

Supplementary Materials

Linacotide treatment reduces endometriosis-associated vaginal hyperalgesia and mechanical allodynia through viscerovisceral cross-talk

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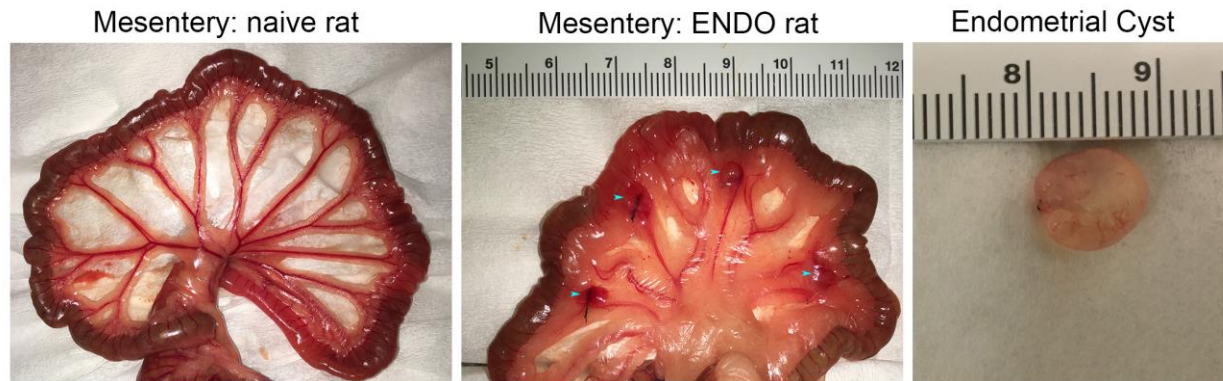
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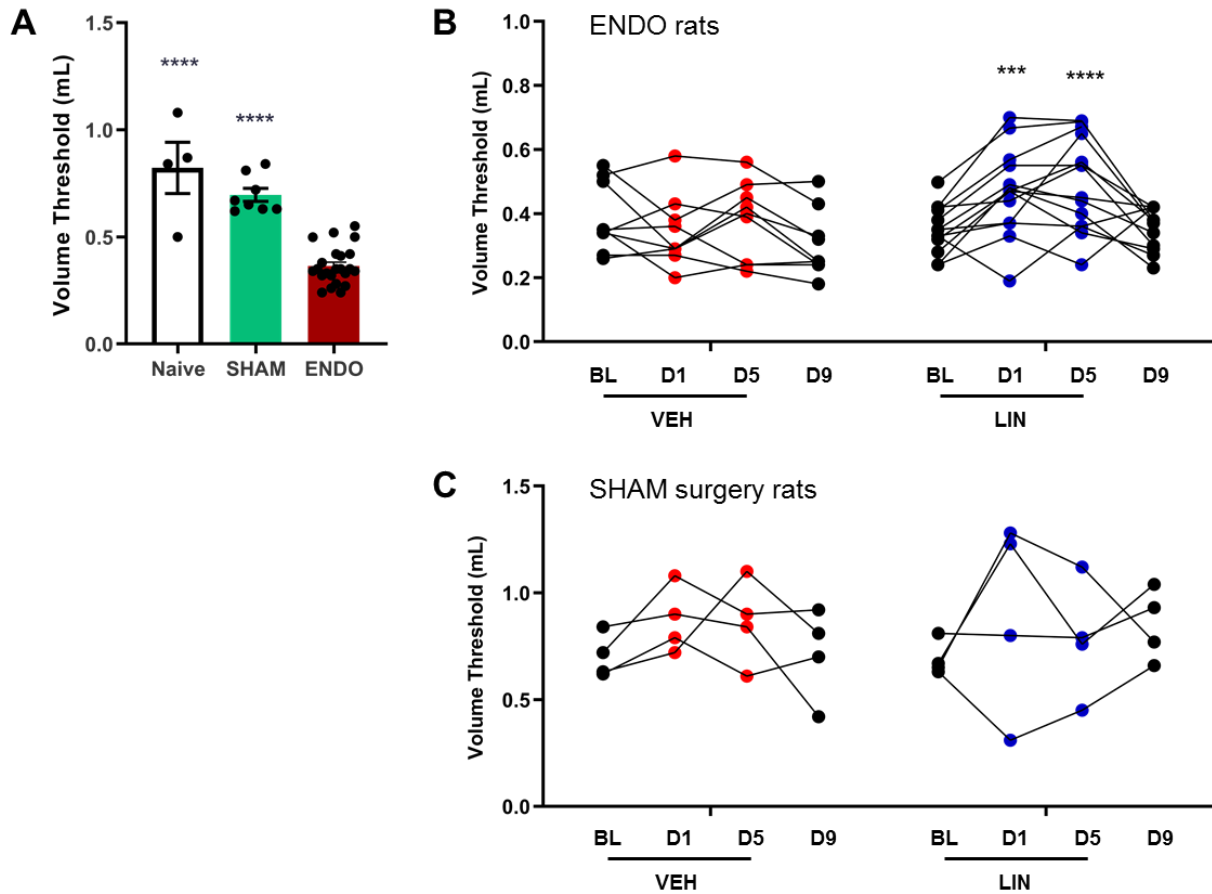
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Supplementary Fig. 1. Ectopically growing endometrial cysts. Left: mesentery of a naïve rat. Center: endometrial cysts (the location of growing cysts is indicated by blue arrowheads) growing ectopically on mesenteric arteries (photo: 8 weeks after ENDO surgery). The sutures attaching endometrial cysts to the mesenteric arteries are clearly visible. Right: surgically resected endometrial cyst (photo: 8 weeks after ENDO surgery). Ruler units: cm.

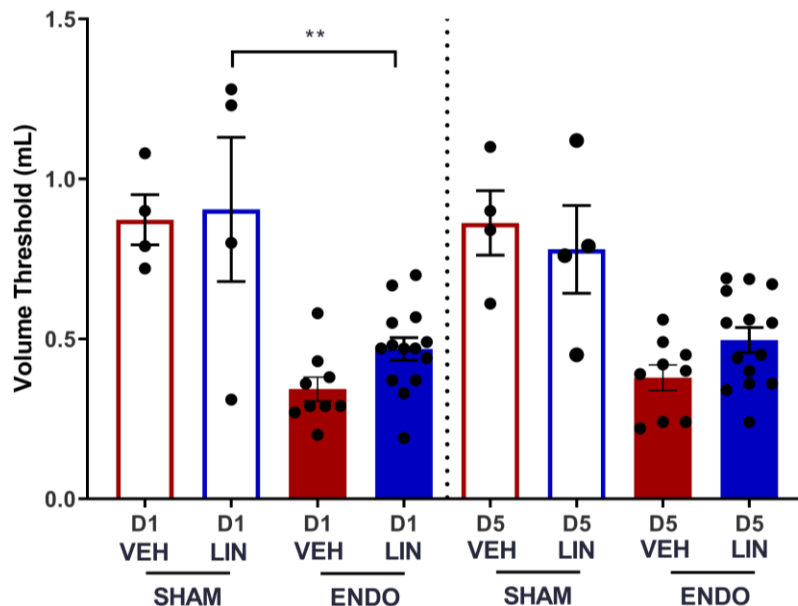


Supplementary Fig. 2. Daily oral linacotide treatment reduces endometriosis-associated vaginal hyperalgesia. (A) Vaginal volume thresholds in naïve, SHAM surgery, and ENDO rats. The effects of linacotide (3 $\mu\text{g/kg/day}$, p.o.) (N=14, day 1 and 5; N=12, day 9) or vehicle (sterile water, p.o.) treatment (N=9) on vaginal volume thresholds in ENDO rats (B) and (C) SHAM surgery rats (linacotide (3 $\mu\text{g/kg/day}$, p.o., N=4); vehicle (sterile water, p.o., N=4) were measured as electromyographic recordings of evoked visceromotor responses (VMR) to vaginal distension 2 hours after the last dose either of linacotide or vehicle on day 1 and day 5, and on day 9 following a 4-day withdrawal period from treatment after the last dose either of vehicle or linacotide on day 5. All measurements were performed in proestrus. Data are expressed as the mean \pm SEM. (A) **** $P < 0.0001$: naïve and SHAM surgery rats vs. ENDO rats; (B) *** $P < 0.001$, **** $P < 0.0001$ ENDO rats (LIN) vs. baseline. Two-way repeated measures ANOVA followed by Tukey's

multiple comparison test: linacotide and vehicle treatment (day 1, 5) vs. baseline, and day 9 following a 4-day withdrawal period from treatment vs. baseline; one-way ANOVA followed by Tukey's multiple comparison test: naïve rats and SHAM surgery rats vs. ENDO rats; one-way ANOVA followed by Tukey's multiple comparison test: naïve rats vs. SHAM surgery rats. BL: baseline, D: day, LIN: linacotide, VEH: vehicle.

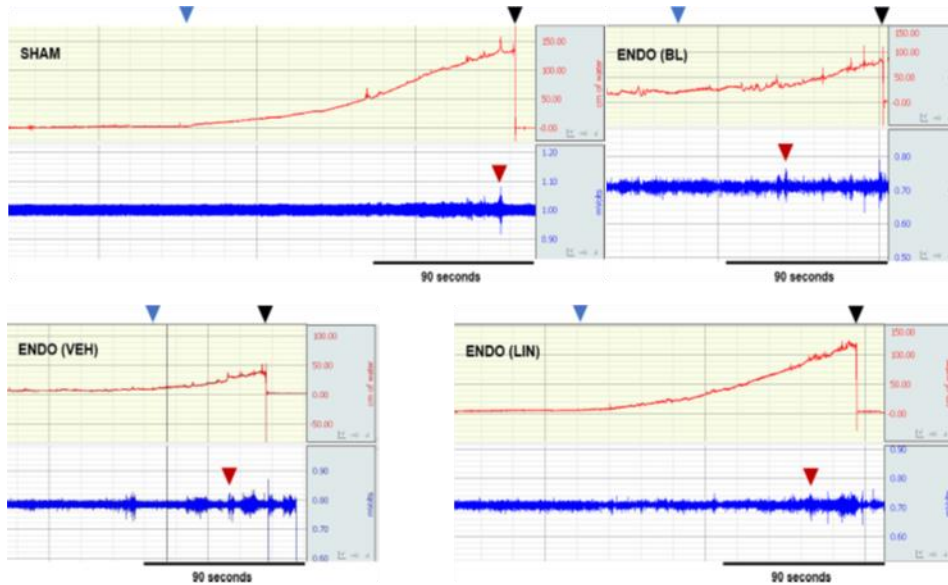


Supplementary Fig. 3. Comparison of daily oral linaclotide treatment on endometriosis-associated vaginal hyperalgesia between SHAM surgery rats and ENDO rats. The effects of linaclotide (3 $\mu\text{g/kg/day}$, p.o.) (N=14) or vehicle (sterile water, p.o.) treatment (N=9) on vaginal volume thresholds in ENDO rats SHAM surgery rats (linaclotide (3 $\mu\text{g/kg/day}$, p.o.) (N=4); vehicle (sterile water, p.o.) (N=4) were measured as electromyographic recordings of evoked visceromotor responses (VMR) to vaginal distension 2 hours after the last dose either of linaclotide or vehicle on day 1 and day 5. All measurements were performed in proestrus. Data are expressed as the mean \pm SEM. $**P < 0.01$. One-way ANOVA followed by Tukey's multiple comparison test: linaclotide treated SHAM surgery rats (day 1, 5) vs. linaclotide treated ENDO rats (day 1, 5). LIN: linaclotide, VEH: vehicle.



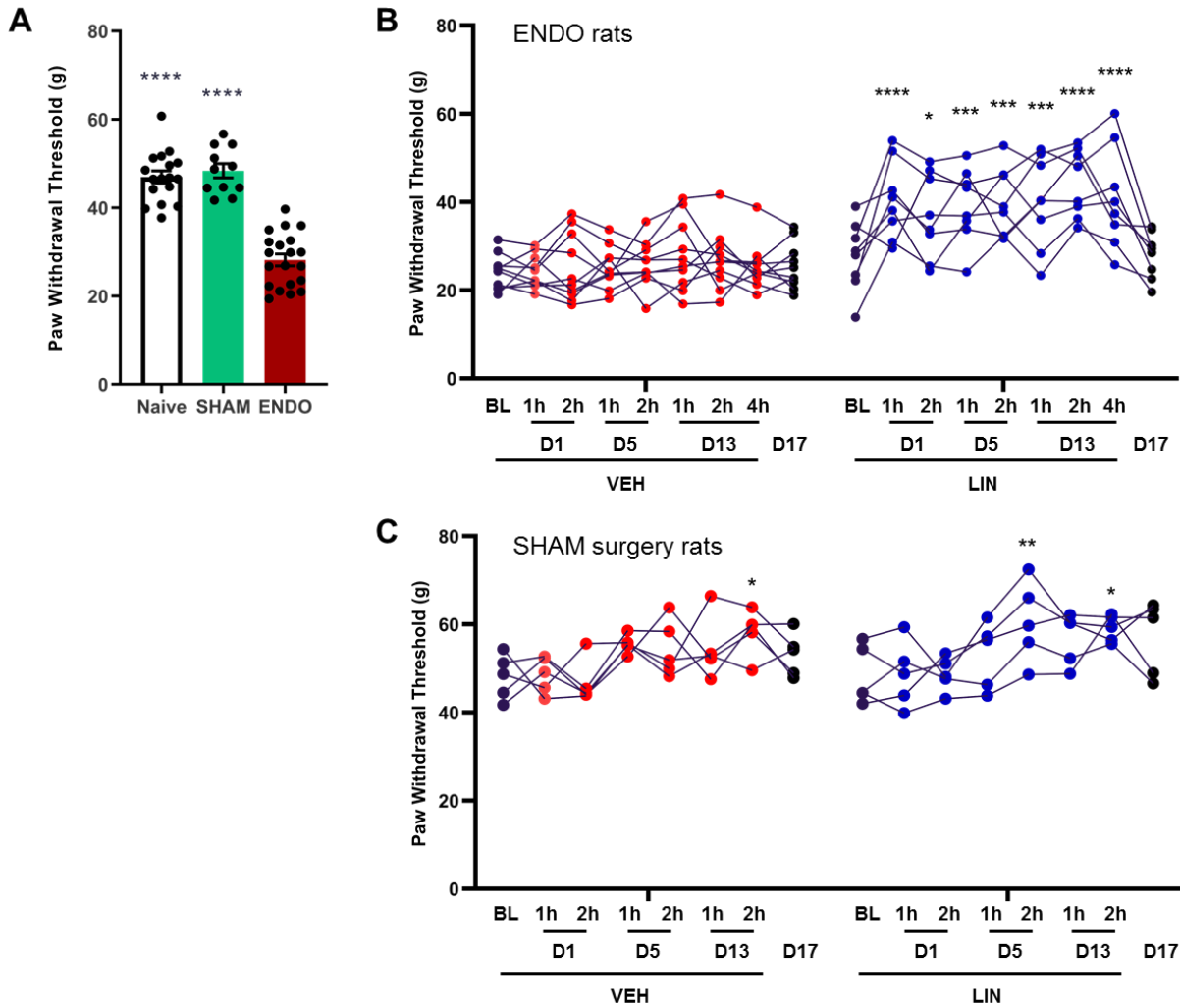
Supplementary Fig. 4. Representative VMR recordings from SHAM surgery rats, ENDO rats (baseline) and ENDO rats on day 5 (2 hours after the last dose either of vehicle or linaclotide). The threshold for discriminating activity from baseline EMG activity was preset at 200% of maximum amplitude of the baseline activity (red arrow). Inflation of the vaginal balloon

was initiated following the establishment of a stable baseline (blue arrow). Black arrows indicate termination of balloon inflation. The prolonged time period in linacotide treated versus vehicle treated ENDO rats (distance between the blue and red arrows) graphically illustrates the increased vaginal volume threshold in linacotide treated ENDO rats.



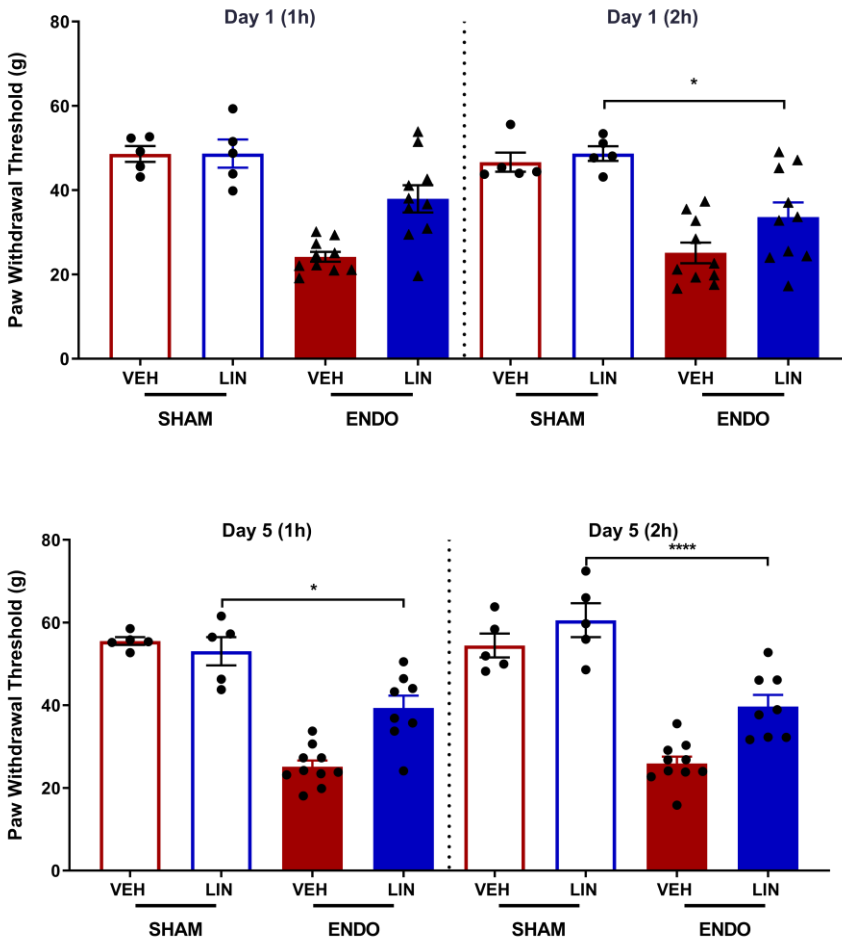
Supplementary Fig. 5. Daily oral linacotide treatment reduces endometriosis-associated mechanical hindpaw allodynia. (A) Hindpaw withdrawal thresholds in naïve, SHAM surgery, and ENDO rats. The effects of linacotide (3 μ g/kg/day, p.o.) or vehicle (sterile water, p.o.) treatment in ENDO rats (B) and (C) SHAM surgery rats were measured as hindpaw withdrawal thresholds in response to applied force (g) using electronic von Frey. Measurements were performed on day 1 (1 hour and 2 hours after a single dose either of linacotide or vehicle, day 5 (1 hour and 2 hours after the last dose either of linacotide or vehicle, day 13 (1 hour, 2 hours, and 4 hours after the last dose either of linacotide or vehicle). The effects of a 4-day withdrawal period from treatment after the last dose of either linacotide or vehicle on day 13 were measured on day

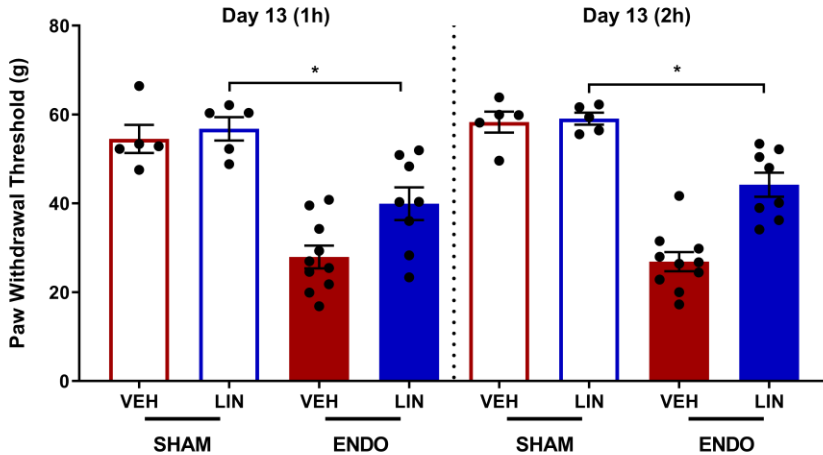
17. Naïve: N=17, vehicle treated SHAM surgery rats: N=5, linacotide treated SHAM surgery rats: N=5, vehicle treated ENDO rats: N=10, linacotide treated ENDO rats: N=8. Data are expressed as the mean \pm SEM. (A) **** $P < 0.0001$: naïve and SHAM surgery rats vs. ENDO rats; (B) * $P < 0.05$, *** $P < 0.001$, **** $P < 0.0001$: ENDO rats (LIN) vs. baseline; (C) * $P < 0.05$: SHAM surgery rats (VEH) vs. baseline; * $P < 0.05$, ** $P < 0.01$: ENDO rats (LIN) vs. baseline. Two-way repeated measures ANOVA followed by Tukey's multiple comparison test: linacotide and vehicle treatment (day 1, 5, 13) vs. baseline, and day 17 following a 4-day withdrawal period from treatment vs. baseline; one-way ANOVA followed by Tukey's multiple comparison test: naïve rats and SHAM surgery rats vs. ENDO rats (baseline); one-way ANOVA followed by Tukey's multiple comparison test: naïve rats vs. SHAM surgery rats (baseline); one-way ANOVA followed by Tukey's multiple comparison test: linacotide treated ENDO rats vs. linacotide treated SHAM surgery rats; BL: baseline, D: day, LIN: linacotide, VEH: vehicle.



Supplementary Fig. 6. Comparison of daily oral linacotide treatment on endometriosis-associated mechanical hindpaw allodynia between SHAM surgery rats and ENDO rats. The effects of linacotide (3 μ g/kg/day, p.o.) or vehicle (sterile water, p.o.) treatment were measured as hindpaw withdrawal thresholds in response to applied force (g) using electronic von Frey. Measurements were performed on day 1 (1 hour and 2 hours after a single dose either of linacotide or vehicle, day 5 (1 hour and 2 hours after the last dose either of linacotide or vehicle, and day 13 (1 hour and 2 hours after the last dose either of linacotide or vehicle). Naïve: N=17, vehicle treated SHAM surgery rats: N=5, linacotide treated SHAM surgery rats: N=5, vehicle treated ENDO rats:

N=10, linacotide treated ENDO rats: N=8. Data are expressed as the mean \pm SEM. $*P < 0.05$, $****P < 0.0001$. One-way ANOVA followed by Tukey's multiple comparison test: linacotide treated SHAM surgery rats (day 1, 5, 13) vs. linacotide treated ENDO rats (day 1, 5, 13). LIN: linacotide, VEH: vehicle.





Supplementary Fig. 7. GC-C mRNA expression is absent in the uterus. (A) Using qRT-PCR, GC-C mRNA expression was measured in the uterus of ENDO rats following daily oral treatment either with linaclotide (3 $\mu\text{g/kg/day}$) or vehicle (sterile water) for 5 days and compared to GC-C mRNA expression in the uterus of naïve and SHAM surgery rats (N=5-8). GC-C mRNA expression was absent in the uterus under all treatment conditions, demonstrating that GC-C mRNA expression is not differentially regulated in normal uterine tissue after transplantation onto mesenteric arteries and grown into endometrial cysts. GC-C mRNA expression in the ileum of naïve rats is shown as a reference. Data are expressed relative to expression of glyceraldehyde 3-phosphate dehydrogenase (GAPDH). (B) *In situ* hybridization showing representative sections of the uterus at 40x (top), 100x (middle) and 200x (bottom) magnification from vehicle treated (left panel) and linaclotide treated ENDO rats (right panel). This data further confirms that GC-C expression is absent in the uterus. Scale bars: 200 μm (40x), 100 μm (100x) and 50 μm (200x) magnification. VEH, vehicle; LIN, linaclotide.

