Pulsatile gonadotropin-releasing hormone release from hypothalamic explants of male marmoset monkeys compared with male rats

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Woller MJ, Tannenbaum PL, Schultz-Darken NJ, Eshelman BD, Abbott DH. Pulsatile gonadotropin-releasing hormone release from hypothalamic explants of male marmoset monkeys compared with male rats. Am J Physiol Regul Integr Comp Physiol 298: R70–R78, 2010. First published November 4, 2009; doi:10.1152/ajpregu.00193.2009.—The present study was conducted to quantify in vitro gonadotropin-releasing hormone (GnRH) release parameters in the male marmoset. We ascertained primary cultures of marmoset hypothalamic tissues for ~2 days (marmosets) to assess GnRH release profiles in vitro in hypothalamic explants from testis-intact and gonadectomized males. Pulsatile GnRH release profiles were readily demonstrated from in vitro hypothalamic explants isolated from adult male marmoset monkeys. Gonadectomy of male marmosets resulted in elevated mean GnRH and pulse amplitude from hypothalamic explants on the 1st day of culture (day 0). GnRH pulse amplitude increased by day 2 in ~67% of hypothalamic explants from testis-intact marmosets, suggesting release from an endogenous regulator of GnRH. We also measured GnRH release profiles in vitro in hypothalamic explants from testis-intact and gonadectomized rats. Male rats showed no changes in any concentration or frequency release parameters for GnRH following gonadectomy or during successive days in culture. The present study represents a unique examination of GnRH release from male marmoset monkey hypothalamic tissue and compares release dynamics directly with those obtained from male rat, suggesting a species difference in feedback regulation of GnRH release.

hypothalamus; tissue culture; monkey; luteinizing hormone releasing hormone

THE HYPOTHALAMIC DECAPEPTIDE gonadotropin-releasing hormone (GnRH) I is responsible for controlling pulsatile gonadotropin release through its binding to pituitary gonadotrope cell membrane type I GnRH receptors in all vertebrates studied (14, 25). In most mammals, the gonadotropins released are luteinizing hormone (LH) and follicle-stimulating hormone (FSH), but New World monkeys, such as common marmosets, utilize pituitary release of chorionic gonadotropin (CG) and FSH to control steroidogenesis and gametogenesis (15).

Our focus in this study is to investigate GnRH release dynamics and testicular hormone-mediated feedback regulation of GnRH release in a small-bodied (350–400 g) male New World primate, the common marmoset (Callithrix jacchus), in which pituitary gonadotropin stimulation of the testis differs from most mammalian species (15). We ascertained GnRH release dynamics from hypothalami isolated from testis-intact and gonadectomized male marmosets. In this regard, testosterone (T) provides the major testicular-mediated negative feedback regulation of gonadotropin release at the anterior pituitary, as exemplified by a number of animal models (14). Although male marmosets demonstrate expected changes in circulating gonadotropin levels following castration, postcastration estradiol replacement, and GnRH antagonist therapy (24), postcastration T replacement studies have not been performed. Investigations in male rhesus monkeys have been limited to measurement of GnRH tissue content; orchidectomy of rhesus monkeys decreases overall hypothalamic GnRH content while increasing GnRH content in the median eminence (35), and T treatment decreases GnRH content in the median eminence while increasing overall hypothalamic GnRH content (34). These studies are consistent with T-regulated negative feedback on GnRH release, but measurements of the effects of orchidectomy on GnRH release, whether in vivo or in vitro, have not been performed in males of any primate species.

The role of T in regulating hypothalamic release of GnRH has been investigated more extensively in model systems such as the rat and ram. The role of T, however, either mediated via androgen receptors or following aromatization to estrogen (17), in regulating hypothalamic GnRH release remains controversial. In an extensive review of this area, Kalra and Kalra (17) made a compelling argument refuting the notion that castration of male rats leads to an increase in hypothalamic GnRH secretion and that T replacement inhibits GnRH release. Our recent findings in male rats (50) are consistent with Kalra and Kalra’s (17) assertion. In vitro GnRH release from hypothalami collected from sham surgery (testis-intact) and gonadectomized male rats was similar in both male groups (50). In the ram, the only other animal model in which the direct effects of T on hypothalamic GnRH release have been assessed, T was demonstrated to show classical negative feedback on GnRH release (45). The issue of T-mediated negative feedback regulation of hypothalamic GnRH release in both male rats and primates is therefore still open to question. The present study employs a proven in vitro method for sequential sampling of GnRH release from hypothalamic explants, developed for both rats (46, 48, 49, 51) and marmosets (42), to initiate an investigation of the regulatory control of reproductive neuroendocrine function in adult male marmoset monkeys and rats.

In this first in vitro measurement of GnRH release from male marmoset hypothalamic explants, we also examine a novel physiological challenge to the neuronal network that regulates GnRH release in males. Oxytocin is implicated in the stimulation of hypothalamic GnRH release in male rats (32) and in men (6). Activation of neural oxytocin release is also implicated in social bonding in rodents (10) that may well have implications for nonhuman primates and humans (11). In this regard, subordinate adult males in marmoset social groups...
either do not form or are prevented from forming highly affiliative sociosexual relationships with adult females (1, 36). Such social bonding constraints may have implications for neural oxytocin release in subordinate males that might compromise GnRH release. Because there is indirect evidence that oxytocin stimulates GnRH release in marmosets (27) and rats (38), we challenged both marmoset and rat hypothalamic preparations with a physiological dose of oxytocin to demonstrate its stimulation of GnRH release.

In vivo measurement of GnRH release has been accomplished in a number of species, including rats (23), rabbits (28), pigs (19), sheep (7, 18, 22), horses (21, 33, 43), and marmoset monkeys (2, 3, 41) but remains technically challenging (13). While in vitro methods have limitations, they can be useful alternatives in terms of readily investigating GnRH release profiles in a highly controlled environment (49). These findings provide a direct comparison of GnRH release from hypothalamic explants isolated from both testis-intact and gonadectomized male marmosets and rats and illustrate clear species differences in testicular hormone-mediated negative feedback effects on GnRH in a male primate compared with a male rodent.

MATERIALS AND METHODS

Animals. Hypothalamic explants were harvested from 14 male-female, pair-housed, adult (>2–7 yr old) male common marmosets that were captive-born and male-female, pair-housed at the Wisconsin National Primate Research Center at the University of Wisconsin, Madison, as previously described (23, 38). Three socially dominant testis-intact males were used for experiment 1. Eight socially dominant testis-intact males and three long-term gonadectomized males (>3 mo) were used for experiments 2 and 3. Immediately before death, each male was removed from its home cage and placed in an airtight chamber where it received an anesthetic dose of isoflurane (2% Forane; 1.6 l/min oxygen) for airtight chamber where it received an anesthetic dose of isoflurane (2% Forane; 1.6 l/min oxygen) for anesthetic dose of isoflurane (2% Forane; 1.6 l/min oxygen) for anesthetic dose of isoflurane (2% Forane; 1.6 l/min oxygen) for hour 1–6; 1000–1500), day 1 (hour 24–30; 0900–1500), and day 2 (hour 49–54; 0900–1500) similar to the method described above for gonadectomized adult males were perfused continuously for ~54 h. Samples were collected on day 0 (hour 1–6; 1000–1500), day 1 (hour 24–30; 0900–1500), and day 2 (hour 49–54; 0900–1500) by placing chamber exit tubing in the fraction collector for 5- to 6-h periods while sampling at 10-min intervals. At the end of the experiment, 56 mM KCl was infused in each chamber to test tissue viability through induction of exocytosis and release of GnRH.

In a parallel study, six chambers containing rat hypothalamic explants isolated from three testis-intact adult males were perfused continuously for ~78 h. Samples were collected on day 0 (hour 1–7; 1000–1600), day 1 (hour 24–30; 0900–1500), day 2 (hour 49–54; 0900–1500), and day 3 (hour 73–78; 0900–1500) similar to the method described above for gonadectomized adult males. The slight differences in the total duration of marmoset and rat perfusion, and the time windows for sample collection, were because of logistical constraints. Previous studies in male rats and female marmosets suggest such minor differences are inconsequential (42, 49).

Experiment 2: Effect of gonadectomy of hypothalamic tissue donor on subsequent pulsatile GnRH release from explants isolated from marmosets and rats. Sixteen chambers containing marmoset hypothalamic explants isolated from eight testis-intact adult males and six chambers containing explants isolated from three gonadectomized adult males were perfused continuously for ~30 h. Samples were collected on day 0 (hour 0–5; 1000–1500 for experiment 2) and on day 1 (hour 24–30; 0900–1500 for experiment 3).

Experiment 3: Oxytocin stimulation of GnRH release from marmoset and rat hypothalamic explants isolated from both gonad-intact and gonadectomized animals. Nine chambers containing marmoset hypothalamic explants from five testis-intact adult males and six chambers containing marmoset explants from three gonadectomized adult males as well as six chambers containing rat hypothalamic explants from three testis-intact adult males and six chambers containing rat hypothalamic explants from three gonadectomized adult males were perfused for ~30 h. Samples were collected on day 1 (hour 24–30; 0900–1500). The tissues were challenged with 10 min infusion of culture medium (vehicle), oxytocin (10 nM in culture media), and KCl (56 mM in culture media). At ~1 h into sample collection (hour 25; 1000), vehicle was infused for 10 min. Two hours later (hour 27; 1200), a 10-min infusion of oxytocin was performed, followed 2 h after that (hour 29; 1400) by a 10-min infusion of KCl.

GnRH radioimmunoassay. GnRH concentrations in perfusates were determined by a previously described radioimmunoassay (46–49, 51). GnRH concentration in perfusates was estimated using anti-GnRH provided by Dr. T. Nett (R1245). Synthetic GnRH used as trace and the reference preparation was purchased from Richelieu Laboratories (Montreal, Canada). Sensitivity of the assay was 0.1

hemibothalamicus) was placed in 10 ml culture medium, comprising a modified Krebs-Ringer bicarbonate buffer containing 2.2 mM CaCl₂, 154 mM NaCl, 5.6 mM KCl, 1.0 mM MgCl₂, 6.0 mM NaHCO₃, 10 mM glucose, 2 mM HEPES, 0.1% BSA, and 0.006% bacitracin, pH = 7.40 (15, 17, 28). Each hypothalamic explant was loaded in a perifusion chamber (200 l), and stainless steel mesh screens were added to the in-flow and out-flow ports of the chambers. Perifusion procedures. An Endotronics Accusyst S cell perfusion system was used to pump culture medium at 100 l/min in chambers containing marmoset or rat hypothalamic explants. A mixture of 95% O₂-5% CO₂ was maintained in a hood over the manifold area immediately in front of the perfusion chambers where gas-permeable lines containing media flowing in the chambers were exposed to the O₂/CO₂ mix to maintain media pH at 7.4. The perfusion system operated continuously throughout all experiments. Daily sampling was performed by collecting 1-ml fractions at 10-min intervals using a refrigerated fraction collector as described in each experiment below. Samples were frozen at ~80°C until radioimmunoassay.

Experiment 1: GnRH release profiles from marmoset and rat hypothalamic explants in culture. Six chambers containing marmoset hypothalamic explants isolated from three testis-intact adult males were perfused continuously for ~54 h. Samples were collected on day 0 (hour 1–6; 1000–1500), day 1 (hour 24–30; 0900–1500), and day 2 (hour 49–54; 0900–1500) by placing chamber exit tubing in the fraction collector for 5- to 6-h periods while sampling at 10-min intervals. At the end of the experiment, 56 mM KCl was infused in each chamber to test tissue viability through induction of exocytosis and release of GnRH.
pg/tube at 92% binding. The intra-assay and interassay coefficients of variation were 5.7 and 9.2%, respectively. Oxytocin was tested for crossreactivity in our GnRH RIA at doses of 100, 10, 1, and 0.1 nM and demonstrated no detectable antibody binding activity.

**Pulse detection.** Pulsatile release of GnRH from hypothalamic explants was ascertained by the PULSAR computer algorithm (25). The parameters used for GnRH pulse detection were similar to those previously reported for GnRH release in vivo (37, 46, 49, 51). The cut-off criteria for G1, G2, G3, G4, and G5 were 3.8, 2.6, 1.9, 1.5, and 1.2 standard deviations, respectively. The intra-assay coefficient of variation for GnRH was described by the formula $\frac{y}{H11005X}{H110013.38}$. Statistical assessments of characteristics of pulsatile GnRH release (mean, baseline, pulse amplitude, and pulse frequency) were performed following PULSAR analysis.

**Statistics.** For all experiments, the data were log transformed to diminish the large variability of values within groups and to increase linearity of the data (47). A repeated-measures ANOVA was performed to examine for differences among days for several variables. Data are reported as backtransformed means $95\%$ confidence limits for GnRH mean, nadir, and pulse amplitude. A one-way ANOVA was performed on the marmoset and rat data to test for differences in GnRH pulse parameters in experiment 2 and in GnRH release in response to challenges of oxytocin, KCl, and vehicle in experiment 3 (40).

**RESULTS**

**Experiment 1: GnRH release dynamics in cultured marmoset explants from testis-intact males and explants from testis-intact male rats.** Figure 1 shows unequivocal pulsatile GnRH release throughout the experiment from two hypothalamic explants obtained from two testis-intact marmosets. Male marmoset in vitro hypothalamic explants clearly maintain functional viability, in terms of pulsatile GnRH release, with pulses occurring every 78.1 (84.6–71.1) min [mean [95% confidence interval (CI)], all males and days 0–2 combined] for at least 54 h (into day 3; the maximum duration of the perfusion). In two explants obtained from the same testis-intact male marmoset, out of a total of six explants from three testis-intact males, GnRH release dynamics remained stable throughout the experiment (Fig. 1). In the remaining four explants (from the remaining 2 males), GnRH release dynamics increased from day 0 to day 2, in terms of pulse amplitude and mean concentrations, but not in terms of GnRH pulse frequency or nadir concentrations (Fig. 1). There was a large degree of individual variability (both within and between males) in the amount of GnRH released from these cultures, and this variability became more pronounced by day 2 of culture (~48–54 h), as reflected by the progressively increasing 95% CI across the experimental days (Fig. 2).

Figure 2 shows GnRH release profiles from two representative hypothalamic explants obtained from testis-intact male rats demonstrating invariant GnRH pulsatility throughout the 78-h culture. GnRH pulsatile release occurred every 46.5 (52.2–40.8) min [mean (95% CI); all males and days combined]. GnRH release dynamics remained stable during all days of culture (Fig. 3), and mean, pulse amplitude, pulse nadir, and pulse frequency values were unchanged throughout the course
of the experiment (Fig. 3) with no evidence for within- or between-male variability in rats. These relatively invariant male rat GnRH parameters were in contrast to those obtained from four out of six marmoset explants (Fig. 3).

Figure 3 summarizes the overall GnRH data collected from six marmoset and six rat hypothalamic explants obtained from testis-intact males. Comparison of GnRH pulse frequency between marmoset and rat hypothalamic explants reveals that marmoset pulse frequencies (individual explant means ranged from 0.72 ± 0.03 to 0.86 ± 0.05 pulses/h) were ~56.9% slower than those observed in rats (individual explant means ranged from 1.29 ± 0.18 to 1.47 ± 0.16 pulses/h; P < 0.02).

Experiment 2: Effect of gonadectomy of hypothalamic tissue donor on subsequent pulsatile GnRH release from explants isolated from marmosets and rats. Using a similar approach to that taken in experiment 1, we measured GnRH release from hypothalami isolated from both testis-intact and gonadectomized marmosets and rats on day 0 and compared release parameters between the groups to contrast the differences between the presence and absence of testicular hormone-mediated negative feedback, respectively. Representative examples of GnRH release from each animal preparation are shown in Fig. 4, and the release parameters are summarized for each group in Fig. 5. Mean GnRH values and GnRH pulse amplitude were significantly higher from hypothalamic explants obtained from gonadectomized compared with testis-intact male marmosets (Fig. 5).

Rat hypothalamic explants from both testis-intact and gonadectomized males had significantly more frequent GnRH pulse frequencies compared with GnRH release dynamics of corresponding marmoset tissues (Fig. 5). There were no differences in GnRH pulse parameters when gonadectomized rats were compared with testis-intact rats (Fig. 5).

DISCUSSION

This study represents the first characterization of GnRH release in male common marmosets. Hypothalamic explants from male marmosets exhibited pulsatile GnRH release in vitro for 54 consecutive hours. GnRH pulse frequencies demonstrated by male marmoset hypothalamic explants were unexpectedly 50–60% slower (1.0–1.1 pulses/h) than those observed in male rat hypothalamic explants in this and previous studies (50). Such different hypothalamic GnRH dynamics may reflect primate-rodent species differences, although in
vivo hypothalamic studies of another male nonhuman primate, gonadectomized rhesus monkeys (~1.3 pulses/h), produced GnRH pulse frequencies comparable to those found in gonadectomized male rats (~1.3 pulses/h) (9, 46, 47) and GnRH pulse frequencies obtained from both testis-intact (~1.5 pulses/h) and gonadectomized (~1.2 pulses/h) male rats used in this study (Fig. 5). Whether the slower frequency of episodic GnRH release in male marmosets is related to regulation of pituitary CG rather than LH release, as speculated for female marmosets (41, 42) remains to be determined.

Mean GnRH release and GnRH pulse amplitude were significantly higher in gonadectomized compared with testis-intact male marmosets (Fig. 5), consistent with the notion of testicular hormone-mediated negative feedback at the hypothalamic level and previous studies of male gonadotropin regulation in this primate (24). These results are also consistent with those obtained from hypothalamic explants removed from testis-intact male marmosets after 2 days in culture: both mean GnRH and pulse amplitude increased in 67% of explants. Removal of the hypothalamic explants from in vivo testicular hormone-mediated negative feedback regulation would thus be expected to result in such increased GnRH release in vitro.

There are surprisingly few studies demonstrating increases in hypothalamic GnRH release following removal of testicular hormone-mediated negative feedback. In rams, clear increases in hypothalamic GnRH release following bilateral orchidectomy (5, 44) have been reported. An increase in hypothalamic GnRH release has been reported in only a few studies of male rats (9, 20), whereas, in a review of the effects of gonadectomy in male rats, Kalra and Kalra (17) concluded that the preponderance of evidence in this rodent supports the idea that removal of T does not increase GnRH release in males and that subsequent replacement does not decrease GnRH release. Nevertheless, gonadectomy has been shown to increase the frequency of GnRH neuron firing rate in brain slices obtained from male mice (30).

In vivo assessment of endogenous hypothalamic GnRH release has been accomplished using push-pull perfusion. This technique has proved to be a powerful tool for examining in vivo hypothalamic GnRH release over time in a number of species. While in vivo push-pull perfusion is a proven sampling tool to determine pulse frequency, it is not ideal for making comparisons between absolute concentrations of GnRH release since placement of the cannula within the hypothalamus has a profound effect on the absolute levels of GnRH levels measured (13). The closer the placement of the push-pull cannula to dense populations of GnRH neuron terminals in the median eminence, the greater the concentration of GnRH measured in the perfusate (13). In vivo push-pull perfusion studies of hypothalamic GnRH release in male rats have yielded varying results. One study (9) reports a faster GnRH pulse frequency in gonadectomized rats (~1.3 pulses/h) compared with testis-intact rats (~0.8 pulses/h). Another comparable study, however, concludes that GnRH pulse frequency and amplitude are not different in gonadectomized and intact rats (20). The latter study is consistent with our present in vitro findings and with the conclusions of the Kalra and Kalra (17) review.
In our previous studies, rat hypothalamic tissues cultured from testis-intact or gonadectomized male rats exhibited the same patterns and levels of GnRH release, and thus did appear not to reflect any major negative feedback effect of T, other gonadal or extra-gonadal, or neuroendocrine factors, on GnRH release in this species (50). Interestingly, infusion of dextran-coated T in hypothalamic explants obtained from either testis-intact or gonadectomized male rats resulted in only an acute stimulation of GnRH release, consistent with induction of a single GnRH pulse (50). Pielecka and Moenter (30) obtained similarly paradoxical results when treating brain slices from castrated male mice with estradiol and DHT. GnRH neuron firing rate increased in the in vitro preparation, whereas similar in vivo treatment resulted in suppressing circulating LH levels in the normal, testis-intact male range. In male rodents, steroid hormones may have transient or long-lasting direct activational effects on hypothalamic GnRH release (17, 30). Thus the rat in vitro hypothalamic explant model used in this study has proved resistant to exhibiting GnRH release characteristics consistent with classic T negative feedback that has been reported in experiments using rams in vivo (44, 45) and in the present experiment using marmoset explants in vitro. Marmoset hypothalamic explants obtained from testis-intact males display GnRH release characteristics consistent with removal from inhibitory negative feedback regulation after 2 days in culture and are also consistent with the in vivo time course reported for increased circulating CG levels following gonadectomy in this species (by day 3 postgonadectomy) (16). In contrast, rat hypothalamic explants obtained from either testis-intact or gonadectomized males do not exhibit GnRH release characteristics consistent with the hypothalamus being affected by the presence or absence of testicular hormone-mediated negative feedback. Our results suggest a possible difference in testis-regulated inhibitory control of the reproductive neuroendocrine hypothalamus between male marmosets and male rats.

To further evaluate the effect of steroid environment on induction of GnRH release, we challenged hypothalamic explants isolated from testis-intact and gonadectomized marmosets and rats with known GnRH secretagogues, oxytocin, and KCl. Oxytocin is a neurotransmitter that is associated with control of hypothalamic GnRH release in several model systems (6, 10, 11, 27, 32, 38), including male rats (29, 31). In marmosets, oxytocin administered centrally as an intracerebroventricular bolus of 5–500 ng stimulates an increase in plasma LH levels, presumably through an increase in release of hypothalamic GnRH (27). These results are consistent with our direct observation of increased GnRH release from cultured hypothalamic explants in both testis-intact and gonadectomized male marmosets and testis-intact and gonadectomized male rats in response to a 10-min infusion of a 10 nM bolus of oxytocin. GnRH responses to oxytocin are comparable to those following a 56 mM KCl infusion. This manipulation occurred at a time point (24–30 h of culture) when rat and marmoset GnRH release profiles had yet to diverge (Fig. 3). Oxytocin has been implicated in other species as a regulator of hypothalamic GnRH release. Oxytocin stimulates GnRH release from rat medialbasal hypothalamic explants in vitro (31). In contrast to these studies, treatment of static (not perfused) cultures of rat mediobasal hypothalamic explants and median eminence explants with oxytocin results in inhibition of GnRH release (12), although this latter result may be an artifact due to the lack of continuous perfusion with fresh medium in static cultures. Our present observations are consistent with a central role for oxytocin in contributing to the stimulation of GnRH secretion.
in both male rats and marmosets and suggest that oxytocin may act centrally to regulate GnRH release.

**Perspectives and Significance**

The present study demonstrates a valid in vitro model for observing continuous GnRH release from marmoset hypothalamic explants over 3 days. This approach will dramatically increase the experimental manipulation possible from each individual marmoset compared with in vivo push-pull perfusion techniques. We are further intrigued by the potential implications of increased levels of mean GnRH and GnRH pulse amplitude associated with both gonadectomized marmosets compared with testis-intact marmosets and with explants from intact marmosets that were maintained for >54 h of culture. This observation, when contrasted with a lack of gonadectomy effect on GnRH release from hypothalamic explants obtained from male rats, illustrates the need for a further refinement of our understanding of different negative feedback effects on hypothalamic function in various laboratory animal models used to study male reproductive neuroendocrine function.

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**Fig. 5.** A–C: summary GnRH data from experiment 2 comparing mean GnRH, nadir GnRH, GnRH pulse amplitude, and GnRH pulse frequency from day 0 explants isolated from testis-intact marmosets, gonadectomized marmosets, testis-intact rats, and gonadectomized rats. Values for mean, baseline, and pulse amplitude are expressed as backtransformed means ± 95% confidence limits. D: values for pulse frequency are expressed as means ± SE. There was a significant difference between testis-intact marmosets and gonadectomized marmosets for mean GnRH (P < 0.05) and pulse amplitude (P < 0.01), but no difference in any parameter comparing testis-intact rats with gonadectomized rats. GnRH pulse frequency for testis-intact rats was significantly faster than testis-intact marmosets (P < 0.01), and pulse frequency for gonadectomized rats was significantly faster than for gonadectomized marmosets (P < 0.05). Bars with the same letters are not significantly different from each other.

**Fig. 6.** Change in GnRH was calculated as mean area under the curve (AUC) and plotted as mean ± 95% confidence limits of the peak response within 60 min postchallenge of vehicle, oxytocin (10 mM), and KCl (56 mM). A 10-min oxytocin infusion increased GnRH release in testis-intact and gonadectomized marmoset (A) and rat tissues (B) compared with a 10-min vehicle infusion. A 10-min KCl infusion also increased GnRH release in both marmoset (A) and rat (B) tissue compared with vehicle infusion.
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GRANTS
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DISCLOSURES
No conflicts of interest are declared by the authors.


