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Low COMT and Stress Potentiate Functional Pain and Depressive Behavior, Especially in Female Mice

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1. Introduction

Functional pain syndromes (FPS), including fibromyalgia (FM), temporomandibular disorder (TMD), tension-type headache (TTH), irritable bowel syndrome (IBS), and low back pain (LBP) represent a significant healthcare problem that affect nearly one in three Americans, predominantly females.[18; 25; 73; 77; 89; 102] These conditions are characterized by persistent pain in the absence of tissue damage and often co-occur, thereby affecting multiple body sites. Accumulating evidence suggests that the origins of FPS are linked to abnormalities in pathways that regulate catecholamine bioavailability. Patients with FPS have increased circulating basal and stress-induced levels of the catecholamines epinephrine and norepinephrine [9; 27; 70; 87], and reduced activity of catechol-O-methyltransferase (COMT),[60; 80] a ubiquitously expressed enzyme that metabolizes catecholamines.[59] An estimated 66% of FPS patients have variants in the *COMT* gene that result in lower COMT activity.[49; 53; 66; 82; 94] The 'low COMT activity' variants are associated with the onset of FM[5; 20; 34; 62; 92] and TMD[24], increased pain in response to experimental stimuli,[24; 113] and pain-related comorbidities such as depression.[6; 28; 41; 49; 52; 76; 94]

In previous pre-clinical studies, we demonstrated that genetic knockout or pharmacologic inhibition of COMT results in pain at multiple body sites that persists for months, pain-related volitional behaviors (eg. avoidance of bright light), and systemic inflammation *via* activation of adrenergic receptor β_2 and β_3 . [17; 46; 47; 111] This animal model of COMT-dependent functional pain, defined as increased pain following COMT inhibition, has provided valuable mechanistic insight into how genetically-driven increases in basal catecholamine levels lead to chronic functional pain. However, it is important to consider how the effects of the low COMT activity genetic predisposition are modified by

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environmental events, such as stress. Stress activates the sympathetic nervous system, stimulating the release of catecholamines so as to induce pain in healthy individuals while exacerbating pain in those with FPS.[61] In fact, individuals with the low COMT activity genotype report increased pain after stressful events, such as a motor vehicle accident.[64; 78] The independent and joint contributions of low COMT and stress to FPS and related comorbidities such as depression, which also predominantly affect females, have remained unclear.

Thus, the present study sought to examine the contributions of low COMT and/or stress to functional pain and depressive-like behavior, as well as molecular correlates of these behaviors. We hypothesized that stress would exacerbate the degree of COMT-dependent functional pain and that COMT inhibition would exacerbate stress-dependent depressive-like behavior. Further, we hypothesized that low COMT and stress would differentially affect the expression of brain-derived neurotrophic factor (BDNF; a factor involved in nociception and depression) and glucocorticoid receptor (GR; a stress-related receptor) in the spinal cord and hippocampus, in a sex-dependent manner. To test these hypotheses, separate groups of mice receiving sustained delivery of the COMT inhibitor OR486 or vehicle were subjected to repeated forced swim stress or sham swim. Pain behavior and depressive-like behavior were measured over the course of 14 days. BDNF and GR expression in the spinal cord and hippocampus CA1 area was measured on day 14 using immunohistofluorescence and Western blot.

2. Materials and Methods

2.1. Animals

Adult 8–12 week-old male (N=56) and female (N=62) C57BL/6 mice weighing 18–30g were bred in-house. Mice had ad libitum access to standard laboratory chow and water, and were maintained under a 10 hr light / 14 hr dark cycle. All animal procedures were approved by the Institutional Animal Care and Use Committee at Duke University and conformed to the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

2.2. General experimental design

Here, we aimed to determine the independent and joint effects of low COMT and stress on pain and comorbid depressive-like behavior, as well as molecular correlates of these behaviors in male and female mice. Male and female mice were randomly assigned to one of four experimental groups; 1) OR486 + swim sham (OR486+Sham), 2) OR486 + swim stress (OR486+Stress), 3) Vehicle + swim sham (Vehicle+Sham), and 4) Vehicle + swim stress (Vehicle+Stress). The COMT inhibitor OR486 or vehicle was delivered over 14 days, and mice were subjected to the forced swim or sham paradigm on days 8–10 during OR486 or vehicle delivery.

Mice were handled and habituated for 4 days prior to minipump implantation. Pain behaviors evoked by mechanical and thermal stimuli were evaluated at baseline (on day 0), prior to swim stress or swim sham (on days 6–7), during swim stress or swim sham (on days 9–10), and following swim stress or swim sham (on days 12–13). Depressive-like behavior

during the tail suspension test was evaluated on day 13. On day 14, mice were euthanized and spinal cord and hippocampal tissues were collected to measure the expression of BDNF and GR. Experimenters performing pain assessments and quantifying BDNF and GR expression were blinded to both the drug and stress conditions.

2.3. COMT-dependent functional pain

We modeled COMT-dependent functional pain in mice as described previously.[17; 46; 111] In brief, Alzet osmotic minipumps (model 1002, Durect Corporation, Cupertino, CA, USA) were implanted subcutaneously in the intrascapular region for systemic delivery of vehicle or the COMT inhibitor OR486 (Tocris Bioscience, Bristol, UK) over 14 days at a dose sufficient to cross the blood brain barrier.[67] The OR486 was dissolved in a 5:2:3 ratio mixture of dimethyl sulfoxide: ethanol: 0.9% sterile saline and injected into the pump which was subsequently incubated overnight at 37°C in a 15mL conical tube containing sterile saline.

2.4. Forced swim stress

As described previously,[13] mice were subjected to the forced swim or sham stress paradigm on days 8–10 (10 min on day 8, 20 min on days 9 and 10). Mice were placed in their respective glass swimming chambers (45.72 cm in height × 20.23 cm in diameter) that contained 20 cm (swim stress) or 2 cm (swim sham) of water maintained at 24–26°C. Following each swim session, mice were dried with a towel and provided with a heat source until dry.

2.5. Pain behavior testing

Paw withdrawal threshold to punctate mechanical stimuli was assessed using the von Frey up–down method. A series of nine von Frey monofilaments with logarithmically increasing stiffness (0.008–1.5 g, Stoelting, Wood Dale, IL, USA) was presented perpendicularly to the central plantar surface of the hindpaw. First, the middle filament (#5, 0.17 g) was applied to the hindpaw for 3 s. If the mouse responded with a withdrawal, an incrementally lower filament was applied. In the absence of a withdrawal, an incrementally higher filament was applied. A series of six total responses were recorded for each paw. Results were determined using a logarithmic algorithm to determine the gram-force value that would elicit paw withdrawal in 50% of trials ($10(X_f + k\delta)/10,000$, where X_f = value [in log units] of the final von Frey filament used; k = tabular value of positive and negative responses, and δ = mean difference [in log units] between stimuli). Mechanical allodynia was defined as a significant decrease in paw withdrawal threshold from baseline.

After determining paw withdrawal threshold, paw withdrawal frequency to a noxious von Frey monofilament was assessed. A higher gram force filament (#7, 0.7 g) was applied to the hind paw 10 times for a duration of 1 s, with an inter-stimulus interval of 1 s. The number of paw withdrawals (ranging from 0–10) was recorded for each hindpaw. Mechanical hyperalgesia was defined as a significant increase in the number of paw withdrawals from baseline.

Finally, paw withdrawal latency to thermal heat was assessed using the Hargreaves method. [36] Mice were placed in plexiglass chambers, and a radiant beam of light was applied to the hindpaw through a glass floor. Paw withdrawal latencies were recorded in duplicate per paw. If the second latency recorded was not within ± 4 seconds of the first, a third measure was recorded. The two latencies closest in value were averaged to determine overall latency to withdrawal. Thermal hyperalgesia was defined as a decrease in paw withdrawal latency from baseline.

2.6. Depressive-like behavior

Depressive-like behavior was assessed using the tail suspension test (TST).[14] In brief, mice were suspended for 6 minutes from their tails, taped to a table 20–25 cm from the floor, such that they could not escape or hold onto nearby surfaces. Immobility time, which serves as an indicator of despair/depression, was recorded and quantified. Immobility time was also recorded during the forced swim stress sessions as previously described.[106]

2.7. Immunohistochemical staining

On day 14, mice were deeply anesthetized with an intraperitoneal injection of 300mg/kg Fatal Plus (Vortech Pharmaceuticals, Dearborn, MI, USA), and subsequently perfused intracardially with 50 mL of ice-cold heparinized 0.1 M phosphate-buffered saline. This was followed by 25–50 ml fixative (4% paraformaldehyde in 0.1 M sodium phosphate buffer) applied at a rate of 20 ml/min. The lumbar-sacral segment of the spinal cord and the hippocampus were isolated, post-fixed in 4% paraformaldehyde for 12 hr, and finally immersed in a 30% sucrose solution for storage at 4°C until sectioning. Transverse sections were cut to a thickness of 30 μ m using a cryostat (HM 550, Microm International GmbH, Walldorf, Germany), incubated in 5% fetal bovine blocking serum in 0.01 M phosphate-buffered saline at room temperature for 1 hr, and stained overnight at 4°C with the rabbit anti-BDNF primary antibody (ab108319, 1:500; Abcam, Cambridge, United Kingdom), the rabbit anti-GR primary antibody (1:500; Thermo Scientific, Waltham, MA, USA), or with the mouse anti-NeuN antibody (1:500; Millipore Sigma, St. Louis, MO, USA). The following day, the sections were incubated with the anti-rabbit Alexa-594 IgG (1:500; Jackson ImmunoResearch Inc., West Grove, PA, USA) and anti-mouse Alexa-488 IgG (1:500; Jackson ImmunoResearch Inc., USA) secondary antibodies for 1 hr at room temperature. The sections were then mounted on gelatin-coated slides (VWR, Radnor, PA, USA), allowed to air-dry until complete dehydration, and coverslipped. The specificity of immunohistochemical staining was verified by omission of the primary antibody or omission of the secondary antibody from the staining protocol for each set of experiments.

Histological features of the spinal dorsal horn and hippocampus CA1 area were captured using a Zeiss 780 upright confocal microscope (Oberkochen, Germany). Expression of BDNF and GR were quantified by optical density using ImageJ software (National Institutes of Health, Bethesda, MD, USA).

2.8. Western blot

Flash frozen spinal cord tissue was harvested on day 14 from a separate cohort of mice to confirm BDNF and GR antibody specificity. The tissue was homogenized in a Precellys 24

tissue homogenizer (Bertin Instruments, Montigny-le-Bretonneux, France) with tissue protein extraction reagent (TPER, ThermoFisher). To accommodate for variation in tissue homogenates, protein concentrations were normalized following bicinchoninic acid (BCA; Pierce, Grand Island, NY, USA) measurement of protein content to evenly load protein into wells of 4–20% SDS-PAGE gels (BioRad). Gels were run using standard SDS-PAGE methods and transferred onto PVDF membranes using the iBlot2 dry blotting system (Life Technologies). Membranes were then blocked, probed with primary antibodies for GAPDH (1:1000; Cell Signaling, Danvers, MA, USA), BDNF (ab108319, 1:1000; Abcam, Cambridge, United Kingdom) or GR (1:1500; Thermo Scientific, Waltham, MA, USA), and subsequently probed with a corresponding secondary antibody. Specific bands were visualized with enhanced chemiluminescence (Thermo Scientific, Waltham, MA, USA) and quantified with Image J. The relative levels of BDNF and GR were normalized to GAPDH.

2.9. Statistical analyses

Group differences in behavioral responses and immunohistochemical fluorescent intensities were analyzed by 1-way or 2-way ANOVA. *Post hoc* comparisons were performed using the Bonferroni test, which corrected for multiple comparisons. Statistical significance was defined as $P < 0.05$. All statistical analyses were performed using GraphPad Prism 7 (GraphPad Software, La Jolla, CA, USA).

2. Results

3.1. Effect of Stress on COMT-dependent Functional Pain in Males and Females

Here, we sought to examine the impact of COMT- and stress-dependent increases in catecholaminergic tone on chronic pain and depressive-like behavior as well as molecular correlates of these behaviors. First, we examined the independent and joint contributions of low COMT and stress to pain. In line with our previous work, we found that sustained systemic delivery of the COMT inhibitor OR486 produced pronounced increases in pain behavior in both males and females (Figure 1). [46; 111] Compared to males and females in the Vehicle+Sham group, those in the OR486+Sham group exhibited mechanical allodynia (Two-way ANOVA for group \times time. Males: $F_{3,76} = 8.61$, $P < 0.001$ (Fig 1A) and females: $F_{3,97} = 5.07$, $P < 0.001$ (Fig 1B)), mechanical hyperalgesia (Two-way ANOVA for group \times time. Males: $F_{3,76} = 18.47$, $P < 0.001$ (Fig 1C) and females: $F_{3,97} = 15.18$, $P < 0.001$ (Fig 1D)), and thermal heat hyperalgesia (Two-way ANOVA for group \times time. Males: $F_{3,76} = 29.44$, $P < 0.001$ (Fig 1E) and females: $F_{3,97} = 18.89$, $P < 0.001$ (Fig 1F)) over the 14-day time course. The degree of COMT-dependent pain did not differ between males and females in the OR486+Sham group (Figure 1s).

Similarly, we found that a 3-day repeated swim stress on days 8–10 produced moderate increases in pain behavior on days 12–13 post-stress in both males and females (Figure 1). Compared to males and females in the Vehicle+Sham group, those in the Vehicle+Stress group exhibited mechanical allodynia (Two-way ANOVA for group \times time. Males: $F_{3,76} = 7.335$, $P < 0.001$ (Fig 1A) and females: $F_{3,90} = 4.84$, $P < 0.005$ (Fig 1B)), mechanical hyperalgesia (Two-way ANOVA for group \times time. Males: $F_{3,76} = 21.76$, $P < 0.001$ (Fig 1C) and females: $F_{3,90} = 20.34$, $P < 0.001$ (Fig 1D)), and thermal heat hyperalgesia (Two-way

ANOVA for group \times time. Males: $F_{3,76} = 10.98$, $P < 0.001$, (Fig 1E) and females: $F_{3,90} = 9.8$, $P < 0.001$ (Fig 1F)). The degree of stress-dependent pain did not differ between males and females in the Vehicle+Stress group (Figure 1s).

Next, we found that sustained systemic OR486 delivery together with repeated swim stress produced significantly greater pain on days 12–13 post-stress than either OR486 delivery or swim stress alone (Figure 1). Compared to males and females in the OR486+Sham and Vehicle+Stress groups, those in the OR486+Stress group exhibited significant increases in mechanical allodynia (Two-way ANOVA for group \times time. Males: $F_{2,37} = 14.21$, $P < 0.001$ (Fig 1A) and females: $F_{2,48} = 23.42$, $P < 0.001$ (Fig 1B)), and mechanical hyperalgesia (Two-way ANOVA for group \times time. Males: $F_{2,37} = 23.56$, $P < 0.001$ (Fig 1C) and females: $F_{2,48} = 30.39$, $P < 0.001$ (Fig 1D)). Of note, females in the OR486+Stress group exhibited greater thermal heat hyperalgesia compared to those in the OR486+Sham and Vehicle+Stress groups (Two-way ANOVA for group \times time. $F_{2,48} = 199.7$, $P < 0.001$ (Fig 1F)), while males in the OR486+Stress group only exhibited greater thermal heat hyperalgesia compared to those in the Vehicle+Stress group, but not the OR486+Sham group (Two-way ANOVA for group \times time. $F_{2,37} = 52.32$, $P < 0.001$ (Fig 1E)). A direct comparison of pain behavioral responses post-stress revealed a sex difference, such that thermal heat hyperalgesia was exacerbated in females. Thus, stress exacerbates COMT-dependent functional pain, especially among females.

3.2. Effect of Stress on COMT-dependent increases in Spinal BDNF Expression in Males and Females

Spinal BDNF increases the net excitatory drive of nociceptive neurons, and has been shown to play a key role in the onset and persistence of numerous chronic inflammatory and neuropathic pain conditions.[23; 84] Here, for the first time, we evaluated spinal BDNF expression in our mouse model of functional pain in the presence and absence of stress. We found that sustained systemic delivery of OR486 led to increases in spinal BDNF expression in both males and females, and swim stress potentiated this increase in females, but not males (Figure 2). Compared to the Vehicle+Sham group, male and female mice in the OR486+Sham group exhibited increased BDNF expression in lamina I-VI of the spinal dorsal horn (Two-way ANOVA for group \times lamina. Males: $F_{1,74} = 40.17$, $P < 0.001$ (Fig 2A,C) and females: $F_{1,74} = 22.8$, $P < 0.001$ (Fig 2B,D)). Compared to their respective OR486+Sham groups, females, but not males, in the OR486+Stress group exhibited significantly higher increases in spinal BDNF expression (Two-way ANOVA for group \times lamina. $F_{1,74} = 32.27$, $P < 0.001$ (Figure 2B,D)). Analysis of between-subjects factors in the same ANOVA revealed that the potentiation of stress-dependent increases in spinal BDNF expression by OR486 in female vs male mice is significant (One-way ANOVA for group. $F_{5,42} = 11.79$, $P < 0.001$ (Figure 2E)). Consistent results were obtained by Western blot analysis (Figure 6s). This finding is consistent with the ability of swim stress to potentiate COMT-dependent thermal heat hyperalgesia in female, but not male mice. In the absence of OR486 delivery, swim stress did not alter spinal BDNF expression (Figure 2s).

3.3. Effect of Stress on COMT-dependent Increases in Spinal GR Expression in Males and Females

Glucocorticoid-GR interactions promote neuronal plasticity and maintain pain following injury and/or stress.[1; 54; 55] Here, we evaluated spinal GR expression in our mouse model of functional pain in the presence and absence of stress. We found that sustained systemic delivery of OR486 led to increases in spinal GR in both males and females. Compared to the Vehicle+Sham group, male and female mice in the OR486+Sham group exhibited increased GR expression in lamina I-VI of the spinal dorsal horn (Two-way ANOVA for group \times lamina. Males: $F_{1,90} = 95.33$, $P < 0.001$ (Fig 3A,C) and females: $F_{1,90} = 96.07$, $P < 0.001$ (Fig 3B,D)). In contrast to BDNF, GR expression was not potentiated by stress in females or males, and no differences were observed between males and females (Figure 3E). In the absence of OR486 delivery, swim stress did not alter spinal GR expression (Figure 3s). Again, consistent results were obtained by Western blot analysis (Figure 6s).

3.4. Effect of COMT Inhibition on Stress-induced Depressive-like Behavior in Males and Females

We evaluated the independent and joint contributions of low COMT and stress on depressive-like behavior. We found that that the 3-day repeated swim stress paradigm, but not sustained systemic delivery of OR486, produced a significant increase in depressive-like behavior during the TST, but only among females (One-way ANOVA for group. $F_{3,35} = 9.8$, $P < 0.001$ (Figure 4A)). Compared to females in the Vehicle+Sham group, those in the Vehicle+Stress group exhibited significant increases in immobility time. Next, we found that repeated swim stress together with sustained OR486 delivery produced significantly greater depressive-like behavior than swim stress alone. Females in the OR486+Stress group exhibited significant increases in immobility time during the TST compared to those in the Vehicle+Stress group. Males in the OR486+Stress group did not differ from those in the Vehicle+Stress group, although they did exhibit increased immobility time during the TST compared to those in the Vehicle+Sham group. The greater impact of repeated swim stress and sustained OR486 delivery on depressive-like behavior in females was also observed during the final 10 minutes of the third and final day of the swim stress paradigm (One-way ANOVA for group. $F_{3,45} = 5.7$, $P < 0.003$ (Figure 4B)). During the swim task, immobility time for females in the OR486+Stress group was 40% greater than that for males. Thus, OR486 delivery exacerbates stress-induced depressive-like behavior, especially in females.

3.5. Effect of COMT Inhibition on Stress-dependent Decreases in Hippocampal BDNF Expression in Males and Females

BDNF not only acts as a pronociceptive factor, but also serves in depression. Here we assessed the expression of BDNF in the hippocampus CA1 area from the four different groups. We found that swim stress led to decreases in hippocampal BDNF in both male and females (One-way ANOVA for group. Males: $F_{2,30} = 11.16$, $P < 0.001$ (Fig 5B,D) and females: $F_{2,27} = 32.71$, $P < 0.001$ (Fig 5C,E)), and OR486 delivery exacerbated this decrease in females, but not males. Compared to the Vehicle+Sham group, mice in Vehicle+Stress group exhibited decreased BDNF expression in the hippocampus. Compared to mice in the Vehicle+Stress group, female, but not male mice, in the OR486+Stress group exhibited even

lower decreases in hippocampal BDNF expression. This finding is consistent with the ability of COMT inhibition to exacerbate stress-dependent depressive-like behaviors mostly in female, but not male mice. Analysis of between-subjects factors in the same ANOVA revealed that the potentiation of OR486-dependent increases in hippocampal BDNF expression by stress in female vs male mice is significant (One-way ANOVA for group. $F_{5,57} = 18.67$, $P < 0.001$ (Figure 5F)). In the absence of swim stress, OR486 delivery also resulted in a modest decrease in hippocampal BDNF expression (One-way ANOVA for group. $F_{1,38} = 20.64$ (Figure 4s)), with no sexual dimorphism observed (Figure 4s). Consistent results were obtained by Western blot analysis (Figure 6s).

3.6. Effect of COMT on Stress-dependent Increases in Hippocampal GR Expression in Males and Females

Finally, we examined hippocampal GR expression in a stress-induced depression model in the presence and absence of COMT inhibition. We found that swim stress resulted in increased hippocampal GR expression in both males and females (One-way ANOVA for group. males: $F_{2,18} = 7.246$, $P < 0.01$ (Fig 6A,C) and females: $F_{2,18} = 14.95$, $P < 0.01$ (Fig 6B,D)). Compared to the Vehicle+Sham group, mice in the Vehicle+Stress group exhibited higher hippocampal GR expression. In contrast to BDNF, hippocampal GR expression was not potentiated by COMT inhibition either in females or in males, and no differences were observed between males and females (Figure 6E). In the absence of swim stress, COMT inhibition did not alter hippocampal GR expression (Figure 5s). Again, consistent results were obtained by Western blot analysis (Figure 6s).

4. Discussion

Accumulating evidence implicates COMT and stress in the etiology of FPS and comorbid depression, which are conditions that predominantly affect females. However, the independent and joint contributions of COMT and stress to these conditions have not been examined. Here, we demonstrated that repeated swim stress potentiates the effect of low COMT on functional pain and that low COMT potentiates the effect of stress on depressive-like behavior. The joint effects of low COMT and stress on functional pain and depressive-like behavior were significantly greater in females relative to males. Consistent with behavioral data, we found that stress potentiates COMT-dependent increases in spinal BDNF and that low COMT potentiates stress-dependent decreases in hippocampal BDNF in females, but not males. Thus, females are more susceptible to the additive effects of factors that increase catecholaminergic tone and alter BDNF signaling.

4.1. Swim Stress Exacerbates COMT-dependent Functional Pain

Many patients with FPS express low COMT activity genetic variants,[53; 66; 81] which are associated with increased circulating catecholamine levels and increased pain evoked by mechanical and thermal stimuli.[24; 113] In previous pre-clinical studies we demonstrated that increased basal catecholamine levels resulting from low COMT drive pain through activation of peripheral β ARs and downstream inflammatory mediators that increase nociceptor and glial cell activity.[38; 46; 111] As pain responses in patients with FPS are enhanced following stressful events which potentiate catecholamine release from

sympathetic nerves, here we sought to investigate the influence of stress in our mouse model of FPS.[64; 78] First, we evaluated the effects of stress in vehicle-treated mice, and found that swim stress produced mechanical and thermal heat pain in males and females comparable to that produced by OR486. This finding is in line with results from clinical studies demonstrating a causative role for stress in FPS such as fibromyalgia, [104] and pre-clinical studies demonstrating that repeated stress results in long-lasting pain *via* activation of sensory neurons at spinal and supraspinal sites.[39; 71; 72]

Next, we evaluated the effects of stress in OR486-treated mice, and found that swim stress significantly increased COMT-dependent mechanical and thermal heat pain in males and females. In line with our previous studies, the degree of pain in the OR486+Sham group did not differ between males and females.[17] However, the degree of thermal heat pain in the OR486+Stress group was significantly greater in females. While stress has been shown to enhance pain responses in models of neuropathic pain,[11; 51] this is the first pre-clinical study to demonstrate the significant and sexually dimorphic impact of stress on functional pain.

4.2. COMT Inhibition Exacerbates Stress-induced Depressive-like Behavior

Among patients with chronic FPS, the prevalence of depression ranges from 30–80%. [15; 32; 93; 107] As depression is a major comorbidity of FPS, we sought to investigate the influence of COMT inhibition and stress on depressive-like behavior. First, we evaluated the effects of stress in vehicle-treated mice. In line with results from clinical studies demonstrating that stress is a major contributing factor to the occurrence of depression, [19; 63; 75] we found that swim stress led to depressive-like behavior, but only in females.

Next, we evaluated the effects of stress in OR486-treated mice. We found that COMT inhibition significantly increased stress-dependent depressive-like behavior in females, but not males. This finding is consistent with a growing literature that individuals with low COMT activity variants are more susceptible to the negative consequences of stress on depression. Individuals with the low COMT activity variant are at greater risk for depression during childhood[22] and adulthood,[21; 45; 58] and their depressive symptoms are associated with precipitating stressors. Meanwhile, transgenic mice overexpressing the human high COMT activity variant exhibit diminished stress responses.[68] Our results together with published findings suggest that depression and FPS share a common etiology linked to abnormalities in catecholaminergic tone.[30; 48; 88; 108]

4.3. COMT Inhibition and Stress, Together, Unmask Sexual Dimorphism in Functional Pain and Depressive Behavior

FPS and depression predominantly affect females.[3; 7; 8; 33; 56; 65] Females are, on average, 3 times more likely to be diagnosed with FPS[29; 40; 95] and 2 times more likely to experience depression.[12; 83; 100; 101] Here, we found that OR486 and swim stress together lead to enhanced thermal heat hyperalgesia and depressive-like behavior only in females. The unmasking of sexual dimorphism in pain and depressive phenotypes required both COMT inhibition and stress. This interesting discovery could be due to inherent sex differences in catecholamine tone. COMT activity levels are lower in peripheral and brain

tissues of females,[10; 16] and COMT-related sex differences in nociception, cognition, anxiety and other behavioral traits have also been observed.[7; 31; 37; 68; 103] The co-occurrence of catecholamine-dependent pain and depressive-like behavior, which is more pronounced in females, suggests common neurobiologic alterations in regions that mediate these behaviors.

4.4. BDNF is a Sex-specific Biomarker Correlated with Functional Pain and Depressive-like Behavior

Following assessment of behavior on day 14, spinal and hippocampal tissues were collected to determine the independent and joint effects of COMT inhibition and stress on BDNF and GR expression. BDNF is a neurotrophin essential for many aspects of central nerve system (CNS) function, including activity-dependent forms of synaptic plasticity.[43] A large body of literature has also demonstrated a major role for BDNF in pain[57; 79] and depressive disorders.[44; 69; 109] Here, we first examined the independent effects of COMT inhibition and stress on BDNF expression in pain-relevant spinal regions. Compared to mice in the Vehicle+Sham group, males and females in the OR486+Sham, but not the Vehicle+Stress, group exhibited significant increases in spinal BDNF expression. This suggests that spinal BDNF is a marker of pain versus stress, and is consistent with findings from published reports demonstrating that increases in spinal BDNF expression contribute to pain hypersensitivity in pre-clinical models of surgical incision,[50] orthopedic surgery,[110] and neuropathic pain.[112]

Next, we examined the independent effects of COMT inhibition and stress on BDNF expression in the depression-relevant CA1 hippocampal region. Compared to mice in the Vehicle+Sham group, males and females in the Vehicle+Stress, but not OR486+Sham, group exhibited significant increases in hippocampal BDNF expression. This finding suggests that hippocampal BDNF is a marker of stress/depression versus pain, and is in line with findings from clinical and pre-clinical studies demonstrating that depressed individuals exhibit decreased BDNF expression in the hippocampus [26; 74; 85]. The utility of hippocampal BDNF as a marker of stress/depression is underscored by its correlation with disease severity and antidepressant treatment response.[4; 90; 109]

Finally, we examined the joint effects of COMT inhibition and stress on BDNF expression in spinal and hippocampal tissues. We found that mice in the OR486+Stress group exhibited marked increases in spinal BDNF expression compared to the OR486+Sham group and marked decreases in hippocampal BDNF expression compared to the Vehicle+Stress group. Consistent with our behavioral data, this effect was only observed in females. Region- and sex-specific alterations in BDNF expression have been shown to occur in different neurological disorders. For example, chronic prenatal stress leads to increased spinal BDNF expression in correlation with increased visceral hypersensitivity in female, but not male rats.[105] Similarly, maternal separation leads to decreased hippocampal BDNF expression alongside anhedonia in mature female but not male rats.[42] Here, we provide the first demonstration that BDNF expression is altered in a region- and sex-specific manner following COMT inhibition and stress. Thus, BDNF might act as a predicative marker for sexual dimorphism.

4.5. GR Expression is Correlated with Functional Pain and Depressive-like Behavior

Glucocorticoid activation of GR plays a critical role in neuroplasticity related to both neuropathic pain [97–99] depression.[2] Here, we first examined the independent effects of COMT inhibition and stress on GR expression in the spinal cord. Compared to mice in the Vehicle+Sham group, males and females in the OR486+Sham, but not Vehicle+Stress, group exhibited significant increases in spinal GR expression. This finding suggests that spinal GR is a marker of pain versus stress, and is consistent with findings from studies demonstrating increased GR expression in the spinal dorsal horn of rodents following nerve injury.[86; 98; 99]

Next, we examined the independent effects of COMT inhibition and stress on GR expression in the CA1 hippocampal region. Compared to mice in the Vehicle+Sham group, males and females in the Vehicle+Stress, but not OR486+Sham, group exhibited significant increases in hippocampal GR expression. This finding suggests that hippocampal GR is a marker of stress/depression versus pain, and is consistent with those from the previous studies showing increased hippocampal GR expression in different types of depression.[35; 91; 96] Interestingly, GR can down-regulate BDNF expression in the hippocampus. Thus, the crosstalk between GR and BDNF signaling pathways might play a major role in physiology and pathology of depression.[2]

Finally, we assessed the joint effects of COMT inhibition and stress on GR expression in spinal and hippocampal tissues. We found that males and females in the OR486+Stress group exhibited comparable increases in spinal GR expression compared to the OR486+Sham group and in hippocampal GR expression compared to the Vehicle+Stress group. Thus, stress does not influence COMT-dependent increases in spinal GR and COMT inhibition does not influence stress-dependent increases in hippocampal GR expression.

5. Conclusions

Our findings suggest that functional pain syndromes and depressive disorders, which predominantly affect females, share a common etiology linked to heightened catecholaminergic tone. Thus, therapies that normalize catecholamine signaling may prove useful in the treatment of comorbid functional pain and depression. Further, we identified BDNF as a sex-specific biomarker correlated with functional pain and depressive behavior.

Supplementary Material

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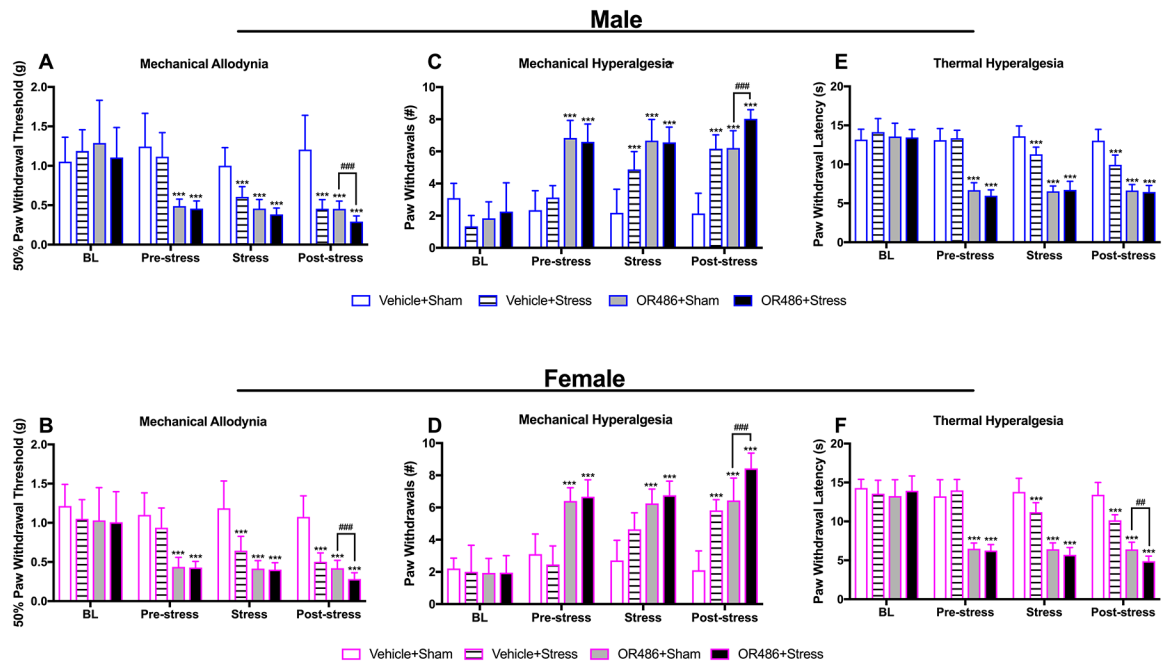


Figure 1. Stress exacerbates COMT-dependent functional pain, especially among females. Sustained systemic delivery of the COMT inhibitor OR486 or 3 days of swim stress each produce pronounced (A, B) mechanical allodynia, (C, D) mechanical hyperalgesia, and (E, F) thermal heat hyperalgesia in both males and females. OR486 and swim stress together lead to significantly greater mechanical allodynia and mechanical hyperalgesia in males and females and greater thermal hyperalgesia only in females. Data are shown as Mean \pm SD, N=6–9 per group. ***P < 0.001 different from the Vehicle+Sham group; ##P < 0.01, ###P < 0.001 different from the OR486+Sham group.

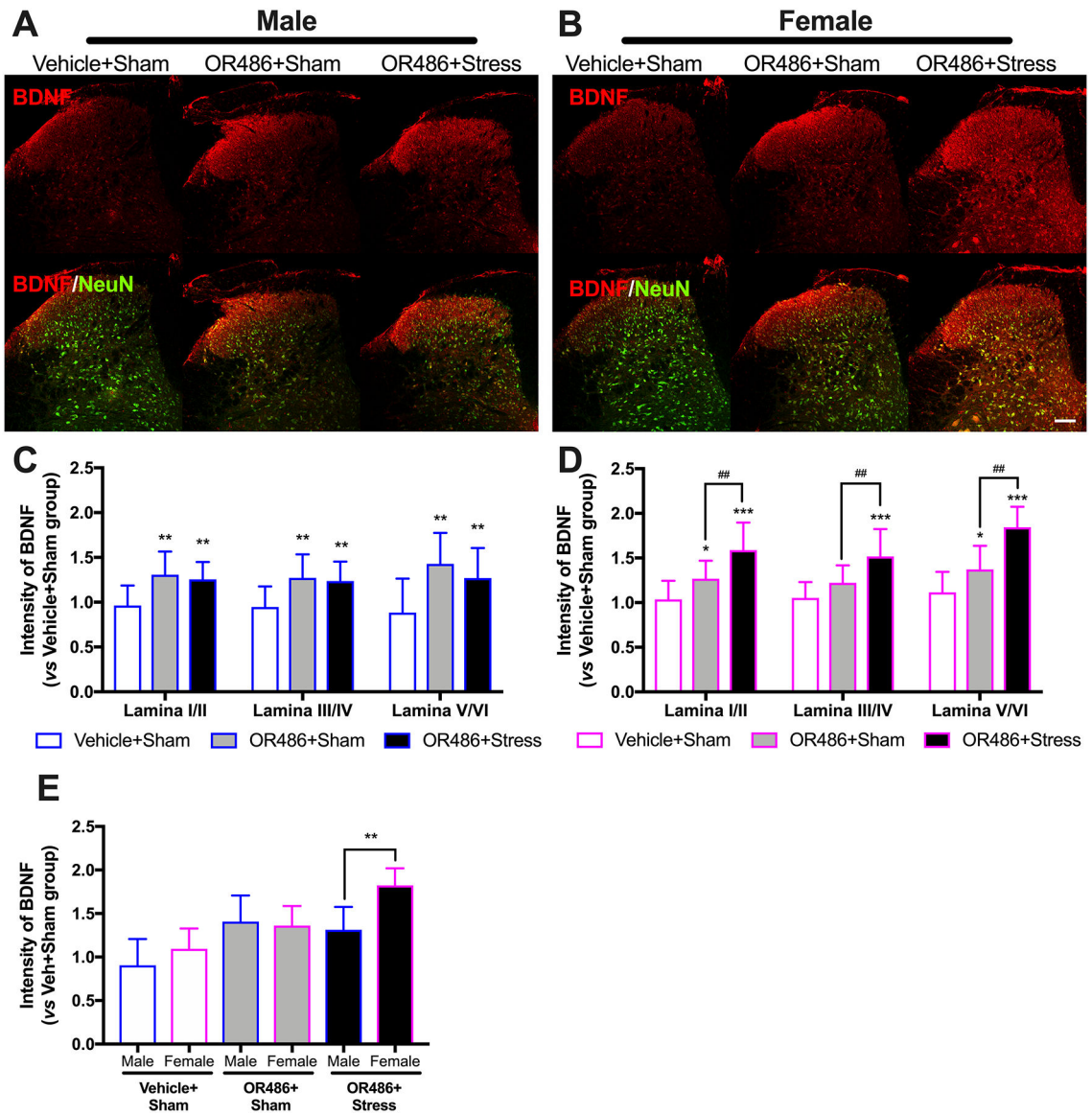


Figure 2. Stress exacerbates COMT-dependent increases in spinal BDNF expression in females, but not males.

Immunohistochemical staining shows expression of spinal BDNF on day 14 in (A) males and (B) females receiving the COMT inhibitor OR486 or vehicle in the absence or presence of swim stress. Quantitative analysis of immunofluorescence intensity demonstrates that sustained OR486 delivery results in increased expression of BDNF in spinal dorsal horn in both (C) males and (D) females, and that swim stress potentiates COMT-dependent increases in spinal BDNF expression in females, but not males. (E) The potentiation of COMT-dependent increases in spinal BDNF expression by stress in females vs males is confirmed by analysis of between-subjects factors in the same ANOVA. Data are shown as Mean \pm SD, N=6–8 per group, *P < 0.1, **P < 0.01, ***P < 0.001 different from the Vehicle+Sham group; ##P < 0.01, different from the OR486+Sham group. Scale bar = 100 μ m.

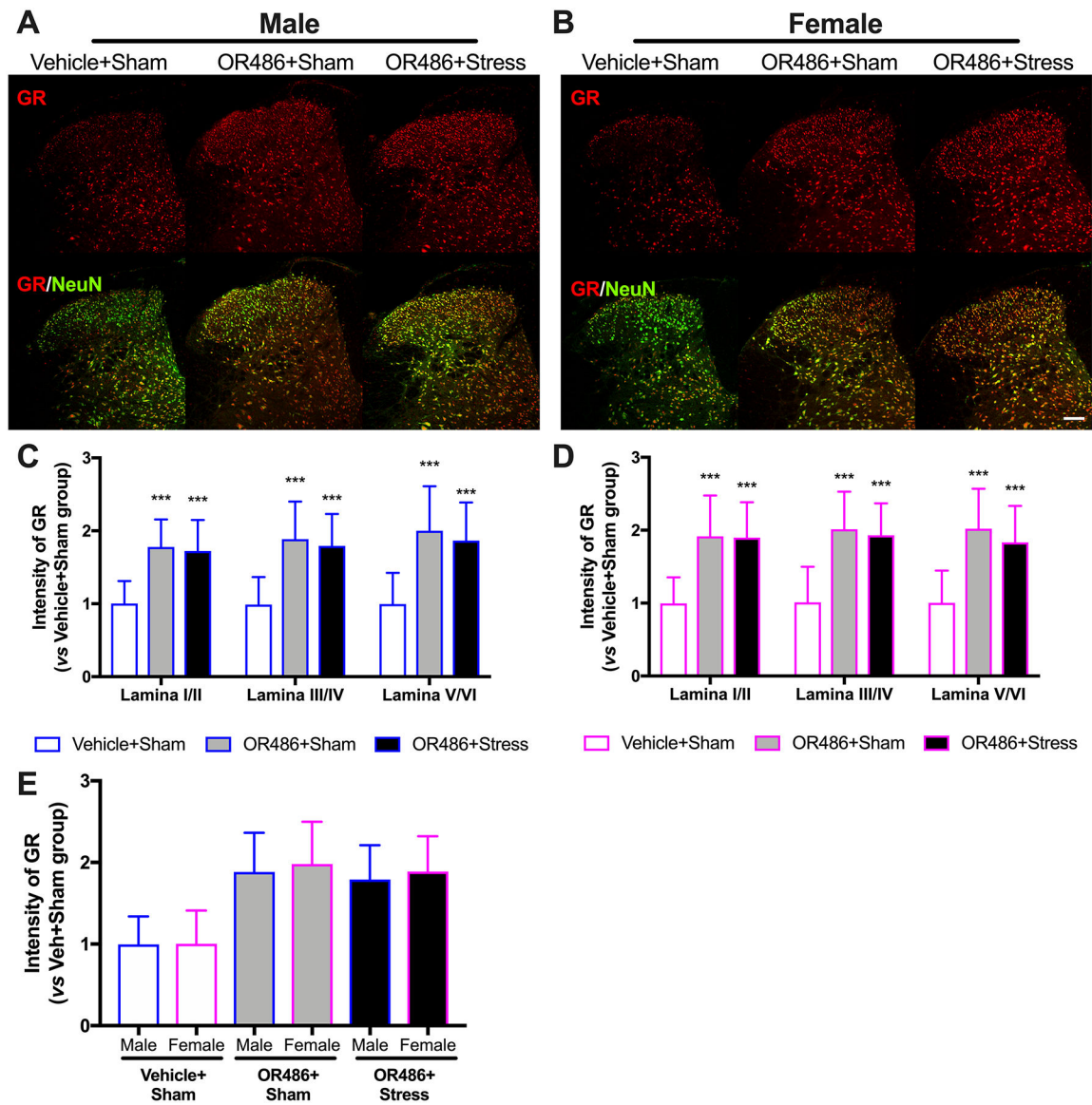


Figure 3. COMT inhibition leads to increases in spinal GR expression in a stress- and sex-independent manner.

Immunohistochemical staining shows expression of spinal GR on day 14 in (A) males and (B) females receiving the COMT inhibitor OR486 or vehicle in the absence or presence of swim stress. Quantitative analysis of immunofluorescence intensity demonstrates that sustained OR486 delivery leads to increased expression of GR in spinal dorsal horn in both (C) males and (D) females, and that swim stress does not alter COMT-dependent increases in spinal GR expression. (E) No sexually dimorphic effects were observed following analysis of between-subjects factors in the same ANOVA. Data are shown as Mean \pm SD, N=6–8 per group, ***P < 0.001 different from the Vehicle+Sham group. Scale bar = 100 μ m.

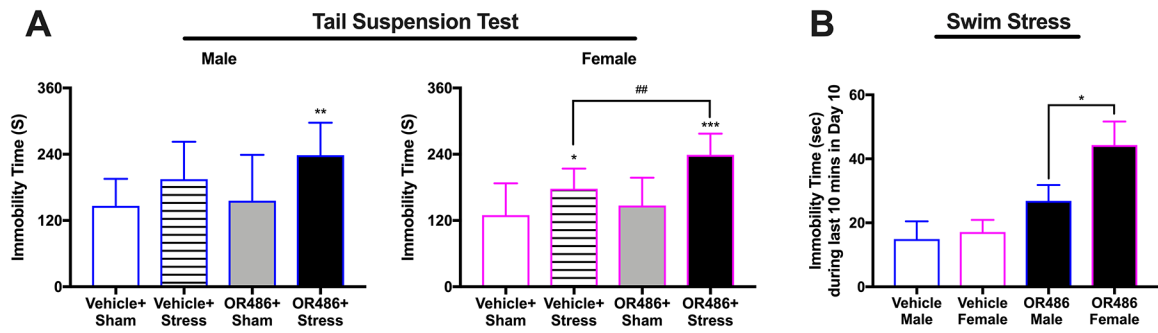


Figure 4. COMT inhibition exacerbates stress-induced depressive-like behavior, especially in females.

(A) Swim stress leads to increased immobility time during the tail suspension test, which is potentiated by sustained OR486 delivery, in males and females. Data are shown as Mean \pm SD, N=8–13 per group, *P < 0.05, **P < 0.01, ***P < 0.001 different from the Vehicle +Sham group; ##P < 0.01, different from the Vehicle+Stress group.

(B) Compared to males, female mice in the OR486+Stress group exhibit a 4-fold greater increase in immobility time during the swim task on day 3. Data are shown as Mean \pm SD, N=8–13 per group, *P < 0.05.

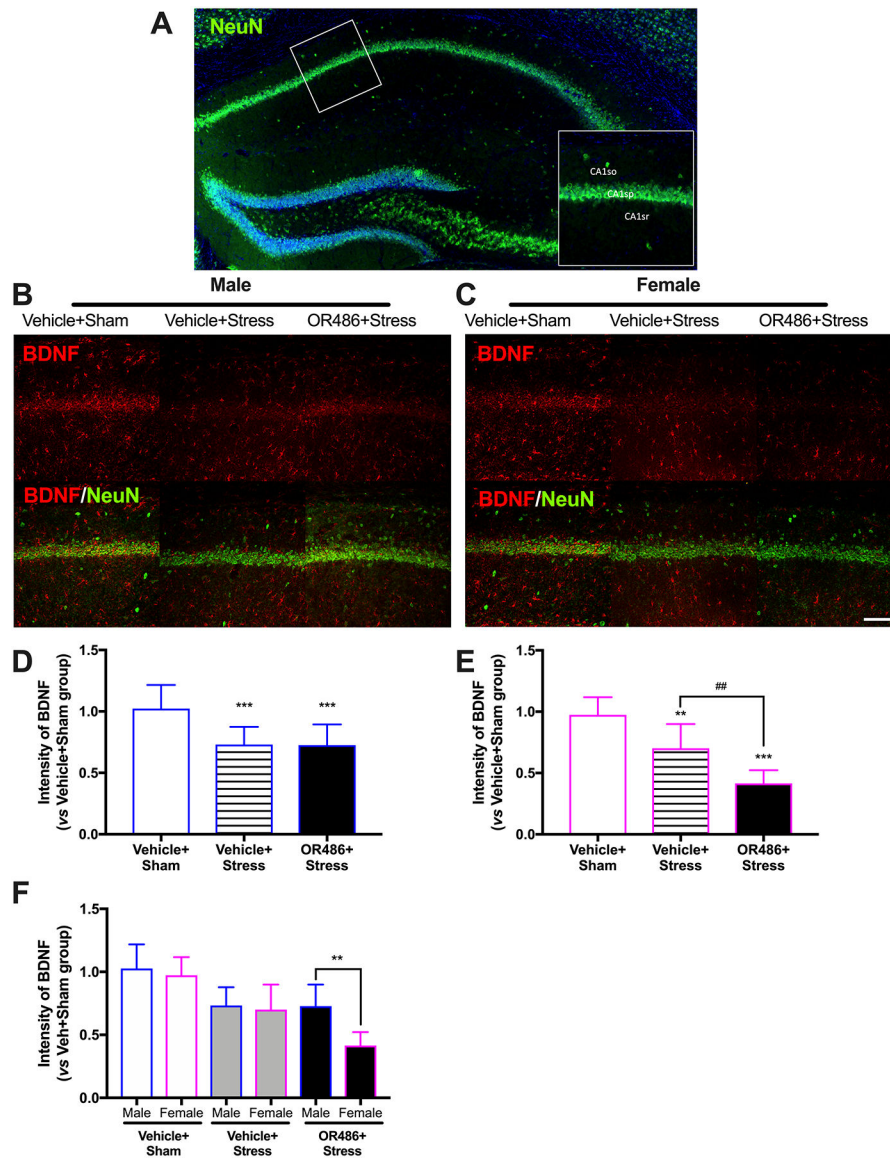


Figure 5. COMT inhibition exacerbates stress-dependent decreases in expression of hippocampal BDNF in females, but not males.

(A) A gross hippocampal image stained with NeuN (Green) depicts the CA1 area where we focused our analyses. The pyramidal layer (CA1sp) is shown as the transverse dot line in the middle, the area below CA1sp is stratum radiatum (CA1sr), and the area above CA1sp is stratum oriens (CA1so). Immunohistochemical staining shows expression of BDNF in the hippocampal CA1 area on day 14 in (B) males and (C) females receiving the COMT inhibitor OR486 or vehicle in the absence or presence of swim stress. Quantitative analysis of immunofluorescence intensity demonstrates that swim stress leads to decreased expression of hippocampal BDNF in both (D) male and (E) female mice, and that sustained OR486 delivery potentiates stress-induced decreases in hippocampal BDNF expression in females, but not males. (F) The potentiation of stress-dependent increases in spinal BDNF expression by OR486 in females vs males is confirmed by analysis of between-subjects factors in the same ANOVA. Data are shown as Mean \pm SD, N=6–8 per group, **P < 0.01,

***P < 0.001 different from the Vehicle+Sham group; ##P < 0.01 different from the Vehicle +Stress group. Scale bar = 100 μ m.

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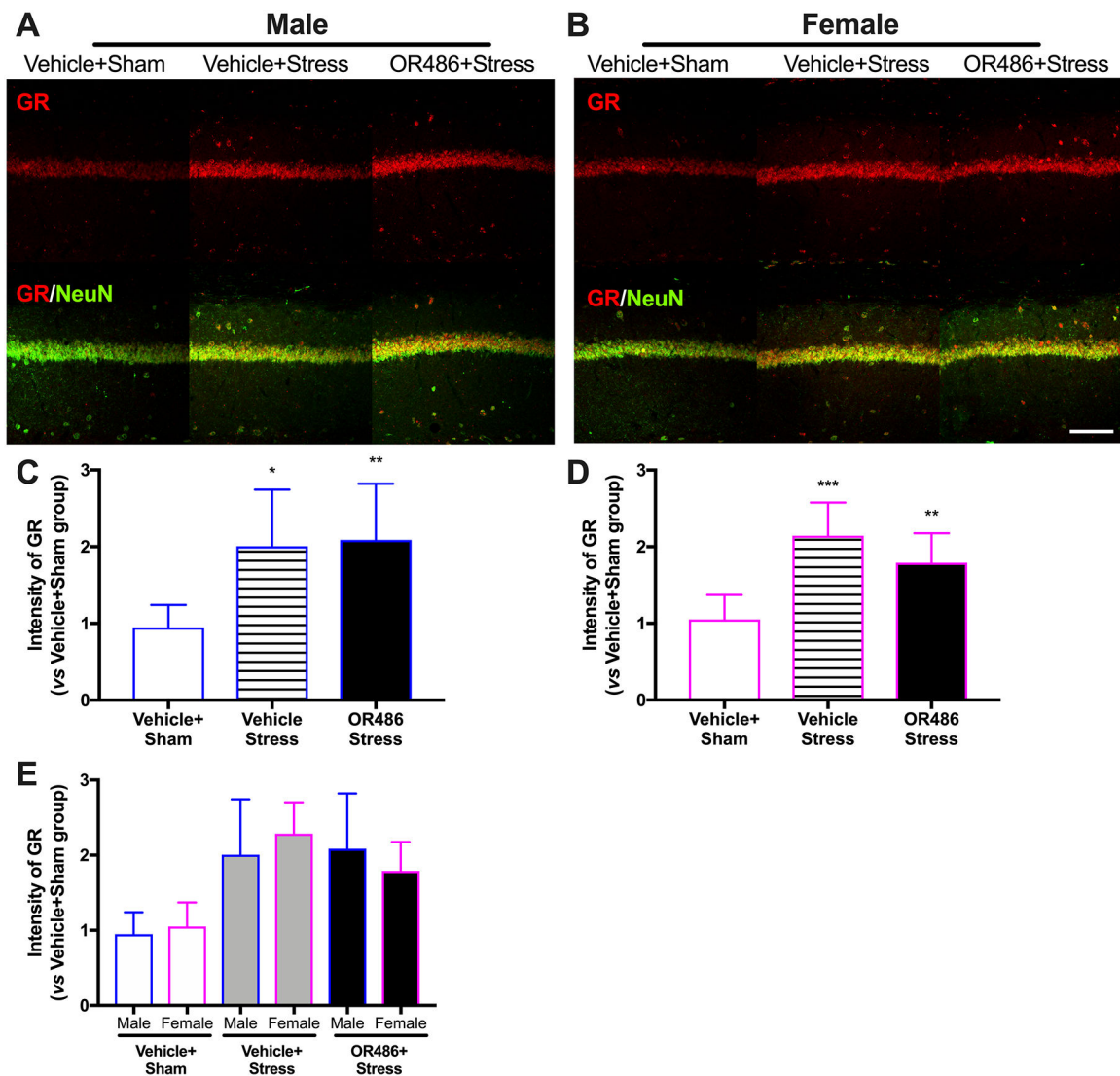


Figure 6. Stress leads to increases in hippocampal GR expression in a COMT- and sex-independent manner.

Immunohistochemical staining shows expression of GR in the hippocampal CA1 area on day 14 in (A) males and (B) females receiving the COMT inhibitor OR486 or vehicle in the absence or presence of swim stress. Quantitative analysis of immunofluorescence intensity demonstrates that swim stress leads to increased expression of hippocampal GR in both (C) male and (D) female mice, and that sustained OR486 delivery does not alter stress-dependent increases in hippocampal GR expression. (E) No sexually dimorphic effects were observed following analysis of between-subjects factors in the same ANOVA. Data are shown as Mean \pm SD, N=7 per group, *P < 0.1, **P < 0.01, ***P < 0.001 different from the Vehicle+Sham group. Scale bar = 100 μ m.