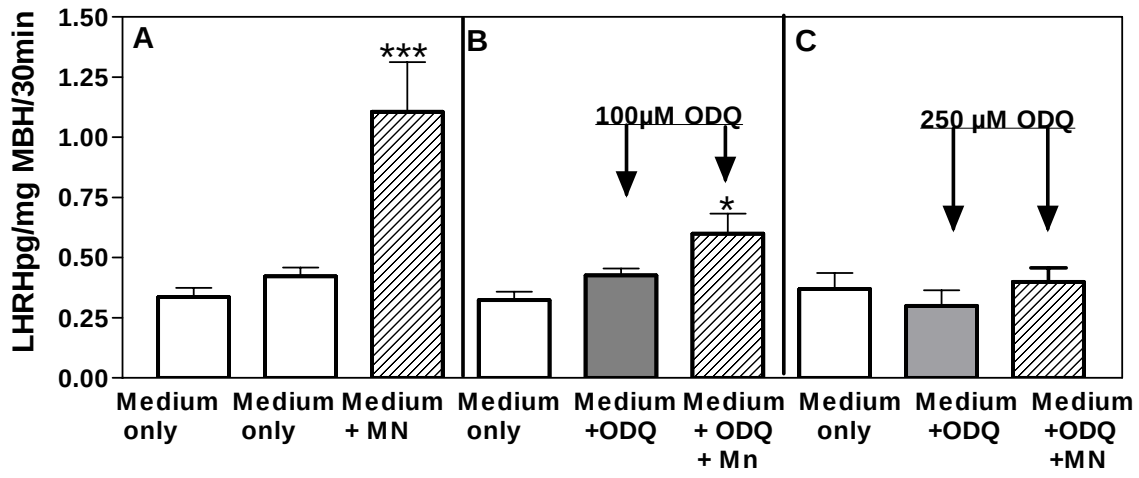

Figure 4.6. Effect of GC inhibition using ODQ on Mn-induced LHRH release.
Open bars represent basal LHRH release. Grey bars represent LHRH released in the presence of ODQ. Hatched bars represent LHRH released following 250µM $MnCl_2$ stimulation in the absence (panel A) or presence (panels B and C) of the inhibitor. Note that the Mn stimulated LHRH release from control tissues (panel A), but this stimulatory effect was dose dependently blocked (panels B and C) by the presence of the sGC inhibitor in the medium. ***$p<0.01$ vs medium only. *$p<0.05$ vs medium only and medium plus ODQ. Each bar represents the mean (±SEM). N=20, 11 and 15 for panels A, B and C respectively.

Effect of $MnCl_2$ on cGMP release

Basal and $MnCl_2$-stimulated release of both cGMP and LHRH were assessed from the same MBH tissue incubates. The 50µM dose of $MnCl_2$ induced an increase (p

<0.05) in the amount cGMP released over basal levels (Fig 4.7 A), an effect that was accompanied by an increase ($p<0.01$) in the release of LHRH (Fig 4.7 B). Similar stimulations were observed for both cGMP and LHRH following addition of the 250µM dose of $MnCl_2$ (not shown).

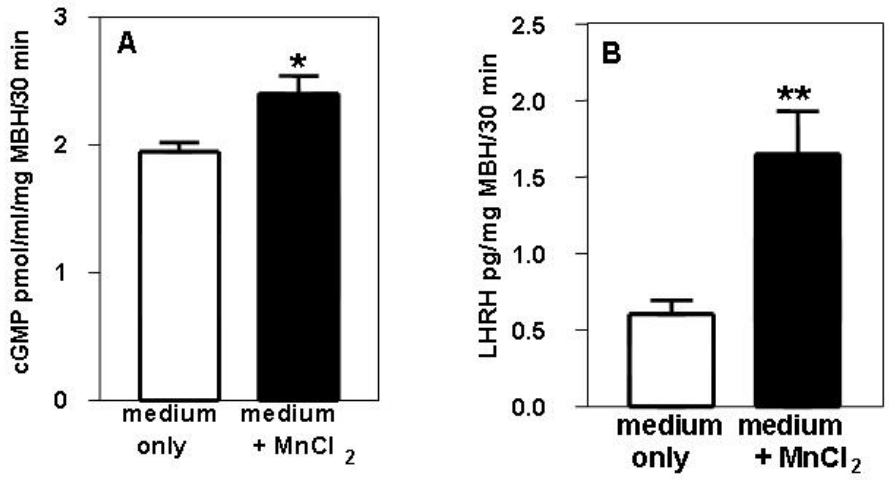

Figure 4.7. Manganese stimulates cGMP and LHRH release.
Open bar represents basal cGMP and LHRH release. Solid bars represent cGMP and LHRH released following addition of 50µM MnCl2 into the medium. Note that Mn stimulated both cGMP (panel A) and LHRH (panel B) secreted from the same tissue incubates. *p < 0.05 and **p < 0.01 versus basal levels. Each bar represents the mean (±SEM). N=8 for panels A and B, respectively.

Effect of PKG inhibition on $MnCl_2$-induced LHRH release

Figure 4.8 A and B shows that basal secretion of LHRH was not affected by medium alone or medium containing KT5823. The addition of 250µM of $MnCl_2$ to the medium elicited an increase ($p<0.05$) in LHRH released from control tissues, but failed to induce stimulation of the peptide in the tissues exposed to the inhibitor.

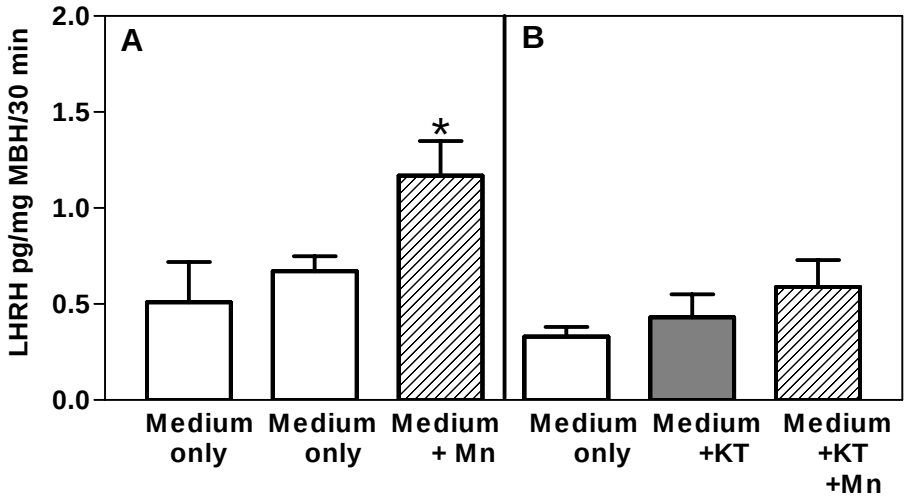

Figure 4.8. Effect of PKG inhibition using KT5823 on Mn-induced LHRH release.
Open bars represent basal LHRH release. Grey bar represents LHRH released in the presence of 10 μM KT5823. Hatched bars represent LHRH released following 250 μM $MnCl_2$ stimulation in the absence (panel A) or presence of (panel B) of the inhibitor. Note that Mn stimulated LHRH release in control tissues (panel A), but was unable to stimulate LHRH release when the PKG blocker was present in the medium. *$p<0.05$ vs medium only. Each bar represents the mean (±SEM). N=8 for panels A and B, respectively.

DISCUSSION

We showed previously that $MnCl_2$ is capable of acting at the hypothalamic level to stimulate LHRH release in prepubertal animals. Specifically, in our initial study, we showed in prepubertal female rats that Mn, when administered into the third ventricle, caused a dose-dependent release in LH. Furthermore, prior exposure to acyline, an LHRH receptor antagonist, blocked the central action of Mn to stimulate LH, and that Mn dose-dependently stimulated LHRH release directly from medial basal hypothalami incubated *in vitro* (Pine *et al*., 2005). In that study, we also demonstrated that the serum levels of LH, FSH and E_2 were elevated significantly, and that the day of vaginal

opening (VO) was advanced when the diet of developing female rats was supplemented with a low dose of Mn beginning when the rats were 12 days old.

The present results demonstrate the site and mechanism by which Mn induces prepubertal LHRH/ LH secretion in female rats. In this regard, we showed that blocking receptors such as NMDA-R or IGF1-R did not alter Mn-induced LH release in vivo. We also demonstrated that the blocking NOS did not alter Mn-induced LH release in vivo, nor did it alter Mn-induced LHRH release in vitro. Importantly, at doses of 50 and 100μM, Mn did not induce total NO_2^-, a marker of NO production, yet these doses were capable of stimulating LHRH release. Only the 250μM dose caused increased NO_2^- accumulation. Thus, taken together these results suggest that low doses of Mn do not induce LHRH release after first acting to stimulate NOS/NO. Conversely, ODQ, a specific blocker of sGC, dose dependently inhibited the release of LHRH induced by Mn, demonstrating that GC is the site of Mn action to facilitate LHRH secretion. Furthermore, Mn stimulates directly the release of cGMP and LHRH from the same MBH tissue, and finally, a downstream PKG inhibitor, KT5823, blocked Mn-induced LHRH release. Overall, these results demonstrate that the principal action of Mn is to facilitate activation of GC, subsequently stimulating the cGMP/ PKG pathway controlling LHRH secretion in prepubertal female rats. The fact that Mn can stimulate GC in prepubertal animals is supportive of an earlier report indicating that Mn is a natural element that is particularly important during development and capable of activating more than 50 enzyme systems, including GC and protein kinases (Wedler,1993). Furthermore, even though it has been known since the1970's that the Mn

cation is the preferred cofactor for GC and that $MnCl_2$ can increase GC activity either directly or as a co-factor with NO (Garbers, 1979; Murad, 1994), to our knowledge, the present report is the first to demonstrate a Mn stimulation of sGC in a physiological setting relevant to neuropeptide hormone secretion.

The results generated by this study further indicate that Mn, through its ability to stimulate prepubertal LHRH/ LH secretion, may contribute to events leading to puberty. The age at which normal puberty occurs depends on a complex series of events within the hypothalamus that culminate in the increased release of LHRH. This timely increase appears to require the interactive participation of neuronal circuitries and glial cells within the hypothalamus (Ojeda and Ubanski, 1994) which are likely influenced by specific metabolic signals, as well as genetic and environmental influences. Signals that can activate excitatory amino acid receptors (Nyberg *et al*., 1993; Urbanski and Ojeda 1990; Gay and Plant, 1987), and the peptides, insulin-like growth factor-1 (Hiney *et al*., 1996; Danilovich *et al*., 1999; Wilson, 1998), and kisspeptin (Navarro *et al*., 2004; Shahab *et al*., 2005) are all examples of influences capable of stimulating LHRH release and advancing signs of sexual maturity. We have indicated here, and in previous work (Pine *et al*., 2005), that Mn is an essential nutrient that can also stimulate LHRH/ LH release and advance puberty, suggesting that it may represent an important environmental component of the pubertal process.

Mn is able to enter the brain either through the cerebral vasculature or via the cerebral spinal fluid (CSF). The mechanism by which Mn crosses the blood brain barrier is not well understood, but apparently involves binding of the metal to transport systems

such as transferrin (Aschner and Aschner, 1990; Aschner and Aschner, 2000). As the blood levels of Mn rise, its influx into the CSF rises and entry across the choroid plexus becomes more important (Murphy *et al.*, 1991). The preoptic area and hypothalamus, which are adjacent to the third ventricle, receive Mn from both capillaries and the CSF. It has been shown that Mn crosses the blood brain barrier over four times more efficiently in the young (Mena, 1974), and younger animals do not have the full capacity to eliminate the Mn (Fechter, 1999). Furthermore, the element accumulates in the hypothalamus (Deskin *et al*., 1980; Pine *et al*., 2005), and is known to be taken up by both neurons and glial cells (Tholey *et al*., 1990); thus, suggesting a possible role in neuronal/glial communications within the developing hypothalamus.

Evidence has been presented in recent years suggesting that puberty may be occurring at an earlier age, especially in females (Herman-Giddings *et al*., 1997; Parent *et al*., 2003). The cause of this apparent trend is not known, but pubertal onset before 8 years of age in girls and 9.5 years in boys is considered precocious (Lee, 1996). The central form of precocious puberty is LHRH-dependent, and is characterized by hormonal changes similar to those occurring at the normal time of puberty. Importantly, in central precocious puberty there is a premature activation of the LHRH secretory system. In boys, this is usually accounted for by hypothalamic harmatomas, other CNS lesions, or familial disease with less that 10% considered idiopathic. Conversely, in girls, over 65% of the cases of precocious puberty are considered idiopathic. Thus far, research suggests that in addition to a possible action of Mn during normal pubertal events, a risk to certain individuals for precocious puberty could develop should they be

exposed to elevated levels of the element during the juvenile or early adolescent years. This possibility is supported by several lines of evidence. As mentioned above, we have shown that Mn acts centrally to stimulate LHRH secretion and it causes elevated gonadotropin and gonadal steroid levels, and can advance puberty in both sexes, although females appear to be more sensitive to low but elevated levels (Pine *et al*., 2005; Lee *et al*., 2006). Environmental sources of Mn are abundant since it is found in water, food, soil and air, with some regions having higher levels than others, and some cultures consuming greater amounts in their diets than others. Infants and children have been classified as being more sensitive to Mn exposure (EPA, 2002), mainly because the minimum level of exposure is not well defined (Greger, 1999). We suggest that should moderately elevated levels of Mn accumulate in the hypothalamus too early in life and reach levels not normally attained until later, then a potential concern for precocious development may occur. Epidemiological research in children and experimental studies in primates may be able to further address this question.

In summary, our results clearly show that Mn can induce prepubertal LHRH secretion via a hypothalamic action to activate sGC, causing stimulation of the cGMP/PKG pathway. Because of this hypothalamic action, and the fact that Mn administration can advance puberty, we suggest that this essential nutrient is a potential environmental factor involved in the pubertal process.

CHAPTER V

DISCUSSION AND CONCLUSIONS

This study is the first to show that Mn is capable of acting at the hypothalamus to stimulate the secretion of puberty related hormones and advance the time of puberty in animals. Specifically, our initial study using prepubertal female rats, showed in vitro that Mn induces luteinizing hormone releasing hormone (LHRH) secretion from nerve terminals in the basal hypothalamus, that the central administration of Mn increased luteinizing hormone (LH) release dose-dependently, and that this action was blocked by pretreatment with acyline, an LHRH receptor antagonist. Chronically, we demonstrated that low level Mn supplementation caused elevated levels of serum LH, follicle stimulating hormone (FSH), and estradiol (E_2), and advanced vaginal opening (VO). Together, the results indicate a hypothalamic action of Mn to activate the female reproductive system at the time of puberty.

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. At puberty, the pituitary and gonad are capable of function at any age after a short period of exposure to LHRH (Lee 1996). 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 fact that this study shows that low doses of Mn can act centrally to elicit prepubertal LHRH/LH secretion is important, and it suggests a facilitative role of the metal at the time of puberty. These results also indicate that an early elevation of Mn could pose a risk for precocious sexual development. This may not necessarily mean high levels, but 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. In this regard, Mn-induced activation of the LHRH releasing system early in childhood may place an individual at risk for precocious pubertal development.

Puberty may be occurring at an earlier age especially in females, yet the cause of this trend is not known. The onset of puberty before age 8 in girls and 9.5 in boys is usually considered precocious (Lee 1996). The central form of precocious puberty is LHRH-dependent and characterized by hormonal changes similar to those that occur at the normal time of puberty. In the central form of 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.

Mn is able to enter the brain either through the cerebral vasculature or via the cerebral spinal fluid (CSF). 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 (Mena 1974), and is known to be taken up by both neurons and glial cells

(Tholey *et al*., 1990); thus, suggesting a possible role in neuronal/ glial communications within the developing hypothalamus. The mechanism of crossing the blood brain barrier is not completely understood, but likely involves binding of Mn to transport systems such as transferrin (Aschner and Aschner, 1990; Aschner, 2000). As the blood levels of Mn rise, its influx into the CSF rises and entry across the choroid plexus becomes more important (Murphy *et al*., 1991). The preoptic area (POA) and hypothalamus, which are adjacent to the third ventricle, receive Mn from both capillaries and the CSF. Importantly, this study revealed the accumulation of Mn in both of these brain regions that control reproductive function in immature females, further supporting the potential risk for developmental alterations caused by early Mn exposure.

Knowing the effects of Mn on female development, a logical question was whether Mn could cause the same effects on the development of prepubertal male rats. In this regard, we showed that the acute action on LHRH/LH release, both in vivo and in vitro were similar to that which we described in females, although the dose required was somewhat higher. Chronically, the serum levels of LH, FSH, and testosterone were elevated after Mn supplemental treatment, and again, a higher dose was observed compared with females. It is well-known that LH facilitates spermatogenesis by stimulating production of testosterone, and both FSH and testosterone are capable of stimulating all phases of spermatogenesis (Simoni *et al*., 1999). Importantly, the present study showed that chronic, low level of Mn exposure advanced spermatogenesis as indicated by increased daily sperm production and efficiency of sperm production.

Comparing results from the male and female studies, we suggest a gender difference in responsiveness to Mn. A 10 mg/kg dose of Mn was effective in significantly elevating serum gonadotropins and advancing the time of vaginal opening in females, while a 25 mg/kg dose of Mn was required to significantly elevate serum gonadotropins and accelerate spermatogenesis in males. These results suggest that males are less sensitive to the hypothalamic effect of Mn than females. Male rats require higher level of Mn exposure and it appears to be due to gender differences in Mn metabolism; male rats clearing Mn two times faster than female rats (Zheng *et al*., 2000). 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 (Newland 1999).

Overall, our results clearly indicate that Mn is capable of acting within the hypothalamus to induce puberty related hormones, and to advance the timing of puberty in both sexes. Thus, it became important to discern the hypothalamic site and mechanism by which Mn acts to stimulate hypothalamic function at puberty. Therefore, we used both in vitro and in vivo methods to investigate the potential pathways that could be involved in Mn-stimulated LHRH secretion in prepubertal female rats.

In this regard, we showed that blocking the N-methyl-DL- aspartic acid receptors (NMDA-R), the insulin-like growth factor-1 receptors (IGF1-R), or inhibiting nitric oxide synthase (NOS) was ineffective in altering Mn induced LHRH/LH release. Dose response, pharmacological blockade and nitrite (NO_2^-) assessments indicated that the lowest doses of Mn used stimulated LHRH release, but did not induce nitric oxide (NO)

production, while only the highest dose stimulated NO. Conversely, dose dependent inhibition of Mn-induced release of LHRH by ODQ, a specific blocker of soluble guanylyl cyclase (sGC), demonstrated that sGC is the site of this Mn action. Furthermore, the study showed that Mn stimulated the release of cyclic GMP (cGMP) and LHRH from the same medial basal hypothalamic (MBH) tissue, and a downstream protein kinase G (PKG) inhibitor, KT5823, blocked Mn-induced LHRH release. Collectively, these data demonstrate that the principal action of Mn is to facilitate activation of GC, subsequently stimulating the cGMP/PKG pathway controlling LHRH secretion in prepubertal female rats.

Mn is a natural element that is particularly important during development and capable of activating more than 50 enzyme systems, including GC and protein kinases (Wedler, 1993). Importantly, even though it has been known since the1970's that the Mn cation is the preferred cofactor for GC, and that $MnCl_2$ can increase GC activity either directly or as a co-factor with NO (Garbers, 1979; Murad, 1994), the present study is the first to demonstrate a Mn stimulation of sGC in a physiological setting relevant to neuropeptide hormone secretion

The results generated by this study further support that Mn contributes to events leading to puberty by stimulating LHRH/LH secretion. As mentioned above, the age at which normal puberty begins depends on a complex series of events within the hypothalamus that culminate in the increased release of LHRH. This timely increase appears to require the interactive participation of neuronal circuitries and glial cells within the hypothalamus (Ojeda and Ubanski, 1994), which are likely influenced by

specific metabolic signals, as well as genetic and environmental influences. Signals that can activate excitatory amino acid receptors (Nyberg *et al*., 1993; Urbanski and Ojeda 1990; Gay and Plant, 1987), and the peptides, insulin-like growth factor-1 (Hiney *et al*., 1996; Danilovich *et al*., 1999; Wilson, 1998), and kisspeptin (Navarro *et al*., 2004; Shahab *et al*., 2005) are other examples of influences capable of stimulating LHRH release and advancing signs of sexual maturity. Our results suggest that Mn, through its ability to stimulate prepubertal LHRH release, may be an environmental factor, which may work in concert with the above signals, as well as with genetic factors to influence pubertal development.

In conclusion, our results clearly show that exposure to low levels of Mn during juvenile development can induce prepubertal LHRH/LH secretion at the hypothalamic level. The mechanism of Mn action is through activation of sGC, which causes stimulation of the cGMP/PKG pathway, resulting in the stimulated secretion of LHRH and thereby playing an early role in the pubertal process. Based on these results, we suggest that Mn may be an important environmental component of the pubertal process; however, should levels of this metal accumulate in the hypothalamus prior to the normal time of puberty, then this may put an individual at a higher risk for precocious pubertal development.

REFERENCES

Amann, R.P., Johnson, L., Thompson, D.L., and Pickett, B.W. (1976). Daily spermatozoal production, epididymal spermatozoal reserves and transit time of spermatozoa through the epididymis of the Rhesus monkey. *Biol. Reprod.* **15,** 586-592.

Antunes-Rodrigues, J., and McCann, S.M. (1970). 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.* **133,** 1464-1469.

Aschner, M., Erikson, K.M., and Dorman, D.C. (2005). Manganese dosimetry: Species differences and implications for neurotoxicity. *Crit Rev Toxicol.* **35**, 1-32.

Aschner, M. (2000). Manganese: Brain transport and emerging research needs. *Environ. Health Perspec.t* **108**, 429-432.

Aschner, M., and Aschner, J.L. (1990). Manganese transport across the blood brain barrier: relationship to iron homeostasis. *Brain Res. Bull.* **24**, 857-860.

Agency for Toxic Substances and Disease Registry (ATSDR). (2000). Toxicological Profile for Manganese, U.S. Department of Health And Human Services Public Health Service (available at http://www.atsdr.cdc.gov/toxprofiles/tp151.html),

Beuter, A., Mergler, D., de Geoffroy, A., Carriere, L., Belanger, S., Varghese, L., Sreekumar, J., and Gauthier, S. (1994). Diadochokinesimetry : A study of patients with Parkinson's disease and manganese exposed workers. *Neurotoxicology* **15**, 655-664.

Boyer, P.H., Shaw, J.H., and Phillips, P.H. (1942). Studies on manganese deficiency in the rat. *J Biol Chem* **143**, 417-425.

Brann, D.W., and Mahesh, V.B. (1991). Endogenous excitatory amino acid regulation of the progesterone-induced LH and FSH surge in estrogen-primed ovariectomized rats. *Neuroendocrinology* **53**, 107-110.

Calne, D.B., Chu, N.S., Huang, C.C., Lu, C.S., and Olanow, W. (1994). Manganism and idiopathic parkinsonism: Similarities and differences. *Neurology* **44**, 1583-1586.

Chehab, F.F., Mounzih, K., Lu, R., and Lim, M.E. (1997). Early onset of reproductive function in normal female mice treated with leptin. *Science* **275**, 88-90.

Claypool, L.E., Terasawa, E. (2000). N-methyl DL-aspartate induces the release of luteinizing hormone releasing hormone in the prepubertal and pubertal female rhesus

monkey as measured by in vivo push-pull perfusion in the stalk median eminence. *Endocrinology* **141**, 219-228**.**

Danilovich, N., Wernsing, D., Coschigano, K.T., Kopchick, J.J., and Bartke, A. (1999). Deficits in female reproductive function in GH-R-KO mice; Role of IGF-1. *Endocrinology* **140**, 2637-2640.

da Silva, W.G.P., Campos, R.C., and Miekeley, N. (1998). A simple digestion procedure for the determination of cadmium, copper, molybdenium and vanadium in plants by graphite furnace atomic absorption spectrometry and mass inductively coupled plasma spectrometry. *Anal Lett,* **31**, 1061-1070.

Dees, W.L., and Skelley, C.W. (1990). Effects of ethanol during the onset of female puberty. *Neuroendocrinol* **51**, 64-69.

Delemarre-Van De Waal, H.A., Wennink, J.M.B., Odink, R.J.H. (1991). Gonadotropin and growth hormone secretion throughout puberty. *Acta Paediatr Scand* **372**, 26-331.

Deskin, R., Bursain, S.J., and Edens, F.W. (1980). Neurochemical alterations induced by manganese chloride in neonatal rats. *Neurotoxicology* **2**:65-73.

Elbetieha, A., Bataineh, H., Darmani, H., and Al-Hamood, M.H. (2001). Effects of long-term exposure to manganese chloride on fertility of male and female mice. *Toxicol Lett.* **119,** 193-201.

Environmental Protection Agency (EPA). (2002). Health effects support document for manganese. EPA report 02-029. US Environmental Protection Agency, Wash., D.C.

Fechter, LD. (1999). Distribution of manganese in development. *Neurotoxicology* **20,** 197-201.

Freeland-Graves, J.H., and Llanes, C. (1994). Models to study manganese deficiency in manganese. In *Health and Disease* (Klimis-Tavantzis,D.J., ed.), pp. 59-86, CRC Press, Boca Raton.

Garbers, D.L. (1979). Purification of soluble guanylate cyclase from rat lung. *J Biol Chem* **254**, 240-243.

Gay, V.L., and Plant, T.M. (1987). N-methyl-D,L-aspartate elicits hypothalamic gonadotropin-releasing hormone release in prepubertal male rhesus monkeys. *Endocrinology* **120,** 2289-96.

Gerber, G.B., Leonard, A., and Hantson, P. (2002). Carcinogenicity, mutagenicity and teratogenicity of manganese compounds. *Crit Rev Oncol Hematol.* **42,** 25-34.

Gray, L.E. and Laskey, J.W. (1980). Multivariate analysis of the effects of manganese on the reproductive physiology and behavior of the male house mouse. *J Toxicol Environ Health* **6,** 861-867.

Greger, J.L. (1998). Dietary standards for manganese: Overlap between nutritional and toxicological studies. *J. Nutri.* **128**, 368S-371S.

Greger, J.L. (1999). Nutrition versus toxicology of manganese in humans: evaluation of potential biomarkers. *Neurotoxicology* **20,** 205-212.

Harms, P.G. and Ojeda, S.R. (1974). Method for cannulation of the rat jugular vein. *J Appl Physiol* **309,** 261-263.

Hartter, D.E., and Ramirez, V.D. (1985). Responsiveness of immature versus adult male rat hypothalami to dibutyryl cyclic AMP- and forskolin-induced LHRH release in vitro. *Neuroendocrinology* **40**, 476-482.

Herman-Giddens, P.A., Slora, E., Wasserman, R.C., Bourdony, C.J., Bhapkar, M.V., and Koch, G.G. (1997). Secondary sexual characteristics and menses in young girls seen in office practice: a study from the pediatric research office settings network. *Pediatrics* **88,** 505-512.

Hiney, J.K., Sower S.A., Yu, W.H., McCann, S.M., Dees, W.L. (2002). Gonadotropin-releasing hormone neurons in the preoptic-hypothalamic region of the rat contain lamprey gonadotropin-releasing hormone III, mammalian luteinizing hormone-releasing hormone, or both peptides. *Proc Natl Acad Sci U S A.* **19**, 2386-91.

Hiney, J.K., Dearth, R.K., Lara, F., Wood, S., Srivastava, V., and Dees W.L. (1999). Effects of ethanol on leptin secretion and the leptin-induced luteinizing hormone (LH) release from late juvenile rats. *Alcohol Clin. Exp. Res.* **23,** 1785-1792.

Hiney, J.K., Srivastava, V., Lara, T., Dees, W.L. (1998). Ethanol blocks the central action of IGF-1 to induce luteinizing hormone secretion in the prepubertal female rat. *Life Sci.* **62**, 301-8.

Hiney, J.K., Srivastava, V., Nyberg, C.L., Ojeda, S.R., and Dees, W.L. (1996). Insulin-like growth factor I of eripheral origin acts centrally to accelerate the initiation of female puberty. *Endocrinology.* **137,** 3717-3727.

Hurley, L. (1981). Teratogenic aspects of manganese, zinc and copper nutrition. *Physiol. Rev.* **61,** 249-295.

Ibanez, L., Potau, N., Zampolli, M., Rique, S., Saenger, P., and Carrascosa, A. (1997). Hyperinsulinemia and decreased insulin-like growth factor-binding protein-1 are common features in prepubertal and pubertal girls with a history of premature pubarche. *J Clin Endocrinol Metab*. **82,** 2283-2288.

Jankovic, J. (2005). Searching for a relationship between manganese and welding and Parkinson's disease. *Neurology*. **64,** 2021-8.

Johnson, L., Lebovitz, R.M., and Simson, W.K. (1984). Germ cell degeneration in normal and microwave-irradiated rats: potential sperm production rates at different developmental steps in spermatogenesis. *Anat. Record* **209,** 501-507.

Johnson, L., Petty, C.S., and Neaves, W.B. (1980). A comparative study of daily sperm production and testicular composition in humans and rats. *Biol. Reprod.***22,** 1233-1243.

Karanth, S., Yu, W.H, Mastronardi, C.A., and McCann, S.M. (2004). Inhibition of Melatonin-induced ascorbic acid and LHRH release by nitric oxide synthase or cyclic GMP inhibitor. *Exp Biol Med* **229**, 650-656.

Keen, C.L., Ensunsa, J.L., and Clegg, M.S. (2000). Manganese metabolism in animals and hymans including the toxicity of manganese. In *Manganese and its Role in Biological Processes* (Sigel, A., Sigel, H., eds), pp. 89-121, New York: Marcel Dekker, Inc.

Keen, C.L., Ensunsa, J.L., Watson, M.H., Baly, D.L., Donavan, S.M., and Monaco, M.H. (1999). Nutritional aspects of manganese from experimental studies. *Neurotoxicology* **20,** 213-224.

Knobil, E. (1980) The neuroendocrine control of the menstrual cycle. *Recent Prog Horm Res* **36**, 53-88.

Laskey, J.W., Rehnberg, G.R., Hein, J.F., and Carter, S.D. (1982). Effects of chronic manganese (Mn_3O_4) exposure on selected reproductive parameters in rats. *J Toxicol Environ Health* **9**, 677-687.

Lee, P.A. (1996). Disorders of puberty. In *Pediatric Endocrinology* (Lifshitz F, ed), pp. 175-195, New York: Marcel Dekker, Inc.

Leblond, C.P., and Clermont, Y. (1952). Definition of the stages of the cycle of the seminiferous epithelium in the rat. *Ann. N.Y. Acad. Sci.* **55,** 548-573.

Lee, B., Hiney, J.K., Pine, M.D., Srivastava, V., and Dees, W.L. (2006). Manganese stimulates luteinizing hormone releasing hormone secretion in prepubertal female rats: Hypothalamic site and mechanism of action. *J Physiol.* (in press)

Lee, B., Pine, M., Johnson, L., Rettori, V., Hiney, J.K. and Dees, W.L. **(**2006). Manganese acts centrally to activate reproductive hormone secretion and pubertal development in male rats. *Reprod Tox*. **22**, 580-585.

Lioy, P.J. (1983). Air pollution emission profiles of toxic and trace elements from energy related sources: Status and needs. *Neurotoxicology* **4**, 103-112.

Lonnerdal, B., Keen, C.L., and Bell, J.G. (1987). In *Nutritional Bioavailability of Manganese* (Kies C, ed), pp. 9-20, CRC Press, Boca Raton.

Macdonald, M.C., and Wilkinson, M. (1990). Peripubertal treatment with N-methy-D-aspartic acid or neonatally with monosodium glutamate accelerates sexual maturation in female rats, an effect reversed by MK-801. *Neuroendocrinology* **52**, 143-149.

Malecki, E.A., Devenyi, A.G., Beard, J.L., and Connor, J.R. (1999). Existing and emerging mechanisms for transport of iron and manganese to the brain. *J Neurosci Res* **56,** 113-122.

Matsumaoto, A., and Arai, Y. (1977) Precocious puberty and synaptogenesis in the hypothalamic arcuate nucleus in pregnant mare serum gonadotropin (PMSG) –treated immature female rats. *Brain Research* **129,** 375-378.

Mena, I. (1974). The role of manganese in human disease. *Annals of Clin Lab Sci* **4**, 487-491.

Mayer, M.L., and Westbrook, G.L. (1987) Permeation and block of N-mrthyl-D-aspartic acid receptor channels by divalent cations in mouse cultured central neurons. *J Physiol* **394,** 501-527.

Murad, F. (1994). Regulation of cytosolic guanylyl cyclase by nitric oxide: The NO cyclic-GMP signal transduction system. *Adv Pharmacol* **26,** 19-33.

Murphy, V.A., Wadhwami, K.C., Smith, Q.R., and Rapoport, S.I. (1991). Saturable transport of manganese across the rat blood brain barrier. *J Neurochem* **57,** 948-954.

Navarro, V.M., Fernandez-Fernandez, R., Castellano, J.M., Roa, J., Mayen, A., Barreiro, M.L., Gaytan, F., Aguilar, E., Pinilla, L., Dieguez, C., and Tena-Sempere, M. (2004). Advanced vaginal opening and precocious activation of the reproductive axis by KiSS-1 peptide, the endogenous ligand of GPR54. J Physiol. **561**, 379-86.

Newland, M.C. (1999). Animal models of manganese neurotoxicity**.** *Neurotoxicology* **20**, 415-432.

Newland, M.C., and Weiss, B. (1991). Persistent effects of Mn on effortful responding and their relationship to manganese accumulation in the primate globus pallidus. *Toxicol Appl Pharmacol* **113**, 87-97.

Newland, M.C., Cox, C., Hamada, R., Oberdorster, G., and Weiss, B. (1987). The clearance of manganese chloride in the primate. *Fund and Appl Tox* **9**, 314-328.

Nyberg, C.L., Hiney, J.K., Minks, J.B., and Dees, W.L. (1993). Ethanol alters N-methyl-DL-aspartic acid-induced secretion of luteinizing hormone releasing hormone and the onset of puberty in the female rat. *Neuroendocrinology* **57**, 863-868.

Ojeda, S.R. and Urbanski, H.F. (1994). Puberty in the rat. In *The Physiology of Reproduction* (Knobil E, Neill JD., Eds), pp. 363-409. Raven Press, New York.

Pal, P.K., Samii, A., and Calne, D.B. (1999). Manganese neurotoxicity: A review of clinical features, imaging and pathology. *Neurotoxicology* **20**, 227-238.

Parent, A.S., Teilman, G., Fuul, A., Skakkebaek, N.E., Toppar, J., and Bourguignon, J.P. (2003). The timing of normal puberty and the age limits of sexual precocity: variations around the world, secular trends and changes after migration. *Endo. Reviews* **24**, 668-693.

Pathomvanich, A., Merke, D. P., and Chrousos, G.P. (2000). Early Puberty: A Cautionary Tale. *Pediatrics* **105**, 115-116.

Pine, M., Lee, B., Dearth R., Hiney, J.K., and Dees, W.L. (2005). Manganese acts centrally to stimulate luteinizing hormone secretion: a potential influence on female pubertal development. *Toxicol Sci* **85**, 880-885.

Ponnapakkam, T.P., Bailey, K.S., Graves, K.A., and Iszard, M.B. (2003). Assessment of male reproductive system in the CD-1 mice following oral manganese exposure. *Reprod Toxicol.***17**, 547-551.

Quesada, A., and Etgen, A.M. (2002). Functional interactions between estrogen and insulin-like growth factor-1 in the regulation of alpha-1 B-adrenoceptors and female reproductive function. *Journal of Neuroscience* **22**, 2401-2408.

Rettori, V., Belova, N., Dees, W.L., Nyberg, C.L., Gimeno, M., and McCann, S.M. (1993). Role of nitric oxide in the control of lutinizing hormone releasing hormone release *in vivo* and *in vitro*. *Proceedings of the National Academy of Science* **90**, 10130-10134.

Robb, G.W., Amann, R.P., and Killian, G.J. (1978). Daily sperm production and epididymal sperm reserves of pubertal and adult rats. *J. Reprod. Tertil.* **54**,103-107.

Sarkar, D.K., Smith, G.C. and Fink, G. (1981) The effect of manipulating central catecholamines on puberty and the surge of luteinizing hormone and gonadotropin – releasing hormone induced by pregnant mare serum gonadotropin in female rats. *Brain Research* **213**, 335-349.

Shahab, M., Mastronardi, C., Seminaara, S., Crowley, W.F. Ojeda, S.R., and Plant, T.M. (2005). Increased hypothalamic GRP54 signaling: A potential mechanism for initiation of puberty in primates. *Proc Natl Acad Sci* **102**, 2129-2134.

Simoni, M., Weinbauer, G.F., Gromoll, J., and Niesclag, E. (1999). Role of FSH in male gonadal function. *Ann. Endocrinol. (Paris)* **60**, 102-106.

Smith, S.E., Medlicott, M., and Ellis, G.H. (1944). Manganese deficiency in the rabbit. *Arch Biochem Biophy*s **4**, 281-289.

Smith, Q.R. and Rapoport, S.I. (1991). Saturable transport of manganese across the rat blood brain barrier. *J Neurochem* **57**, 948-954.

Takeda, A. (2003). Manganese action in brain function. *Brain Res. Brain Res. Rev*. **41**, 79-87.

Thibault, C., Kennedy, G., Gareau, L.,and Zayed, J. (2002). Preliminary assessment of atmospheric manganese tricarbonyl and particulate manganese in selected urban sites. *J Toxicol Environ Health A.* **65,** 3-11.

Tholey, G., Megias-Megias, L., Wedler, F.C., and Ledig, M. (1990). Modulation of Mn accumulation in cultured rat neuronal and astroglial cells. *Neurochem Res* **15**, 751-754.

Urbanski, H. F., and Ojeda, S. R. (1990). A role for N-methyl D-aspartate (NMDA) receptors in the control of LH secretion and initiation of female puberty. *Endocrinology*. **126**, 1774-1776.

US Environmental Protection Agency (USEPA). (2001). Contaminant candidate list preliminary regulatory determination support document for manganese (EPA 815-R-01-013).

US Environmental Protection Agency (USEPA). (2002). Health effects support document for manganese. (EPA Report 02-029).

Wedler, F.C. (1993). Biological significance of manganese in mammalian systems. In *Progress in Medicinal Chemistry* (Ellis GP, Luscombe DK, Eds), pp. 89-133, Elsevier Science

Wedler, F.C. and Denman, R.B. (1984). Glutamine synthetase: The major Mn (II) enzyme in mammalian brain. *Curr Top Cell Regul* **24**, 153-169.

Wheeler, M.D. and Styne, D.M. (1990) Diagnosis and management of precocious puberty. *Pediatr Clin North Am*. **37**, 1255-71.

Wilson, M.E. (1998). Premature elevation in serum IGF-1 advances first ovulation in monkeys. *J Endocrinology* **158**, 247-257.

Zheng, W., Kim, H, and Zhao, Q. (2000). Comparative toxicokinetics of manganese chloride and methylcyclopentadienyl manganese tricarbonyl (MMT) in Sprague-Dawley rats. *Toxicol Sci* **54**, 295-301.

VITA

NAME:	Bo Yeon Lee
ADDRESS:	Department of Toxicology c/o Dr. W. L. Dees Texas A&M University College Station, 77843-4461
E MAIL ADDRESS:	bo495@hotmail.com

EDUCATION:

Ph.D. Toxicology,Texas A&M University, College Station, Texas. May 2007.

B.S. Veterinary Medicine, Chonnam National University, Kwangju, Korea, February 2001.