

The ARRIVE Guidelines Checklist

Animal Research: Reporting In Vivo Experiments

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	ITEM	STATEMENT
Title	1	Estrogen re-enhanced visceral hyperalgesia
Abstract	2	Chronic stress during pregnancy may increase visceral hyperalgesia of offspring. We established a chronic prenatal stress (CPS) + chronic adult stress (CAS) mice model. The single fiber recording and patch-clamp experiments were used to study the effects. We found that chronic stress induced visceral hypersensitivity is estrogen dependent and the hypersensitivity is estrogen dependent sensitization of primary afferent colon neurons
INTRODUCTION		
Background	3	Clinical studies show that early life adverse experiences are risk factors for the development of IBS symptoms, including visceral pain and ongoing chronic stress, especially abdominal pain. A critical molecular event in the development of this female-enhanced visceral hypersensitivity is up-regulation of brain-derived neurotrophic factor (BDNF) expression in the lumbar-sacral spinal cord of female CPS + CAS rats.
Objectives	4	We predicted that chronic prenatal stress (CPS) + chronic adult stress (CAS) will maximize visceral hyperalgesia; and estrogen plays an important role in colonic hyperalgesia.
METHODS		
Ethical statement	5	The Institutional Animal Care and Use Committee of the University of Texas Medical Branch at Galveston, TX approved all animal procedures.
Study design	6	Pregnant dams were subjected to a CPS protocol that consisted of a random sequence of twice-daily applications of one of three stress sessions, one hour water avoidance stress, 45 min cold restraint stress or 20 min forced swim stress starting on 6th day and continuing until delivery (21st day). Male and female offspring from the stressed dams were designated CPS rats. Control dams received sham stress and their offspring were designated control rats. As adults (8-16 wks), control and prenatally stressed offspring were challenged by the same CAS protocol for nine days. Ovariectomy (OVX) or sham surgery was performed on female prenatal stress offspring in the 56th day. Daily Letrozole treatment was initiated on the 49th day, 2 weeks prior to initiation of adult stress. Treatment was continued through the stress protocol.
Experimental procedures	7	The OVX or sham surgery was performed on female prenatal stress offspring in the 56th day. The aromatase inhibitor Letrozole (4,4'-(1H-1,2,4-triazol-1-yl-methylene)-bis-benzonitrile; 1.0 mg/kg, oral administration; Novartis) was used in experiment group; and Vehicle (hydroxypropyl

		<p>cellulose 0.3% in water) was used in contral group once daily for 14 days.</p> <p>Multiunit afferent discharges were recorded from the distal ends of L6-S2 dorsal rootlets decentralized close to their entry into the spinal cord. A bundle of multiunit fibers was distinguished into 2-6 single units off-line using wave mark template matching in Spike 2 software that differentiates spikes by shape and amplitude. Colonic afferent fibers were identified by their response to graded colorectal distention (CRD). The balloon was used to distend colorectum. Isoflurane, 2.5%, followed by 50 mg/kg, i.p. sodium pentobarbital induced general anesthesia that was maintained by infusing a mixture of pentobarbital sodium + pancuronium bromide + saline by intravenous infusion through the tail vein. Adequacy of anesthesia was confirmed by the absence of corneal and pupillary reflexes and stability of end-tidal CO₂ level. A tracheotomy tube connected to a ventilator system provided a mixture of room air and oxygen. Expired CO₂ was monitored and maintained at 3.5%. Body temperature was monitored and maintained at 37 °C by a servo-controlled heating blanket. A laminectomy from T12 to S2 exposed the spinal cord. The head was stabilized in a stereotaxic frame. The dura was gently opened and a warm mineral oil pool, contained by skin flaps, covered the exposed spinal cord and roots.</p>
Experimental animals	8	Experiments were performed on pregnant Sprague-Dawley rats and their 8-16 week old male and female offspring.
Housing and husbandry	9	Rats were housed individual cages and accessed to food and water in a room with controlled conditions (22 ± 2°C, relative humidity of 50% ± 5%), a 12 h light/12 h dark cycle.
Statistical methods	10	Single fiber activity data were analyzed using ANOVA with repeated measures; CRD intensity was the repeated factor and experimental group as the between group factor. If significant main effects were present, the individual means were compared using the Fisher post-hoc test.
RESULTS		
Baseline data AND Number analysed	11	<ol style="list-style-type: none"> 1. Spontaneous activity (SA) of afferent single units in male and female control rats (n=70 fibers in 6 rats for each group) 2. Average responses to graded CRD of afferent fibers in male and female control rats (male: n=56 fibers in 6 rats; female: n=70 fibers in 6 rats; two-way ANOVA, *P<0.05 vs. the same pressure male group). 3. Responses of low threshold (LT) fibers to CRD in male and female control rats (male: n=42 fibers in 6 rats; female: n=40 fibers in 6 rats; two-way ANOVA, *P<0.05 vs. the same pressure male group). 4. Responses of high threshold (HT) afferent fibers to CRD in male and female control rats (male: n=14 fibers in 6 rats; female: n=29 fibers in 6 rats; two-way ANOVA, *P<0.05 vs. the same pressure male group). 5. Effects of CAS on afferent fiber responses to CRD from control and CPS female rats (n=6 rats, 59 fibers for control and 99 fibers for CPS female individual group, two-way ANOVA, *P<0.05 vs. the same pressure control group, #P<0.05 vs. the same pressure CPS group).

		<ol style="list-style-type: none"> 6. Effects of CAS on afferent fiber responses to CRD from control and CPS male rats (n=6 rats, 57 fibers for control and 95 fibers for CPS female group, two-way ANOVA, *P<0.05 vs. the same pressure control group). 7. Membrane input resistance from all four groups (n=5 rats, 45 cells in each group, one-way ANOVA, *P<0.05 vs. control, #P<0.05 vs. CPS). 8. Rheobase from all four experimental groups (n=5 rats, 45 cells in each group, one-way ANOVA, *P<0.05 vs. control or as the graph shown, #P<0.05 vs. CAS). 9. Number of action potentials elicited by current injection at either 2x and 3x the rheobase in all four experimental groups (two-way ANOVA, *P<0.05 vs. control, #P<0.05 vs. CPS). 10. Action potential overshoot recorded from all four experimental groups (*P<0.05 vs. control). 11. Plasma estrogen level changes in control and CPS rats by estrus cycle phases (n=8 rats, one-way ANOVA, *P<0.05 vs. control proestrus/estrus (P-E) phase, #P<0.05 vs. CPS diestrus (D) phase). 12. Plasma estrogen levels increase in CPS rats and following CAS 24 hours after last adult stressor (n=8 rats, one-way ANOVA, *P<0.05 vs. control, #P<0.05 vs. CPS). 13. OVX significantly reduced CPS female rat plasma estrogen levels before and after CAS (n=5 rats, one-way ANOVA, *P<0.05 vs. sham group or as the graph shown). 14. Letrozole treatment significantly reduced CPS female rat plasma estrogen levels before or after CAS (n=5 rats, one-way ANOVA, *P<0.05 vs. vehicle group or as the graph shown). 15. Plasma norepinephrine (NE) levels from control, CAS, CPS and CPS+CAS group female rats (n=5 rats, one-way ANOVA, *P<0.05 vs. control, #P<0.05 vs. CPS). 16. Plasma adrenocorticotrophic hormone (ACTH) levels from control and CPS+CAS group female rats (n=5 rats, t-test, *P<0.05 vs. control). 17. Rheobase (n=45 cells in 6 rats in each group, t-test, ***P<0.0001 vs. Veh.+CAS+CPS). 18. Membrane input resistance (RIn) (t-test, *P<0.05). C: Action potential overshoot (t-test, *P<0.05). 19. Number of action potentials (APs) elicited by current injection at 2x and 3x rheobase (two-way ANOVA, *P<0.05). 20. Plasma estrogen levels in cycling females that received a bolus estradiol (E2) infusion on day 1 (n= 8 rats in each group, two-way ANOVA, *P<0.05 vs. vehicle group). 21. Quantification of NGF levels from colon wall in IHC (n=4 rats in each group, one-way ANOVA, *P<0.05 vs. control group, #P<0.05 vs. CPS group).
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		22. Western blotting of NGF protein level from control and CPS+CAS female rats' colon wall tissue (n=6 rats in each group, t-test, *P<0.05 vs. control group).
DISCUSSION		
Interpretation/scientific implications	12	Acute blockade of the endogenous synthesis of estrogens in rat spinal cord may significantly reduce visceral hypersensitivity, suggesting that locally produced estrogen can regulate nociceptive neurons to modulate visceral hypersensitivity. Chronic stress-estrogen-BDNF axis sensitizes visceral hypersensitivity in female offspring subjected to CPS. The development of chronic stress induced visceral hypersensitivity in female rats is estrogen dependent. Our findings provide key scientific evidence in a preclinical model in support of developing gender-based treatment for abdominal pain in IBS.
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