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Research article

Changes in the 5-HT2$_A$ receptor system in the pre-mammillary hypothalamus of the ewe are related to regulation of LH pulsatile secretion by an endogenous circannual rhythm

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Abstract

Background: We wanted to determine if changes in the expression of serotonin 2A receptor (5HT2$_A$ receptor) gene in the premammillary hypothalamus are associated with changes in reproductive neuroendocrine status. Thus, we compared 2 groups of ovariectomized-estradiol-treated ewes that expressed high *vs* low LH pulsatility in two different paradigms (2 groups per paradigm): (a) refractoriness (low LH secretion) or not (high LH secretion) to short days in pineal-intact Ile-de-France ewes (RSD) and (b) endogenous circannual rhythm (ECR) in free-running pinealectomized Suffolk ewes in the active or inactive stage of their reproductive rhythm.

Results: In RSD ewes, density of 5HT2$_A$ receptor mRNA (by in situ hybridization) was significantly higher in the high LH group (25.3 ± 1.4 *vs* 21.4 ± 1.5 grains/neuron, $P < 0.05$) and ^{3}H-Ketanserin binding (a specific radioligand) of the median part of the premammillary hypothalamus tended to be higher in the high group (29.1 ± 4.0 vs 24.6 ± 4.2 fmol/mg tissu-equivalent; $P < 0.10$). In ECR ewes, density of 5HT2$_A$ receptor mRNA and ^{3}H-Ketanserin binding were both significantly higher in the high LH group (20.8 ± 1.6 *vs* 17.0 ± 1.5 grains/neuron, $P < 0.01$, and 19.7 ± 5.0 vs 7.4 ± 3.4 fmol/mg tissu-equivalent; $P < 0.05$, respectively).

Conclusions: We conclude that these higher 5HT2$_A$ receptor gene expression and binding activity of 5HT2$_A$ receptor in the premammillary hypothalamus are associated with stimulation of LH pulsatility expressed before the development of refractoriness to short days and prior to the decline of reproductive neuroendocrine activity during expression of the endogenous circannual rhythm.

Background

Seasonal reproductive activity is a common feature of many mammalian species of temperate latitudes [1]. In ewes, ovulatory activity is suppressed for several months in spring and summer during the anestrous period. These seasonal changes in ovulatory activity result from changes in LHRH and LH pulsatile secretion [2,3]. Seasonality of neuroendocrine reproductive activity in ewes is under the

control of an endogenous circannual rhythm [4–6] synchronized by photoperiod through its control of the circadian rhythm of melatonin secretion [7,8]. Expression of the endogenous circannual rhythm can be observed under two types of experimental conditions : prolonged exposure to constant photoperiod (i.e. constant duration of melatonin secretion) or elimination of melatonin secretion by pinealectomy. In the case of pineal-intact ewes exposed to constant short days following constant inhibitory long days, three successive phases of reproductive neuroendocrine activity can be distinguished: (a) initially, a short-day induced stimulation of LH pulsatile activity after a time-lag of about 45 days, (b) second, inhibition of LH pulsatility, resulting from «refractoriness» to short days after about 150 short days [9], probably the initial event of the expression of the endogenous rhythm, and (c) third, circannual changes between periods of high and low LH pulsatility reflecting expression of the endogenous circannual rhythm [5]. In pinealectomized ewes that are functionally uncoupled from their photoperiodic environment, the endogenous circannual rhythm is expressed and induces alternations of periods of high and low LH pulsatility, which are not synchronous among ewes or in phase with the seasons [8,10].

Basic mechanisms responsible for the generation and the expression of this endogenous rhythm are unknown. Several neuromediator systems, such as catecholamines and serotonin (5HT) [11,12] (review [13]), and neuroplastic remodeling of GnRH neurons [14,15] seem to play a role in seasonal and photoperiodic regulation of LH pulsatile secretion. However, in most of these cases, it is not known whether these mechanisms are involved in the expression of the endogenous rhythm or whether they simply mediate the effect of photoperiod and melatonin. In the case of serotonergic pathways, however, it is clear that the serotonergic component plays a role in LH suppression during the refractory state to short days [16,17]. Indeed, systemic administration of serotonin antagonists can temporarily reverse the suppression of LH pulsatile secretion in ewes that are refractory to short days [18]. The use of specific antagonists, and especially ketanserin, led to the suggestion that $5HT2_A$ receptors are probably involved in this inhibitory effect of serotonin [19]. The inhibition of LH pulsatile secretion during the establishment of refractoriness to short days is associated with changes in $5HT2_A$ receptor binding capacities (^{3}H-ketanserin binding activity) in specific area of the ventral hypothalamus [20]. However, it is worth noting that although an increase in $5HT2_A$ receptor binding capacities was expected during this period of low secretion to be consistent with the inhibitory role of serotonin, the contrary was found, i.e. an increased ^{3}H-ketanserin binding. This specific hypothalamic area has been identified as the premammillary hypothalamus as confirmed by the expression at this site of $5HT2_A$ receptor mRNA [21].

Collectively, the foregoing observations prompted us to investigate whether $5HT2_A$ receptors in the premammillary hypothalamus may play a role in the expression of the endogenous rhythm. As an initial step, we have determined if expression of the $5HT2_A$ receptor gene in the premammillary hypothalamus changes during the course of expression of the endogenous rhythm independently of photoperiodic or melatonin influence. In addition, we have examined if transcriptional regulation of the $5HT2_A$ receptor gene is associated with changes in binding capacities of this receptor. For this purpose, we employed in situ hybridization for mRNA of $5HT2_A$ receptor and binding of ^{3}H-Ketanserin in the premammillary hypothalamus of ewes under two experimental conditions enabling the expression of the rhythm: (a) pineal-intact ewes that were either refractory or not to short days (RSD), and (b) pinealectomized ewes during either the active and inactive stages of their endogenous circannual rhythm (ECR) of reproductive neuroendocrine activity.

Results

Experiment 1: Refractoriness to short days

Mean plasma LH during photoperiodic treatments varied as expected (Fig. 1) with low LH plasma concentrations during long days, high values resulting from the stimulatory effect of short days and low values during continued exposure to short days due to the establishment of a refractory state to short days. On the day before sacrifice, LH pulse frequency was significantly higher in the High group than in the Low group (mean ± S.E.M. 2.83 ± 0.40 *vs* 0.50 ± 0.22 pulses/5 hours, $P < 0.001$).

In situ hybridization analysis for $5HT2_A$ receptor mRNA revealed that the density of silver grains was significantly higher in the neurons of ewes from the High group (25.3 ± 1.4 *vs* 21.4 ± 1.5 grains/neuron, $P < 0.05$). Overall distribution of neurons per class of number of grains was significantly different between the two groups (Chi2= 52, $P < 0.0001$); neurons bearing more than 20 grains were more numerous in the High than in the Low group (Figure 2). An example of in situ hybridization $5HT2_A$ receptor mRNA labeling is given in Figure 3.

^{3}H-Ketanserin binding was not significantly different between groups, but tended to be significantly higher in the High group than in the Low group (29.1 ± 4.0 vs 24.6 ± 4.2 fmol/mg tissue-equivalent; $P < 0.10$).

Experiment 2: Expression of the endogenous circannual rhythm

Endogenous LH cycles were desynchronized among ewes and appeared to free run in all ewes included in the

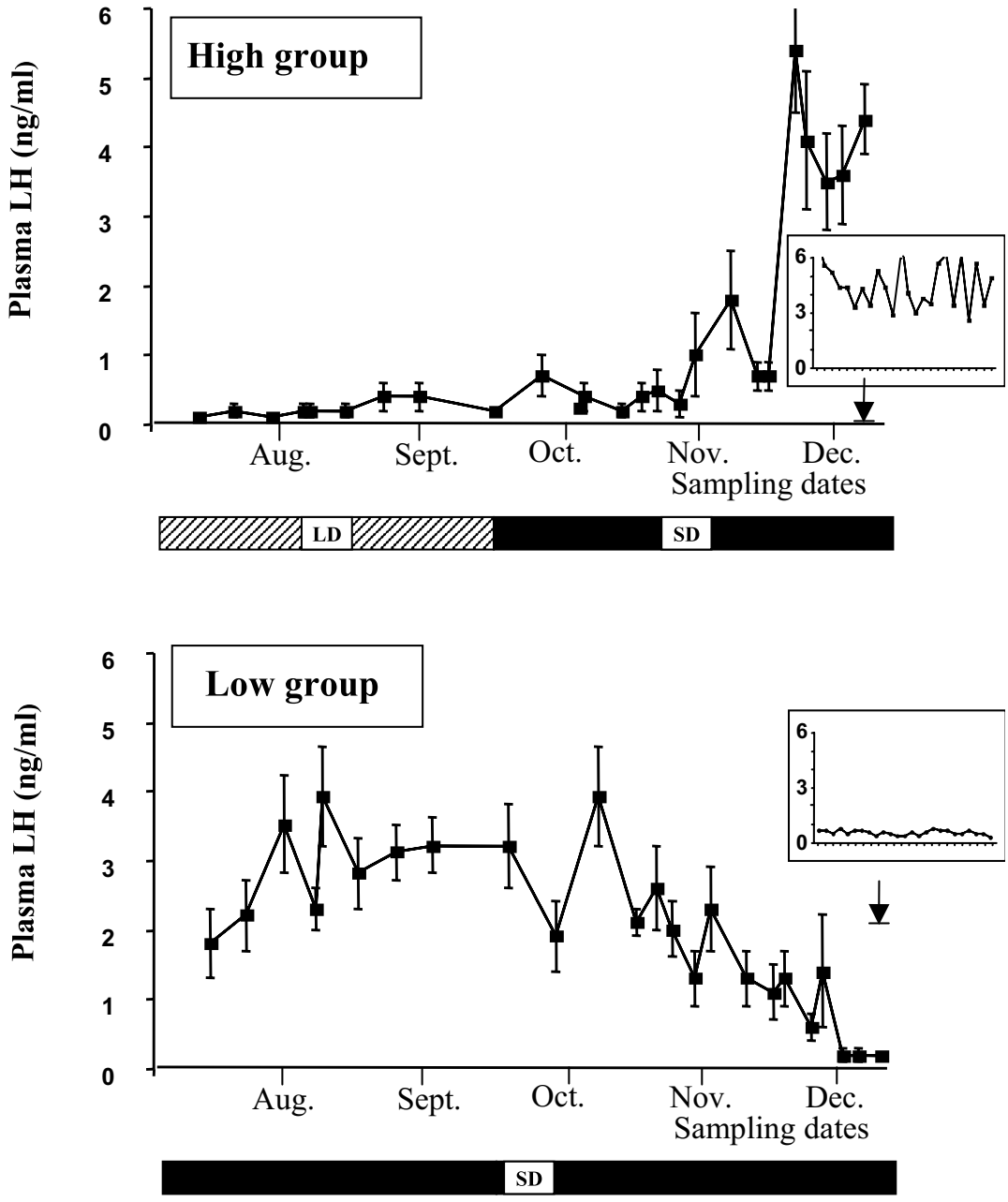

Figure 1

Experiment 1 (RSD): Mean (± SEM) plasma LH concentration in 2 groups of ovariectomized estradiol-treated Ile-de-France ewes subjected to the artificial photoperiodic regimens indicated by the horizontal bars. High ewes (n = 6; upper graph) were subjected to 3 months of short days (8L: 16D; April to July, data not shown) – 3 months of long days (16L: 8D, July to October), and 3 months of short days (8L: 16D, October to January). Low ewes (n = 6) were subjected to 3 months of long days (April to July, data not shown), and 6 months of short days (July to January). Inserts depict representative LH pulsatile secretory profiles the day before sacrifice (blood sampled every 12 min for 5 hours).

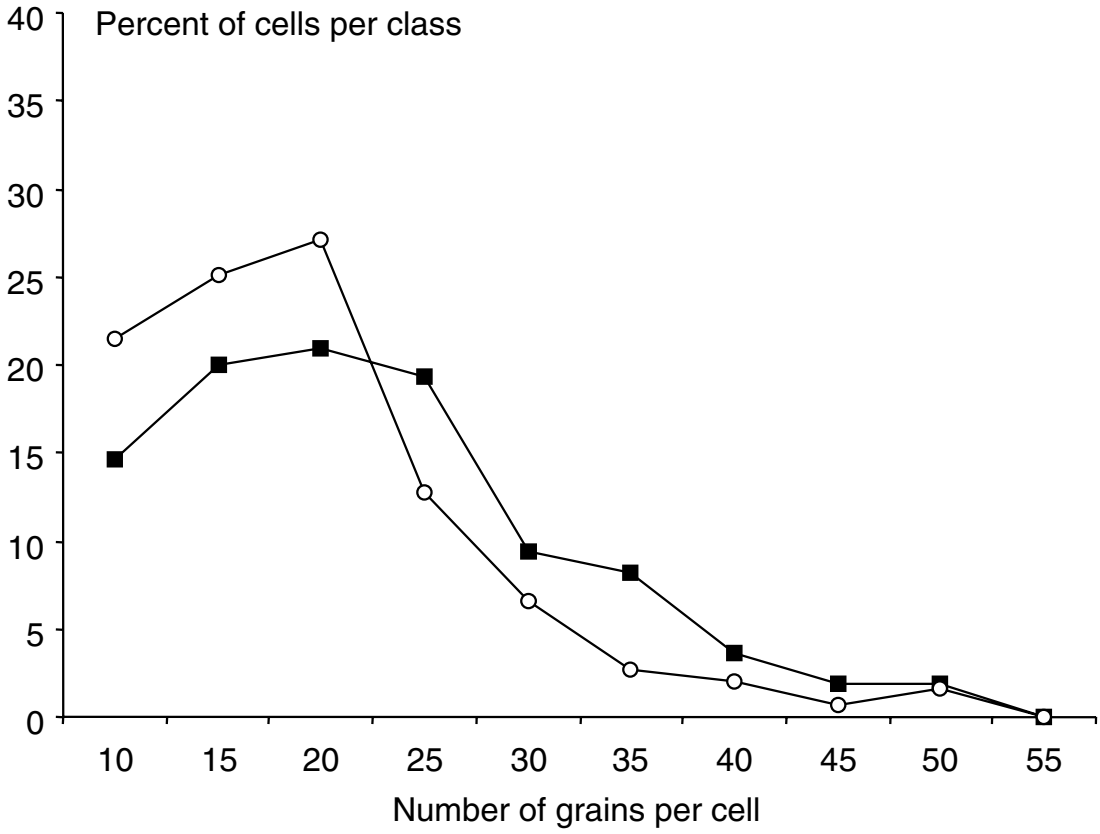

Figure 2
Frequency distribution of labelled neurons in frontal brain sections after in situ hybridization with riboprobes against sheep 5HT2$_A$-receptor, according to the number of silver grains per neuron, in the premammillary hypothalamus of Ile-de-France ewes during the establishment of refractoriness to short days (experiment 1).
Closed and open symbols depict ewes with high or low LH pulsatile activity, respectively.

analysis [for more details see ref. [10]]. Figure 4 illustrates the LH profiles for one ewe in the High and Low groups; data were not pooled for presentation of mean values because the circannual LH cycles were not synchronized among ewes. On the day before sacrifice, LH pulse frequency was significantly higher in the High group than in the Low group (3.50 ± 0.72 *vs* 0.83 ± 0.40 pulses/4 hours, $P < 0.01$). One ewe which was detected as being high with the cluster cycle detection algorithm, did not show a high pulsatile activity (1 pulse/5 hours); this animal was included in the analysis as she had high overall LH values.

Expression of 5HT2$_A$ receptor gene was different between groups. Density of silver grains was significantly higher in the neurons of ewes from the High compared to the Low group (20.8 ± 1.6 *vs* 17.0 ± 1.5, $P < 0.05$). Overall distribution of neurons classified by number of grains per neuron was significantly different between the two groups (Chi2= 52, $P < 0.0001$); neurons bearing more than 20 grains were more numerous in the High as compared with the Low group (Figure 5).

^{3}H-Ketanserin binding in the premammillary hypothalamus was significantly higher in the High group than in the Low group (19.7 ± 5.0 *vs* 7.4 ± 3.4 fmol/mg tissue-equivalent; $P < 0.05$). An example of ^{3}H-Ketanserin binding sites on sections of the premammillary hypothalamus is shown in Figure 6.

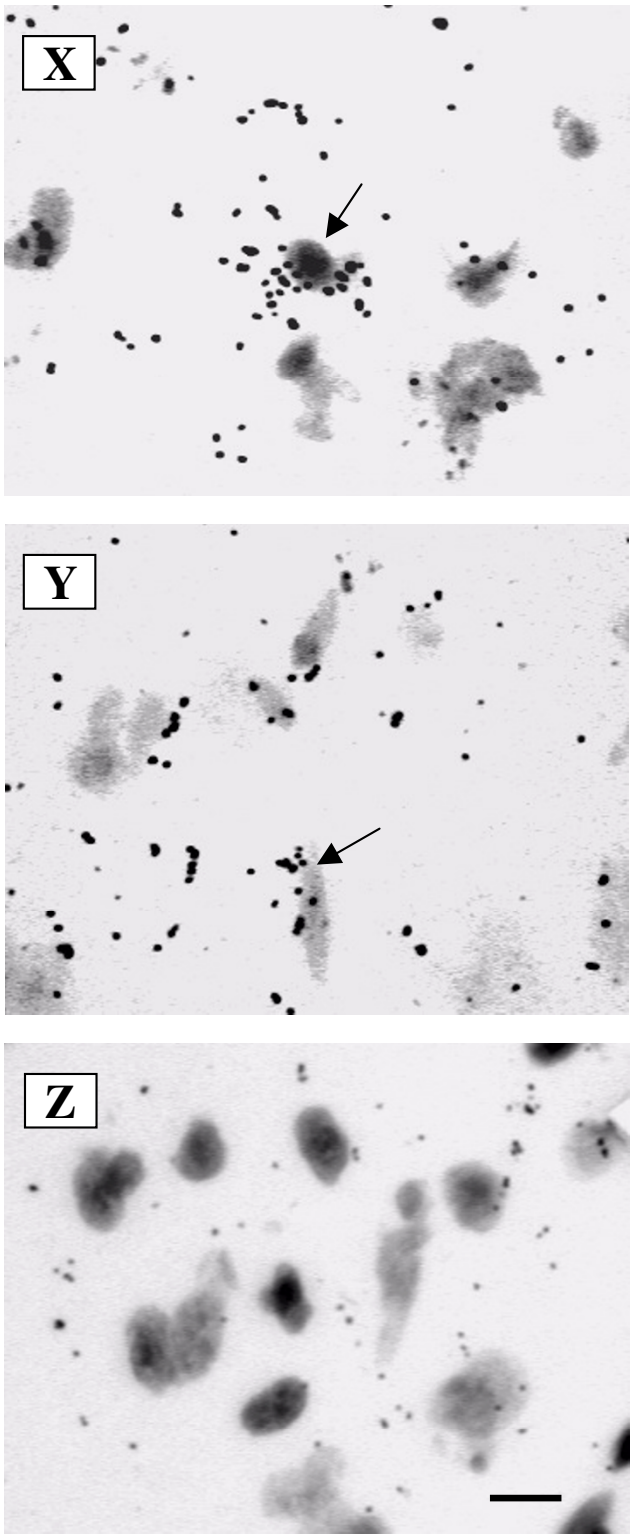

Figure 3
Bright-field microscopy images of premammillary hypothalamus after in situ hybridization with antisense (X, Y) or sense (Z) riboprobes against sheep 5HT2A receptor. Arrows indicate labeled neurons. Scale bar 15 μm. X is a ewe from the high group while Y is a ewe from the low group.

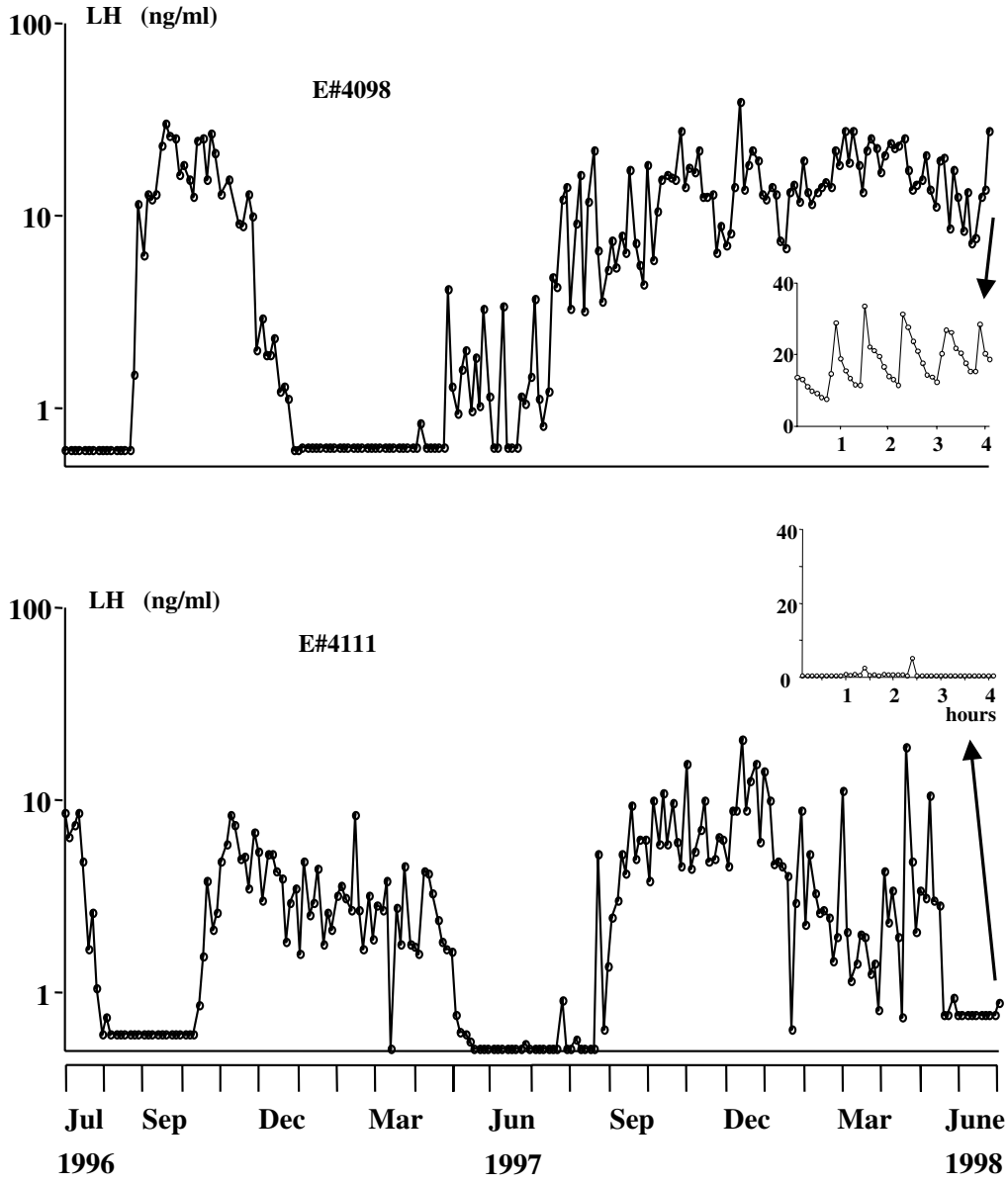

Figure 4

Experiment 2 (ECR): plasma LH concentration measured in bi-weekly blood samples obtained from two pinealectomized Suffolk OVX+E ewes. Animals were maintained melatonin-free under simulated natural photoperiodic conditions in a light-proof building for 2 years. The upper graph depicts LH profiles in one ewe in the High stage of the endogenous rhythm at the end of the experiment and the lower graph LH profile of one ewe in the Low stage. Inserts depict LH pulsatile secretory profiles at the end of the experiment (blood sampled every 6 min for 4 hours).

Discussion

Coordinated changes in the expression of the $5HT2_A$ receptor gene and in the density of $5HT2_A$ receptors were observed in the premammillary hypothalamus; both gene expression and binding activity were greater in ewes expressing a high LH pulsatile secretion compared to those expressing a low LH pulsatile secretion. This relationship was observed both during the establishment of refractoriness to short days (RSD) and during the course of the endogenous circannual rhythm (ECR) of neuroendocrine activity. The results obtained with ketanserin binding, in ewes refractory or not to short days (experiment 1) tended to be significant, this tendency confirms earlier results obtained in the same breed of ewes, placed in the same experimental conditions [20]. Our current results brought interesting new insights by showing that this increase in binding capacity results at least in part from an up-regulation of expression of the $5HT2_A$ receptor gene.

It is important to emphasize that the two experiments described here were conducted in two different locations (France and USA), in two different seasonal breeds of sheep (Ile-de-France and Suffolk), and using two different animal models (pineal-intact ewes maintained in short days and pinealectomized ewes under normal photoperiodic changes). Yet, the results were comparable regarding changes in the $5HT2_A$ receptor system in the premammillary hypothalamus according to LHRH/LH pulsatile activity. The observation that expression of the gene and density of receptors varied in the same direction in both experiments (even if a non-significant tendency in the binding density was observed in the RSD experiment) is of major importance. This provides strong evidence that the enhanced activity of the $5HT2_A$ receptor system in ewes expressing stimulated *vs* inhibited LH pulsatility can occur in the absence of daily changes in melatonin input and thus photoperiodic influence. This suggests one or two possibilities. Either (a) the changing activity of the $5HT2_A$ receptor system is the consequence of changes in the LHRH/LH pulse generating mechanisms, or (b) changes in the $5HT2_A$ receptor system are a fundamental component of the circannual processes that cause changes in LHRH/LH pulsatility. In this case, the establishment of refractoriness to short days could be considered as an early step in expression of the endogenous circannual rhythm [5], and changes of activity in the $5HT2_A$ receptor system could be considered as part of the endogenous mechanisms involved in circannual rhythms of reproductive neuroendocrine activity.

At present, it is not known whether changes in the $5HT2_A$ receptor system are a cause or a consequence of the circannual rhythm of LHRH and LH secretion. Nevertheless, it is of interest to consider a causal relationship and, for this purpose, it is useful to consider certain parallelisms between circadian and circannual mechanisms. Regarding circadian rhythms, three major points could be raised : (a) The suprachiasmatic nucleus is the brain area where the master circadian clock is located, and where several «clock» genes are expressed at different precise times of the cycle (reviews [22] and [23]). (b) Serotonergic fibers project from raphe nuclei to the suprachiasmatic nucleus [24], onto $5HT1_B$ receptors [25], probably to modulate response of the suprachiasmatic nucleus activity to light stimulation [26]. (c) The suprachiasmatic nucleus is the nucleus where photoperiodic and/or light inputs act to synchronize the circadian system to fit with the external environment, directly via nerve fibers and/or by the way of melatonin receptors (review [23]).

How might these three points relate to the circannual rhythms? (a) In contrast with circadian rhythms, there is to date no formal identification of a site for a clock driving circannual rhythms. In the ground squirrel, which was extensively studied in this context [27–33], a brain site involved in generation of circannual cycles was not discovered. (b) With respect to serotonergic innervation, medium or high density of serotonin fibers are present in the premammillary hypothalamus in sheep [34], white-footed mouse [35], monkey [36] and rat [37,38]; these fibers probably originate from the raphe nuclei, as in the cat [39]. Changes in gene expression and ketanserin binding capacities of $5\text{-}HT2_A$ receptor in the pre-mammillary hypothalamus of the ewe were observed in the present study. (c) Finally, the site in which we observed changes in $5HT2_A$ receptor seems to be located in the same area than the site of action of melatonin to stimulate LHRH/LH pulsatile secretion in the ewe [40], probably via MT1 receptors [41,42]. This stimulation of the LHRH pulsatility by melatonin may be one way by which melatonin enters the circannual system to synchronize it to the photoperiodic cue of the outside environment [review [43]]. This may explain that in the results presented here, ewes in the absence of any photoperiodic/melatonin input, expressed de-synchronized rhythmicity of LH secretion. Thus, the suprachiasmatic nucleus contains a circadian clock and is the site where the external cues are treated to reset the clock via melatonin receptors or/and via serotonergic system, and the premammillary hypothalamus may give rise (or help give rise) to circannual rhythmicity and may be a site where melatonin synchronizes the endogenous circannual rhythm and where changes in the serotonergic system take place.

The involvment of the posterior hypothalamus in reproductive function has received relatively little attention in the rat where it has only been shown to inhibit LH release and that it may participate in timing and amplitude of the pro-oestrus surge of the hormone [44]. In the macaque posterior hypothalamus, ovarian steroids altered expres-

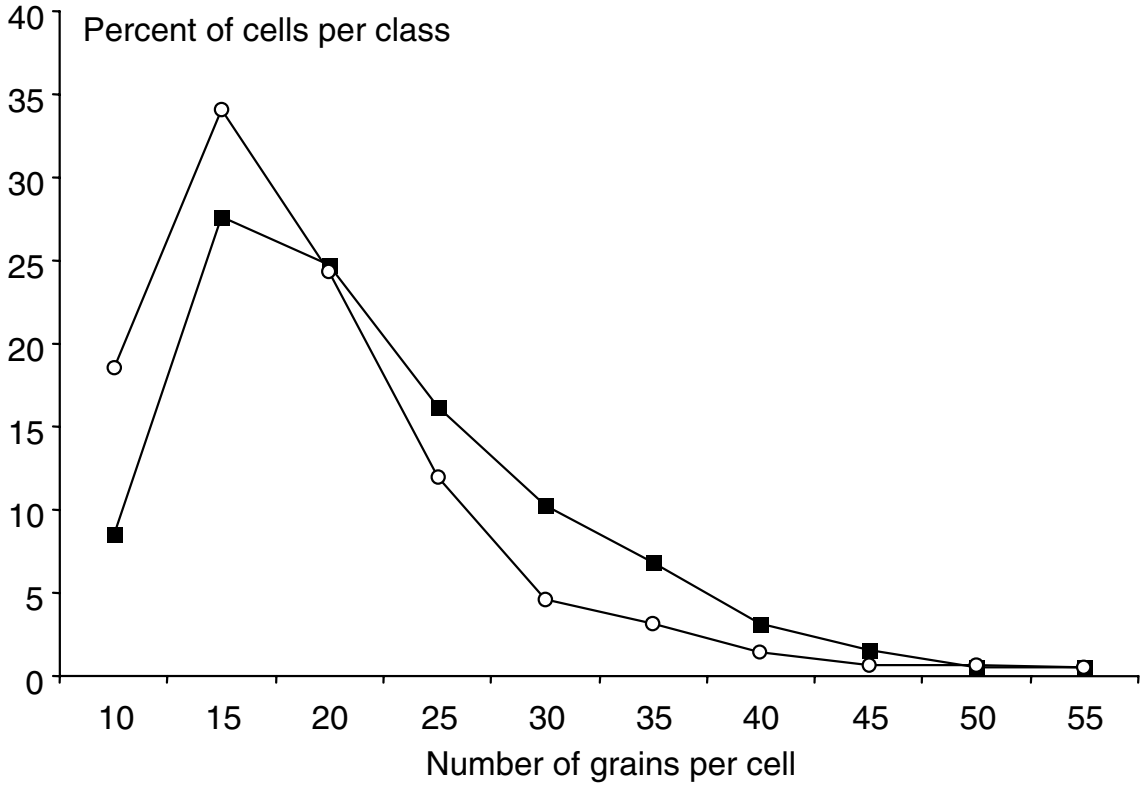

Figure 5
Frequency distribution of labelled neurons in frontal brain sections after in situ hybridization with riboprobes against sheep $5HT2_A$-receptor, according to the number of silver grains per neuron, in the premammillary hypothalamus of pinealectomized Suffolk ewes at different stages of the expression of the endogenous circannual reproductive rhythm (experiment 2). Closed and open symbols depict ewes with high or low LH pulsatile activity, respectively.

sion of $5HT2_C$ receptor gene, but not of $5HT2_A$ receptor gene [45]. Recent preliminary observations in Suffolk ewes suggest that thyroid hormones, which are required for the expression of endogenous rhythms, may be required for the day-night rhythm of fos expression in the premammillary hypothalamus [46], which re-inforces the suggestion of a potential role of this particular structure in the mechanisms involved in the circannual endogenous rhythm of reproductive activity in sheep. More generally, the posterior hypothalamus is considered as playing an important role in the maintenance of circadian wakefulness, especially via its histaminergic neurons [39,38].

The divergent actions of serotonin are achieved through seven distinct families of receptors encoded by more than seven different genes, among them the previously mentionned $5HT2_A$, $5HT2_B$ and $5HT2_C$ receptors, each of which exhibit subtypes or isoforms [reviews [47,48]]. How these different receptors mediate the multiple action of serotonin and whether individual neurons express multiple receptors are still subject of debate [review [48]]. In the present experiment, the riboprobes used were specific of the sheep $5HT2_A$ receptor. They showed a low homology with $5HT2_B$ and $5HT2_C$ receptors (60% for both), and no homology at all for other subtypes of recep-

tors, as it was shown by Pelletier et al. [21]. This situation ensures that the mechanisms described here are specific of the ovine $5HT2_A$ receptors.

Regarding the functional role of $5HT2_A$ receptors, in ovariectomized rats, they seem to be involved in the negative feedback of oestrogens on LH release [49] and in the triggering of LH surge by oestradiol [50]. As previously exposed, it was shown in ewes, that the use of specific antagonists of $5HT2_A$ receptors liberates the inhibition of pulsatile LHRH/LH activity during refractoriness to short days [18,19]. However, this effect is contradictory with the results presented here showing that $5HT2_A$ receptor gene expression and binding activity were lower during this specific stage. More generally, the information on the potential role of these $5HT2_A$ receptors in mammals is scarce but suggests that they could be involved in depression [reviews [51,52]] and/or learning [53], which are both long-term neurobiological events. Regarding the interaction between $5HT2_A$ receptor system and thyroid hormones, it is interesting to note that ^{3}H-Ketanserin binding capacity, is decreased in thyroidectomized rats, and that this effect of thyroidectomy can be reversed with low doses of T4 [54]. Thus, the fact that, $5HT2_A$ receptor activity in rats and the transition into anestrus in sheep [55–58], are dependent of thyroid hormones may suggest that it would be interesting to look for an eventual relationship between $5HT2_A$ receptors and thyroid hormones in sheep.

Conclusions

In conclusion, we demonstrated in two different paradigms that the expression of a change in circannual rhytmicity in LHRH/LH neuroendocrine activity is associated with regulation of $5HT2_A$ receptor gene expression in the premammillary hypothalamus of the ewe. This anatomical and molecular evidence for an implication of the $5HT2_A$ receptor system of the premammillary hypothalamus in circannual rhythmicity provides new insight in the field of circannual rhythms. In particular, the present findings provide novel evidence for a putative site that may contribute to the generation of endogenous circannual rhythms. This possibility is further strengthened by evidence that the premammillary hypothalamus could also be a site for integration of the circannual mechanisms with the melatonin signal which translates photoperiodic cues synchronizing the endogenous rhythm. Thus, our present results pave the way for studies to investigate the functional significance of the changes in $5HT2_A$ receptor and of the premammillary hypothalamus as a component of circannual mechanisms.

Methods

Animal model

Two experiments were conducted on adult ovariectomized ewes treated with constant release estradiol implant (1.7 cm for experiment 1 and 3 cm for experiment 2; [59]). In this model, plasma LH concentration in blood sampled twice a week provides a robust index of seasonal changes in reproductive neuroendocrine responsiveness to estradiol negative feedback on LHRH and LH pulsatile secretion [60]. High LH concentration reflects high frequency of LHRH and LH pulses, which is indicative of the breeding season, whereas low LH reflects infrequent LHRH and LH pulses typical of anestrus [61]. Animals were fed hay and pellets and had free access to water and mineral licks. Surgeries were performed under aseptic conditions.

Experimental design

Experiment 1: Refractoriness to short days (RSD)

This study was conducted on 12 adult (3–7 years old) Ile de France ewes maintained at the INRA Research Center of Nouzilly France (47°N). All ewes were housed in a light-proof building under artificial lighting (300 lux at animal eye level). Temperature was not regulated. Ewes were allocated to two groups (n = 6/group). The first group (high LH group) was exposed to short days (8L:16D) for 3 months starting in April; 3 months of long days (16L: 8D; July to October) and 3 months of short days (8L: 16D; October to January) after which brains were collected. With this photoperiodic regimen, ewes remain sensitive to the stimulatory effect of short days resulting in high pulsatile LH secretion at the time of sacrifice. The second group (low LH group) was exposed to long days for 3 months starting in April followed by 6 months of short days. This photoperiodic regimen enables the photorefractory state to occur resulting in low pulsatile LH secretion at the time of sacrifice. Blood samples were collected twice a week throughout photoperiodic treatments. LH pulsatile secretory profiles on the day before sacrifice were determined from LH concentration measured in blood samples collected every 12-min for 5 hours. Ewes from both groups were sacrificed in pairs by decapitation between 10:00 am and 2:00 pm on the same day. All procedures were performed in accordance with French legal requirements, and with the authorization for animal experimentation nb A37801 of the Ministry of Agriculture.

Experiment 2: Endogenous circannual rhythm (ECR)

Seventeen pinealectomized Suffolk ewes (of which 12 were used, see below) were maintained at the Sheep Research Facility near Ann Arbor, MI U.S.A. (42°18'N). These ewes had previously been used in another experiment aimed at synchronizing their circannual reproductive rhythm by infusions of different melatonin patterns at specific periods of the circannual endogenous cycle [10]. Upon completion of that study, the ewes were made available to the present study. The ewes were pinealectomized in December 1994 during the late breeding season [62,10] and housed in light-proof rooms where

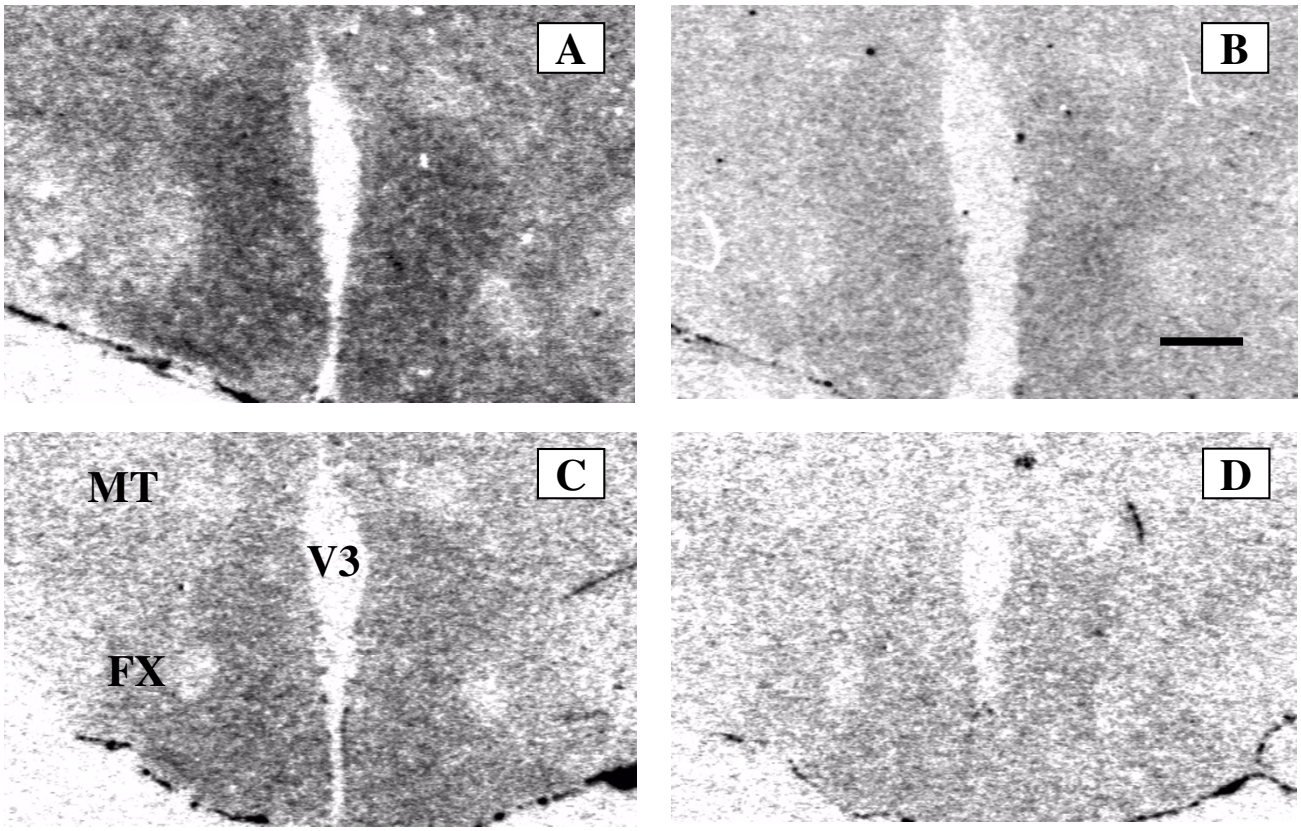

Figure 6
[^{3}H] ketanserin binding sites on coronal sections of premammillary hypothalamus from 2 representative ewes. A and C: total binding and B and D: remaining binding after incubation with 10^{-6}M methysergide. A and B are sections from a ewe exhibiting high LH pulsatility whereas C and D are from a ewe exhibiting low LH pulsatility. Bar = 2mm. FMT, mammillothalamic tract; FX, fornix. Third ventricule (V3).

lighting (350 lux at eye level) was adjusted twice a week to simulate natural photoperiod, including 60 min for civil twilight. Temperature was not regulated. Ewes were allocated to 3 groups, two of which received nightly i.v. infusion of physiological amounts of melatonin for 70 days/year for two consecutive years [10]. Starting in July 1996, all the animals were not treated with melatonin for two consecutive years, to allow free running circannual cycles of pulsatile LHRH and LH secretion to be expressed. LH concentrations were determined in blood samples collected twice a week thoughtout the experiment and every 6-min for 4 hours the day before sacrifice. At the end of the study (July 1998), all ewes were sacrificed by barbiturate overdose during daytime on the same day. Procedures were approved by the University of Michigan Committee on the Use and Care of Animals.

Brain processing, in situ hybridization and autoradiography

Brain processing

Following sacrifice, brains were rapidly removed, dissected out in several blocks and frozen on liquid nitrogen vapors (experiment 1) or by immersion in isopentane maintained at -30° in dry ice (experiment 2). Brains were used as fresh ones and were never perfused nor perifused before or after being removed from the skull. All blocks were stored at -72°C. Frozen frontal brain sections (15 μm) were generated between the infundibular and premammillary recesses using a microtome-cryostat (Leitz

Kryostat 1720) at -20°C and were thaw-mounted on slides pretreated with 1% 3-aminopropyltriethoxysilane (Aldrich Chemical Co, Saint-Quentin, France) in acetone as indicated by Sibony et al. [63]. Consecutive sections were used for in situ hybridization and autoradiography sections for in situ hybridization were post fixed by immersion for 10 min at 4°C in 4% paraformaldehyde in phosphate buffer (0.1 M, pH 7.4), washed in 4 × SSC (4x sodium citrate 0.15 M, sodium chloride 1.2 M), dried under an air-stream, and stored at -20°C. Sections for autoradiography were stored at -20°C immediately after sectioning.

After in situ hybridization, brain sections were stained with neutral red 0.1% (Fluka, Sigma Aldrich, Switzerland) for histological verification and compared with the ewe atlas of Richard [64] and with the rat atlas of Paxinos and Waston [65].

In situ hybridization

In situ hybridization was performed as described by Pelletier et al. [21]. Sense and antisense strand riboprobes (546 bp) were obtained from two clones containing the sheep $5HT2_A$ receptor cDNA insert in the opposite section. The two constructs were linearized by *Hind*III and the transcripts were generated using T7 polymerase in the presence of ^{35}S-UTP (Amersham, UK). Free nucleotides were separated from labelled probes by filtration through a 1 ml Sephadex G50 column previously equilibrated with 20 micrograms tRNA dissolved in TED buffer (10 mM TRIS, 1 mM ED-TA, 10 mM dithiothreitol).

Series of slices were treated by pairs of one ewe from each group. In situ hybridization labelling was performed according to Sibony et al. [63] modified as follows: each section was incubated with 19 microlitters hybridization solution containing approximately 200 000 dpm sense or antisense riboprobe and was treated with Rnase (10 micro litters/ml) for 1 h at 37°C.

After different washings [63], sections were air dried and coated with a liquid NBT2 emulsion (Kodak, Integrabiosciences, Eaubonne, France) diluted twice with sterile demineralized water, and exposed for 6 weeks.

The number of silver grains per neuron marked in the premammillary hypothalamus was counted with a computerized image-analysis system (Biocom Histo 500, Les Ulis, France). A cell was considered as positively labeled if it contained at least 5 silver grains. A minimum number of 100 labeled neurons was recorded for each ewe distributed on both sides of the third ventricle. All sections were included in an area which fitted the maximum of ^{3}H-Ketanserin binding sites [20] and of silver grains in in situ hybridization with the same probes [21]. All determinations were done under blind observations. Results are expressed as the mean number of silver grains per neuron and as the distribution of neurons per class of number of silver grains.

Autoradiography for ^{3}H-Ketanserin binding studies

Autoradiographies were perfomed as described by Le Corre et al. [20]. Six pairs of animals (each pair with one ewe from each group) were constituted at random. Incubation conditions for ^{3}H-Ketanserin were performed according to Pazos et al. [66]. Briefly, slide-mounted tissue sections were preincubated for 15 min in 0.17 M Tris-HCl (pH = 7.7) buffer at room temperature. After dripping, the sections were incubated for 2 h at room temperature with 3 nM ^{3}H-Ketanserin (64.1 Ci/mmol) in 0.4 ml preincubation buffer on each section. The incubation was terminated by washing the sections twice for 5 min in ice-cold preincubation buffer (4°C). The non-specific binding was defined in the presence of 10^{-6}M methysergide. At the end of the washing period, tissues were dried with a stream of air. Sections and tritiated standards (specific activity 0.06–35 nCi/mg tissue equivalent, Amersham, France) were then placed in X-ray cassettes and apposed to Hyperfilm [^{3}H] (Amersham, France) for 4 weeks at -72°C. Films were developed in D19 (Kodak) for 6 min at room temperature, rapidly rinsed with running water, fixed for 20 min (AL4, Kodak) and washed with distilled water.

Quantitative determination of ^{3}H-Ketanserin binding sites were performed using densitometric measurements of autoradiographs with the computerized image-analysis system (Biocom). Reference curves derived from the [^{3}H] standard were used to convert gray level readings into fmol/mg tissue equivalent [67]. Multiple readings (4 to 8) were made in a tissue section and the mean optical density was measured from at least 4 sections per ewe. Non specific binding was similarly determined in adjacent sections. Specific binding was obtained by substracting non-specific from total binding. All determinations were done under blind observations.

LH assays and LH pulse identification

In experiment 1, LH was measured in duplicate in a double-antibody radioimmunoassay [68] as modified by Montgomery et al. [69]. The sensitivity of the assay was 0.1 ng/ml and the intra- and inter-assay coefficients of variation were 9.2 and 9.8 %, respectively. LH values are expressed in terms of CY-LH-S12. In experiment 2, LH was measured in duplicate in 10- to 200-µl aliquots of serum using a modification [70] of a previously described radioimmunoassay [71,72]. LH values are expressed in terms of NIH-LH-S12. Assay sensitivity averaged 0.65 ± 0.19 ng/ml. Within and between assay coefficients of variation (CV) averaged 6% and 10%, respectively. In both experiments, pulses were identified by an adaptation of

the method described by Wallace and MacNeilly [73]. Briefly, a pulse was defined as a value exceeding 1 ng/ml of plasma that had a concentration greater than the mean of the two previous concentrations by at least 3 standard deviations. Standard deviation was estimated for each point from the mean intraassay coefficient of variation. If consecutive samples were identified as a pulse, only the one having the highest concentration was considered as a pulse.

In experiment 2, circannual LH cycles in each ewe were identified by a cluster cycle detection algorithm [74]. LH cycles were divided into high and low stages using a probability level of 5 % or less to discriminate between contiguous clusters of high and low LH values. For analysis, ewes were allocated to the low or high group according to their neuroendocrine stage (low or high LH secretion) at the time of sacrifice as determined by the cluster analysis. Only ewes showing a well defined state using this algorithm were kept in the study (12 ewes, 6 with high LH and 6 with low LH).

Statistical tests

LH pulse frequency, and density labeling in in situ hybridization and binding density of ^{3}H-Ketanserin were compared between groups using unpaired T-test. Distribution of neurons according to staining density was analyzed by the way of the Chi^2 method. (Statview®, Abacus Concept, Berkeley, Ca, USA).

Authors' contributions

All authors contributed equally to this work. PC, FJK & CV conceived the study, and participated in its design and coordination. PC & AD followed experiment 1. AD carried out brain preparation, the autoradiographic and in situ hybridization studies, and the radioimmunoassays of experiment 1. FJK & CV followed experiment 2 and perfomed the radioimmunoassays; CV carried out brain preparation of experiment 2. JP provided the probes for in situ hybridization and gave technical advices for the tissue preparation. BM participated in the design of the study and in its interpretation. PC drafted the manuscript with CV, FJK & BM. All authors read and approved the final manuscript.

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