Pulsatile GnRH secretion from primary cultures of sheep olfactory placode explants

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The aim of this study was to investigate the development of pulsatile GnRH secretion by GnRH neurones in primary cultures of olfactory placodes from ovine embryos. Culture medium was collected every 10 min for 8 h to detect pulsatile secretion. In the first experiment, pulsatile secretion was studied in two different sets of cultures after 17 and 24 days in vitro. In the second experiment, a set of cultures was tested after 10, 17 and 24 days in vitro to investigate the development of pulsatile GnRH secretion in each individual culture. This study demonstrated that (i) primary cultures of GnRH neurones from olfactory explants secreted GnRH in a pulsatile manner and that the frequency and mean interpulse duration were similar to those reported in castrated ewes, and (ii) pulsatile secretion was not present at the beginning of the culture but was observed between 17 and 24 days in vitro, indicating the maturation of individual neurones and the development of their synchronization.

Introduction

Investigation of GnRH pulsatility and its direct regulation remains a challenge (Levine, 1999). Experiments in vivo measuring the concentration of GnRH in the hypothalamo-hyphophyseal portal blood in ovine species have established the pulsatile secretion of this decapeptide (Levine et al., 1982; Clarke et al., 1987; Locatelli et al., 1987) and have shown several putative regulatory pathways (for a review see Thiéry et al., 1991). Electrophysiological recordings from the terminals of these neurones have also contributed to the current theory of regulation of GnRH secretion (Mori et al., 1995). However, these techniques have not been able to locate the anatomical locus of pulsatility or discriminate between direct and indirect modulation of GnRH neurones.

GnRH neurones have three anatomical characteristics. Firstly these neurones are scarce: it has been estimated that there are 800 in mice (Wray et al., 1989), 1200 in rhesus monkeys (Terasawa et al., 1993) and 3000 in ewes (Caldani et al., 1988). Secondly, these neurones do not form a distinct anatomical structure as do other neuroendocrine structures; they are located mainly in the septo–preoptico region in non-primate species, and in the medio–basal hypothalamus in primates (for a review see Silverman et al., 1979). Finally, GnRH neurones originate outside the brain (Schwanzel-Fukuda et al., 1989; Wray et al., 1989; Caldani et al., 1995; Duittoz et al., 1997). The first two characteristics have impeded the direct study of these neurones. In vitro methods using explants of the hypothalamus or the preoptic area have yielded interesting results, but do not enable GnRH neurones to be studied directly. In 1989, the first in vitro model (GT1 cell lines) of GnRH neurones was obtained by producing tumours of GnRH neurones in transgenic mice (Mellon et al., 1990). These cell lines have been studied extensively and have shown the presence of intrinsic pulsatility and numerous mechanisms that control GnRH secretion. However, these results should be interpreted with caution as the metabolism of these cells may be modified by tumour production (for a critical review see Selmanoff, 1997). Terasawa et al. (1999) and Funabashi et al. (2000) demonstrated pulsatile release from cultures of olfactory explants taken from rhesus monkey fetuses and from olfactory placode organotypic cultures from rat fetuses. This model presents several advantages. Firstly, GnRH neurones that originate from the placode have not been in contact with hypothalamic neurones or glial cells. Secondly, these neurones have not established a physiological state of secretion. This type of culture represents an interesting model for studying the mechanisms underlying the onset and the control of pulsatility in GnRH neurones. The aim of the present study was to investigate the onset of GnRH secretion in primary culture of GnRH neurones from olfactory placode explants from sheep (Ovis aries) embryos.

Materials and Methods

Ile de France ewes from an INRA flock were housed in a barn and oestrus cycles were synchronized with a fluogestone acetate intravaginal sponge (30 mg) for 12 days. On the day the sponge was removed, ewes received an i.m. injection of 500 iu equine chorionic gonadotrophin (eCG) (Chrono-gest®, Intervet International, Boxmeer) and 2 days later the ewes were mated. The day after mating was designated as E1.
Pregnancy was assessed by determining plasma progesterone concentrations. At E26 pregnant ewes were anaesthetized with an i.v. injection of 14 mg thiopentone kg$^{-1}$ (Nesdonal®, Sanofi Santé Animale, Paris) followed by halothane (Halothane, Laboratory Belamont, Paris). Caesarean sections were performed under sterile conditions and embryos were collected and maintained in sterile PBS with 5% glucose at 4°C. All animal procedures and care were carried out in agreement with the European legislation for animal experimentation (authorization A37801 of the French Ministry of Agriculture). The entire olfactory placode was dissected out and cut into halves. Each hemi-placode had a volume of approximately 1 mm$^3$ (Duittoz et al., 1997). Explants were placed on to plastic coverslips (Thermanox 13 mm, Nunc, Naperville) in 24-well culture dishes (Nunclon™, Nunc, Naperville). Cultures were maintained in Ham’s F10 medium (Eurobio, Les Ulis) supplemented with 10% fetal bovine serum (FBS), 100 μg gentamycin ml$^{-1}$ and 100 μg mycostatin ml$^{-1}$ (Eurobio, Les Ulis) for up to 35 days at 37°C in a water saturated 5% CO$_2$ atmosphere (Incubator Jouan, Nantes). Medium was changed every 3 or 4 days. The culture medium was collected every 10 min for 3 h in 14 cultures and for 8 h in 30 cultures. The 3 h collection period was insufficient to give a good estimate for pulsatility parameters, therefore the duration of the collection was increased to 8 h. Each culture well contained 550 μl Ham’s F10 and 10% FBS; 500 μl culture medium was collected from each culture well, extracted immediately with methanol (Caraty et al., 1995) and replaced with 500 μl fresh medium at 37°C and maintained under 95% air and 5% CO$_2$. Methanol extracts were maintained at –20°C until radioimmunoassay. In the first set of experiments pulsatile secretion was investigated after 17 and 24 days in vitro to estimate average pulsatility parameters. After the results were obtained, a second set of experiments was conducted to investigate pulsatile secretion in individual cultures after 10, 17 and 24 days in vitro to determine whether there are changes in the pulsatility parameters with increasing time in culture.

GnRH concentration was measured by radioimmunoassay (Caraty et al., 1995) in duplicate aliquots after methanol extraction. GnRH assay sensitivity was 0.5 pg ml$^{-1}$; the mean inter- and intra-assay coefficients of variation were 13% and 9%, respectively (n = 4 assays). GnRHBDS037 antibody is specific for the C-terminal moiety and also binds pro-GnRH and Hyp9-GnRH.

Pulsatility was determined by the PULSAR computer algorithm (Merriam et al., 1982). Cut-off criteria for G1, G2, G3, G4 and G5 were equal to 3.8, 2.6, 1.9, 1.5 and 1.2 standard deviations, respectively. Baxter parameters estimated from intra-assay coefficients of variation from 25 assays were: y = (2.86 ± 0.57x)/100.

Culture wells were fixed in 4% (w/v) paraformaldehyde and incubated with 1% (v/v) H$_2$O$_2$ in PBS with 0.3% (v/v) Triton-X100 for 2 h at 4°C to inhibit the activity of endogenous peroxidase. The samples were incubated with sheep or rabbit normal serum (1:15) in PBS with 0.3% Triton-X100 and 0.1% gelatin for 15 min at 4°C according to the origin of the primary antibody to inhibit non-specific binding of immunoglobulin (IgG). Two antibodies against synthetic GnRH peptide were used: anti-2-10 GnRH (no. 19900) obtained from rabbits after coupling the peptide to human serum albumin (HSA) with glutaraldehyde and anti-1-10 GnRH (no. BDS 037) obtained from sheep after coupling the peptide to BSA using carbodiimide (generously provided by B. D. Schanbacher, USDA Clay Center, NE), see Caldani et al. (1988) for characterization. Both antibodies were used at a dilution of 1:3000. The primary antibody was applied at the given dilution in PBS with 0.3% Triton-X100 and 0.1% (w/v) gelatin for 24 h at 4°C. Culture wells were rinsed three times with PBS at 4°C and incubated with biotinylated anti-rabbit or anti-goat IgG for 30 min at room temperature. Culture wells were rinsed three times with PBS at 4°C and incubated with the ABC Elite complex (Vectastain™, Vector, Burlingame). The culture wells were rinsed twice with PBS and once with 0.05 mol Tris–HCl 1$, pH 7.6. For visualization, 0.02% (w/v) 3, 3’diaminobenzidine (DAB) or DAB with 0.05% (w/v) nickel ammonium sulfate or VIP (Vectastain™, Vector, Burlingame) with 0.003% (v/v) H$_2$O$_2$ in Tris–HCl were applied for 1 or 2 min. The culture cells were dehydrated and mounted with depep. As a positive control, GnRH neurones from the adult sheep hypothalamus were stained using both anti-GnRH9900 and anti-GnRHBDS037. Three negative controls were performed: incubation without the primary antibody, without the secondary antibody, or with inactivated primary antibody. In the inactivated primary antibody control, the primary antibody was preincubated on nitrocellulose membrane saturated with GnRH coupled with HSA using glutaraldehyde. After 2 h, the primary antibody was applied to the culture. The number of positive GnRH cells was counted under a light microscope (Axioplan, Zeiss) with computer-aided image analysis (Histostar, Biocam). Only those cells with a dark stained cytoplasm and well developed neurites were counted. GnRH immunoactive neuroblasts and groups of GnRH immunoreactive migrating neurones were not counted.

Pulsatility parameters were compared using Student’s t test.

Results

Between April and September 1996, 33 explants from four different sets of cultures were used. This corresponds to 12 ewes providing an average of two embryos per Caesarean section. Fourteen of the cultures had a pulsatile secretion profile after 17 or 24 days in vitro. Parameters of pulsatility (number of pulses, mean amplitude of pulses, mean duration of pulses, mean interpulse interval) were not significantly different between cultures after 17 (n = 7) or 24 days in vitro (n = 7) (Table 1). The average number of pulses was 1.03 ± 0.06 pulses h$^{-1}$; the mean amplitude of the pulse was 0.97 ± 0.11 pg ml$^{-1}$; the mean pulse duration was 24.51 ± 1.08 min and the mean interpulse interval was 50.55 ± 2.53 min. Although no significant difference was detected among the parameters of pulsatility after 17 and 24 days in vitro, there was some variability between the cultures, which may mask the development of pulsatile secretion. This hypothesis was tested by monitoring the same wells at 10, 17 and 24 days in vitro. Ten wells were used in one set of cultures and were tested after 10, 17 and 24 days in vitro. None of the cultures showed detectable GnRH secretion after
The present study demonstrates that embryonic GnRH neurones in primary cultures of olfactory explants secrete GnRH in a pulsatile manner and that the parameters are comparable to those expected in castrated adults. This characteristic is apparent in vitro between weeks 2 and 3 of culture.

Other in vitro studies on GT1 cell lines (Martinez de la Escalera et al., 1992; Wetzel et al., 1992) and hypothalamic neurone suspension (Woller et al., 1998) have indicated that pulsatility may be an endogenous property of GnRH neurones. However, the use of modified cell lines that are not fully differentiated may not be of physiological relevance (for a review see Selmanoff, 1997). Suspensions of hypothalamic neurones release GnRH in a pulsatile manner for a

Table 1. Parameters of GnRH pulsatility estimated from primary cultures of olfactory placodes from ovine embryos after 17 and 24 days in vitro

<table>
<thead>
<tr>
<th>Number of days in vitro</th>
<th>Mean concentration of GnRH (pg ml⁻¹)</th>
<th>Mean number of pulses</th>
<th>Mean amplitude of pulses (pg ml⁻¹)</th>
<th>Mean duration of pulses (min)</th>
<th>Mean duration of interpulse interval (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>17 (n = 7)</td>
<td>0.80 ± 0.13</td>
<td>8.0 ± 0.25</td>
<td>0.92 ± 0.04</td>
<td>25.4 ± 0.6</td>
<td>52.6 ± 1.4</td>
</tr>
<tr>
<td>24 (n = 7)</td>
<td>0.91 ± 0.05</td>
<td>8.5 ± 0.30</td>
<td>1.03 ± 0.07</td>
<td>23.6 ± 0.4</td>
<td>48.5 ± 1.1</td>
</tr>
<tr>
<td>Mean (n = 14)</td>
<td>0.85 ± 0.02</td>
<td>8.25 ± 0.13</td>
<td>0.97 ± 0.03</td>
<td>24.5 ± 0.3</td>
<td>50.5 ± 0.6</td>
</tr>
</tbody>
</table>

Fig. 1. Individual profiles of GnRH secretion in a primary culture of olfactory placodes from ovine embryos after 17 and 24 days in vitro. Arrows indicate the presence of a pulse detected by the pulsar algorithm. (a) After 17 days in vitro, 11 pulses were detected with a mean amplitude of 0.85 pg ml⁻¹, a mean pulse duration of 27.3 min and a mean interpulse interval of 58.6 min; (b) in the same culture after 24 days in vitro, 12 pulses were detected with a mean amplitude of 2.05 pg ml⁻¹, a mean pulse duration of 24.6 min and a mean interpulse interval of 34.2 min.

10 days in vitro, three showed a pulsatile secretion after 17 days in vitro and four showed pulsatile secretion profile after 24 days in vitro. Secretion was not observed in six individual cultures. Among the GnRH secreting wells, two had a pulsatile profile after 17 and 24 days in vitro (Fig. 1), and two were not pulsatile after 17 days in vitro but showed pulsatile secretion after 24 days in vitro (Table 2). In cultures that showed pulsatile secretion after both 17 and 24 days in vitro, there was a small decrease in the duration of the interpulse interval (~5 min on average), a small decrease in the mean duration of secretion (~2.6 min on average), and in one culture there was an increase in the pulse amplitude (from 1.04 to 1.86 pg ml⁻¹). Furthermore, in one culture there was an increase in pulse frequency (from 1.4 to 1.6 pulses h⁻¹), whereas in another culture a decrease was observed (from 1.1 to 0.5 pulses h⁻¹) (Table 1). The main conclusion from this individual analysis is that pulsatile secretion of GnRH is established between 17 and 24 days in vitro, and that pulsatility parameters remain stable.

All the cultures tested were processed for GnRH immunocytochemistry (Fig. 2). Cultures that had a basal secretion of GnRH displayed GnRH immunoreactive neurones, some of which were organized into networks. Cultures that showed pulsatile secretion had 80–150 GnRH immunoreactive neurones (107 ± 25, mean ± SEM, n = 3). In some cultures in which GnRH secretion was not detected, immunocytochemistry revealed that GnRH neurones were absent or present in a small number. However, in some cultures in which GnRH secretion could not be detected, immunocytochemistry revealed numerous neurones, some of which were organized into a network. For example, in one culture in which pulsatile GnRH secretion was not detected, there were over 150 neurones immunoreactive for GnRH.
short period. Since hypothalamic neurones are acutely dispersed, it is possible that they retain a rhythm conferred \textit{in vivo} by other neuronal systems. A major advance in this area has been the demonstration of spontaneous pulsatile secretion from GnRH neurones derived from monkey olfactory placode cultures (Terasawa \textit{et al.}, 1999) and from rat olfactory placode organotypic cultures (Funabashi \textit{et al.}, 2000). Whether pulsatile secretion of GnRH is an endogenous property of GnRH neurones remains to be determined as non-GnRH neurones and other types of cell are also present in this type of culture. However, pulsatile GnRH secretion can be obtained in these models using embryonic neurones that have not established a physiological state of secretion. These neurones may be considered ‘naive’. In the present study, pulse frequencies and mean interpulse intervals were in the same range as those obtained in castrated ewes or during the follicular phase of intact ewes. Pulse frequencies and mean interpulse intervals estimated from monkey olfactory placode cultures (Terasawa \textit{et al.}, 1999) are comparable to those obtained in our model of castrated rhesus monkey females and castrated ewes. Studies on GT1 cell lines (Wetzel \textit{et al.}, 1992) and rat olfactory placode organotypic cultures (Funabashi \textit{et al.}, 2000) reported a shorter mean interpulse interval (20–30 min) that corresponds to the value obtained in castrated mice and rats. Such a high pulse frequency is observed in castrated ewes in which negative feedback is not

Table 2. Individual values of GnRH pulsatility parameters of four olfactory placode explants from ovine embryos tested after 10, 17 and 24 days \textit{in vitro}

<table>
<thead>
<tr>
<th>Culture</th>
<th>Number of days in vitro</th>
<th>Mean concentration of GnRH (pg ml\textsuperscript{-1})</th>
<th>Pulse frequency</th>
<th>Mean amplitude of pulses (pg ml\textsuperscript{-1})</th>
<th>Mean duration of pulses (min)</th>
<th>Mean duration of interpulse interval (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>17</td>
<td>1.04 ± 0.07</td>
<td>1.4</td>
<td>0.85 ± 0.54</td>
<td>27.3 ± 12.7</td>
<td>43.0 ± 13.3</td>
</tr>
<tr>
<td>B</td>
<td>17</td>
<td>1.86 ± 0.14</td>
<td>1.6</td>
<td>2.05 ± 0.73</td>
<td>24.6 ± 13.3</td>
<td>34.2 ± 17.3</td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>0.87</td>
<td>1.1</td>
<td>1.67 ± 0.85</td>
<td>30.0 ± 15.0</td>
<td>41.2 ± 16.4</td>
</tr>
<tr>
<td>C</td>
<td>17</td>
<td>0.68 ± 0.06</td>
<td>0.5</td>
<td>1.63 ± 0.64</td>
<td>27.5 ± 20.6</td>
<td>40.0 ± 17.3</td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>0.52 ± 0.08</td>
<td>–</td>
<td>–</td>
<td>30.0 ± 18.5</td>
<td>58.6 ± 35.3</td>
</tr>
<tr>
<td>D</td>
<td>17</td>
<td>0.52 ± 0.01</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>0.73 ± 0.05</td>
<td>0.9</td>
<td>0.63 ± 0.45</td>
<td>25.7 ± 12.7</td>
<td>48.3 ± 17.3</td>
</tr>
</tbody>
</table>

None of the cultures was pulsatile after 10 days \textit{in vitro}. Pulsatile secretion was observed in cultures A and B after 17 and 24 days \textit{in vitro} and in C and D after 24 days \textit{in vitro} but not after 17 days \textit{in vitro}.

Fig. 2. Immunocytochemistry of GnRH neurones in primary cultures of olfactory placodes from ovine embryos. (a) Migrating GnRH neurones which have a bipolar elongated cell body and are often found in groups of two to three co-migrating; (b) GnRH neurones forming a network on top of fibroblasts (after 24 days \textit{in vitro} culture). Scale bars represent 20 \textmu m.
exerted by steroids (Diekman et al., 1973; Barrell et al., 1992). Ham’s F10 medium supplemented with 10% fetal calf serum from the same batch was used in the present study. Fetal calf serum is a potential source of gonadal steroids, and the final concentration of oestradiol in the medium was 31 pg ml⁻¹. This concentration of oestradiol is in the same range as that detected in the plasma of pregnant ewes between day 20 and day 30 of gestation (Terqui, 1978). Although it has been shown that oestradiol receptors are present in mouse GnRH neurones (Skyttn er et al., 1999), in vivo studies on ovine species provide evidence that the effects of oestradiol on GnRH pulsatility involve other neuronal pathways. During anoestrus, subcutaneous implants of oestradiol in castrated ewes giving plasma concentrations of 1–2 pg ml⁻¹ inhibited GnRH pulse frequency, and this mechanism involves hypothalamic dopaminergic neurones (Thiéry et al., 1995; Goodman, 1996). Therefore, oestradiol has an inhibitory effect at low plasma concentrations. During the reproductive season, hypothalamic oestradiol implants located in the mediobasal hypothalamus have a stimulatory effect and induce the preovulatory surge, but oestradiol implants located in the preoptic area have a transitory inhibitory effect on GnRH pulsatility (Caraty et al., 1998). Thus, the main effect of oestradiol on GnRH pulse frequency is inhibitory and appears to involve other neuronal populations. In the present study, relatively high oestradiol concentrations were included in the culture medium and frequency values comparable to those observed in castrated ewes were obtained indicating that oestradiol does not interfere with GnRH pulsatility.

The sampling method used may interfere with hormone release. In the present study a static sampling procedure was used similar to that described by Funabashi et al. (2000) in rat olfactory placode organotypic cultures, whereas Terasawa et al. (1999) used a perifusion system. The static procedure may have modified the pulse frequency, as there was an accumulation of GnRH in the culture medium between two changes of medium. There is evidence that GnRH inhibits its own secretion (Krsmanovic et al., 1993; Padmanabhan et al., 1995); however, whether GnRH acts directly on the GnRH neurones and whether there is a physiological relevance for its action has not been resolved. Nevertheless, in the present study pulsatility parameters comparable to GnRH measurements in vivo in the portal blood of castrated ewes were obtained, as in rhesus monkey olfactory placode experiments in which Terasawa et al. (1999) used a perifusion system. Funabashi et al. (2000) reported that the sampling procedure had an effect on rat GnRH associated peptide (rGAP) immunoreactivity: a high frequency of sampling decreased rGAP immunoreactivity, indicating that the cytoplasmic concentration of GnRH decreased. However, in the model used in the present study there was no apparent link between the number of GnRH immunoreactive cells and pulsatile secretion.

In other pulsatility parameters, the mean amplitude of the pulse was lower in olfactory explant cultures from both sheep and rhesus monkey embryos than in GnRH neurones in vivo. This discrepancy may result from the lack of hypothalamic inputs or from the immaturity of the neurones used in vivo, and there were also fewer GnRH neurones in these cultures than in the adult animal. Conversely, pulse amplitude in GT1 cell culture was higher than those in vivo. However, in these experiments, there were about 2 × 10⁵–10⁶ cells ml⁻¹, indicating that individual GT1 cells have a smaller secretory capacity than native GnRH neurones. The mean duration of a pulse can be interpreted differently according to the type of experiment. In experiments in vivo, the pulse corresponds to a combination of the duration of secretion and half-life of the hormone. In experiments in vitro, there is little or no degradation of GnRH within the time of the experiment (results not shown), thus the pulse duration would correspond only to secretion. In sheep, the estimated duration of GnRH secretion in vivo is approximately 20 min (Locatelli et al., 1987) as also found in the present study. Therefore, there is agreement between results obtained in vivo and in vivo in various mammalian species with different reproductive physiology. This finding is important as there are large differences in reproductive physiology among mammals. In the three species studied, mice have a long follicular phase, a short luteal phase and ovulation that is dependent on the circadian rhythm; rhesus monkeys have a primate type menstrual cycle, and ewes have seasonal reproduction with a short follicular phase and a long luteal phase. Comparative studies of the regulation of GnRH neurones in vivo from these three species may provide some evidence to account for these differences.

There are two requirements for pulsatile GnRH: firstly individual GnRH neurones should secrete GnRH in a discrete manner, and secondly, some or all of the GnRH neurones should be able to synchronize secretion. Synchronization of individual neurones may result from individual coupling (synapses, gap junctions) (Wetzel et al., 1992) or from diffusible substances (Martinez de la Escalera et al., 1992; Terasawa et al., 1999). In the model used in the current study, the presence of GnRH neurones organized into a network does not guarantee pulsatile secretion of GnRH. Only 20% of the cultures were pulsatile; the remaining 80% of cultures showed little or no secretion. Duittoz et al. (1997) showed that GnRH neurones migrate from 10–17 days in vitro, and settle down and form networks between 17 and 24 days in vitro. Electron microscope studies on migrating GnRH neurones in the nasal septum of the mouse embryo have shown that GnRH was present but no secretory vesicles were observed (Zheng et al., 1992). Thus, migrating GnRH neurones may not be able to release GnRH. The fact that some cultures between 17 and 24 days in vitro have detectable but non-pulsatile GnRH concentrations indicates that GnRH neurones have acquired the ability to release GnRH, but lack synchronization. In all the species studied, at the end of the fetal life GnRH neurones are activated, which results in an increase in LH secretion and gonadal steroids, and the GnRH system then remains inactive until puberty. This inhibition is dependent on inhibitory neuronal systems, which may include γ-aminobutyric acid (GABAergic) neurones. A change in the expression of GABA A receptor subunits in individual GnRH neurones has been reported between prepubertal animals and adult animals. These changes are coupled with the transition of a population of GnRH neurones from electrical inactivity to spontaneous activity (Herbison et al., 1999). In the system used in the
current study, most of the cultures were inactive. However, the presence of inhibitory GABAergic neurones in these cultures cannot be eliminated, but individual maturation and development of the synchronization process are important developmental steps that GnRH neurones must achieve before establishing pulsatile secretion of GnRH. The study of the development of Ca²⁺ signalling in individual GnRH neurones and groups of neurones may help to resolve this question.

In conclusion, the present study demonstrates that (i) primary cultures of ovine GnRH neurones from olfactory placodes release GnRH in a pulsatile manner with characteristics comparable to those of castrated adult sheep; and (ii) this characteristic is acquired during weeks 2–3 in vitro. Many questions regarding the onset of pulsatility remain to be answered. Are all GnRH neurones involved in the onset of pulsatility? How does pulsatility develop between weeks 2 and 3 of culture? What is the messenger involved in synchronization? Is there a role for the non-GnRH neurones found in these cultures? Cellular models similar to the primary culture of olfactory placodes will help to answer some of these questions.

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