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ORIGINAL ARTICLE

Gonadotrophin-Releasing Hormone (GnRH) Release in Marmosets I: In Vivo Measurement in Ovary-Intact and Ovariectomised Females

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In vivo hypothalamic gonadotrophin-releasing hormone (GnRH) release was characterised for the first time in a New World primate. A nonterminal and repeatable push-pull perfusion (PPP) technique reliably measured GnRH in conscious common marmoset monkeys. Nineteen adult females (n = 8 ovary-intact in the mid-follicular phase; n = 11 ovariectomised) were fitted with long-term cranial pedestals, and a push-pull cannula was temporarily placed in unique locations within the pituitary stalk-median eminence (S-ME) 2 days prior to each PPP session. Marmosets underwent 1–3 PPPs (32 PPPs in total) lasting up to 12 h. Plasma cortisol levels were not elevated in these habituated marmosets during PPP, and PPP did not disrupt ovulatory cyclicity or subsequent fertility in ovary-intact females. GnRH displayed an organised pattern of release, with pulses occurring every 50.0 ± 2.6 min and lasting 25.4 ± 1.3 min. GnRH pulse frequency was consistent within individual marmosets across multiple PPPs. GnRH mean concentration, baseline concentration and pulse amplitude varied predictably with anatomical location of the cannula tip within the S-ME. GnRH release increased characteristically in response to a norepinephrine infusion and decreased abruptly during the evening transition to lights off. Ovary-intact (mid-follicular phase) and ovariectomised marmosets did not differ significantly on any parameter of GnRH release. Overall, these results indicate that PPP can be used to reliably assess *in vivo* GnRH release in marmosets and will be a useful tool for future studies of reproductive neuroendocrinology in this small primate.

Key words: gonadotrophin-releasing hormone (GnRH), median eminence, push-pull perfusion, marmoset monkey.

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The common marmoset (*Callithrix jacchus*), a small (weighing approximately 400 g) New World monkey, is used increasingly as a nonhuman primate model in basic and applied research (1, 2). These anthropoid primates make excellent animal models for studies in neurobiology (1), neuroendocrinology (3) and behaviour (4) not only because of their small size and ease of use, but also because of their ready adaptation to a variety of experimental approaches, including venipuncture (5), venous cannulation and long-term restraint (3), operant testing (6), functional magnetic resonance imaging (7) and social grouping and testing procedures (8, 9). In common marmoset groups containing more than one adult female, typically only the most socially dominant female reproduces (10, 11). Subordinate females are often anovulatory due to insufficient gonadotrophin secretion from the anterior pituitary (12–14), which occurs in response to behavioural, visual and olfactory cues from the dominant female (15). Socially-mediated changes in fertility occur rapidly (1–2 days) and are reversed just as quickly with subsequent social change (12, 13).

The neuroendocrine mechanisms underlying social regulation of reproduction in female marmosets are unknown, and the implementation of long-term neuroendocrine monitoring systems in marmosets has been problematic. Whereas electrophysiological recordings of hypothalamic multiunit activity (MUA), presumably underlying gonadotrophin-releasing hormone (GnRH) pulses, can be continuously monitored for up to a year in rhesus monkeys (16), this technique has proved unsuccessful in marmosets because rapid

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hypothalamic gliosis causes failure of recording electrode transmission before electrophysiological assessment can begin (K. T. O'Byrne, pers. comm.). In other laboratories, cerebral microdialysis has been used successfully to sample striatal dopamine in anaesthetised (17, 18) and conscious (19) marmosets, but this technique has been employed only in single, terminal sampling sessions. It was our aim to develop a nonterminal and repeatable *in vivo* neuroendocrine sampling technique to enable experimental manipulation of neuroendocrine mechanisms regulating female reproduction.

As an alternative to microdialysis, we opted to develop a repeatable and nonterminal push-pull perfusion (PPP) technique, which would allow for multiple *in vivo* sampling sessions of GnRH release in conscious marmosets. A successful PPP technique will ultimately allow the direct comparison of GnRH release profiles in the same marmoset under multiple social and reproductive conditions. Such comparisons are not possible with microdialysis because repeated and/or long-term microdialysis probe penetration has typically not been successful or recommended (20). Microdialysis also yields very low recovery rates, especially for peptides (e.g. only 6% GnRH recovery rate using microdialysis in rhesus monkeys) (21), often resulting in data best represented as a percentage of baseline from an individual microdialysis session (20). By contrast, PPP allows higher GnRH recovery rates that are expressed in absolute concentrations (20), which is more desirable for between-subject comparisons and for the future characterisation of anticipated low GnRH levels in hypogonadotrophic subordinate female marmosets. Finally, given this small monkey's neuroanatomy and diminutive size of the pituitary stalk-median eminence (S-ME; approximately 1 mm × 1 mm), the 0.5-mm length of the PPP cannula's collection tip is preferable to the 2.0–4.0 mm length of the microdialysis probe's collection membrane for highly localised (20) and repeated sampling within the marmoset S-ME.

We report here the first characterisation of GnRH release from the S-ME of common marmoset monkeys, achieved by adapting a PPP technique routinely used in rhesus monkeys (22–24). We further validated the PPP procedure in ovary-intact and ovariectomised marmosets by demonstrating that: (i) GnRH pulse patterns differed from a computerised random pulse model; (ii) GnRH release varied predictably with anatomical location in the S-ME, and (iii) GnRH release increased characteristically in response to norepinephrine infusion and decreased with lights off at night. These results indicate that PPP is a reliable technique for assessing GnRH release in marmosets and will be a valuable tool for future studies examining reproductive neuroendocrinology in this small primate.

Materials and methods

Subjects and housing

Nineteen adult (aged 31–69 months) female common marmosets underwent a total of 32 PPP sessions. Subjects included eight ovary-intact (intact) females and 11 ovariectomised (OVX) females weighing 406 ± 10 g. Intact females were tested during the mid-follicular phase of their ovarian cycle (see below). Ovariectomised females had their ovaries and fallopian tubes bilaterally removed under Saffan anaesthesia (10.8 mg alphaxalone: 2.7 mg alphadolone acetate intramuscularly; Pitman-Moore, Harefield, Uxbridge, Middlesex, UK) 7.8 ± 1.4 months prior to study.

All subjects were captive-born and housed at the National Primate Research Center at the University of Wisconsin–Madison. Between PPP sessions, intact female subjects were pair-housed with unrelated males or lived in stable social groups with up to three other unrelated males and females, as previously described (8). The subject animal was always the socially dominant female in the group that was pair-bonded with the dominant male. This pair exhibited most of the sexual behaviour and male–female social interactions observed in such social groupings (12). Ovariectomised females were pair-housed with unrelated males. Social groups and pairs were housed indoors on a 12 : 12 h light/dark cycle (lights on 06.30 h) in wire mesh cages (61 cm × 61 cm to 122 cm × 183 cm) fitted with stainless steel nestboxes and perches, wooden dowels, and hanging ropes. Marmoset rooms held multiple social groups and were maintained at approximately 27 °C with 50% humidity. Animals were fed ZuPreem Marmoset Diet (Hills Pet Products, Topeka, KS, USA); Provim and Nutra-Plus vitamin/mineral/protein supplements (Nutra-Vet Research Corp., Poughkeepsie, NY, USA); plain yoghurt; and fruits and/or vegetables once daily. Water was available *ad libitum*.

Monitoring and regulating ovarian cycles

In ovary-intact females, blood samples (0.1–0.3 ml) were collected twice weekly from the femoral vein during brief restraint without anaesthesia (5). Blood samples were immediately placed on ice, centrifuged at 3400 r.p.m. for 10 min, and the plasma fraction aspirated and stored at −20 °C. Samples were assayed for progesterone using a heterologous enzyme immunoassay (see Hormone Assays) to monitor ovarian activity. To control ovarian cycles in intact females undergoing PPP, cloprostenol sodium (a prostaglandin-F2-alpha analogue, 1.0 μg i.m. Estrumate, Mobay Corp., Shawnee, KS, USA) was injected for 1–3 consecutive days during the luteal phase (14–39 days following ovulation) to induce luteolysis and terminate the luteal phase or early pregnancy (25, 26). A similar method of ovarian cycle regulation was successfully employed prior to hypothalamic GnRH sampling in sheep (27). In our marmoset colony, ovulation reliably occurs 9–15 days after cloprostenol treatment (8). Subjects in the present study received cloprostenol 4–5 days prior to each PPP session. A blood sample taken 2 days before PPP confirmed that each marmoset responded to cloprostenol and was in the follicular phase (plasma progesterone < 10 ng/ml) of the ovarian cycle.

Cranial pedestal implantation and hypothalamic cannulation

Cranial pedestal implantation and hypothalamic cannulation procedures were adapted from those developed for the female rhesus monkey (22–24), with marmoset stereotaxic placements based on coordinates provided by Lipp (28). Prior to either procedure, the health of each marmoset was confirmed by haematological screening followed by veterinary consultation. Subjects received presurgical (3 days) and postsurgical (10 days) antibiotic coverage (enrofloxacin: Baytril, 2.27 mg i.m./day, Bayer Corp., Shawnee Mission, KS, USA).

Pedestal implantation

Marmosets were implanted with a stainless steel cranial pedestal (three-prong base with a 6 mm diameter cylindrical chamber tube; custom-manufactured by Narishige Scientific, Tokyo, Japan) under isofluorane gaseous anaesthesia (Forane 2%; 1.6 l/min oxygen) at least 2 months (3.4 ± 0.4 months) in advance of the first PPP procedure. Iohesol (Omnipaque, 25 μl i.c.v.; Nycomed Inc., Princeton, NJ, USA), a radiopaque medium,

was infused into the foramen of Monroe to visualise the third ventricle and create an X-ray ventriculogram. Accurate placement of the pedestal for each individual was achieved using X-ray ventriculography and a stereotaxic apparatus to position the pedestal over the infundibular recess at the midline level. The pedestal was attached to the skull with small bolts and dental acrylic. Pedestal chambers were filled with 1% silver sulfadiazine cream (Par Pharmaceutical Inc., Spring Valley, NY, USA). Animals received anxiolytics (buprenorphine hydrochloride: Buprenex, 0.004 mg i.m., Reckitt & Coleman Inc., Richmond, VA, USA) for the first 24 h after surgery, and the incision surrounding the implant was rinsed daily with sterile water for the first week after surgery.

Cranial pedestals typically remained implanted for 2–4 years. Twice weekly, the pedestal and surrounding acrylic headcap were inspected, topically disinfected with chlorhexidine diacetate (Nolvasan, 1 : 20 dilution; Fort Dodge Laboratory, Fort Dodge, IA, USA), and refilled with fresh silver sulfadiazine cream. Ultimately, the pedestal and headcap were removed under Saffan anaesthesia, and former subjects were returned to the breeding or experimental colony population.

Hypothalamic cannulation

Two days before PPP sampling began, a push-pull cannula was inserted into the pituitary S-ME of the hypothalamus under Saffan anaesthesia. Advance placement of the cannula allowed clearance of cellular debris and minimised potential congestion of the PPP system (23, 24). For cannula insertion, the marmoset was placed into a stereotaxic apparatus and a micromanipulator unit (custom-modified MO-99; Narishige Scientific) was attached to the cranial pedestal. Using the micromanipulator for three-dimensional positioning of the cannula tip, an outer guide 'pull' cannula (23-guage, 77.5 mm) with an inner stylet (27-guage , 78.0 mm; Plastics One Inc., Roanoke, VA, USA) was lowered into place. Accurate placement of the cannula into the desired location within the S-ME was achieved using X-ray visualisation, the individual-specific ventriculogram obtained during cranial pedestal implantation and Lipp's (28) marmoset hypothalamic intracerebral co-ordinate system.

Marmosets underwent up to three hypothalamic cannulations (one cannulation: $n = 9$; two cannulations: $n = 7$; three cannulations: $n = 3$) at intervals of 1–20 months (4.5 ± 0.9). Each cannula tip placement avoided previous cannulation sites (> 1 mm), the third ventricle, and lateral placements beyond 1 mm of the midline (22). After cannulation, animals were placed in a sling-harness (a soft harness, specifically designed in our laboratory, attached to a metal support bar along with a loose-fitting plastic neck collar) (3) until the end of the PPP procedure. At the end of each PPP, the cannula was raised using the micromanipulator, the micromanipulator unit was removed from the pedestal, and the subject and her groupmate(s) were returned to their home cage.

Housing during PPP

Immediately following hypothalamic cannulation 2 days prior to PPP, the female subject and her male mate (plus any socially subordinate female groupmates) were moved to a private PPP procedure room. All groupmates except the subject were housed in a 61 cm × 46 cm × 61 cm wire mesh cage equipped with a cardboard nest-box and wooden dowels. This cage always permitted visual, vocal and olfactory contact with the subject.

The subject was restrained in a sling-harness within 30 cm of her groupmate(s). Vertical locomotion, climbing, and jumping represent a large proportion of marmoset activity patterns in the homecage, making the sling-harness a necessary safety precaution following cannula placement. The sling-harness prevented the subject's manual access to the PPP cannula and limited the range of vertical head movements, but allowed the marmoset to maintain a natural arboreal-like posture, eat, drink, curl into a sleeping position, and freely move all but the head. Apart from sleeping, animals remained conscious while in the sling-harness both before and during the PPP procedure.

Potential subjects were habituated to the PPP procedure room, the investigator and the sling-harness during a series of trials increasing in length (from 1–24 h) under careful monitoring (3). Only animals that habituated well to the process were used as subjects. In addition to the regular diet, harnessed animals were treated to a sweet nutrient liquid (Ensure, Abbott Laboratories, Columbus, OH, USA) and small marshmallow pieces throughout the day. Prior to PPP, harnessed animals were monitored at least hourly during lights on and once during the night; indices of comfort and behavioural activity, liquid and food intake, and excretory output were recorded. During PPP, harnessed animals were monitored continuously.

Perfusate collection

Two days after hypothalamic cannula insertion, the cannula's inner stylet was replaced with an inner 'push' cannula (28-guage, 78.0 mm; Plastics One Inc.), thereby creating a 0.5-mm cannula tip protrusion area. The cannula unit was attached to two identically calibrated 'push' and 'pull' peristaltic pumps (Minipuls 2; Gilson, Middleton, WI, USA) by sterile polyethylene tubing (PE10 tubing; Becton Dickinson, Sparks, MD, USA). The 'push' pump infused a filtered (0.2 μm) modified Krebs-Ringer phosphate buffer solution [artificial cerebrospinal fluid (CSF), pH 7.4] containing bacitracin (10 μg/ml) at 21–24 μl/min. Perfusate was collected on ice in successive 10-min fractions; 175 μl of each fraction was immediately frozen on dry ice and stored at − 40 °C until assayed for GnRH. PPP tubing was monitored continuously and perfusion was stopped immediately at any sign of system congestion. PPP sampling continued for up to 12 h, commencing at various times between approximately 09.00 h and 13.00 h.

Norepinephrine challenge

The ability of norepinephrine to stimulate GnRH release during PPP was tested in three intact females in the mid-follicular phase and one ovariectomised female. An additional three intact mid-follicular females served as controls. Norepinephrine (10^{-4} M; Sigma, St Louis, MO, USA) was dissolved in artificial CSF within 20 min of use and at least 330 min into each PPP. Norepinephrine or vehicle (artificial CSF) was directly infused through the push cannula for 20 min. Perfusates were collected continuously before, during and after infusion. Perfusion transit time through the tubing and pumps was calculated prior to each PPP, allowing the neuroendocrine response to the experimental infusion to start appearing in the collected perfusate at the beginning of a 10-min sample collection period.

Blood sampling

To monitor plasma cortisol concentrations during PPP as an index of generalised stress (29), four subjects (one intact, three OVX) had their right internal jugular vein catheterised [polyethylene PE10 tubing; Becton Dickinson, Franklin Lakes, NJ, USA; modified from (30)] under Saffan anaesthesia at least 1 week prior to PPP. After catheterisation, the subject returned to her home cage wearing a soft lightweight vest specifically designed in our laboratory, with the ventrally exteriorised catheter tubing stored in a velcro-sealed pocket (3). To maintain patency, the jugular catheter was filled with heparin (30 U/ml) and flushed daily. Twelve 0.1-ml samples, approximately one per hour, were collected for cortisol measurement during each of these PPPs once per individual female.

Hormone assays

Plasma progesterone levels were measured in duplicate aliquots by heterologous enzyme immunoassay as described previously (31). The assay sensitivity

was 3.6 pg/tube, with a 6.0% intra-assay coefficient of variation (CV) and a 11.3% interassay CV.

GnRH concentrations in 175 μl PPP perfusates were measured by radioimmunoassay using antiserum R1245 (provided by T. Nett, Colorado State University, Fort Collins, CO, USA) as described previously (24). Synthetic GnRH (Richelieu Biotechnologies, Montreal, Quebec, Canada) was used for the radiolabelled antigen and the reference standard. The assay sensitivity was 0.04 pg/tube, with a 7.4% intra-assay CV and a 12.4% interassay CV.

Plasma cortisol levels were measured in duplicate aliquots in a single radioimmunoassay using a GammaCoat RIA kit (DiaSorin Inc., Stillwater, MN, USA) as described previously (31). The assay sensitivity was 0.015 ng/tube and the intrassay CV was 2.5%.

Histological analysis

In order to confirm cannula placements in the S-ME, a relevant neural location for neuroendocrine GnRH neurones and nerve terminals, two ovariectomised and one ovary-intact female marmosets were sedated with an intramuscular injection of ketamine (15 mg/kg) followed by an intravenous injection of 25–30 mg/kg sodium pentobarbital to induce deep anaesthesia. Each monkey was perfused via the left ventricle, with the descending aorta clamped. The perfusion commenced with 0.9% NaCl containing 2% sodium nitrite to flush blood from the vascular system, followed by 4% paraformaldehyde in 0.1 M sodium phosphate buffer solution (pH 7.4). The brain was removed and further fixed for 2 h at 4 °C. The brain was then placed in a cryoprotectant solution consisting of 30% sucrose in PBS (pH 7.6) until fully saturated as evidenced by sinking. The brain was sectioned coronally at 50 μm thickness by a sliding microtome. The procedure for GnRH immunostaining was similar to that described previously (32). After endogenous peroxidase was inactivated with methanol-H_2O_2, tissues were exposed to the polyclonal antiserum LR-1, which recognises amino acids 3, 4, 7, 8, 9, and 10 of mammalian GnRH (a gift from Dr R. A. Benoit, University of Montreal, Montreal, Canada, 1 : 15 000) or GF-6, which cross-reacts with N-terminus peptide (a gift from Dr N. M. Sherwood, University of Victoria, Victoria, Canada, 1 : 9000) for 48 h at 4 °C, and subsequently to a biotinylated 2nd antibody with avidin-biotinylated peroxidase complex (ABC reagent, Vector Laboratory, Burlingame, CA, USA). For visualisation, 3, 3′-diaminobenzidine (DAB) was used. Some tissues stained with LR-1 were also double stained for c-fos with Vector SG for an unrelated study. The tissue was mounted with glycerine-jelly. In addition to the immunocytochemistry, two marmoset brains were stained with haematoxyline and eosin.

Statistical analysis

Data are expressed as mean ± SEM unless otherwise noted. GnRH pulse peaks were statistically identified using the PULSAR computer algorithm (33) as described previously for use with GnRH (22, 24, 34). Parameters of pulsatile GnRH release were also calculated by PULSAR, including mean GnRH concentration (the average of all GnRH concentrations in a PPP session); GnRH pulse amplitude (the average difference between pulse peak and interpulse trough values); baseline GnRH release (the average of all interpulse trough values); and interpulse interval (IPI: the average time interval between GnRH peaks). Differences in pulsatile GnRH release parameters between intact mid-follicular phase females and ovariectomised females were evaluated by t-tests (unpaired, two-tailed).

Only PPP perfusates collected during procedures lasting more than 240 min were included in analyses of pulsatile GnRH release (intact: ten PPP, n = 6 subjects; OVX: ten PPP, n = 6 subjects), thereby yielding a minimum of four GnRH pulses (three IPIs) per procedure for statistical analysis. For analyses of GnRH baseline release (a factor independent of GnRH pulse number) as a function of cannula location, 12 additional PPP (durations of 180–230 min; intact: three PPP, n = 2 additional subjects; OVX: nine PPP, n = 5 additional subjects) were included in the data set (overall total of 32 PPP).

GnRH pulse parameters (IPI and pulse length) obtained by PPP were compared to a random pulse model. The random model was created by generating a set of random numbers ranging between the lowest mean PPP baseline value and the highest mean PPP pulse peak value, spanning a sampling period equal in time (and number of data points) to the length of the average PPP. Ten random model data sets for intact females and ten random model data sets for OVX females were created and compared to actual data by t-tests (unpaired, two-tailed).

For subjects undergoing multiple PPPs, the presence of any systematic influence of cannulation order on GnRH concentration was tested by chi-square analysis. A within-animal CV was also calculated for each multiple-PPP subject. Differences in the CV between GnRH parameters were evaluated by repeated-measures one-way ANOVA, with Scheffé post-hoc analyses.

GnRH release parameters between different anatomical locations of PPP sampling within the S-ME were compared by one-way factorial ANOVA and Scheffé post-hoc analyses. The mean concentration of GnRH release in response to a norepinephrine or control infusion was normalised as a percentage of the mean pulse peak level specific to that PPP session and was compared to the subject's average GnRH pulse peak level (100%) by a Fisher's exact test for small samples (35). GnRH release during the transition to lights off was evaluated by comparing (paired t-test, two-tailed) the last 60 min of GnRH release during lights on to the first 60 min of release during lights off; a simple regression verified that GnRH release did not decrease during a 240-min period immediately prior to lights off.

Results

Influence of PPP on cortisol levels, ovulation and subsequent fertility

Surgical implantation of the cranial pedestal had no long-term adverse effect on marmoset reproductive competence or behaviour. Intact females that underwent pedestal surgery during the follicular phase ovulated in 16.5 ± 2.8 days after surgery (n = 5). Of seven intact females that had pedestals implanted during early pregnancy or the luteal phase, all five pregnant females maintained pregnancies whereas the two nonpregnant females sustained a normal luteal phase, subsequently ovulating 14 and 24 days after surgery. All ovary-intact subjects with cranial pedestals maintained ovulatory cycles between PPP sessions.

Six intact subjects ultimately returned to our marmoset colony's breeding population after undergoing multiple PPPs and cranial pedestal removal. All of these former subjects subsequently conceived and maintained full-term pregnancies. To date, 18 infants have been born, nursed and successfully reared to adulthood by these six former PPP subjects.

PPP did not result in major disruption of ovulatory cycles in intact females: subjects ovulated 10.6 ± 1.5 days after PPP, corresponding to 14.9 ± 1.4 days (within a normal follicular-phase length (8) after receiving prostaglandin). During the actual PPP procedure, subjects appeared mostly calm and alert. Subjects interacted visually and vocally with their groupmates, ate, napped and slept during the night. Plasma cortisol levels during PPP were within the range of previously reported morning basal cortisol levels in this colony (Fig. 1), although no obvious circadian pattern was detected.

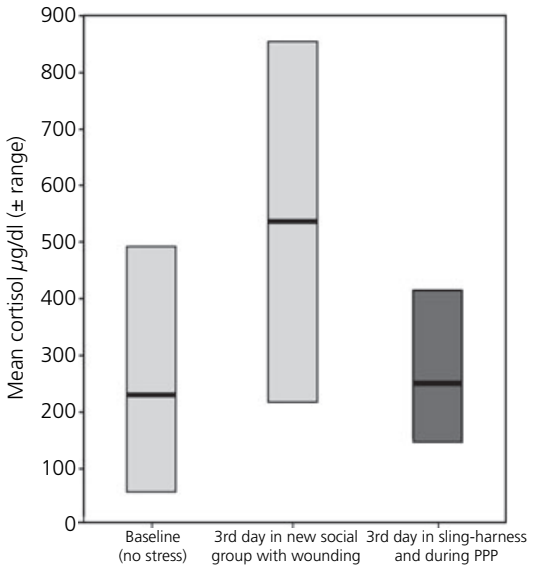

Fig. 1. Plasma cortisol concentrations of adult female marmosets during different conditions in the same colony using the same assay methodology: baseline at 09.00 h [mid-follicular and ovariectomised females, n = 14; derived from (8)]; in new social group, with aggression and wounding, at 09.00 h [mid-follicular phase females, n = 5; derived from (31)]; and during PPP while in sling-harness (mid-follicular and ovariectomised females, n = 4). Note that basal cortisol concentrations in marmosets are naturally much higher than those typically seen in Old World primates (36, 37). A dark bar indicates mean cortisol value.

GnRH pulse profile

PPP of the marmoset S-ME demonstrated similar patterns of pulsatile GnRH release in intact mid-follicular phase and ovariectomised animals. Pulses of GnRH release occurred every 40–80 min, averaging one pulse every 50.0 ± 2.6 min (intact: 52.4 ± 4.3 min; OVX: 47.6 ± 3.1 min, NS). Figure 2 illustrates pulsatile GnRH release in representative intact and ovariectomised females.

GnRH pulse parameters obtained by PPP in female marmosets differed significantly from random pulse model (Random) predictions, consistent with an organised pattern of release. Endogenous GnRH pulses occurred less frequently than randomly generated pulses, with significantly longer interpulse intervals than random [$intact_{Random}$: 33.4 ± 4.3 min, $t(18) = 4.2$, $P = 0.001$; OVX_{Random}: 36.4 ± 1.7 min, $t(18) = 3.2$, $P = 0.005$].

Within-animal variation in GnRH release

Seven subjects underwent multiple PPP procedures (n = 3 intact and n = 4; OVX, each with 2–3 PPP), allowing us to analyse variation in GnRH release within the same animal, but across different PPPs (separated by 6–80 weeks), corresponding to different cannula placements in the S-ME. For animals that underwent multiple PPPs, GnRH release parameters did not vary systematically across individual animals' first, second, and third PPP procedures ($\chi^2 \geq 5.2$, d.f. = 4, $P \geq 0.2$ for all parameters). The temporal patterning of GnRH release, represented by IPI, was relatively consistent within individuals across multiple PPPs (Fig. 3). By contrast, parameters reflecting the amount of GnRH release (mean concentration, baseline concentration, and pulse amplitude) were not consistent within individual subjects (Fig. 3). Random pulse model-derived GnRH release parameters showed no such distinction among CVs [$Intact_{Random}$: $F(2,12) = 2.0$, $P = 0.2$; OVX_{Random}: $F(3,16) = 1.0$, $P = 0.5$].

GnRH release and PPP cannula location

Although parameters reflecting the amount of GnRH release were not consistent within subjects, these parameters did vary predictably with anatomical location of the push-pull cannula tip in the S-ME. As approximated in Fig. 4(A), GnRH release was highest within Area 1 (grey area) that encompassed most of the median eminence and pituitary stalk. Within Area 1, GnRH release did not differ between intact and ovariectomised females (mean concentration: intact: 2.5 ± 0.6 pg/ml/10 min versus OVX: 1.8 ± 0.2 pg/ml/10 min, NS; baseline concentration: intact: 1.4 ± 0.4 pg/ml/10 min versus OVX: 1.8 ± 0.4 pg/ml/10 min, NS; pulse amplitude: intact: 1.3 ± 0.4 pg/ml/10 min versus OVX: 1.1 ± 0.1 pg/ml/10 min, NS). GnRH release was lower when the cannula tip was located in areas more rostral (Area 2A, dotted area in Fig. 4A) or caudal (Area 2P, hatched area) compared to high-release Area 1. Within Area 2P, baseline GnRH concentrations did not differ between intact and ovariectomised females (intact: 0.5 ± 0.2 pg/ml/10 min versus OVX: 0.4 ± 0.1 pg/ml/10 min, NS). Sample sizes were inadequate to statistically compare other GnRH parameters in Areas 2P and 2A between intact and ovariectomised females.

Because GnRH release amounts also did not differ overall between intact and ovariectomised females (mean concentration: intact: 1.5 ± 0.4 pg/ml/10 min versus OVX: 1.3 ± 0.2 pg/ml/10 min, NS; baseline concentration: intact: 1.2 ± 0.3 pg/ml/10 min versus OVX: 1.0 ± 0.2 pg/ml/10 min, NS; pulse amplitude: intact: 1.2 ± 0.3 pg/ml/10 min versus OVX: 0.9 ± 0.1 pg/ml/10 min, NS), data from both groups of animals were combined for analyses of differences in GnRH release with cannula tip location. Overall, GnRH mean concentration [$F(2,17) = 5.0$, $P = 0.01$] and baseline concentration [$F(2,29) = 3.7$, $P = 0.03$] differed significantly with anterior-posterior cannula tip location (Fig. 4B), being highest in Area 1 (Fig. 4A). Mean GnRH pulse amplitude showed similar, but nonsignificant, differences among cannula tip locations (Area 1 = 1.3 ± 0.2 pg/ml; Area 2A = 0.5 ± 0.1 pg/ml; Area 2P = 0.8 ± 0.3 pg/ml).

In all but two PPPs, cannula tip location was within 1 mm of the midline. Across these tightly clumped lateral cannula placements, GnRH release did not vary systematically with distance from the midline ($r^2 \leq 0.03$, NS for all parameters).

Immunocytochemical staining of GnRH neurones in the basal hypothalamus are shown in Fig. 5(A–C). Cell bodies of GnRH neurones were found in the base of the hypothalamus, periventricular region, and dense fibres were found in the median eminence and pituitary stalk (Fig. 5A–C), which are essentially very similar to those found in rhesus monkeys. However, there was one minor difference between rhesus and marmoset GnRH neurone presentation in the

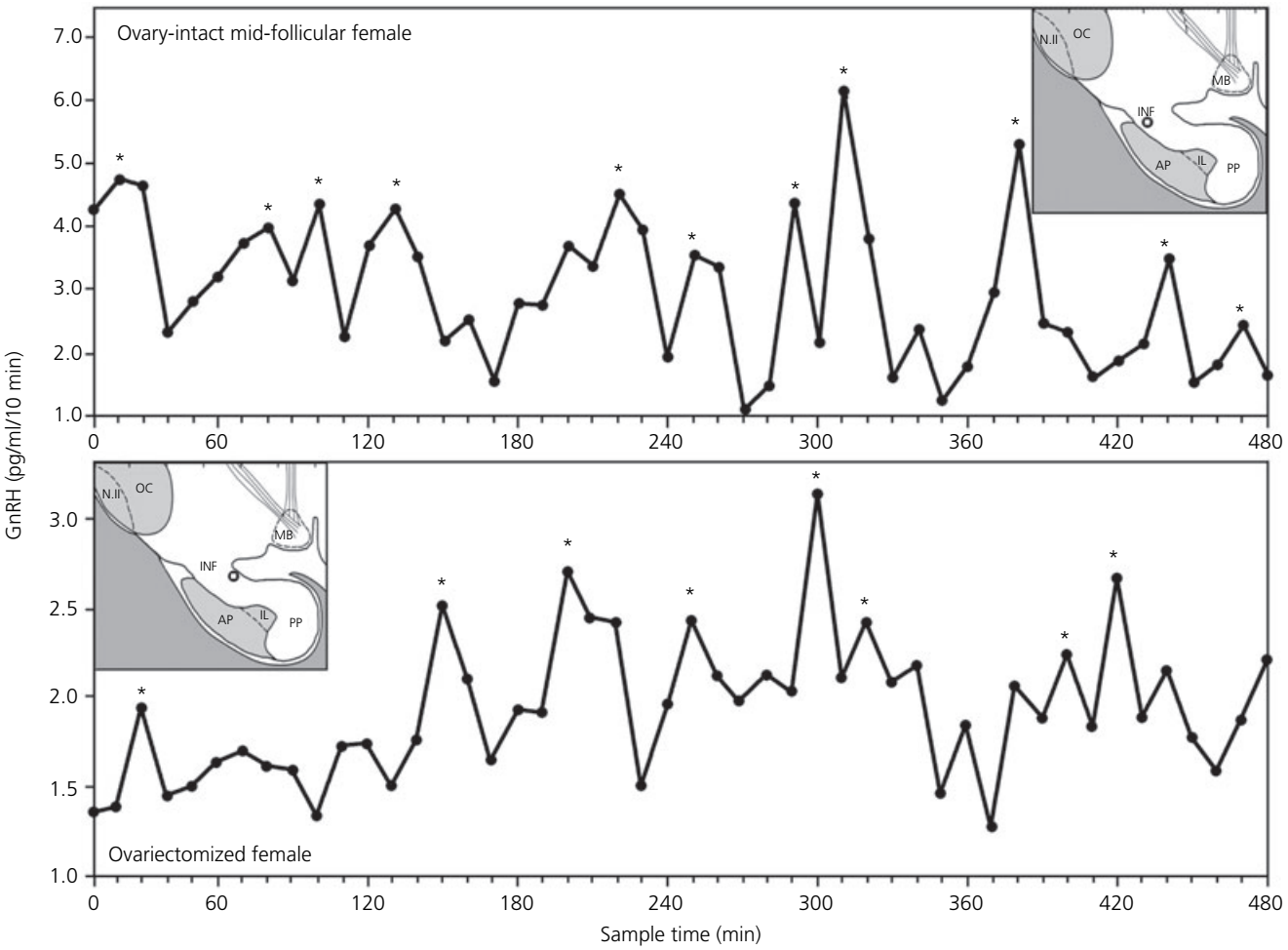

Fig. 2. Examples of pulsatile gonadotrophin-releasing hormone (GnRH) release profiles in an ovary-intact female in the mid-follicular phase and an ovariectomised female. GnRH concentrations, but not frequency of episodic release (see text), differ between the two reflecting different push-pull perfusion (PPP) cannula locations (a circle in each inset) within the pituitary stalk-median eminence. The PPP location for the ovary-intact female lies within the area of highest PPP concentrations for GnRH (Area 1, Fig. 4A), whereas the PPP location for the ovariectomised female lies within an area of lower PPP GnRH concentrations (Area 2P, Fig. 4B). Parasagittal representation modified from Lipp (28): INF, infundibulum; AP, anterior pituitary; PP, posterior pituitary; IL, intermediate lobe of pituitary; VMH, ventromedial hypothalamus; MB, mammillary body. Asterisks indicate GnRH pulse peaks identified by the PULSAR programme.

S-ME. GnRH cell bodies in marmosets were distributed over an extended area anteriorly and posteriorly along the base of the hypothalamus in comparison to rhesus monkeys. In an example from one marmoset that was involved in three separate PPP experiments (Fig. 5D), the cannula tracks produced in each PPP procedure parallel the right-hand side wall of the third ventricle and extend into the S-ME.

GnRH response to norepinephrine

Direct infusion of norepinephrine (10^{-4} M) through the push-pull cannula into the S-ME for 20 min resulted in a corresponding increase in GnRH release. Figure 6(A) shows the response to norepinephrine in representative intact and ovariectomised females. Although the magnitude of GnRH response to norepinephrine varied considerably, GnRH release peaked within 20 min of the onset of norepinephrine infusion in all subjects. Peak GnRH release in response to norepinephrine was always greater than the average GnRH pulse peak for the corresponding PPP (Fisher's exact test, $P < 0.05$), whereas GnRH release was not affected by control vehicle infusions (Fisher's exact test, NS; Fig. 6B).

GnRH response to night-time lights off

In PPP sessions in eight subjects (Intact, $n = 4$; OVX, $n = 4$), PPP continued uninterrupted from daytime lights on through at least 1 h into night-time lights off. Mean GnRH concentration decreased from the last hour of lights on (1.8 ± 0.5 pg/ml) through the first hour of lights off [1.2 ± 0.4 pg/ml; $t(7) = 2.6$, $P = 0.03$]. During this transition, GnRH release declined by $40.2 \pm 6.2\%$ in seven of the eight subjects. By contrast, regression analysis revealed no lights on decrease in GnRH release over a 4-h period immediately preceding lights off (slope $= -0.0009$, $r = 0.05$, $P = 0.9$).

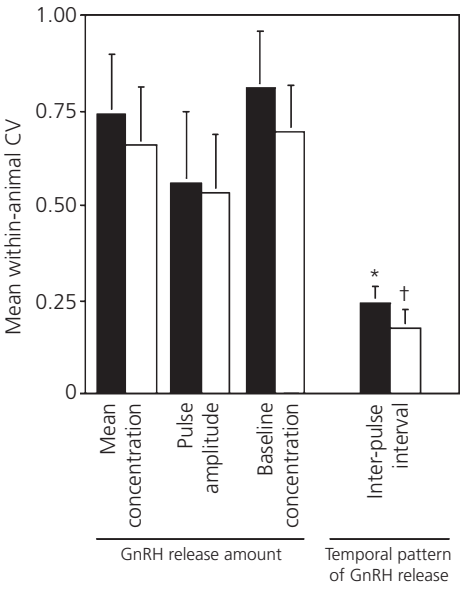

Fig. 3. Within-animal coefficients of variation (CVs) for gonadotrophin-releasing hormone (GnRH) release parameters in ovary-intact (filled bars) and ovariectomised (open bars) females undergoing two to three push-pull perfusion (PPP) sessions. CVs differed significantly across GnRH release parameters [intact: $F(4,12) = 13.11$, $P = 0.001$, $n = 3$; OVX: $F(4,16) = 1.60$, $P = 0.0004$, $n = 4$]. The parameter characterising temporal patterning of GnRH pulses (interpulse interval, IPI) was consistent within individual marmosets across PPP sessions, but the remaining parameters characterising amounts of GnRH release varied across PPP sessions and were associated with differences in cannula tip location. Significant ($P < 0.05$) differences between the IPI parameter for GnRH release pattern and each CV parameter for amount of GnRH released are indicated by an asterisk (*) for ovary-intact females and (†) for ovariectomised females.

Discussion

The present study demonstrates that endogenous hypothalamic release of GnRH can reliably be measured and characterised in female common marmoset monkeys using push-pull perfusion. GnRH release is detectable and pulsatile when directly sampled from the S-ME both in ovariectomised female marmosets and in ovary-intact females in the mid-follicular phase of the ovarian cycle. GnRH release is clearly organised: GnRH pulses have significantly longer interpulse intervals than predicted by a random pulse model, as we have previously demonstrated for female rhesus monkeys (36). With pulses occurring approximately every 50 min, GnRH release frequency in marmosets is comparable to *in vivo* GnRH release frequencies found previously using various techniques in well-studied animal models, including female rhesus monkeys (22, 24, 37–39), sheep (40, 41), rabbits (42, 43) and rats (44, 45), although more rapid pulse frequencies have been demonstrated in postpubertal male and female rats (46, 47). When comparing GnRH pulse frequency results from different studies, it is important to account for the method of sample collection, the method for pulse analysis and the physiological state of the model organism.

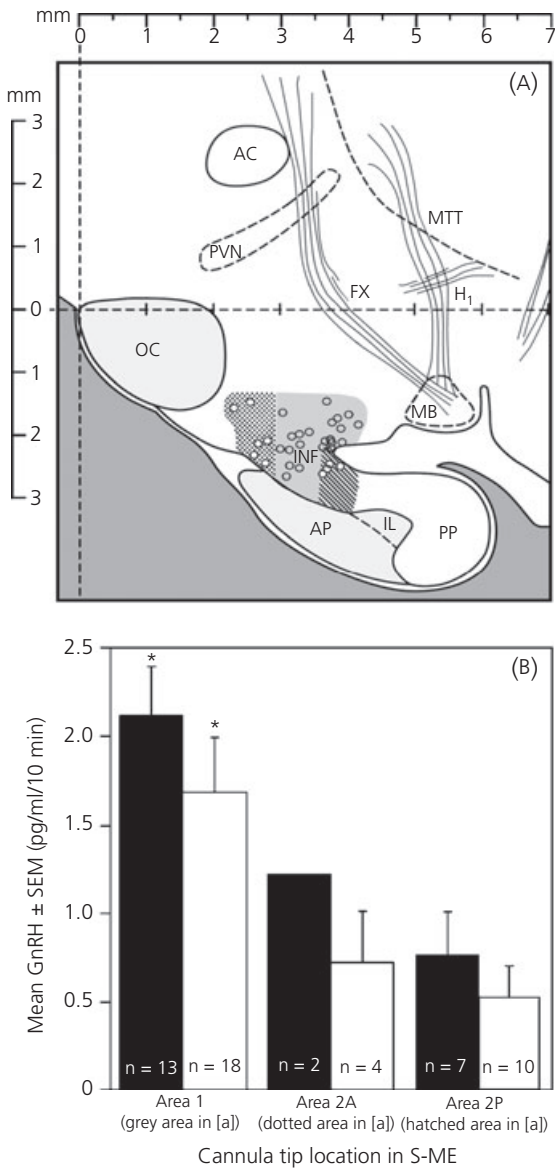

Fig. 4. (A) Mean gonadotrophin-releasing hormone (GnRH) levels that correlate with approximate push-pull perfusion (PPP) cannula tip locations within the stalk-median eminence (S-ME): GnRH levels from 32 individual experiments (open circles) are schematically plotted on the midsagittal plane. Area 1 (grey area) yielded the highest GnRH concentrations, with lower concentrations resulting from more anterior (Area 2A, dotted area) or posterior (Area 2P, hatched area) cannula tip placements. The parasagittal representation of the marmoset hypothalamus and surrounding structures were modified from Lipp (28): INF, infundibulum; AP, anterior pituitary; PP, posterior pituitary; IL, intermediate lobe of pituitary; OC, optic chiasm; FX, fornix; VMH, ventromedial hypothalamus; MB, mammillary body; PVN, paraventricular nucleus; AC, anterior commissure; MTT, mammillothalamic tract; H1, field H1. All cannula placements were within 1 mm of midline. (B) Variation in GnRH concentrations with PPP cannula tip location: overall mean GnRH concentrations (closed bars) [$F(2,17) = 5.02$, $P = 0.01$] and baseline GnRH concentrations (open bars) [$F(2,29) = 3.73$, $P = 0.03$]. *$P < 0.05$ versus Area 2P.

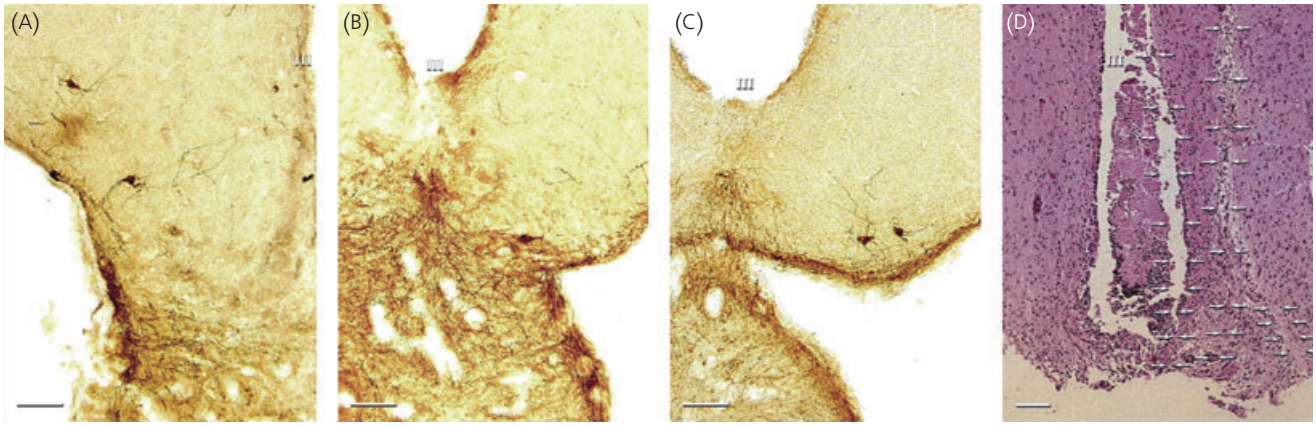

Fig. 5. Serial sections at 150–300 μm intervals are shown of individual (A–C) immunopositive gonadotrophin-releasing hormone (GnRH) fibres and cell bodies in the basal hypothalamus of a female marmoset. Note that immunoreactive GnRH cell bodies in different shapes were seen in each section and GnRH fibres terminate in the pituitary stalk. III, Third ventricle. Scale bar = 100 μm for (A–C). (D) Push-pull perfusion (PPP) cannula tracks in the stalk-median eminence region of a female marmoset. Three cannula tracks (shown by white arrows) from three separate PPP procedures are identifiable because of gliosis in this example. Mean GnRH concentrations (left to right) determined from these three PPP cannula locations were: 2.45 pg/ml, 1.33 pg/ml and 0.46 pg/ml, respectively. Scale bar = 100 μm.

Because the present PPP technique involves temporary cannula placements at different locations in the S-ME, we could further analyse GnRH release parameters across discrete PPP procedures within an individual monkey. Pulse frequency (IPI) is quite consistent within an individual across different PPP procedures, even when the PPP procedures are separated by up to 20 months. These findings suggest that individual female marmosets, whether in the mid-follicular phase of the ovarian cycle or ovariectomised, have characteristically stable GnRH pulse profiles. GnRH pulse frequency has also been found to be consistent within ovariectomised rhesus monkeys undergoing multiple PPPs (22). Additional confirmation of hypothalamic GnRH release dynamics from concurrent assessment of pulsatile pituitary gonadotrophin release is confounded in marmosets, as our preliminary data suggest incomplete or partial concordance of pituitary gonadotrophin release with hypothalamic GnRH release (48). At issue here may be the release of chorionic gonadotrophin (CG) from the marmoset anterior pituitary. Marmosets are New World primates, and as such do not release luteinising hormone (LH) from the anterior pituitary, but rather release CG under control of hypothalamic GnRH (49–51). The LH-beta subunit gene is not expressed in marmoset gonadotrophs, in contrast to Old World primates and humans (52). Conventional understanding of neuroendocrine regulation of pituitary gonadotrophin release may thus not apply in these primates since pituitary CG release stimulated by exogenous GnRH is tardy and prolonged in comparison to GnRH-stimulated LH release in rats (48). Incomplete or partial concordance between endogenous GnRH and CG pulse peaks, and prolonged exogenous GnRH stimulation of CG release, both stand in contrast to the timely temporal relationship found between pulsatile release of endogenous hypothalamic GnRH and pituitary LH in rats (48) and rhesus monkeys (36). In the marmoset, such incomplete or partial concordance between endogenous GnRH and CG confounds the use of episodic release of CG as an accurate temporal biomarker for episodic GnRH release.

Not surprisingly, parameters reflecting concentrations of GnRH are not consistent between PPP procedures within the same marmoset. The differing concentrations of GnRH released cannot be attributed to the total number or sequential order of PPP cannulations within an animal. Instead, the amount of GnRH in perfusate samples varies reliably with cannula tip location within the S-ME, a phenomenon we previously described in female rhesus monkeys (22). GnRH concentrations in marmosets are highest when the cannula tip was located within the S-ME region encompassing the infundibular area tapering towards the anterior pituitary gland. Sampling from more ventral and caudal regions of the S-ME yields lower GnRH concentrations. Such variation in PPP perfusate concentrations of GnRH sampled from differing push-pull cannula tip locations correlate with known distributions of GnRH neurones and terminals. For example, in ovariectomised rhesus monkeys, GnRH release varies with push-pull cannula tip locations in the S-ME and parallels GnRH nerve terminal concentrations (22). However, in rabbits, GnRH neuroterminals are evenly distributed in the basal hypothalamus; accordingly, concentrations of GnRH release do not vary with PPP cannula location in this species (42). To date, there are no publications as to the distribution pattern of GnRH neurones in female marmosets. Our preliminary immunocytochemistry (Fig. 5) suggests that the discrete areas of differing GnRH release that we report in the present study approximately correspond to the distribution pattern of GnRH fibres and neuroterminals in the marmoset S-ME. Moreover, the concentration gradient of GnRH release within the marmoset S-ME also is similar to that we previously reported for the S-ME of rhesus monkeys (22). Further neuroanatomical studies are needed to ascertain a definitive relationship between GnRH release, cannula tip location and GnRH neuroterminal distribution within the S-ME of common marmosets.

Unexpectedly, we found no obvious differences in any GnRH parameters between ovary-intact females in the mid-follicular

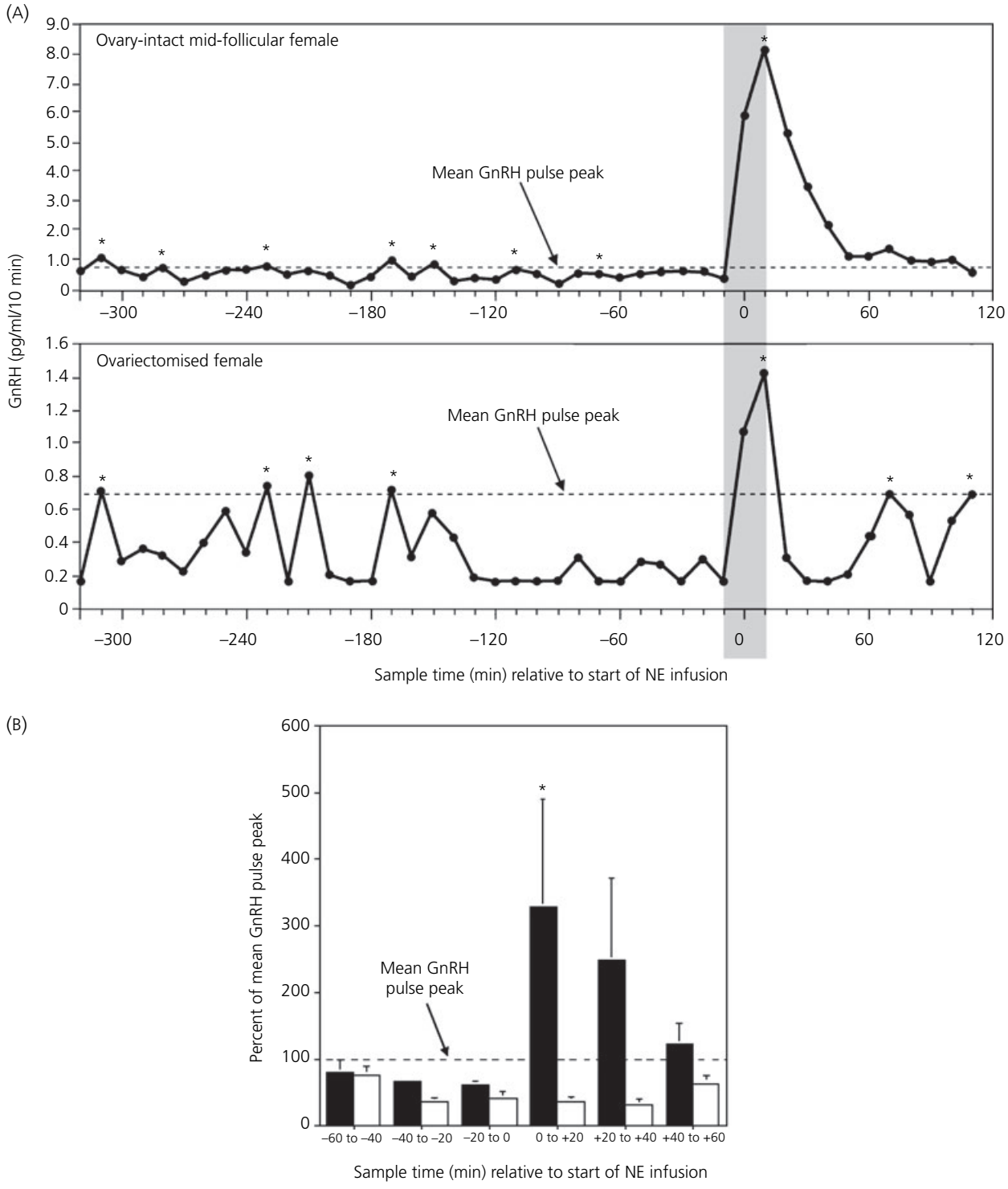

Fig. 6. (A) Gonadotrophin-releasing hormone (GnRH) response to 20-min infusion of norepinephrine (100 μM, shaded bar) in representative ovary-intact and ovariectomised females. Note that the *y*-axis values differ because of different cannula location sites which reflect different magnitudes of response. A dotted line indicates mean GnRH pulse peak; PULSAR-identified GnRH pulses are indicated by asterisks. (B) GnRH response to 20-min infusion of norepinephrine (100 μM) in female marmosets in the mid-follicular phase. GnRH release was significantly elevated above mean GnRH pulse peak levels, shown by the dotted line during norepinephrine (NE) infusions (closed bar, n = 3) but not during control infusions (open bar, n = 3). *P < 0.05, Fisher's exact test. Insufficient numbers of ovariectomised females had similarly administered norepinephrine and thus prevented statistical analysis of the GnRH responses exhibited by such females.

phase and ovariectomised females. Ovariectomy increases the frequency of GnRH release in many mammalian species, including sheep (53), rats (45), and pubertal (54, 55) and adult (56) female rhesus monkeys. However, in the rhesus monkey, although ovariectomy clearly increased the frequency of GnRH release from the S-ME, comparable GnRH release, as determined by PPP from the pituitary stalk, has been reported in separate studies involving ovariectomised (39) and mid-follicular (57) rhesus monkeys. Furthermore, when sampling from the third-ventricle, Xia *et al.* (58) found that ovariectomised rhesus monkeys had approximately the same GnRH levels as intact females in the mid-follicular phase. The different sampling sites [S-ME (54–56, this study); pituitary stalk (41,57) and third ventricle (58)] may have contributed to these different associations between ovariectomy and GnRH release. In the case of female marmosets, it is possible that negative feedback of oestradiol on GnRH release parameters is not obvious when sampling from the S-ME. This may be a consequence of the length of time after OVX or may reflect confounding influences from increased adrenal steroids in response to OVX (59). In addition, because this monkey is a New World primate, and New World primates release CG and not LH from their anterior pituitary gonadotrophs (49–51), conventional understanding of neuroendocrine regulation of pituitary gonadotrophin release may require modification. Nevertheless, marmosets exhibit pituitary CG responses to ovariectomy, postovariectomy oestradiol replacement and oestradiol-induced ovulatory gonadotrophin surges (13, 14, 60, 61) typical of those expected for pituitary LH.

In the present study, norepinephrine infused through the push-pull cannula potently stimulated GnRH release, further validating the biological relevance of the PPP technique for measuring endogenous hypothalamic release of GnRH in the marmoset. In a number of other species studied, endogenous norepinephrine is released synchronously with GnRH, and central administration of norepinephrine causes a transient increase in GnRH release within 10–20 min of infusion (62). Norepinephrine stimulation of GnRH release is oestradiol-dependent in nonprimate species (42, 63, 64), but does not appear to be oestradiol-dependent in rhesus monkeys (24, 65). Although future studies will be needed to more closely examine the role of endogenous norepinephrine in regulating hypothalamic GnRH release in the marmoset, the results from the present study suggest that GnRH release during PPP clearly responds to a central norepinephrine infusion in a predictable manner.

In PPP sessions that spanned both day- and night-time hours, an abrupt decrease in GnRH release was observed during the first hour of night-time lights off. This is reminiscent of the acute lights off drop in GnRH pulse-generator frequency measured by hypothalamic MUA in rhesus monkeys (66). This response in rhesus monkeys was attributed to the action of ambient light loss superimposed on an independent MUA diurnal rhythm (66). It remains to be determined whether GnRH release or pulse frequency is reduced in marmosets during the night in general, as observed in adult females of other species (66, 67).

Overall, our results indicate that PPP is a reliable, nonterminal method for assessing *in vivo* GnRH release in unanaesthetised common marmoset monkeys. Although early PPP methodologies were criticised for causing expansion lesions in the perfused tissue, modern and well-monitored PPP techniques, such as reported here, cause discrete localised histological damage that is no greater than that observed with cerebral microdialysis (20). Indeed, GnRH recovery rates did not change systematically across repeated cannula placements in different locations with the S-ME of individual marmosets. However, given the nonterminal aspect of this procedure, we did not directly verify the extent of tissue damage caused by each PPP or exogenously challenge the responsiveness of the hypothalamic-pituitary axis after PPP completion. At the very least, we find no evidence that any lesioning caused by our PPP significantly damaged the baseline reproductive neuroendocrine system: all intact subjects ovulated after PPP within the typical length of a follicular phase; all intact subjects exhibited normal ovulatory cycles between PPPs, including regular conceptions that required early termination by prostaglandin (3); and all PPP subjects retired to the breeding colony successfully conceived, maintained full-term pregnancies, nursed and raised offspring to adulthood. Following PPP, female subjects were always successfully reunited with their male pair-mates or male and female groupmates. The robust ovulatory cyclicity exhibited by intact PPP subjects (3) also suggests that any potential physical or psychological stress caused by the PPP procedure did not compromise the outcome of the study. The ovulatory cycles of the marmosets were not even affected by the rigorous initial cranial implant surgery. By contrast, ovary-intact rhesus monkeys exhibited amenorrhea for 3–6 months following a comparable cranial implant surgery prior to PPP (57). During the PPP procedure itself, the habituated marmosets appeared calm, interacted with their groupmates and exhibited plasma cortisol levels within the normal morning basal range (3, 8). The lack of an obvious circadian rhythm in cortisol during PPP may be due to altered feeding patterns (i.e. hourly food and snacks instead of a single daily feeding), altered sleeping patterns and/or mild stress (29). Again, any such perturbation caused by PPP was insufficient to disrupt ovulatory cyclicity (3).

Due to their small size, high potential fecundity (i.e. twins twice per year), tractability and absence of zoonotic diseases such as *Herpesvirus simiae* (Herpes B), the common marmoset monkey is an increasingly popular nonhuman primate research model (1, 2). PPP will be a useful addition to the neuroendocrine methodological repertoire for studying this species. In addition, future PPP experiments comparing GnRH release in ovulatory, socially dominant female marmosets and anovulatory, socially subordinate females will provide a unique opportunity to directly examine neuroendocrine mechanisms associated with psychosocial regulation of fertility in a primate.

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