Involvement of *N*-methyl-*D*-aspartate receptor using excitatory amino acid neurotransmitters in control of pulsatile secretion of LH during sexual development in Holstein bull calves

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The neuroendocrine mechanisms underlying the development of pulsatile release of LH in bull calves are poorly understood. The hypothesis that endogenous excitatory amino acids, working through *N*-methyl-*D*-aspartate (NMDA) receptors, are involved in the generation of pulsatile LH release during sexual maturation of bull calves was tested. Holstein bull calves were administered i.v. 0.001, 0.01 and 0.1 mg kg⁻¹ body mass of MK-801, a specific noncompetitive NMDA receptor antagonist, on alternate days at 1, 12 and 24 weeks of age (*n* = 6 per dose), using a replicated 3 × 3 latin square design. Blood samples were obtained from jugular catheters at intervals of 10 min for 2 h before and 2 h after MK-801 treatment at 1 week and for periods of 4 h before and after MK-801 administration at 12 and 24 weeks of age. Plasma concentrations of LH were measured by a specific radioimmunoassay and pulsatile LH secretion was analysed using the Pulsar algorithm. Basal LH secretion at 1 week of age was low but was increased by 12 weeks (0.38 ± 0.01 versus 1.38 ± 0.11 ng ml⁻¹; *P* < 0.001) with the establishment of frequent high amplitude pulses. Mean LH concentrations and pulse amplitude, but not frequency, were lower at 24 than at 12 weeks of age (0.88 ± 0.08 versus 1.38 ± 0.11 ng ml⁻¹; *P* < 0.001, 1.74 ± 0.14 versus 2.82 ± 0.36 ng ml⁻¹; *P* < 0.01, and 1.70 ± 0.30 versus 1.70 ± 0.41 in 4 h; *P* > 0.05, respectively). Administration of MK-801 did not affect LH secretion in 1- and 12-week-old calves. In contrast, the antagonist at the highest dose tested caused a significant decrease in mean LH concentrations (0.30 ± 0.01 versus 0.70 ± 0.10 ng ml⁻¹; *P* < 0.05), which was associated with a reduction in the number of LH pulses (0.70 ± 0.30 versus 1.50 ± 0.30 in 4 h; *P* < 0.01) and pulse amplitude (0.54 ± 0.04 versus 1.72 ± 0.20 ng ml⁻¹; *P* < 0.001), in 24-week-old calves. These data suggest that the initiation of pulsatile secretion of LH during the juvenile phase in bull calves is not mediated by the endogenous excitatory amino acids operating through NMDA receptors. However, maintenance of the pulsatile secretory pattern of LH in prepubertal bull calves, which is required for continued testicular maturation, does involve an NMDA-receptor-mediated neuroexcitatory drive to GnRH-releasing neurones.

Introduction

Initiation of high amplitude LH discharges during the shift from the juvenile to prepubertal phase of development in bull calves (Amann and Walker, 1983; Amann *et al.*, 1986; Deaver and Peters, 1988) is critical for establishing pubertal testicular function (McCarthy *et al.*, 1979; Amann, 1983; Deaver *et al.*, 1988). This attainment of a pulsatile LH secretory pattern has been related to an increase in frequency of GnRH release into hypophyseal portal blood (Rodriguez and Wise, 1989, 1991). However, the mechanisms responsible for the activation of the GnRH neurosecretory system remain elusive. Stimulation of dormant GnRH neurones in calves may occur after the inhibitory input decreases, or the development of an excitatory drive to GnRH neurones, at the transition from the infantile to

juvenile phases of development. Glutamate is a major excitatory amino acid (EAA) neurotransmitter that induces LH secretion in a wide variety of species (Mason *et al.*, 1983; Gay and Plant, 1987; Urbanski, 1990; Lincoln and Wu, 1991; Sesti and Britt, 1992) and plays a role in the surge mode of LH release (López *et al.*, 1990; Meijis-Roelofs *et al.*, 1991; Ping *et al.*, 1994). Evidence indicates that EAAs affect secretion of LH through the release of GnRH (Bourguignon *et al.*, 1989a; Petersen *et al.*, 1991; Spergel *et al.*, 1994). Glutamate-elicited GnRH/LH release is mediated by NMDA and, to some extent, by non-NMDA receptor subtypes to EAAs (Bourguignon *et al.*, 1989a; López *et al.*, 1992; Mahachoklertwattana *et al.*, 1994) located at different hypothalamic sites (Meeker *et al.*, 1994).

We have shown that systemic administration of *N*-methyl-*D*-L-aspartic acid, an agonist of glutamate, results into an acute increase in peripheral LH concentrations in 24-week-old

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prepubertal bull calves (Shahab *et al.*, 1993). In the present study, we tested the hypothesis that the development of pulsatile secretion of LH is a consequence of an EAA-dependent excitatory input to GnRH neurones in bull calves, by looking at the effects of a blockade of EAA ligands specific to NMDA receptors on the development of pulsatile secretion of LH in bull calves.

Materials and Methods

Animals

Twelve Holstein bull calves were used in this study. The calves were obtained at 3–5 days of age and raised at the Dairy Breeding Research Center of The Pennsylvania State University. All the calves were housed in individual pens (1.5 m × 2 m) and subjected to the feeding and management protocols described by Curtis and Amann (1981).

Drugs

The specific noncompetitive NMDA receptor antagonist (+)-5-methyl-10,11-dihydro-5H-dibenzo[a,d]-cyclohepten-5,10-imine maleate (MK-801) was purchased from Research Biochemicals Int. (Natick, MA). MK-801 was dissolved in sterile 0.9% (w/v) NaCl 12 h before administration and kept at 4°C. The solution was used within 5 days of preparation and was passed through a 0.22 µm Millipore filter (Millipore Corp., Bedford, MA) at the time of injection.

Blood collection procedure

Calves were fitted with an indwelling Teflon catheter (16-gauge × 5 $\frac{1}{2}$ ”; Abbocath-T; Abbott Hospitals, Inc., North Chicago, IL) in the external jugular vein to permit frequent blood sampling, 12–18 h before injection of MK-801. The catheters were fixed in place by a dermal silk thread suture and kept patent with 0.9% (w/v) NaCl containing 0.05% (w/v) heparin. At the time of initiation of blood sampling, a polyethylene tubing extension (inner diameter 1.14 mm; Intramedic; Becton Dickinson and Co., Parsippany, NJ) was connected to the catheter at one end and the blood samples were collected from the other end by a 5 ml syringe. The extensions were taped to the halters to keep them in place. The catheters remained in the vein for 5 days; however, to prevent infection, the calves were injected with 10 ml 1% (v/v) oxytetracycline (Promycin; Phoenix Pharmaceutical, Inc., St Joseph, MO) daily.

Experimental design

The bull calves were randomly assigned to receive 0.001, 0.01 and 0.1 mg kg⁻¹ body mass of MK-801 as single i.v. injections on alternate days ($n=6$ per dose) at 1, 12 and 24 weeks of age in the autumn and winter in a replicated 3 × 3 latin square design. The doses of MK-801 used were similar to those used in other studies to suppress LH secretion (Urbanski and Ojeda, 1990; Meijis-Roelofs *et al.*, 1991). In preliminary experiments, a higher dose (1.0 mg kg⁻¹ body mass) was also

tried but, owing to adverse behavioural reactions, was not used. The same set of calves was used at 1 and 12 weeks, but at 24 weeks, different calves were used. Serial blood samples were obtained at intervals of 10 min through the catheters for a period of 2 h before and 2 h after MK-801 administration at 1 week, while at 12 and 24 weeks, blood samples were taken at intervals of 10 min for periods of 4 h before and 4 h after MK-801 administration. The blood samples were collected in chilled glass tubes containing 100 µl of 38% (w/v) sodium citrate. The samples were centrifuged (2800 g for 20 min) within 1 h of the 4 or 8 h blood sampling period, and the plasma obtained was stored at -20°C until used for the hormone analyses.

Luteinizing hormone assay

The concentrations of LH in plasma were determined by the radioimmunoassay procedures described by Deaver and Peters (1988). The hormone concentrations were measured in duplicate 100 µl aliquots using anti-ovine LH (RAOLH 35, kindly supplied by J. J. Reeves, Pullman, WA) as primary antibody and bovine LH (USDA bLH-B-5) as standard in these assays. The sensitivity of the assays was 0.25 ng ml⁻¹. Intra- and interassay coefficients of variation were 7.5% and 11.4%, respectively.

Statistical analyses

Pulses in LH secretion were detected by using the PC-Pulsar algorithm (Merriam and Watcher, 1982). The effect of MK-801 administration on LH secretion was analysed using the General Linear Model procedures of the Statistical Analysis System (SAS; Freund and Littell, 1982). Plasma concentrations of LH for a period of 4 h in 1-week-old calves, and for a period of 8 h in 12 and 24-week-old calves, were analysed separately. The model included the dose of MK-801, period (pre- and post-antagonist) and dose × period interaction as sources of variation. The effects of dose and period were tested using calf (dose × period) as the error term. When appropriate, characteristics of pulsatile LH secretion were further evaluated using the means (paired *t* test) procedures available in SAS. All data are expressed as means ± SEM.

Results

The concentrations of plasma LH in 1-week-old calves given three different doses of MK-801 are shown (Fig. 1). Basal LH secretion was low (0.38 ± 0.01 ng ml⁻¹) and only one peak was detected in 18 four hour profiles. LH concentrations in periods of 2 h following i.v. injection of 0.001, 0.01 and 0.1 mg MK-801 kg⁻¹ body mass were not different (dose × period: $P>0.05$) from the values before injection with MK-801 (0.37 ± 0.04 versus 0.38 ± 0.04 , 0.48 ± 0.08 versus 0.42 ± 0.05 and 0.39 ± 0.06 versus 0.38 ± 0.05 ng ml⁻¹, respectively). By 12 weeks of age, plasma concentrations of LH increased to 1.40 ± 0.11 ng ml⁻¹ and spontaneous secretory peaks were evident (Table 1). Plasma LH profiles for 8 h for three representative 12-week-old calves challenged with MK-801 are shown (Fig. 2). MK-801 administration did not affect LH

Fig. 1. Concentrations of LH in plasma of representative one-week-old bull calves administered (a) 0.001, (b) 0.01 and (c) 0.1 mg MK-801 kg^{-1} body mass i.v. The arrow indicates the time of administration of MK-801.

secretion in 12-week-old calves (dose $\times$ period: $P > 0.05$). Comparison of the characteristics of pulsatile LH secretion observed during the 4 h periods before and after MK-801 injection revealed no effect of any dose of the antagonist on LH secretion (Table 1). Compared with that at 12 weeks, average plasma concentrations of LH were lower at 24 weeks of age, due in part to a reduction in LH pulse amplitude (Table 2). LH pulse frequency observed before injection of MK-801 in 24-week-old calves was similar to that observed in 12-week-old animals. The effect of MK-801 administration on secretion of LH in three representative 24-week-old calves is illustrated (Fig. 3). Treatment with 0.001 and 0.01 mg MK-801 kg^{-1} body mass did not affect any characteristics of LH secretion (Table 2). However, administration of 0.1 mg NMDA receptor antagonist kg^{-1} body mass significantly suppressed plasma concentrations of LH in the post-treatment period (dose $\times$ period: $P < 0.05$). This inhibition of LH secretion was found to be a direct result of decrements in LH pulse frequency and amplitude ($P < 0.01$ – 0.001 ; Table 2).

Discussion

The paucity of pulsatile LH secretion in infantile bull calves reflects an absence of release of GnRH and relative pituitary insensitivity to GnRH. Hypothalamic concentrations of GnRH do not change with age (Amann *et al.*, 1986) and immature bull calves do respond to exogenously administered GnRH (Rodriguez and Wise, 1991) suggesting that release of the decapeptide is compromised in the earlier phases of sexual development. While the roles of opioids, catecholamines and gonadal steroids in the control of GnRH neurosecretion and the subsequent initiation of pulsatile LH secretion during the course of pubertal development in bull calves have been studied (Amann *et al.*, 1986; Deaver and Peters, 1988;

MacDonald *et al.*, 1990; Rodriguez *et al.*, 1993), to our knowledge, the present study is the first attempt to examine the contribution of EAA neurotransmitters that use NMDA receptors in the control of pubertal activation of the bovine hypothalamo–gonadotrope axis.

Developmental changes in the pattern of secretion of LH observed in this study are similar to those observed by Amann and Walker (1983) and Amann *et al.* (1986). At 12 weeks of age, peripheral LH concentrations were high, with frequent spontaneous discharges driven by GnRH (Rodriguez and Wise, 1989). However, administration of MK-801 at this age did not inhibit the secretory function of GnRH neurones or the pituitary release of LH. MK-801 has been shown to be an antagonist that is dependent on use (Bourguignon *et al.*, 1990) which blocks only activated receptors, and hence, gives a measure of endogenous agonistic activity at the NMDA receptor complex. Therefore, the results of this study indicate that NMDA receptor-mediated EAA stimulatory input to the GnRH-releasing neural circuitry is not significantly involved in the initiation of pulsatile secretion of LH in 12-week-old bull calves. The results do not support our hypothesis that the development of LH pulsatile secretion is triggered by an NMDA receptor-selective EAA excitatory drive to dormant GnRH neurones. This is in contrast to the situation in rats, in which stimulation of GnRH release from hypothalamic explants at the onset of puberty have been related to an increased activation of NMDA receptors (Bourguignon *et al.*, 1990). The NMDA receptor system has also been demonstrated to play a role in the development of enhanced LH secretion at the onset of puberty in female rats (Urbanski and Ojeda, 1990) and the initiation of seasonal breeding in hamsters (Urbanski, 1990). Similarly, chronic administration of an agonist of glutamate has been shown to induce precocious puberty in primates (Plant *et al.*, 1989). This evidence suggests a role for NMDA receptors in sexual maturation in the species studied; however, the specific effect of endogenous EAA on pulsatile LH secretion has not been assessed.

Gonadal steroids have been shown to potentiate the stimulatory effects of EAA on LH secretion in rodents (Luderer *et al.*, 1993; Brann and Mahesh, 1992; Carbone *et al.*, 1992) and sheep (Estienne *et al.*, 1990), possibly attributed to steroid-mediated increases in neuronal responses to EAA (Smith, 1989) or a steroid-dependent increase in EAA ligands in the hypothalamus (Carbone *et al.*, 1992). In 12-week-old bull calves, plasma concentrations of both oestradiol and testosterone are low (Amann *et al.*, 1986). Therefore, it is possible that the lack of a role for NMDA receptor-sensitive EAA neurotransmission in the control of pulsatile LH secretion may have been due to inadequate circulating steroid titres. It may also be argued that, at this stage of growth, NMDA-specific EAA pathways are not developed. This appears less likely as we have reported that the activation and blockade of NMDA receptors significantly affect prolactin secretion in bull calves as early as 1 week of age (Shahab *et al.*, 1994).

Perhaps the most important observation of the present study was the finding that blockade of endogenous EAA ligands specific to NMDA receptors by a single injection of MK-801 completely abolished pulsatile secretion of LH in the 24-week-old bull calves. As MK-801 is an open NMDA receptor channel blocker, this finding suggests that endogenous EAAs,

Table 1. Effect of intravenous administration of MK-801, a specific noncompetitive antagonist of the NMDA receptor, on pulsatile secretion of LH in 12-week-old bull calves

MK-801 (mg kg ⁻¹ body mass)	Before MK-801 administration			After MK-801 administration		
	Mean (ng ml ⁻¹)	Number of peaks	Amplitude (ng ml ⁻¹)	Mean (ng ml ⁻¹)	Number of peaks	Amplitude (ng ml ⁻¹)
0.001	1.3 ± 0.2	1.7 ± 0.5	2.3 ± 0.2	1.7 ± 0.2	1.7 ± 0.5	2.7 ± 0.3
0.01	1.6 ± 0.2	1.7 ± 0.5	3.3 ± 1.0	1.8 ± 0.2	1.8 ± 0.4	3.5 ± 0.8
0.1	1.3 ± 0.2	1.7 ± 0.3	2.9 ± 0.5	1.3 ± 0.1	1.3 ± 0.3	2.9 ± 0.4

NMDA: *N*-methyl-D-aspartate.
Values are means ± SEM for periods of 4 h; *n* = 6.
Values before and after MK-801 administration were not different.

working via NMDA receptors, are significantly involved in the intermittent stimulation of GnRH pulse-generating mechanisms. This is the first demonstration of a regulatory role of NMDA-specific EAA neurotransmission in the pulsatile secretion of LH in a gonad intact model. Although NMDA receptor antagonists have been shown to inhibit pulsatile GnRH release from hypothalamic explants *in vitro* (Bourguignon *et al.*, 1989b), evidence for a role of endogenous EAA in the control of pulsatile LH secretion is limited to only two reports. In these investigations, multiple systemic or intracerebroventricular administrations of AP5, a competitive NMDA receptor antagonist, led to a significant suppression of LH pulses in adult castrated rats (Arslan *et al.*, 1988) or a slight inhibition in ovariectomized lambs (Hileman *et al.*, 1992). In the last mentioned study, AP5 decreased mean serum LH concentrations without affecting the LH pulse frequency or the amplitude. This could be attributed to an inappropriate steroid environment, as the lambs were used 18 weeks after ovariectomy. Arslan *et al.* (1988) used adult rats 1–3 days after castration. By 24 weeks of age, peripheral concentrations of testosterone are increasing in bull calves (Amann *et al.*, 1986; Deaver and Peters, 1988). These observations support our hypothesis that gonadal steroids have a facilitatory role in the stimulation of GnRH pulse generator by endogenous NMDA receptor-mediated EAA neurotransmission.

There are two possible mechanisms of NMDA receptor-dependent stimulation of GnRH neurones in prepubertal bull calves. First, there may be a direct interaction. This hypothesis is supported by the demonstration of the existence of glutamate-immunoreactive neuronal connections with GnRH neurones (Goldsmith *et al.*, 1994) and by glutamate-induced stimulation of release of GnRH from GT1-7 neurones *in vitro* (Mahachoklertwattana *et al.*, 1994; Spergel *et al.*, 1994). Second, an indirect NMDA-specific EAA stimulation of GnRH neurones via noradrenaline may occur, as observed in rats (MacDonald and Wilkinson, 1992). In addition, there is evidence of EAA-stimulated release of dopamine at several cerebral sites (Moghaddam *et al.*, 1990; Youngren *et al.*, 1993). An indirect endogenous EAA stimulatory influence may be more relevant to the situation in bull calves, in which hypothalamic noradrenaline (Deaver and Peters, 1988) and dopamine (Rodriguez *et al.*, 1993) activities have been related to maturational changes in the pulsatile secretion of LH.

Fig. 2. Plasma profiles of LH for 8 h in 12-week-old bull calves injected i.v. with (a) 0.001, (b) 0.01 and (c) 0.1 mg MK-801 kg⁻¹ body mass. The arrow indicates the time of administration of MK-801. Pulses in secretion of LH, as detected by PC-Pulsar algorithm, are identified by asterisks.

The NMDA receptor activation, in addition to stimulating changes in GnRH secretion, may also induce central release of other neuropeptides such as corticotrophin releasing factor

Table 2. Effect of intravenous administration of MK-801, a specific noncompetitive antagonist of the NMDA receptor, on pulsatile secretion of LH in 24-week-old bull calves

MK-801 (mg kg ⁻¹ body mass)	Before MK-801 administration			After MK-801 administration		
	Mean (ng ml ⁻¹)	Number of peaks	Amplitude (ng ml ⁻¹)	Mean (ng ml ⁻¹)	Number of peaks	Amplitude (ng ml ⁻¹)
0.001	1.1 ± 0.1	1.8 ± 0.3	1.9 ± 0.4	0.7 ± 0.1	1.7 ± 0.3	1.6 ± 0.4
0.01	0.8 ± 0.1	1.8 ± 0.3	1.5 ± 0.2	0.8 ± 0.1	1.7 ± 0.3	1.6 ± 0.6
0.1	0.7 ± 0.1	1.5 ± 0.3	1.7 ± 0.2	0.3 ± 0.01 ^a	0.7 ± 0.3 ^b	0.5 ± 0.04 ^c

NMDA: N-methyl-D-aspartate.

Values are means ± SEM for four-hour periods; n = 6.

Superscripts identify values significantly less than the corresponding values before treatment (^aP < 0.05; ^bP < 0.01; ^cP < 0.001). **Fig. 3.** Plasma profiles of LH for 8 h in 24-week-old bull calves given (a) 0.001, (b) 0.01 and (c) 0.1 mg MK-801 kg⁻¹ body mass i.v. The arrow indicates the time of administration of MK-801 and asterisks identify pulses in secretion of LH.

(CRF) (Reyes *et al.*, 1990), which has the potential to inhibit GnRH secretion. However, most of the evidence suggests that stimulation of CRF/adrenocorticotrophic hormone by NMDA agonists is an independent effect that does not alter

concomitant NMDA-dependent stimulation of GnRH/LH release (Gay and Plant, 1987; Farah *et al.*, 1991; Lincoln and Wu, 1991). In contrast, in long-term ovariectomized monkeys, NMDA-associated stimulation of CRF may inhibit LH secretion (Reyes *et al.*, 1990). No data are available regarding the role of EAA in exciting the hypothalamo-pituitary-adrenal axis in cattle and, based on the evidence in other species, it appears unlikely that, in intact prepubertal calves, stimulation or inhibition of pulsatile LH secretion by the NMDA receptor activation or blockade is secondary to modulations in CRF release.

Taken together, the results of the present study suggest that different mechanisms are operative in the control of pulsatile secretion of LH in the juvenile and prepubertal phases of sexual development in bull calves. This contention may be analogous to the situation in rats, in which qualitative changes occur in the control of GnRH neurosecretion during maturation by different neurotransmitter systems, including EAA (Arias *et al.*, 1990; Moguilevsky *et al.*, 1990; Carbone *et al.*, 1992). Initiation of pulsatile LH secretion in juvenile calves is apparently not dependent on excitatory inputs to GnRH neurones mediated through the NMDA-specific EAA. The influence of EAAs on GnRH neurones at this time may be dampened by a lack of steroids. Rather, the development of characteristic high amplitude LH pulses may reflect the release of GnRH neurones from opioid (MacDonald *et al.*, 1990) or steroid inhibition (Amann *et al.*, 1986). However, maintenance of pulsatile LH secretion in the prepubertal period may involve an intermittent stimulatory NMDA receptor-specific EAA drive to the GnRH perikarya network, potentiated by androgens or by oestrogens aromatized from androgens in neuronal tissue. Endogenous EAA ligands may stimulate GnRH release directly through NMDA receptors located on GnRH neurones or indirectly through receptors present on catecholaminergic neurones. The results of the present study add to the understanding of the hypothalamic regulation of LH secretion during pubertal development in bull calves and support a physiological role for NMDA receptor-specific EAA neurotransmission in the control of gonadotrophin secretion.

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