

Pain-related behavior and brain activation in cynomolgus macaques with naturally occurring endometriosis

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STUDY QUESTION: Can pain be objectively assessed in macaques with naturally occurring endometriosis?

SUMMARY ANSWER: Behavioral, pharmacological and *in vivo* brain imaging findings indicate that pain can be quantified in macaques with endometriosis.

WHAT IS KNOWN ALREADY: Endometriosis is characterized by abdominopelvic hypersensitivity. The mechanism by which endometriosis evokes pain is largely unknown, as currently available analgesics offer limited pain relief. Thus, there is a need for both greater understanding of the *in vivo* mechanism of endometriosis-associated pain and better methods of testing novel therapeutics.

STUDY DESIGN, SIZE, DURATION: Pain-related behavior and brain activation were assessed in five cynomolgus macaques with endometriosis. Three healthy female macaques served as controls.

PARTICIPANTS/MATERIALS, SETTING, METHODS: Abdominopelvic sensitivity to force was assessed with an algometer. Activation of brain areas using block design force stimulation and the effects of a single dose of the analgesic drug morphine and 2-month treatment with the progestin dienogest on brain activation were observed via functional magnetic resonance imaging.

MAIN RESULTS AND THE ROLE OF CHANCE: Pain response thresholds in macaques with endometriosis were significantly less than that of healthy macaques ($P = 0.0003$). In addition, non-noxious force activated the insula and thalamus, which was reduced with morphine and 2-month dienogest treatment.

LIMITATIONS, REASONS FOR CAUTION: The specific role of cysts, such as peritoneal cysts, in endometriosis pain was not explored. While non-noxious stimulation activated the insula and thalamus, macaques were sedated during fMRI scans. Current findings need further confirmation in a larger cohort.

WIDER IMPLICATIONS OF THE FINDINGS: The current study demonstrated central sensitization and related pain behavior in macaques with naturally occurring endometriosis. Altered functioning of the central nervous system could be the focus of future mechanistic studies and for the development of novel therapeutics.

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Key words: non-human primate / chronic pelvic pain / hyperalgesia / translational model / progestin / analgesia / functional magnetic resonance imaging

Introduction

Endometriosis, the presence of endometrium outside of the uterus, occurs in ~5–10% of women of childbearing age and is characterized by infertility and chronic pelvic pain (CPP) (Laux-Biehlmann *et al.*, 2015). The presence and intensity of pelvic pain may fluctuate with the menstrual cycle (e.g. dysmenorrhea), but over time, pain may evolve to be continuous in nature (Crosignani *et al.*, 2006). Patients with endometriosis-associated pain also demonstrate enhanced responsiveness to noxious and innocuous somatic stimulation ('hyperalgesia' and 'allodynia', respectively) within the painful abdominopelvic region, such that a significant negative correlation is observed between patient-rated abdominopelvic pain intensity (e.g. visual analog scale) and pressure (or 'force') threshold (Schliep *et al.*, 2015; Mui *et al.*, 2016). While currently available treatments, including analgesics, hormonal therapy and surgical cyst removal, ameliorate pain in some patients, pain often returns following treatment cessation or following surgery (Stratton and Berkley, 2011). In addition, currently available treatments are not without significant adverse somatic and reproductive side effects (Stratton and Berkley, 2011; Zito *et al.*, 2014).

Significantly altered responsiveness of central nervous system (CNS) neurons to peripheral stimulation (or 'central sensitization') has been hypothesized to underlie a number of chronic pain states. Nociceptive substances that have been observed in other chronic pain states have been identified in the abdominopelvic cavity of preclinical animal models of endometriosis and in patients with endometriosis (Rizner, 2009; Stratton and Berkley, 2011). These substances are believed to sensitize peripheral nociceptive neurons and lead to persistent neurophysiological changes to post-synaptic CNS neurons (Stratton and Berkley, 2011; Morotti *et al.*, 2014). A similar mechanism has been proposed in the case of endometriosis pain, but the exact CNS mechanism has yet to be fully elaborated. Identification of the relevant pain-related brain areas, in endometriosis as well as in other chronic pain states, has potential utility as objective, pharmacodynamic biomarkers for pain and analgesia, which are subjective, individual experiences (Smith *et al.*, 2017; Wanigasekera *et al.*, 2018).

Current mechanistic understanding of clinical endometriosis-associated pain is generally based on rodent models, but rodents are genetically and physiologically distinct from humans. In particular, rodents do not menstruate and do not naturally develop endometriosis. In addition, immunological functioning, which appears to have a key role in the genesis and maintenance of endometriosis, significantly differs between rodents and humans (Mestas and Hughes, 2004). By contrast, non-human primates share a number of physiological similarities with humans, including reproductive and neurological anatomy and physiology, and furthermore, show spontaneous endometriosis (Yamanaka *et al.*, 2012). Thus, non-human primates are an ideal species for preclinical exploration of disease mechanism and evaluating potential therapeutics for endometriosis (Nishimoto-Kakiuchi *et al.*, 2018).

The primary objective of the current study was to demonstrate the presence of central sensitization in *Macaca fascicularis* with naturally occurring endometriosis. Macaques with endometriosis display symptoms suggestive of chronic pain, but whether they exhibit allodynia or hyperalgesia as observed in clinical endometriosis is not known (Maginnis *et al.*, 2008; Nishimoto-Kakiuchi *et al.*, 2018). Thus, a method of applying graded stimulation to the abdominopelvic region was developed. Next, non-noxious abdominopelvic stimulation was used

to observe brain activation with functional magnetic resonance imaging (fMRI).

Finally, preliminary pharmacological characterization of behavioral sensitization and brain activation in macaques with endometriosis was performed. Both hormone modulators and analgesics are first-line agents for the management of endometriosis-associated pain (Stratton and Berkley, 2011; Zito *et al.*, 2014). Standard analgesics, including non-steroidal anti-inflammatory drugs (NSAIDs) and opiates have shown varying degrees of efficacy on central sensitization (Millan, 1999), but such an effect has yet to be described with hormone modulators in endometriosis-associated central sensitization. Thus, the effect of the progestin dienogest, a drug used for the treatment of endometriosis, was examined on behavioral sensitization in macaques with endometriosis (Zito *et al.*, 2014). In addition, the effect of morphine and dienogest treatment on non-painful brain activation in these macaques was assessed. The current findings suggest that macaques with naturally occurring endometriosis exhibit behavioral and central sensitization, which could be utilized to further elaborate the mechanism of endometriosis-associated pain and to develop effective therapeutics.

Materials and Methods

General study plan

- The acute, antinociceptive efficacy of a single dose of morphine, meloxicam and acetaminophen on non-noxious abdominal force hypersensitivity was assessed in five cynomolgus macaques with naturally occurring endometriosis. A 3-day washout interval was used between drugs; each macaque received all three drugs.
- One week following drug testing, brain activation in three macaques with endometriosis in response to non-noxious abdominal stimulation and the effect of a single dose of morphine on brain activation were examined with fMRI. For comparison, the effect of non-noxious abdominal stimulation on brain activation in three healthy, control macaques was also assessed.
- About 1 month following fMRI, pre-dienogest treatment stimulation responses were obtained from five macaques with endometriosis. Following baseline determinations, macaques with endometriosis were treated for 8 weeks with dienogest. Stimulation-evoked brain activation was assessed with fMRI on the eighth week of dienogest treatment. Response thresholds were measured 2 and 4 weeks after cessation of dienogest treatment.

At the end of the study, all five macaques with endometriosis were deeply anesthetized and endometrioma were removed for histological analysis. While unconscious, macaques were euthanized with an overdose of pentobarbital. The three healthy, control macaques were not euthanized.

Studies were performed between June 2016 and May 2017.

Ethical approval

All study procedures were reviewed and approved by the Hamamatsu Pharma Research, Inc., Animal Care and Use Committee and were carried out in accordance with guidelines within the 'Guide for the Care and Use of Laboratory Animals' (Eighth ed., National Academy of Sciences, 2011).

Subjects

Five mature, pre-menopausal, female *Macaca fascicularis* (10–18 years of age) and three healthy female macaques (control; 10 years of age) were used in the current study. Macaques were obtained from EBS's Co., Ltd. (Hashimoto, Japan) colony in Vietnam. For details concerning the initial

field screening of the five macaques with endometriosis, findings on autopsy at the end of the study and the results of histological examination of the cysts from each of the five macaques with endometriosis, see Supplementary methods.

The macaques were housed in adjoining individual primate cages that allowed visual and limited tactile interactions. Room conditions, including humidity, temperature and light were as per the 'Guide'. Animals had free access to tap water and were fed standard non-human primate chow (Oriental Yeast Co., Ltd., Chiba, Tokyo, Japan), which was supplemented weekly with fresh fruits or vegetables. The animals' home cages were supplied with enrichment devices and positive interaction (e.g. hand feeding of treats) between research and animal care staff.

Effects of analgesics on abdominopelvic hypersensitivity

The aim of force (or 'pressure') testing in the current study was to deliver a graded stimulus to quantify changes to somatosensation, and not visceral sensation, in macaques with endometriosis and in healthy controls (See Supplementary methods). Abdominal areas in which a cyst was palpable were not tested—pressure was not applied to endometriomas.

Macaques were acclimated to restraint in a monkey chair. A modified algometer (Matsumiya Medical Co., Ltd., Tokyo, Japan) was used to measure sensitivity to force (kg) applied to the abdominal area. A 2.5-cm-diameter plastic cap covered the standard algometer probe. The algometer was pushed against the skin until a pain-related facial expression (flinching of the facial muscles around the eyes and the muscle at the back of the head) was observed. The 'response force' was measured three times, in the same area, with about 1 min between measurements, and the mean response force was calculated. The maximum stimulation force was set at 3 kg. A previous study found significant activation of the insular cortex with 2.5 kg of force applied to the abdomen in healthy, uninjured macaques (Hama *et al.*, 2018). To avoid possible tissue injury, cutoff was set at 3 kg; if no response was observed, then 3 kg was assigned.

Following the measurement of baseline response to stimulation, either morphine (3 and 6 mg/kg diluted in saline, i.m.; 0.5 ml/kg; Shionogi & Co., Tokyo, Japan), meloxicam (0.5 mg/kg, i.m.; 0.5 ml/kg; Boeringer Ingelheim, Tokyo, Japan) or acetaminophen (10 mg/kg, p.o. in distilled water; 5 ml/kg; Sigma-Aldrich Co., St. Louis, MO, USA) was administered. Response thresholds were measured over time following drug administration. Behavioral testing was conducted in a blinded manner. (Regarding selection of doses, see Supplementary methods.)

Effect of dienogest on abdominopelvic hypersensitivity

Following baseline response measurement, dienogest (0.025 mg/kg in distilled water; purchased from Mochida Pharmaceutical Co., Ltd., Tokyo, Japan) was orally administered, *via* gavage (5 ml/kg), twice per day to five macaques with endometriosis. Macaques with endometriosis were dosed for a total period of 8 weeks and tested once per week and further tested 2 and 4 weeks after the last dosing of dienogest (see Supplementary Fig. S1 for dienogest study schedule).

Effect of drug treatments on stimulation-evoked brain activation visualized with fMRI

While five macaques with endometriosis were initially used to observe the effect of morphine on force-evoked activation, two of the macaques needed to be excluded from further data analysis as respiratory complications developed to anesthesia, while the macaques were in the MRI

scanner. Thus, brain imaging and imaging analysis utilized a total of three macaques with endometriosis. For comparison, three healthy, control macaques also underwent fMRI.

After 8 weeks of dienogest treatment in macaques with endometriosis, stimulation-evoked brain activation was observed with fMRI. While five macaques underwent dienogest treatment, two of the macaques developed anesthesia-related complications while in the MRI scanner. Thus, brain imaging and imaging analysis utilized a total of three dienogest-treated macaques with endometriosis. For comparison, three healthy, control macaques also underwent fMRI.

Brain activation in anesthetized macaques, without and with abdominal stimulation, was assessed using a 3.0 T MRI system (Signa HDxt 3.0 T MRI system (GE Healthcare, Milwaukee, WI, USA)). Macaques were sedated by continuous intravenous infusion of propofol (0.2 mg/kg/min) and heads were fixed within an MR compatible acrylic head holder (Matsui Co., Aichi, Japan). The dose of propofol used has little, if any, analgesic effect (Steinbacher, 2001).

The anatomical MRI protocol consisted of a T1-weighted fast spoiled gradient-recalled (FSPGR) sequence (repetition time (TR)/echo time (TE), 15.8/7.0 ms; number of averages, 1; flip angle, 12°; field of view, 150 mm × 150 mm; matrix, 256 × 224; slice thickness/interval, 1.0/0.5 mm; number of slices, 168). Functional scan sequences consisted of field-echo, echo-planar imaging (EPI; TR/TE, 3000/35 ms; flip angle, 90°; field of view, 140 mm × 140 mm; matrix, 64 × 64; slice thickness, 2.4 mm; number of slices, 30).

During one fMRI scan, animals underwent a block design stimulation protocol: 10 sets of abdominal force stimulations (See Supplementary methods). One stimulation set consisted of 30 s. of an 'OFF' stimulus, a 100 g weight (empty 1.5 l water bottle with a 2.5-cm-diameter smooth cap) applied by hand to rest perpendicularly on the skin, followed by 30 s. of an 'ON' stimulus, a 1.5 kg weight (1.5 l water bottle filled with water) applied by hand to rest perpendicularly on the skin. For each set, 10 frames were acquired, for a total of 100 frames per functional scan. A 30-s interval without stimulation separated each set. One fMRI scan was 14.5 min in duration.

Morphine was dissolved in saline on the day of fMRI scanning. On the day of MRI scanning, macaques underwent a pre-morphine scan and a post-morphine scan. Following a baseline scan of 10 sets of force stimulations, macaques received 3 mg/kg (in 0.5 ml/kg, i.m.) of morphine. MRI scanning was performed again, between 15 and 30 min following dosing, with macaques stimulated with 10 sets of stimuli.

Using the same block design stimulation protocol, stimulation-evoked brain activation in dienogest-treated macaques was observed before and after 8 weeks of dienogest treatment.

MRI data analysis

All subsequent image analyses were conducted with statistical parametric mapping software (SPM12; Wellcome Trust Centre for Neuroimaging, London, UK). The images were realigned and resliced on to the mean EPI image to correct for head motion. The EPI images were co-registered to the corresponding T1-weighted anatomical image and normalized to a macaque brain template (Black *et al.*, 2004). (Stereotaxic coordinates according to Horsley–Clarke's stereotaxic coordinates.) The resulting image was smoothed with a 4 mm × 4 mm × 4 mm full-width at half-maximum Gaussian kernel. Voxel-wise statistical analysis was based on a general linear model. A fixed-effect model was used for group analysis of data from the three macaques with endometriosis and the three healthy control macaques.

Contrast was defined as (1.5 kg stimulation–100 g stimulation) to isolate regions responsive to 1.5 kg stimulation-related signals in the entire brain.

Following drug treatment, contrast was defined as (pre-treatment–post-treatment) and (post-treatment–control), to determine decreased areas of activation following drug treatment and to compare activation following drug treatment in macaques with endometriosis with activation in healthy control macaques.

Statistics

No statistical method was used to determine sample sizes prior to the start of the current study. The fewest number of animals was used on the basis of ethical considerations and on the basis of animal availability. Group sizes were similar to those reported in previous publications (Mann *et al.*, 1986; Nagasaka *et al.*, 2017). Unless otherwise indicated, data are presented as mean \pm SD. Comparisons of behavioral and biochemical parameters between control macaques and macaques with endometriosis at one time-point (e.g. response thresholds) were performed using an unpaired, two-tailed Student's *t*-test (with Welch's correction for uneven variances as needed). For the analysis of the effect of drug treatment on response thresholds over time, a two-way repeated-measures analysis of variance (ANOVA) was performed, followed by Dunnett's test for multiple comparisons. Statistical analysis was performed using Prism 4.02 (GraphPad Software, San Diego, CA, USA). Minimal statistical significance was set at $P < 0.05$.

From individual functional data, SPM12 generated statistical parametric maps. A *t*-score was generated following comparisons between voxels and *t*-scores were transformed to *Z* scores via the corresponding *P*-values. Average *Z* score maps were calculated from individual *Z* score maps. Peak voxels were considered significant at a *Z* score > 1.96 ($P < 0.05$, uncorrected for multiple comparisons, one-tailed *t*-test) or a *Z* score > 2.3 ($P < 0.01$).

Results

Abdominal hypersensitivity in macaques with endometriosis

Prior to analgesic drug administration, macaques with endometriosis demonstrated abdominopelvic hypersensitivity (Fig. 1). Six milligrams per kilogram (i.m.) morphine significantly increased response threshold 10 and 30 min following administration ($F(3, 19) = 10.80, P = 0.001$). One hour after morphine administration, response force thresholds were not significantly different from before administration. While maximal efficacy was observed 10 min after morphine administration, the maximum possible effect ((Post-administration – Pre-administration)/(Control force – Pre-administration) $\times 100$) was about 41%. (The force response threshold in healthy, control macaques were considered 100% maximum possible effect.) Treatment with either meloxicam or acetaminophen did not significantly change response thresholds ($P > 0.05$).

Non-noxious stimulation-evoked activation of the thalamus and insular cortex in macaques with endometriosis

Whole-brain scan during block design non-noxious stimulation (Fig. 2) applied to the abdominopelvic region showed significant bilateral activation of insula (left insula *Z* scores $> 1.96, P < 0.05$; right insula *Z* score $> 2.3, P < 0.01$) and bilateral thalamus (*Z* score $> 2.3, P < 0.01$). Therefore, subsequent investigation of stimulus-evoked brain

activation in the current study focused on the thalamus and insula (*Z* scores and peak voxel coordinates in Table 1).

By contrast, in healthy macaques, abdominopelvic stimulation with 1.5 kg did not evoke significant brain activation ($Z < 1.96, P > 0.05$) (Table 1; Supplementary Fig. S2).

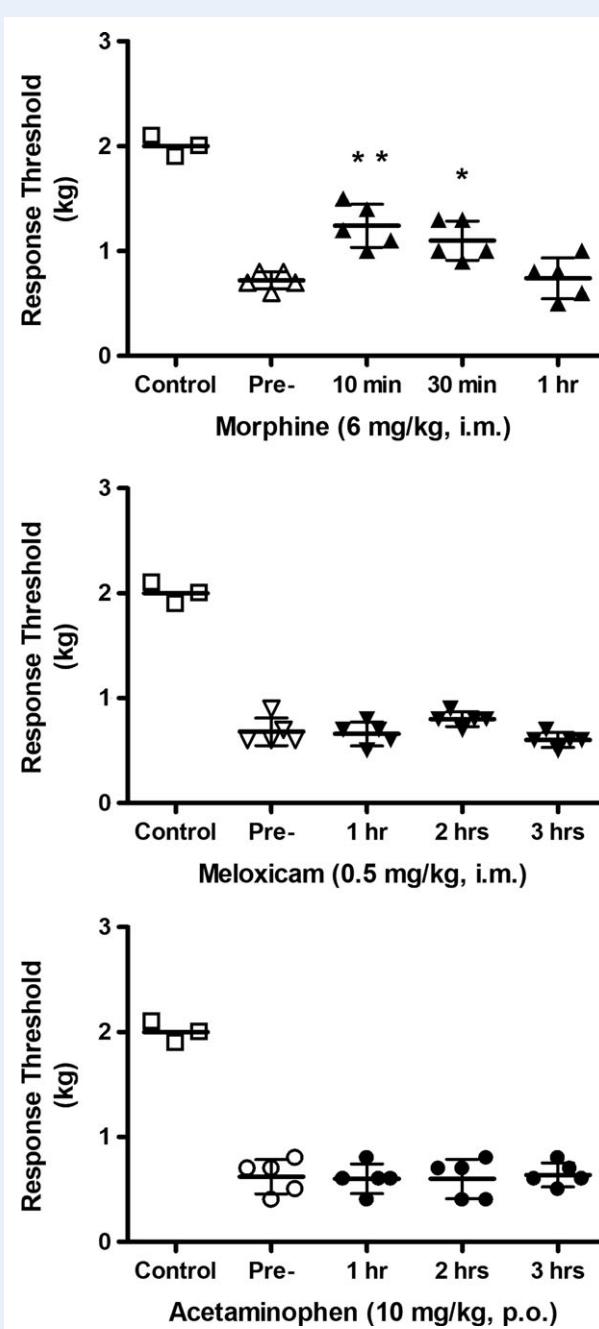


Figure 1 Response threshold (kg) was measured before morphine, meloxicam or acetaminophen administration ('Pre-'). The mean response threshold of healthy ('control') macaques is shown for comparison. Morphine significantly increased response force 10 min and 30 min after administration. By contrast, no increased response threshold was observed following administration of either meloxicam or acetaminophen. Data shown as mean \pm SD. * $P < 0.05$, ** $P < 0.01$ vs. Pre-.

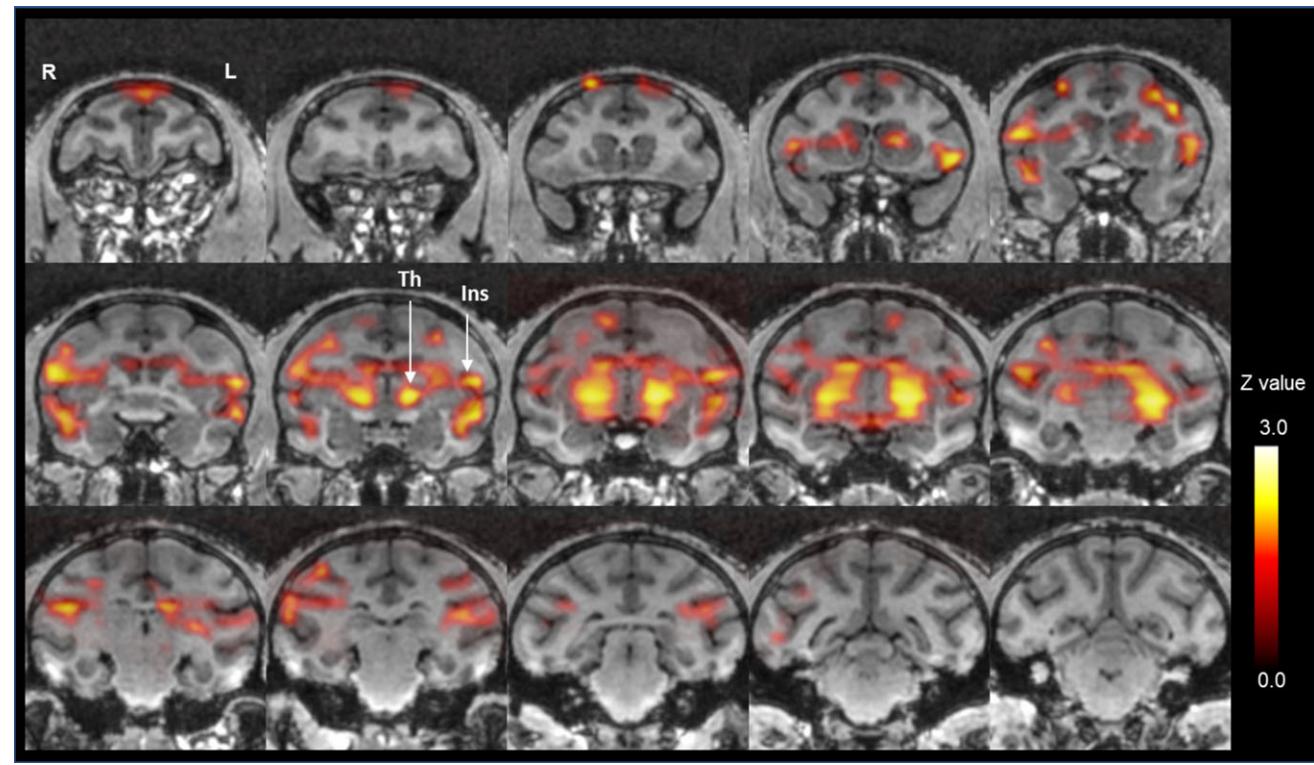


Figure 2 Non-noxious stimulation-evoked brain activation in macaques with endometriosis. Bilateral activation of the thalamus (Th) and the insular cortex (Ins) in macaques with endometriosis observed with non-noxious abdominal stimulation (Table I). Images are averaged brain activation from three macaques with endometriosis. Coronal brain sections are arranged from rostral (upper left) to caudal (lower right) separated by 2.0 mm. L, anatomical left; R, anatomical right.

Morphine reduced thalamic and insular cortex activation in macaques with endometriosis

Administration of an antinociceptive dose of morphine (3 mg/kg, i.m.; $t(2) = 23.24, P = 0.0018$) bilaterally reduced stimulation-evoked thalamic and insular activation in macaques with endometriosis (Fig. 3). Stimulation-evoked activation 'before' morphine treatment was significantly higher in the insula (Z scores $> 1.96, P < 0.05$) and thalamus (Z scores $> 2.3, P < 0.01$) compared to force-evoked activation 'after' morphine treatment ('Pre-morphine $>$ Post-morphine', Table II). The data suggest that morphine treatment suppressed non-noxious evoked activation of the insula and thalamus.

Furthermore, the effect of morphine treatment in macaques with endometriosis was such that stimulation-evoked activation in insula and thalamus was not significantly different from that of stimulation-evoked activation in healthy, control macaques (i.e. no activation; 'Post-morphine $>$ control', Table II).

Dienogest treatment reduced abdominal hypersensitivity in macaques with endometriosis

Before dienogest treatment, the mean response threshold was significantly lower in macaques with endometriosis than that of healthy

macaques ($t(6) = 4.437, P = 0.0044$, Fig. 4). Beginning on the second week of dienogest treatment, mean force thresholds increased over time, compared to pretreatment force thresholds, and remained increased for the duration of the treatment period ($F(10, 54) = 5.789, P < 0.0001$). At 4 weeks of dienogest treatment, the maximum possible effect was about 49%.

Furthermore, response thresholds remained significantly elevated, compared to pretreatment response thresholds, 2 and 4 weeks after the last dose of dienogest. Four weeks after the last dose of dienogest, the maximum possible effect was about 29%.

Dienogest treatment reduced thalamic and insular activation in macaques with endometriosis

Stimulation-evoked brain activation in macaques with endometriosis was assessed with fMRI during the last week of dienogest treatment (Fig. 5). Bilateral activation of the insula (Z scores $> 1.96, P < 0.05$) and thalamus (Z scores $> 2.3, P < 0.01$) 'before' dienogest treatment was significantly higher compared to activation 'after' treatment ('Pre-dienogest $>$ Post-dienogest', Table III). The data suggest that dienogest treatment decreased insular and thalamic activation.

While comparison of insular and thalamic stimulation-evoked activation before and after dienogest treatment suggests an overall decrease in activation, thalamic activation was still significantly higher than that

Table I Whole-brain scanning with fMRI identified significant activation of bilateral insula and thalamus during application of a non-noxious force applied to the abdominopelvic area of macaques with endometriosis.

Endometriosis					
Area	Hemisphere	Z score	Coordinates (mm)		
			x	y	z
Insula	Left	2.29	18	20	6
	Right	2.33	-14	18	4
Thalamus	Left	2.81	-2	-6	-2
	Right	2.74	4	-8	2

Control					
Area	Hemisphere	Z score	x	y	z
Insula	Left	0.41	20	16	6
	Right	0.38	-18	18	4
Thalamus	Left	0.21	-4	-8	8
	Right	0.18	6	-4	10

No significant activation was observed in control (healthy) macaques during application of a non-noxious force to the abdominopelvic area. Z scores of peak voxels are shown. Stereotaxic coordinates (x, y, z) are according to Horsley–Clarke's stereotaxic coordinates. Peak voxels were considered significant at Z score > 1.96 ($P < 0.05$) and Z score > 2.3 ($P < 0.01$).

of control (Z scores > 1.96 , $P < 0.05$; 'Post-dienogest 8 W > control', Table III). Stimulation-evoked activation of the insula, however, was not significantly different from activation observed in control macaques (i.e. no activation; Table III).

Discussion

Cynomolgus macaques with naturally occurring endometriosis demonstrated decreased abdominopelvic response thresholds, suggesting hypersensitivity to non-noxious stimulation. In addition, pain-associated brain areas, the thalamus and the insula, were robustly activated with a stimulus that failed to activate these areas in healthy macaques. Hypersensitivity and activation of brain areas with non-noxious stimulation suggest the presence of central sensitization in macaques with endometriosis. The reduction of thalamic and insular activation in macaques with endometriosis with an antinociceptive dose of morphine further suggest an association between brain activation and endometriosis-associated pain. While dienogest treatment alleviated hypersensitivity and decreased non-noxious stimulation-evoked brain activation, significant residual non-noxious force-evoked activation of the thalamus was nonetheless observed at the end of dienogest treatment. The current study suggests the presence of central sensitization in macaques with naturally occurring endometriosis, which could be utilized to further elaborate the role central sensitization in endometriosis pain.

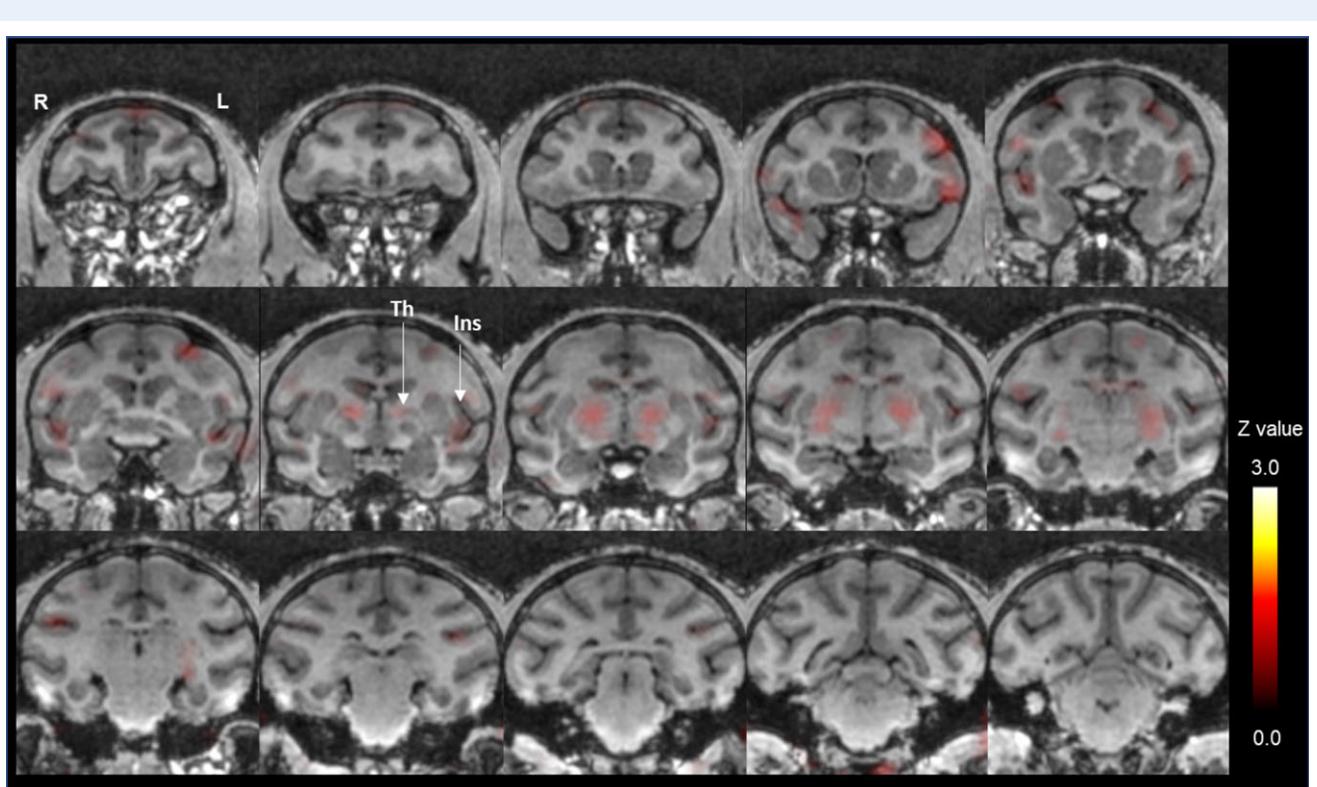


Figure 3 Morphine reduction of stimulation-evoked brain activation in macaques with endometriosis. Morphine reduced stimulation-evoked activation in the thalamus (Th) and insular cortex (Ins). Images are averaged brain activation from three macaques with endometriosis.

Table II Insula and thalamus activation observed with fMRI during non-noxious stimulation of the abdominopelvic area was compared before (pre-morphine) and after (post-morphine) morphine treatment in macaques with endometriosis.

Pre-morphine > Post-morphine					
Area	Hemisphere	Z score	Coordinates (mm)		
			x	y	z
Insula	Left	2.01	18	18	8
	Right	2.11	-14	18	4
Thalamus	Left	2.64	-2	-6	-2
	Right	2.57	4	-8	4
Post-morphine > control					
Area	Hemisphere	Z score	x	y	z
Insula	Left	0.61	18	20	6
	Right	0.71	-14	16	8
Thalamus	Left	0.84	-2	-10	0
	Right	0.64	6	-8	2

Post-morphine activation in bilateral thalamus and insula was significant decreased compared to pre-morphine treatment. Also, insula and thalamus activation of macaques with endometriosis after morphine treatment was compared to that of control (healthy) macaques. No significant difference in brain activation was observed between morphine-treated macaques with endometriosis and control macaques. Z scores of peak voxels are shown. Stereotaxic coordinates (x, y, z) are according to Horsley-Clarke's stereotaxic coordinates. Peak voxels were considered significant at Z score > 1.96 ($P < 0.05$) and Z score > 2.3 ($P < 0.01$).

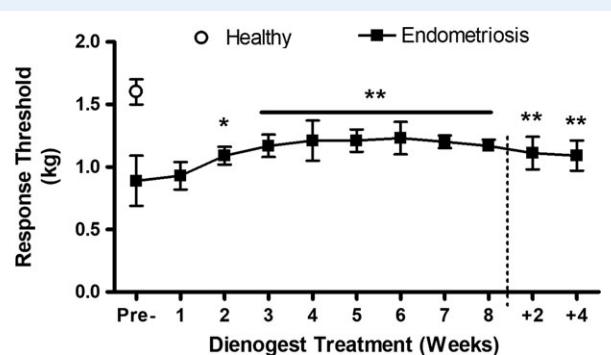


Figure 4 Effect of dienogest treatment over time in macaques with endometriosis. Response thresholds (kg) increased over time with dienogest treatment. Response thresholds remained elevated 2 and 4 weeks following discontinuation of dienogest treatment. The vertical dotted line after 8 weeks indicates the end of dienogest treatment. Mean response threshold of healthy macaques shown for comparison. Data are expressed as mean \pm SD. * $P < 0.05$, ** $P < 0.01$ vs. Pretreatment.

Characterization and quantification of abnormal pain perception, in both humans and non-human animals, could be utilized to uncover underlying neural pathophysiology. Quantitative sensory testing could help to bridge the translational gap as an indicator of treatment efficacy (Rolle et al., 2006; Fenton et al., 2009; Berge, 2011; Smith et al.,

2017). In chronic pain, including endometriosis-associated pain, increased sensitivity to stimulation correlates with subjective ratings of ongoing pain (Schliep et al., 2015; Mui et al., 2016; Smith et al., 2017). Similarly, associations between somatosensation and subjective pain rating are also observed following analgesic treatment. These suggest that the mechanism underlying ongoing pain mediates alterations in somatosensation. Thus, abdominopelvic hypersensitivity in macaques with endometriosis suggests ongoing pain, as do other non-evoked pain behaviors observed in macaques with endometriosis (Maginnis et al., 2008; Nishimoto-Kakiuchi et al., 2018). For example, Maginnis et al. (2008) scored various behaviors (e.g. attitude, activity) that in total suggested pain ('discomfort') in macaques with endometriosis; progesterone treatment apparently improved symptoms. Nishimoto-Kakiuchi et al. (2018) used decreased food consumption as an indirect indicator of endometriosis pain. The advantage of a response threshold is that stimulation can be delivered in a graded and controlled manner.

The pathophysiology that mediates endometriosis-induced hypersensitivity has yet to be fully elaborated but could involve persistent nociceptive input from injured peripheral tissues and changes in central processing of somatosensation. It has been suggested that key factors initiating peripheral nociceptive input include substances released from the endometriomas, such as pro-inflammatory substances and growth factors, which sensitize sensory nerves found within the peritoneum and the endometriomas (Stratton and Berkley, 2011; Laux-Biehlmann et al., 2015). These processes, in turn, lead to alterations in post-synaptic spinal, subcortical and cortical neurons, wherein increased basal activity and responsiveness to subsequent stimuli are observed (Millan, 1999). Sensitization along the neuraxis has been proposed as the basis of ongoing pain and perception of non-noxious stimulation as painful.

The current study suggests sensitization occurred in the thalamus and insula of macaques with endometriosis, since non-noxious stimulation activated these areas, whereas the same stimulus did not activate either thalamus or insula in healthy controls. The thalamus serves as a major pain relay and modulatory center and thalamic neurons project to brain areas which have roles in the affective-cognitive aspects of pain, including the insula, the cingulate cortex and the prefrontal cortex (Millan, 1999). Insular neurons in turn project to nuclei within the limbic system, which is involved in the affective component of pain sensation (Mesulam and Mufson, 1982; Veldhuijzen et al., 2010). Pain-related behaviors observed in macaques with endometriosis (Maginnis et al., 2008; Nishimoto-Kakiuchi et al., 2018) could be mediated by these or other brain areas. Further exploration, for example, in awake macaques, could uncover a role of other brain areas in unevoked as well as evoked endometriosis pain.

Thus far, a few clinical studies suggest possible relationships between brain functioning and endometriosis-associated pain. Volumetry derived from MRI found decreased brain gray matter volume in the thalamus and insula in endometriosis patients (As-Sanie et al., 2012). The loss of gray matter could be due to neuronal loss over time due to overactivation or an extended period of neuronal atrophy. Resting-state functional connectivity mapping in endometriosis patients suggests increased connectivity between the hippocampus and insula, thalamus, caudate nucleus and cerebellum (Beissner et al., 2017). Interestingly, a course of analgesic psychotherapy in these patients reduced functional connectivity between brain regions, indicating that connections as well as discrete brain areas may be amenable to

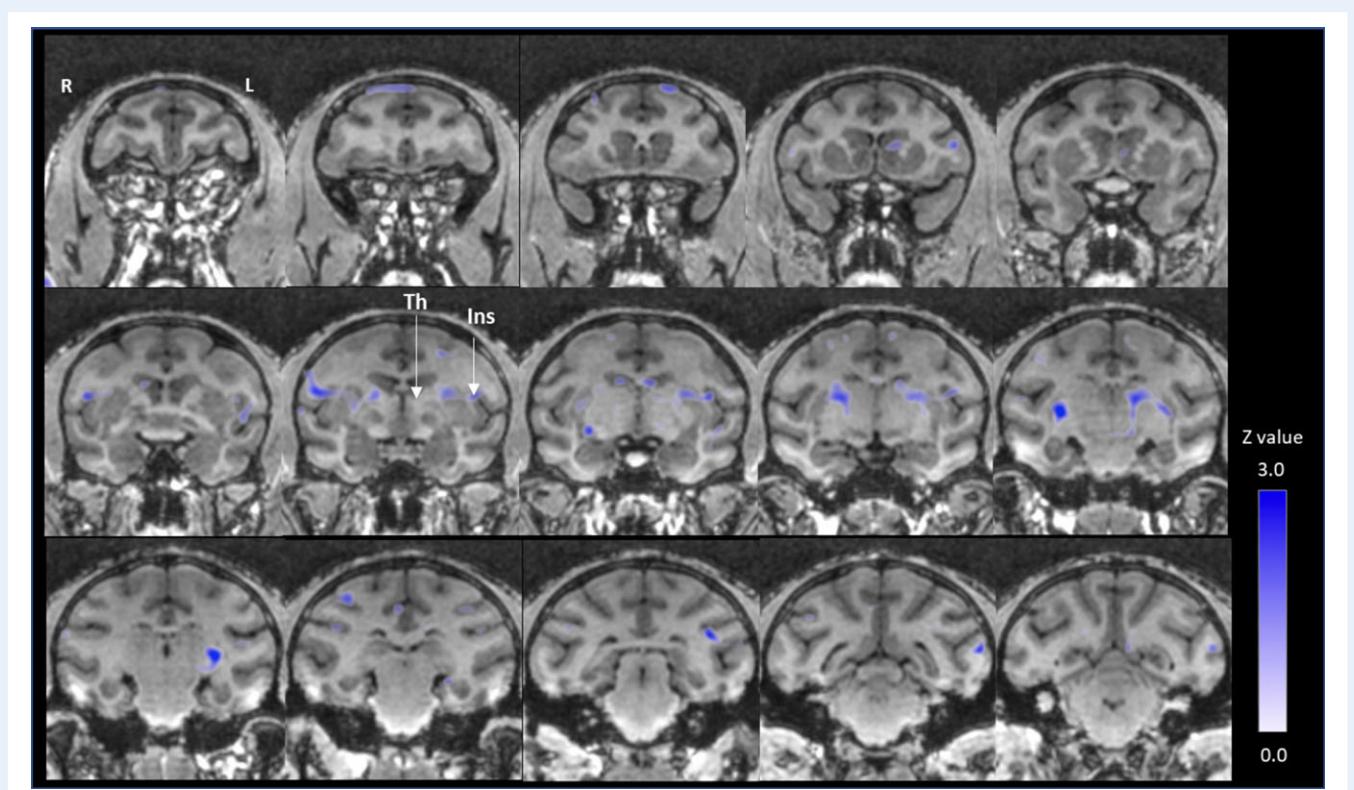


Figure 5 Stimulation-evoked brain activation in dienogest-treated macaques with endometriosis. Non-noxious stimulation-evoked brain activation acquired with fMRI during the eighth week of dienogest treatment from three macaques with endometriosis. Figure shows activation difference obtained from subtracting brain activation of dienogest-treated macaques from brain activation of pre-dienogest treatment macaques (Pre-dienogest > Post-dienogest 8 W; Table III). Images are averaged brain activation from three macaques with endometriosis.

therapeutic intervention (Beissner et al., 2017). Whether endometriosis patients demonstrate stimulus-evoked activation in the brain has yet to be shown—the current preclinical findings suggest this possibility in patients. Clinical studies will be needed to confirm this hypothesis and to further validate the utility of the macaques as a preclinical model of endometriosis.

It may not be entirely surprising, given the various endometriosis-associated pains and their possibly varying mechanisms, that an NSAID and acetaminophen did not show efficacy against hypersensitivity in the current study and morphine showed only modest efficacy. Clinical evidence demonstrating robust efficacy of these classes of drugs in endometriosis pain is lacking (Carey and As-Sanie, 2016; Brown et al., 2017). While opioids are the mainstay for moderate to severe pain, the current study showed at best partial pain relief obtained with a non-sedating dose of morphine. It is possible that some analgesics are effective on certain endometriosis pain and not effective on others, which suggests the need to define specific mechanisms for specific pains (Stratton and Berkley, 2011). If hypersensitivity can be generalized to various pains associated with endometriosis, then macaques with endometriosis could be a useful model with which to develop treatments—the findings with an NSAID and morphine in the current study tend to support the clinical findings of non-robust efficacy. It may be necessary, however, to further define methods of mimicking specific symptoms of endometriosis-associated pain.

Interestingly, modest pain relief was obtained with dienogest treatment, without an overall reduction of cyst volumes (Supplementary Fig. S3; Supplementary Table S1). Pain relief also persisted despite termination of dienogest treatment, which has been observed clinically (Schindler, 2011). The findings do not support a direct association between disease severity and pain (Schindler, 2011; Leonardo-Pinto et al., 2017). Perhaps more effective disease modification could have further reduced pain. Alternatively, it is possible that pain mediated by central sensitization is not sensitive to 2 months of dienogest treatment and that other treatments that directly address central sensitization could have been more efficacious. In either case, the current macaque model could be utilized to understand why some endometrioma are not affected by hormonal treatment, despite reduced estradiol levels in dienogest-treated macaques (Supplementary Fig. S4), but still result in modest pain relief (Leonardo-Pinto et al., 2017).

A potentially crucial extension of the current study would be to evaluate brain activation in awake macaques. In the current study, the dose of propofol was sufficient to retard movement but not to induce deep unconsciousness. In humans, sedating doses of propofol do not appear to markedly suppress brain activity and cerebral blood flow (Veselis et al., 2005; Frolich et al., 2017) and brain activation to noxious and non-noxious stimulation is generally intact with anesthetic doses of propofol (Steinbacher, 2001; Nagasaka et al., 2017). While stimulation-evoked brain activation was largely suppressed with morphine, behavioral efficacy

Table III Insula and thalamus activation observed with fMRI during non-noxious stimulation of the abdominopelvic area was compared before (pre-dienogest) and after (post-dienogest) 8 weeks of dienogest treatment in macaques with endometriosis.

Pre-dienogest > post-dienogest 8 W					
Area	Hemisphere	Z score	Coordinates (mm)		
			x	y	z
Insula	Left	2.08	16	18	8
	Right	2.24	-14	20	4
Thalamus	Left	2.71	-4	-6	-6
	Right	2.86	2	-4	4
Post-dienogest 8 W > control					
Area	Hemisphere	Z score	Coordinates (mm)		
			x	y	z
Insula	Left	1.48	18	18	8
	Right	1.91	-16	20	6
Thalamus	Left	2.19	-4	-8	2
	Right	2.05	2	-4	6

Activation in bilateral thalamus and insula was significantly decreased post-dienogest treatment compared to pre-dienogest activation. Insula and thalamus activation during non-noxious stimulation was also compared between macaques with endometriosis post-dienogest treatment and control (healthy) macaques. While post-dienogest brain activation was decreased compared to pre-dienogest brain activation in macaques with endometriosis, compared to brain activation of control macaques, significant 'residual' activation in macaques with endometriosis was observed. Z scores of peak voxels are shown. Stereotaxic coordinates (x, y, z) are according to Horsley-Clarke's stereotaxic coordinates. Peak voxels were considered significant at Z score > 1.96 ($P < 0.05$) and Z score > 2.3 ($P < 0.01$).

was much less than 100%. It is possible that a pharmacologic interaction with propofol anesthesia may have contributed to the suppression of brain activation (Hendrickx *et al.*, 2008). Thus, the effect of drugs in the awake state will need to be evaluated. Potential interactions between centrally acting drugs and anesthesia need to be understood for proper interpretation of deactivation or inactivation following treatment.

The current findings in macaques with endometriosis suggest that central sensitization underlies chronic endometriosis-associated pain. A few pharmacological agents, such as an opioid and progestin, reduced both pain behavior and evoked brain activation, a potential *in vivo*, physiological marker of central sensitization. Macaques with endometriosis could be used in further elaborating the mechanism of endometriosis pain and devising treatments based on potentially novel mechanisms of action. Given their genetic and anatomical similarities to humans, macaques with endometriosis could be a clinically relevant model of endometriosis pain.

Supplementary data

Supplementary data are available at *Human Reproduction* online.

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Authors' roles

Experiments designed by A.M., T.N., M.Y., Y.A., S.O., I.H., A.H. and H.T. Experiments were performed by A.M., M.Y., T.N., S.O. and Y.A. Data analysis and interpretation performed by T.N., S.O. and A.H. The manuscript was written by A.H. All authors reviewed and approved the manuscript.

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Conflict of interest

All authors are employees of Hamamatsu Pharma Research, Inc. Neither the funders nor Hamamatsu Pharma Research, Inc. had any involvement in the study design, collection, analysis, and interpretation of data, writing of the report and the decision to submit the report for publication.

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