Manganese stimulates luteinizing hormone releasing hormone secretion in prepubertal female rats: hypothalamic site and mechanism of action

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We have shown recently that Mn^{2+} stimulates gonadotropin secretion via an action at the hypothalamic level, and a diet supplemented with a low dose of the element is capable of advancing the time of female puberty. In this study, we used an *in vitro* approach to investigate the mechanism by which Mn^{2+} induces luteinizing hormone-releasing hormone (LHRH) secretion from prepubertal female rats. The medial basal hypothalamus from 30-day-old rats was incubated in Locke solution for 30 min to assess basal LHRH secretion, then incubated with buffer alone or buffer plus either a nitric oxide synthase (NOS) inhibitor (*N*-monomethyl-L-arginine (NMMA); 300 or 500 μM) or a soluble guanylyl cyclase (sGC) inhibitor (1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ); 100 or 250 μM) for another 30 min. Finally, the incubation continued for a further 30 min, but in the presence of $MnCl_2$ (50 or 250 μM) to assess the effect of the blockers on stimulated LHRH secretion. Both 50 and 250 μM $MnCl_2$ stimulated LHRH release ($P < 0.05$ and $P < 0.01$, respectively). The addition of 300–500 μM NMMA to the medium did not block Mn^{2+}-stimulated release of LHRH, even with the higher dose of $MnCl_2$. Furthermore, while 50, 100 and 250 μM $MnCl_2$ all significantly induced LHRH release, the two lowest doses did not stimulate total nitrite released from the same tissue, an effect only observed with the highest dose. Taken together, these data suggest that Mn^{2+} is not an effective stimulator of NO. Conversely, inhibiting sGC with ODQ blocked the Mn^{2+}-stimulated secretion of LHRH in a dose-dependent manner, indicating that GC is the site of action of Mn^{2+}. Additionally, we showed that Mn^{2+} stimulated cGMP and LHRH from the same tissues, and that downstream blocking of protein kinase G formation with KT5823 (10 μM) inhibited Mn^{2+}-induced LHRH release. These data demonstrate that the principal action of Mn^{2+} within the hypothalamus is to activate sGC directly and/or as a cofactor with available NO, hence generating cGMP and resulting in prepubertal LHRH release.

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Mn^{2+} 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), connective tissue and the reproductive system (Greger, 1999; Keen *et al.* 1999). It has been known for many years that Mn^{2+} 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 for Mn^{2+} in the reproductive process. Because Mn^{2+} 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^{2+} 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^{2+} 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 $MnCl_2$ 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

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(FSH) and oestradiol (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^{2+} can accelerate maturation in males (Lee *et al.* 2006), it is important to note that the $MnCl_2$ 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^{2+} is capable of acting within the hypothalamus to influence LHRH release during prepubertal development.

At the present time, the hypothalamic mechanism by which Mn^{2+} acts to facilitate prepubertal LHRH secretion is not known. Because NO stimulates 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^{2+} is the preferred cofactor for activation of this enzyme (Murad, 1994), we questioned whether this pathway was the site of action for Mn^{2+}-induced LHRH secretion. As it is not known whether Mn^{2+} can effectively stimulate hypothalamic NO directly, or whether the principal action may reside in regulating activation of sGC, we investigated whether Mn^{2+}-induced stimulation of LHRH secretion involves an action within the NO–GC–cGMP–protein kinase G (PKG) signalling pathway.

Methods

Animals

Immature female Sprague-Dawley rats 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, 06.00 h; lights off, 18.00 h) and temperature (23°C), with *ad libitum* access to food and water. The diet (Harlan Teklad 2016) contained 94.7 mg kg^{-1} manganese and 149.8 mg kg^{-1} iron as analysed by the Heavy Metal Analysis Laboratory, Department of Veterinary Integrative Biosciences, College of Veterinary Medicine, Texas A & M University. On the day of experiment, animals were killed using decapitation without anaesthesia. All procedures used were approved by the University Animal Care and Use Committee at Texas A & M University and in accordance with the NIH Guidelines for the Care and Use of Laboratory Animals.

Chemicals

N-monomethyl-L-arginine (NMMA), a nitric oxide synthase (NOS) inhibitor, KT5823, a PKG inhibitor, and $MnCl_2$ were purchased from Sigma (St Louis, MO, USA). 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ), a specific sGC inhibitor, was purchase from EMD Biosciences Inc. (San Diego, CA, USA). Both KT5823 and ODQ were dissolved in dimethyl sulphoxide and subsequent dilutions were made using Locke solution. NMMA and $MnCl_2$ were diluted in Locke solution.

Experimental protocol

Female rats (30 days old) 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 previously described (Nyberg *et al.* 1993) with minor modifications. Briefly, each MBH was incubated in a vial containing 350 μl Locke solution containing (mM): Hepes 2, NaCl 154, KCl 5.6, $MgCl_2$ 1, $NaHCO_3$ 6, glucose 10 and $CaCl_2$ 1.25, and 1 mg ml^{-1} bovine serum albumin, pH 7.4; inside a Dubnoff shaker (80 cycles min^{-1}) at 37°C in an atmosphere of 95% O_2–% CO_2. After a 30 min equilibration period, the initial incubation medium was discarded, and all MBHs were incubated in fresh medium for 30 min to establish basal LHRH release. The medium was replaced by medium alone or medium plus NMMA (300 or 500 μM; Karanth *et al.* 2004), ODQ (100 or 250 μM; Karanth *et al.* 2004) or KT5823 (10 μM) for 30 min, then removed and stored in microcentrifuge tubes. The medium was then replaced by 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 min before these samples of medium were collected. All of the samples were boiled for 10 min, and then stored at −80°C 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 by 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 min. The samples of medium were collected, divided into aliquots, boiled for 10 min 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

LHRH was measured as previously described (Nyberg *et al.* 1993) using Antiserum R11B73 kindly provided by Dr V. D. Ramirez. Synthetic LHRH used for the standards and iodinations was purchased from Sigma. The sensitivity of the assay was 0.2 pg $tube^{-1}$, and the intra-assay and inter-assay coefficients of variation were $< 10\%$. Total

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NO_2^- and cGMP were measured by kits purchased from Caymen Chemical (Ann Arbor, MI, USA). The sensitivity of the NO_2^- and non-acetylated cGMP assays were 1 μM and 1 pmol ml^{-1}, respectively.

Statistical analysis

All values are expressed as the means (± S.E.M.). Differences between treatment groups were analysed by Kruskal–Wallis non-parametric analysis of variance (ANOVA) followed by *post hoc* testing using Student–Newman–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 3.0 (GraphPad, San Diego, CA, USA) were used to calculate and plot the results.

Results

Effect of NOS inhibition on Mn^{2+}-induced LHRH release

Figure 1 demonstrates that the basal release of LHRH from the MBH was not altered by medium alone (Fig. 1*A* and *C*) or the medium containing 300 μM NMMA (Fig. 1*B* and *D*). The addition of 50 μM $MnCl_2$ to the medium caused an increase in the amount of LHRH released from both control tissues (Fig. 1*A*, $P < 0.05$) and tissues exposed to 300 μM NMMA (Fig. 1*B*, $P < 0.05$). Furthermore, the addition of 250 μM $MnCl_2$ likewise caused increased LHRH release from control tissues (Fig. 1*C*, $P < 0.01$), as well as from tissues exposed to both 300 μM NMMA (Fig. 1*D*, $P < 0.05$) and 500 μM NMMA ($P < 0.05$, not shown).

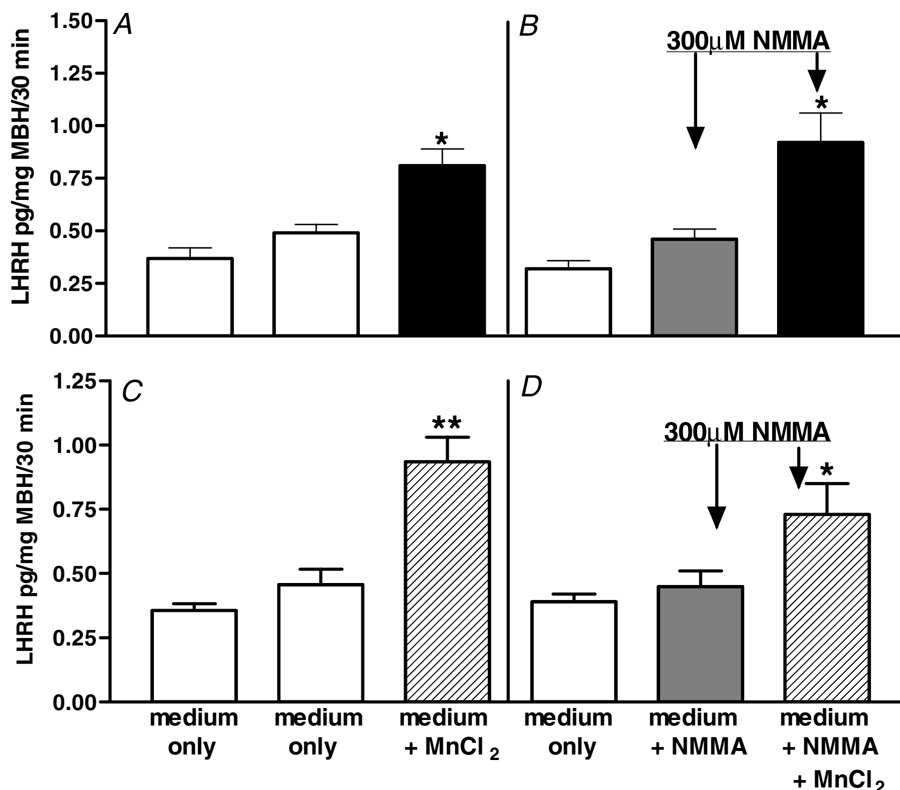

Figure 1. Effect of nitric oxide synthase (NOS) inhibition using *N*-monomethyl-L-arginine (NMMA) on $MnCl_2$-induced luteinizing hormone-releasing hormone (LHRH) release

Open bars represent basal LHRH release. Grey bar represents LHRH release in the presence of NMMA alone. Filled bars represent LHRH released following stimulation with 50 μM $MnCl_2$ in the absence (*A*) or presence (*B*) of NMMA. Hatched bars represent LHRH released following stimulation with 250 μM $MnCl_2$ in the absence (*C*) or presence (*D*) of the inhibitor. Note that Mn^{2+} stimulated LHRH release in control tissues after both doses (*A* and *C*), and that this action was not altered by the presence of the NOS blocker (*B* and *D*). *$P < 0.05$ *versus* medium alone or medium plus NMMA; **$P < 0.01$ *versus* medium alone. Each bar represents the mean (± S.E.M.). $n = 8$, 9, 11 and 10 for *A*, *B*, *C* and *D*, respectively.

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Effect of Mn^{2+} on NO_2^- production

In order to assess more directly a potential effect of Mn^{2+} 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 50 μM $MnCl_2$ dose failed to increase NO_2^- levels into the medium (Fig. 2*A*), but did stimulate a marked increase in LHRH released (Fig. 2*B*, $P < 0.001$). Identical results were noted following a 100-μM dose of $MnCl_2$ (not shown). The 250 μM dose of $MnCl_2$ did, however, increase the production of both NO_2^- (Fig. 2*C*, $P < 0.001$) and LHRH (Fig. 2*D*, $P < 0.001$).

Effect of GC inhibition on Mn^{2+}-induced LHRH release

Figure 3 shows that the basal secretion of LHRH was not affected by medium alone or medium containing doses of 100 and 250 μM ODQ. The subsequent addition of $MnCl_2$ to the medium elicited a marked increase ($P < 0.001$) in LHRH released from control tissues (Fig. 3*A*), but this action was blunted markedly by 100 μM ODQ and blocked by 250 μM dose of the inhibitor (Fig. 3*B* and *C*, respectively).

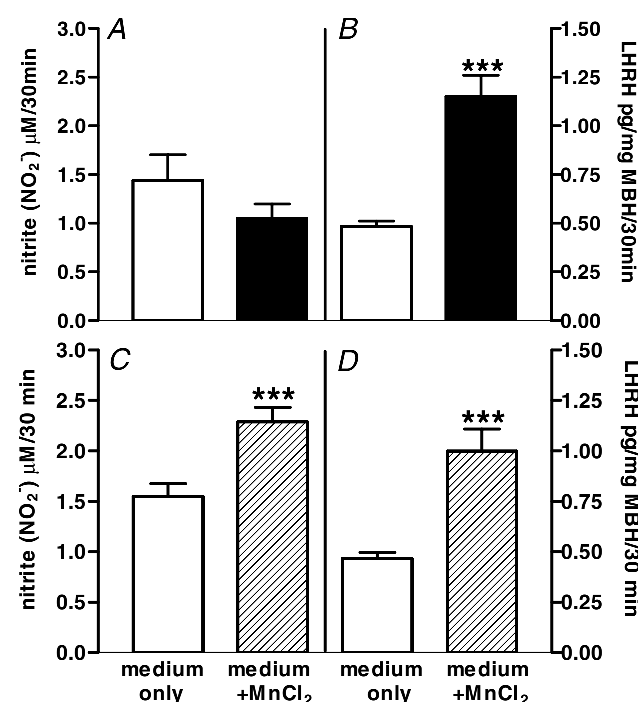

Figure 2. Effect of $MnCl_2$ on NO_2^- production and luteinizing hormone-releasing hormone (LHRH) release from the same tissues

Open bars represent basal NO_2^- and LHRH released into the medium. Filled or hatched bars represent NO_2^- and LHRH released following stimulation by $MnCl_2$ at 50 and 250 μM, respectively. Note that 50 μM $MnCl_2$ did not increase NO_2^- (*A*), but markedly induced LHRH release (*B*). Similar effects were observed with 100 μM $MnCl_2$ (not shown). The 250 μM $MnCl_2$ dose caused increases in both NO_2^- (*C*) and LHRH (*D*). Together, these data suggest that $MnCl_2$ can only stimulate NO production at higher concentrations. Each bar represents the mean (± S.E.M.). The number of tissues per group is depicted within each panel. ***$P < 0.001$ *versus* respective medium. $n = 9$ for *A* and *B*, and $n = 14$ for *C* and *D*.

Effect of Mn^{2+} on cGMP release

Basal and Mn^{2+}-stimulated release of both cGMP and LHRH were assessed from the same MBH tissue incubates. The 50 μM dose of $MnCl_2$ induced an increase ($P < 0.05$) in the amount cGMP released above basal levels (Fig. 4*A*), an effect that was accompanied by an increase ($P < 0.01$) in the release of LHRH (Fig. 4*B*). Similar stimulations were observed for both cGMP and LHRH following addition of the 250 μM dose of $MnCl_2$ (not shown).

Effect of PKG inhibition on Mn^{2+}-induced LHRH release

Figure 5*A* and *B* shows that basal secretion of LHRH was not affected by medium alone or medium containing KT5823. The addition of 250 μM $MnCl_2$ 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.

Discussion

We showed previously that Mn^{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^{2+}, 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^{2+} to stimulate LH, and Mn^{2+} dose-dependently stimulated LHRH release directly from MBHs 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 VO was advanced when the diet of developing female rats was supplemented with a low dose of $MnCl_2$ beginning when the rats were 12 days old.

The present results demonstrate the site and mechanism by which Mn^{2+} induces prepubertal LHRH secretion in female rats. In this regard, we showed that blocking NOS with NMMA was ineffective in blocking LHRH release following doses of 50 and 250 μM $MnCl_2$. Furthermore, these doses of 50 and 100 μM $MnCl_2$ did not increase total NO_2^-, a marker of NO production, yet were capable of stimulating LHRH release. Only 250 μM caused increased NO_2^- accumulation. Thus, taken together these results suggest that low doses of $MnCl_2$ do not induce LHRH

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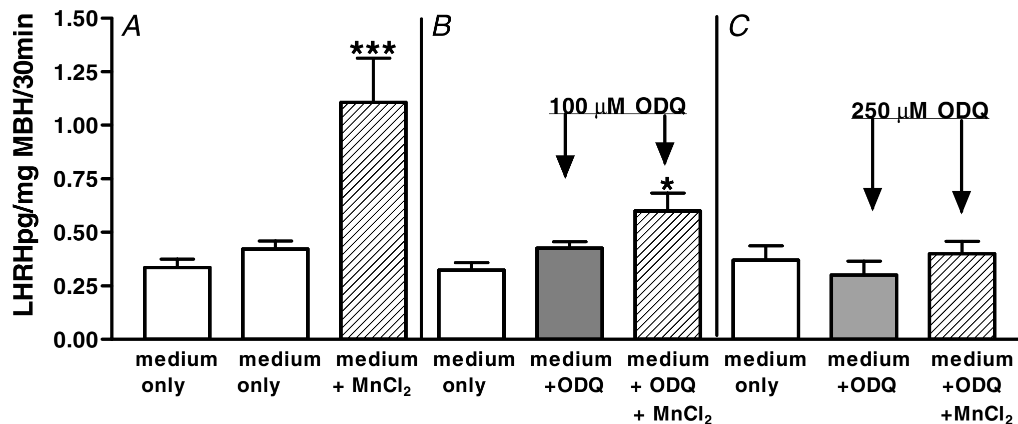

Figure 3. Effect of guanylyl cyclase (GC) inhibition using 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ) on $MnCl_2$-induced luteinizing hormone-releasing hormone (LHRH) release

Open bars represent basal LHRH release. Grey bars represent LHRH released in the presence of ODQ. Hatched bars represent LHRH released following stimulation with 250 μM $MnCl_2$ in the absence (*A*) or presence (*B* and *C*) of the inhibitor. Note that the $MnCl_2$ stimulated LHRH release from control tissues (*A*), but this stimulatory effect was dose-dependently blocked (*B* and *C*) by the presence of the soluble GC inhibitor in the medium. ***$P < 0.01$ *versus* medium alone. *$P < 0.05$ *versus* medium alone and medium plus ODQ. Each bar represents the mean (± S.E.M.). $n = 20$, 11 and 15 for *A*, *B* and *C*, respectively.

release by acting first to stimulate NOS/NO. Conversely, ODQ, a specific blocker of sGC (Zhao *et al.* 2000), inhibited the release of LHRH induced by Mn^{2+} in a dose-dependent manner, demonstrating that sGC is the site of action of Mn^{2+} to facilitate LHRH secretion. Furthermore, Mn^{2+} stimulates directly the release of cGMP and LHRH from the same MBH tissue, and finally, a downstream PKG inhibitor, KT5823, blocked Mn^{2+}-induced LHRH release. Overall, these results demonstrate that the principal action of Mn^{2+} is to facilitate activation of sGC, subsequently stimulating the cGMP–PKG pathway controlling LHRH secretion in prepubertal female rats. The fact that Mn^{2+} can stimulate sGC in prepubertal animals is supportive of an earlier report indicating that Mn^{2+} 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 the 1970s that Mn^{2+} is the preferred cofactor for GC and that it can increase GC activity either directly or as a cofactor with NO (Garbers, 1979; Murad, 1994), to our knowledge, the present report is the first to demonstrate stimulation of sGC by $MnCl_2$ in a physiological setting relevant to neuropeptide hormone secretion.

The results generated by this study further indicate that Mn^{2+}, through its ability to stimulate prepubertal LHRH 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 & Urbanski, 1994) which are likely to be influenced by specific metabolic signals, as well as genetic and environmental influences. Signals that can activate excitatory amino acid receptors (Gay & Plant, 1987; Urbanski & Ojeda, 1990; Nyberg *et al.* 1993), and the peptides insulin-like growth factor-1 (Hiney *et al.* 1996; Wilson, 1998; Danilovich *et al.* 1999) and kisspeptin (Navarro *et al.* 2004*a*; Shahab *et al.* 2005), are all examples of influences capable of stimulating LHRH release and

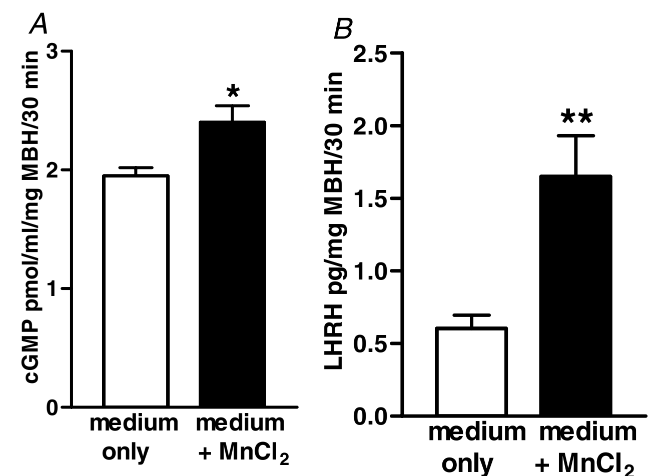

Figure 4. $MnCl_2$ stimulates cGMP and luteinizing hormone-releasing hormone (LHRH) release

Open bar represents basal cGMP and LHRH release. Filled bars represent cGMP and LHRH released following addition of 50 μM $MnCl_2$ into the medium. Note that $MnCl_2$ stimulated both cGMP (*A*) and LHRH (*B*) secreted from the same tissue incubates. *$P < 0.05$ and **$P < 0.01$ *versus* basal levels. Each bar represents the mean (± S.E.M.). $n = 8$ for both *A* and *B*.

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advancing signs of sexual maturity. We have indicated here, and in previous work (Pine *et al.* 2005), that Mn^{2+} is an essential nutrient that can stimulate LHRH release and advance puberty, suggesting that it may represent an important environmental component of the pubertal process.

In addition to the above puberty-related substances, GPR54, a novel G-protein-coupled receptor (Lee *et al.* 1999), has received increasing attention during the last few years for its role in the onset of puberty. In this regard, a mutation of *GPR54* in humans, and a deletion of the *GPR54* locus in mice, caused hypogonadotrophic hypogonadism and delayed puberty (de Roux *et al.* 2003; Seminera *et al.* 2003; Funes *et al.* 2003). Kisspeptins, which are products of the metastasis suppressor gene, *KiSS-1*, have been shown to be ligands to this receptor (Navarro *et al.* 2004*a*). Increases in *KiSS-1* and *GPR54* gene expressions occur during pubertal development (Navarro *et al.* 2004*b*). Furthermore, kisspeptins can act at the hypothalamic level to stimulate LHRH/LH release in immature rats and rhesus monkeys (Navarro *et al.* 2004*a*; Thompson *et al.* 2004; Shahab *et al.* 2005) and advance VO in rats (Navarro *et al.* 2004*b*). Taken together, these data demonstrate that *GPR54* activation by its ligands contributes to LHRH release at the time of puberty. Even though there has been no evidence presented to date demonstrating that Mn^{2+} can bind to or activate this protein receptor, and while this was not part of the present study, this potential action cannot be ruled out and warrants further discussion. *GPR54* is localized in the MBH (Gottsch *et al.* 2004; Shahab *et al.* 2005), as well as on the LHRH neuronal soma in the preoptic area (POA) (Parhar *et al.* 2004; Irwig *et al.* 2005). Although this is speculative, should Mn^{2+} be shown to have the capability to activate this protein receptor by some mechanism, then it is possible that the metal would be able to act at both the terminals in the MBH and the neuronal soma in the POA. The present study was designed to assess only the effect of Mn^{2+} on LHRH secretory mechanisms within the MBH, and we showed conclusively that the metal stimulates LHRH release via a NO-independent, activation of cGMP within the LHRH nerve terminals. Further work will be needed to assess whether Mn^{2+} is capable of activating GPR54 and, if so, whether it will also produce similar effects on the LHRH soma in the POA.

Mn^{2+} is able to enter the brain either through the cerebral vasculature or via the cerebral spinal fluid (CSF). The mechanism by which Mn^{2+} crosses the blood–brain barrier is not well understood, but is thought to involve binding of the metal to transport systems such as transferrin (Aschner & Aschner, 1990; Aschner & Aschner, 2000). As the blood levels of Mn^{2+} rise, its influx into the CSF rises and entry across the choroid plexus becomes more important (Murphy *et al.* 1991). The POA and hypothalamus, which are adjacent to the third ventricle, receive Mn^{2+} from both capillaries and the CSF. Mn^{2+} crosses the blood–brain barrier over four times more efficiently in the young than old animals (Mena, 1974), and younger animals do not have the full capacity to eliminate Mn^{2+} (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. Of importance, 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 than 10% of cases 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^{2+} during normal

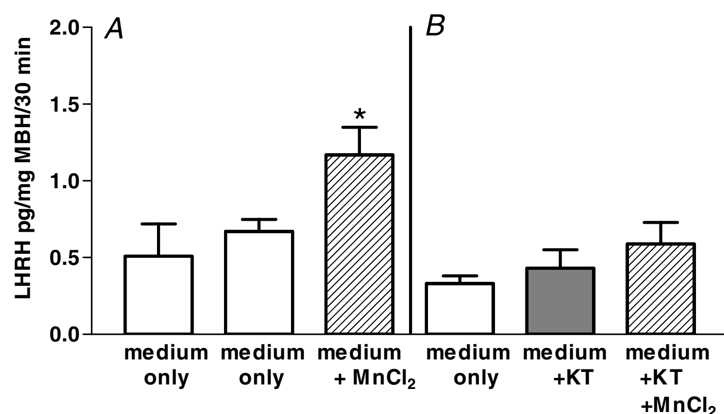

Figure 5. Effect of protein kinase G (PKG) inhibition using KT5823 on $MnCl_2$-induced luteinizing hormone-releasing hormone (LHRH) release
Open bars represent basal LHRH release. Grey bar represents LHRH released in the presence of 10 μM KT5823. Hatched bars represent LHRH released following stimulation with 250 μM $MnCl_2$ in the absence (*A*) or presence (*B*) of the inhibitor. Note that $MnCl_2$ stimulated LHRH release in control tissues (*A*), but was unable to stimulate LHRH release when the PKG blocker was present in the medium. $^*P < 0.05$ *versus* medium alone. Each bar represents the mean (± S.E.M.). $n = 8$ for both *A* and *B*.

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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^{2+} 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^{2+} are abundant, with it found in water, food, soil and air, and 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^{2+} exposure (Environmental Protection Agency, 2002), mainly because the minimum level of exposure is not well defined (Greger, 1999). We suggest that should moderately elevated levels of Mn^{2+} accumulate in the hypothalamus too early in life and reach levels not normally attained until later, then there is the potential for precocious development to occur. Epidemiological research in children and experimental studies in primates may be able to further address this issue.

In summary, our results clearly show that Mn^{2+} 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^{2+} administration can advance puberty, we suggest that this essential nutrient is a potential environmental factor involved in the pubertal process.

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