Sex differences in nociceptor translatomes contribute to divergent prostaglandin signaling in male and female mice

Short title: Sex differences in mouse nociceptor translatomes

Diana Tavares-Ferreira[1], Pradipta R. Ray[1], Ishwarya Sankaranarayanan[1], Galo L. Mejia[1], Andi Wangzhou[1], Stephanie Shiers[1], Ruta Uttarkar[1], Salim Megat[1], Paulino Barragan-Iglesias[1], Gregory Dussor[1], Armen N. Akopian[2], Theodore J. Price[1,*]

[1]University of Texas at Dallas, Department of Neuroscience and Center for Advanced Pain Studies

[2]University of Texas Health San Antonio, Department of Endodontics

*correspondence to Theodore J Price – theodore.price@utdallas.edu
800 W Campbell Rd
Richardson TX 75080
USA
972-883-4311

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ABSTRACT

Background – There are clinically relevant sex differences in acute and chronic pain mechanisms, but we are only beginning to understand their mechanistic basis. Transcriptome analyses of rodent whole dorsal root ganglion (DRG) have revealed sex differences, mostly in immune cells. We examined the transcriptome and translatome of the mouse DRG with the goal of identifying sex differences.

Methods – We used Translating Ribosome Affinity Purification (TRAP) sequencing and behavioral pharmacology to test the hypothesis that nociceptor (Nav1.8 expressing neurons) translatomes would differ by sex.

Results – We found 66 genes whose mRNA were sex-differentially bound to nociceptor ribosomes. Many of these genes have known neuronal functions but have not been explored in sex differences in pain. We focused on *Ptgds,* which was increased in female mice. The mRNA encodes the prostaglandin D_2 (PGD_2) synthesizing enzyme. We observed increased Ptgds protein and PGD_2 in female mouse DRG. The Ptgds inhibitor AT-56 caused intense pain behaviors in male mice but was only effective at high doses in females. Conversely, female mice responded more robustly to another major prostaglandin, PGE_2, than did male mice. Ptgds protein expression was also higher in female cortical neurons, suggesting DRG findings may be generalizable to other nervous system structures.

Conclusions – Nociceptor TRAP sequencing (TRAP-seq) reveals unexpected sex differences in one of the oldest known nociceptive signaling molecule families, the prostaglandins. Our results demonstrate that translatome analysis reveals physiologically relevant sex differences important for fundamental protective behaviors driven by nociceptors.

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INTRODUCTION

For decades, studies in the neuroscience field have been done almost entirely on male animals (1). However, there are physiological and molecular differences in the peripheral and central nervous systems between males and females. Many neurological disorders have been shown to have different incidence proportion, age of onset, symptoms, and response to treatment between males and females. Schizophrenia tends to develop at an earlier age in men (2, 3); Parkinson's disease is twice as common in men than women (4) and it has sex differences in symptoms and response to treatment (5). On the other hand, major depressive disorder (6), anxiety disorders (7), and Alzheimer's disease (8) affect more women than men. There are also sex differences in pain syndromes. Neuropathic pain, osteoarthritis, migraine, and fibromyalgia are more frequently reported in women than in men (9-13). A common thread in all of these neurological disorders is that they are poorly treated by current therapies. The exclusion of one of the sexes in pre-clinical studies has likely been detrimental to the success of translational research. The National Institutes of Health has responded to this issue by mandating consideration of sex as a biological variable in funding proposals (14).

As noted above, there are clear sex differences in pain mechanisms, yet, we are only beginning to understand how these differences emerge (15). Nociceptors of the dorsal root (DRG) and trigeminal ganglion are the neurons that send nociceptive information to the brain and are a possible source of mechanistic diversity that causes sex differences in pain. A previous study suggested that there are some sex differences in sensory neuron transcriptomes (16). However, transcription and translation are not directly coupled in eukaryotes, so there can be important divergences between transcriptomes (the cellular RNA profile) and translatomes (the subset of the transcriptome that is bound to ribosomes for translation) in cells (17). An example in nociceptors is the similar transcription of the prolactin receptor (*Prlr*) in male and female nociceptors, but the female-selective localized translation of the *Prlr* mRNA (18). This sex difference in *Prlr* mRNA translation causes an important sex difference in prolactin-evoked pain responses (18-20).

The primary goal of our study was to identify differences in translatome between male and female nociceptors using Translating Ribosome Affinity Purification (TRAP) methodology (21-23). We demonstrate that there are specific mRNAs that are differentially bound by ribosomes (hence likely differentially translated) in neurons of the DRG between males and females. Notably, we identify differentially translated mRNAs encoding proteins that function in similar pathways between male and female nociceptors. This suggests that different proteins may drive similar functions in male and female neurons. One of the differentially translated mRNAs identified using our TRAP approach was *Ptgds* (Prostaglandin D-synthase). Ptgds is an abundant enzyme in neuronal cells that converts PGH_2 to PGD_2. We find that it is up-regulated in female neurons. Consistent with this, we observed significant differences in behavioral responses between males and females when inhibiting this enzyme. We also noted sex differences in the response to PGE_2. Our use of TRAP technology to delve into sex differences in nociceptor translatomes reveals a fundamental difference in how male and female mice respond to one of the oldest known families of nociceptive signaling molecules, the prostaglandins. (24).

METHODS AND MATERIALS

Detailed methods are provided in *Supplemental Methods*. All animal procedures were approved by the Institutional Animal Care and Use Committee at University of Texas at Dallas. DRGs from Nav1.8-TRAP male and female mice were quickly dissected and homogenized using Precellys® Minilys Tissue Homogenizer. An aliquot of the lysate was saved for use as INPUT (IN; bulk RNA-sequencing), and the remaining was used for immunoprecipitation (IP; TRAP-sequencing) by incubating the lysate with protein G-coated Dynabeads (Invitrogen) bound to anti-GFP antibodies for 3 h at 4°C. RNA was eluted from all samples using the Direct-zol kit (Zymo Research) and cDNA libraries were prepared with total RNA Gold library preparation (Illumina). After standardizing the amount of cDNA, the libraries were sequenced on Illumina NextSeq500 sequencing machine with 75-bp single-end reads. Reads were then mapped against the reference genome and transcriptome (Gencode vM16 and GRCm38.p5) using STAR v2.2.1 (25). Relative abundances in Transcripts Per Million (TPM) for each gene of each sample were quantified by Stringtie v1.3.5 (26). Downstream analyses were restricted to protein-coding genes and excluding mitochondrial chromosome genes. For each expressed coding gene, we report log_2 fold change, Bhattacharyya coefficient (BC) (27) and strictly standardized mean difference (SSMD) (28, 29). We used immunohistochemistry, ELISA assay and behavioral tests to understand the consequences of sex differences in *Ptgds* expression.

RESULTS

Nav1.8[cre] mice were crossed with Rosa26[fs-TRAP] (30) in order to create mice expressing eGFP fused to the ribosomal L10a protein in Nav1.8-positive neurons (Nav1.8-TRAP mice) (**Figure 1A**). The specificity of the TRAP approach has been previously characterized, where it was shown that eGFP-RPL10a is expressed in sensory neurons in the dorsal root ganglion (DRG) including most nociceptors (22, 23). We used Nav1.8-TRAP mice to characterize the translatome of male and female DRG neurons by immunoprecipitating actively translating ribosomes and purifying the associated mRNA (**Figure 1B**). We sequenced bulk RNA (INPUT or IN in later plots), correspondent to total RNA from whole DRGs, and immunoprecipitated (IP) mRNA, correspondent to mRNA bound to GFP-tagged ribosomes in Nav1.8-positive neurons. This approach allowed us to characterize differences at the steady-state transcriptional and active translational levels between female and male DRG.

Heatmap of the correlation coefficients for coding gene TPMs of each biological replicate showed a clear separation between TRAP-seq and bulk RNA-seq as expected given the different cell populations from which the cDNA library is constructed (**Figure 2A**). Consequently, in our hierarchical clustering analysis using these correlation coefficients, we find two distinct clusters of the bulk RNA-seq and TRAP-seq molecular profiles (**Figure 2B**). Subclusters within each cluster are very similar to each other and do not segregate by sex, showing that whole-transcriptome molecular profiles in each assay type (RNA-seq and TRAP-seq) are consistent across sexes for the DRG. All biological replicates for each sex in the input and IP samples showed high correlation coefficients across gene TPMs, suggesting high reproducibility across experiments (**Figure 2C**).

Because TRAP-seq purifies translating mRNAs in a cell type-specific manner, we tested the specificity of our approach using a group of control genes. We analyzed a subset of genes

known to be enriched in specific cell populations in the DRG and verified that neuronal mRNAs, such as *Calca*, *Trpv1, Scn10a, Prph*, had enriched relative abundance in IP fraction. In contrast, non-neuronal genes such as glial markers (*Mpz*, *Mbp, Gfap*) were depleted in IP samples (**Figure 2D**).

Percentile ranks were calculated (**Suppl. File 1 Tabs 1A, 2A**) for gene expression levels (in TPM) for each RNA-seq and TRAP-seq sample. Based on these order statistics, we conservatively determined a set of 15,072 genes (>= 30th percentile) that were consistently detected in at least one sex in the RNA-seq samples, and out of those, 12,542 genes (>= 15th percentile) that were consistently detected in at least one sex in the TRAP-seq samples. These numbers are consistent with previous mouse DRG RNA-seq and TRAP-seq studies (31, 32).

For each biological replicate in INPUT and IP, we plotted the empirical probability densities of coding gene TPMs and noted a distinctly bimodal distribution for genes that are consistently detected versus those that are lowly expressed or undetected in each assay (**Figure 2E, F**). The TPM expression levels were finally quantile normalized (represented as qTPMs) in order to correct for sequencing depth and, thus, ensure comparability between samples.

To determine differentially expressed (DE) genes, we calculated the log2-fold change of TPM across sexes, and two related statistics - strictly standardized mean difference (SSMD) of TPM percentile ranks (28, 29) and Bhattacharyya coefficient (BC) of qTPMs (27) between sexes for quantifying the effect size and controlling for within-group variability. (**Suppl. File 1 Tabs 1A-B** for INPUT; **Suppl. File 1 Tabs 2A-B** for IP). For stringency, we required DE genes to have | log2-fold change | > 0.41 (corresponding to fold change > 1.33), | SSMD | > 0.9, and BD < 0.5. We plotted the SSMD values against the Fold Change (Log$_2$ scale) for the autosomal genes for INPUT and IP (**Figures 3A, D**).

We found a total of 80 genes for INPUT (transcriptome) (**Figure 3A, B; Suppl. File 2**) and 66 genes for IP (translatome) (**Figure 3C, D; Suppl. File 3**) that were DE between sexes. Given that we used mice that had not been experimentally manipulated, we anticipated finding a relatively small number of genes using both approaches. Interestingly, we did not observe a substantial overlap between genes DE in INPUT and IP, except for sex-chromosomal genes such as *Eif2s3x, Eif2s3y, Uty* and *Kdm5d* and a few autosomal genes (**Figure 3E***)*. We found that *Lepr* (Leptin receptor) and *Lbp* (Lipopolysaccharide binding protein) were up-regulated in both INPUT and IP, in females. *Ep400* (E1A Binding Protein P400) was up-regulated in male INPUT and female IP, highlighting discrepancies between transcriptome and translatome. *Atf3* (Activating transcription factor 3), a known nerve injury marker (33), was up-regulated in both INPUT and IP in males. Since *Atf3* can be induced by minor injuries, like scratches (34), this may be explained by more frequent fighting in male cages.

Next, we conducted gene-set enrichment analysis for DE genes using GO Enrichment Analysis resource PANTHER (35). We did not find any statistically significant GO terms for DE genes in INPUT. In contrast, we identified 6 GO terms (Biological Process) statistically significant (FDR < 0.05) for genes DE in IP (**Figure 3F**). The majority of the genes DE in IP were involved in the regulation of cell communication and signaling (**Figure 3G**). We identified several genes (e.g., *Sfrp4*, *Sema6a*) that encode for membrane proteins involved in cell signaling. Genes such as *Fbln1*, an extracellular matrix structural protein, and *Fgf9*, a

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growth factor, are involved in cell to cell communication. Genes such as *Map3k1* and *Mapk1ip1* are involved in the regulation of MAPK cascade. These findings suggest that there may be sex differences in proteins that regulate fundamental signaling pathways in male and female nociceptors. We expanded our functional analysis by manually curating relevant information regarding DE genes for INPUT and IP (**Suppl. Tables 1 and 2** contain detailed information).

Within the DE genes in the INPUT we identified several transcription factors such as *Hoxd4* in females and *Foxd3* (known to be glially expressed) in males. We also noted different immune-related genes identified in male and female DRG INPUT, consistent with previous work in mice (36, 37) and humans (38). These included *Cxcl16* (Chemokine (c-x-c motif) ligand 16), a T-cell signaling molecule, and *Jchain* (Immunoglobulin joining chain), which were up-regulated in female INPUT. *Cd276* (Cd276 antigen) was up-regulated in male INPUT and plays a role in inflammatory responses by regulating cytokine production and T cell receptor signaling.

In the IP fraction, as expected, the identified DE genes were neuronally enriched in expression when compared to the INPUT fraction (**Suppl. Tables 1 and 2**). In the female translatome (IP) we found several up-regulated DE genes that are involved in neuronal functions such as *Pcdha8* (Protocadherin alpha 8), *Zmynd8* (Zinc finger, mynd-type containing 8) and *Slc6a13* (Solute carrier family 6 (neurotransmitter transporter, GABA), member 13). In the male translatome, genes with known neuronal functions such as *Chka* (Choline kinase alpha) and *Sema6a* (Sema domain, transmembrane domain (tm), and cytoplasmic domain, (semaphorin) 6a) were found to be up-regulated. Several enriched gene-set categories were similar between males and females, but these were driven by different genes, suggesting that unique genes may control similar functions in male and female nociceptors.

Since translation efficiency can be controlled by sequence elements within the mRNA (39, 40), we examined whether there were motifs in the 5′ untranslated regions (UTRs) or 3' UTRs of DE mRNAs in IP. We found three enriched motifs in the 3' UTRs of the genes up-regulated in males (**Figure 4A**) and one enriched motif in the 3' UTRs of the genes up-regulated in females (**Figure 4B**). The 3' UTR of a gene is known to influence the localization, degradation, and translation efficiency of an mRNA (6,7). The identified motifs are involved in neuron differentiation and migration, cell communication, and signal transduction, in agreement with the biological functions of DE genes identified in our IP fractions. We did not find any enriched motifs in the 5' UTRs of male or female DE mRNAs.

Next, we proceeded to validate our TRAP-seq approach by linking sex differences in the nociceptor active translatome to functional differences in expression and/or behavior. We decided to focus on a gene that was up-regulated in the female IP: Prostaglandin-D2 synthase (*Ptgds*). Ptgds catalyzes the conversion of prostaglandin H_2 (PGH_2) to prostaglandin D2 (PGD_2) (**Figure 5A**), which is known to be the most abundant prostaglandin in the brain (41, 42) and regulates nociception, sleep and temperature homeostasis (43-53). Prostaglandins are among the most widely studied of pain inducing molecules and many drugs are currently marketed as analgesics that target this class of molecules (54-56). We reasoned that previously unknown sex differences in prostaglandin signaling could have a dramatic impact on further our understanding of sex differences in pain.

First, we reanalyzed single-neuron DRG RNA-sequencing (57) and observed *Ptgds* mRNA expression in several subpopulations of neurons, including ones expressing *Calca* (a marker for peptidergic neurons) and *P2rx3* (a marker for non-peptidergic neurons) (**Figure 5B**). We noted that a receptor for PGD_2, *Ptgdr1* (DP1), was also expressed, especially in non-peptidergic neurons, but *Ptgdr2* (DP2) was not expressed in DRG neurons. We confirmed, using IHC, that Ptgds is expressed in almost all neurons in the mouse DRG (**Figure 5C**). Next, we sought to verify whether there were any sex differences in Ptgds expression at the protein level in the mouse DRG. Ptgds expression was markedly higher in female DRG (**Figure 5D, E**). To verify our previous TRAP experiment, we conducted an independent experiment where we tracked the estrous cycle stage in the female mice. We found that *Ptgds* mRNA associated with ribosomes was substantially higher in this group of female mice with some variation within the estrous cycle, with highest at estrous phase and estrogen levels (**Figure 5F**). In addition, we also investigated whether Ptgds showed sex-differences in expression in brain neurons (**Supp. Figure 1A**). Similar to observations in the DRG, Ptgds expression was higher in female cortical neurons (**Supp. Figure 1B**).

Having confirmed that Ptgds is more highly expressed in female DRG neurons, we investigated whether this would lead to functional sex differences. First, we measured PGD_2 levels in female and male DRGs. Because PGD_2 is highly unstable and can degrade very rapidly, we opted for converting PGD_2 to a more stable derivative PGD_2-MOX (by treating our samples with methoxylamine hydrochloride, MOX HCl) and performed an ELISA assay. Our results demonstrated that PGD_2 levels are higher in female DRGs (**Figure 5G**) in concordance with the higher levels of Ptgds. Next, we tested whether inhibiting Ptgds would produce differential behavioral effects in male and female mice. Unexpectedly, in pilot experiments, we noted intense grimacing behavior in male mice, so we focused on this behavioral output since it is driven by nociceptor input to the CNS (58). We used three different doses (1 mg/kg, 3 mg/kg and 10 mg/kg) of AT-56, a selective and competitive inhibitor of Ptgds (59) observing a robust grimacing effect after intraperitoneal (i.p.) injections of AT-56 (10 mg/kg) in particular in male mice (**Figure 6A**). At each dose, female mice exhibited less grimacing that lasted for a shorter time (**Figure 6B**). This finding suggests that females are protected against Ptgds inhibition-evoked pain because they have higher basal PGD_2 levels and more enzyme.

A previous study demonstrated that Ptgds gene knockout led to a loss of Prostaglandin E_2 (PGE_2) evoked mechanical pain hypersensitivity (43), suggesting an interplay between these closely related molecules (**Figure 5A**) in pain signaling. Moreover, a previous clinical study suggested a sex difference in ibuprofen-induced analgesia in an experimental pain model wherein only males showed analgesia in response to this drug, which lowers PGE_2 levels (60). We investigated whether there were any differences in mechanical behavior or grimace between males and females in response to PGE_2. While intraplantar injection of PGE_2 did not lead to any response to von Frey filaments in male mice at doses of 300 ng or 1 g (**Figure 7A**), it produced mechanical hypersensitivity in female mice (**Figure 7B**). We did not observe any significant grimacing behavior in male mice (**Figure 7C**) after PGE_2 injection. Female mice, however, displayed grimacing up to 60 min after PGE_2 injection (**Figure 7D**).

DISCUSSION

We used nociceptor TRAP sequencing to reveal sex differences in the translatomes of these neurons that are crucial for nociception. We reach several conclusions based on our work. Consistent with previous studies done at the whole transcriptome level for the DRG, most differences can be attributed to immune genes (36, 38, 61), a finding that may be important for chronic pain(15). At the translatome level, we observed differences in mRNAs bound by ribosomes in nociceptors between males and females, and many of these likely play an important role in the function of these cells. The nociceptor specificity of our TRAP-seq approach, did not identify similar sex differences at the transcriptome and translatome levels for autosomal genes, supporting the important role of translation regulation in nociception (62). We validated higher Ptgds expression in female neurons and found that this leads to a profound sex difference in behavioral response to PGD_2. We also found sex differences in behavioral responses to PGE_2 demonstrating that there is a fundamental divergence in prostaglandin signaling between males and females in relation to nociception.

There is a growing body of evidence of mechanistic sex differences in nociception and pain (13, 63, 64). Immune cells have been shown to play an important role in the development and resolution of chronic pain, many of them with sex-specific mechanisms (e.g. macrophages in male mice (65)), where different genes are up-regulated following injury in male and female mice (15). We identified non-neuronal genes with potential roles in inflammatory and immune responses that had differential baseline expression between males and females. These included genes that regulate T cell signaling, such as *Cxcl16* (up-regulated in females) and *Cd276* (up-regulated in males). These findings are consistent with previous literature showing sex differential roles of T cells in opioid analgesia (66) and where distinct proteins produced by T cells are up-regulated following spared nerve injury - PPARα in male mice and PPARγ in female mice (67). Interestingly, in the latter study, it was reported that T cells play a key role in driving neuropathic pain in females (67) whereas monocyte-associated genes such as toll-like receptor 4 (TLR4) contributed to neuropathic pain in males (67, 68).

While sex differences in immune contributions to pain have garnered extensive attention, differences at the neuronal level have not been found in some transcriptomic studies (36), and have not been examined in others (69). However, some recent studies examining specific neuronal populations have found sex differences (16), and some genes, such as the prolactin receptor, show sex differences in expression within neuronal populations (70). Using the TRAP technique, which we have previously used to characterize nociceptor translatome changes in neuropathic pain (22) and between ganglia (23), we have identified a number of sex differences in translation of mRNAs in DRG nociceptors. *Inpp5d* (Inositol polyphosphate-5-phosphatase D) was up-regulated in female nociceptors. This gene has been associated with dementia and Alzheimer's (71, 72), which are both more common in women (8). It would be of interest to investigate whether translation of this gene is also up-regulated in the brain of females. In contrast, *Apln* (Apelin), which is a neuroprotectant neuropeptide and anti-inflammatory protein, is up-regulated in male nociceptors and it has been reported as a promising target to treat Alzheimer's disease (73). We also identified several genes with sex differences in mRNA association with ribosomes at the baseline level that have been previously linked to pain (see table 4). For instance, genes in the MAPK cascade have been linked to inflammatory responses in sensory neurons (74), with *Map3k1* (mitogen-activated protein kinase kinase kinase 1) being up-regulated in a model of carrageenan-induced hyperalgesia (75). *Map3k1* was up-regulated in female nociceptors in our study while *Mapk1ip1* (mitogen-activated protein kinase 1 interacting protein 1) was up-

regulated in male nociceptors. These findings suggest that although MAPK signalling likely plays a key role in pain in both males and females (76), there may be underlying nuances in signalling that are sex-specific, such as the prominent role of p38 in pain signalling in males (77-79).

We saw little overlap in transcriptome differences in the DRG between males and females and nociceptor translatome differences between males and females. Some of the reason for this is almost certainly technical; however, this divergence might also be partially explained by sex differential translation regulation. This area of research has not garnered much attention but may be important for neuronal function given the key role of translation regulation in synaptic plasticity (80). In the context of pain, we recently demonstrated that female-selective translation of the prolactin receptor mRNA in nociceptors is a causative factor in the pain promoting effects of prolactin in female mice (18).

The increased abundance of *Ptgds* mRNA in the active translatome of female nociceptors intrigued us due to the well-known role of prostaglandins in pain signaling. A previous study showed higher Ptgds expression in the neonatal female brain (81) but we are not aware of any other reports of sex differences for this enzyme in neuronal tissue. Ptgds converts PGH_2 to PGD_2, which is one of the most abundant prostaglandins in the brain (41, 42, 82). PGD_2 is known to have important roles in the regulation of nociception (43, 83), temperature (53) and sleep (52). There is also evidence that Ptgds is involved in the transport of retinoids in the brain (84), thus playing essential roles in the nervous system. In behavioral experiments, inhibition of Ptgds caused robust pain behavior in male mice, while female mice showed effects only at high doses of inhibitor. Higher baseline levels of Ptgds and PGD_2 in female nociceptors likely explain these sex differences in response to AT-56. Previous studies have shown both anti- (46) (47) and pro-nociceptive roles of PGD_2 (48). We were not able to test the effect of AT-56 in pain models due to the effect of the drug alone. Interestingly, we also found higher levels of Ptgds protein in female cortex, indicating that our nociceptor findings may be generalizable to other neuronal populations.

Mice lacking the *Ptgds* gene from birth do not develop tactile pain following PGE_2 injection (43). This suggests an interaction between the *Ptgds* gene and PGE_2 that prompted us to look for sex differences in response to PGE_2. We found that female mice responded to lower doses of PGE_2 with mechanical hypersensitivity and grimacing than did male mice, reminiscent of similar recent findings with calcitonin gene-related peptide (85). This suggests a complex balance between PGD_2 and PGE_2 in nociceptive signaling that will take more work to fully understand. Nevertheless, these sex differences in prostaglandin signaling have implications for some of the most commonly used pain relievers – the cyclooxygenase (COX) inhibitors. Studies in rodent arthritis models have described reduced inflammation in COX isoform knockout mice in females but not males (86). In a study using an experimental pain model in healthy human subjects, ibuprofen produced analgesia in men but not women despite equal blood levels of the drug (60). Collectively, these results point to profound sex differences in prostaglandin signaling in the nervous system. Our findings of enhanced translation of *Ptgds* mRNA in the female nervous system can help to understand the molecular underpinnings of these differences better. Nearly a century after the discovery of prostaglandins (87), and centuries after humans started targeting them for pain, mechanistic sex differences in their actions are only beginning to come into focus. In our view, this is a testament to the dire need for consideration of sex as a biological variable in basic neuroscience research.

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Figures and legends
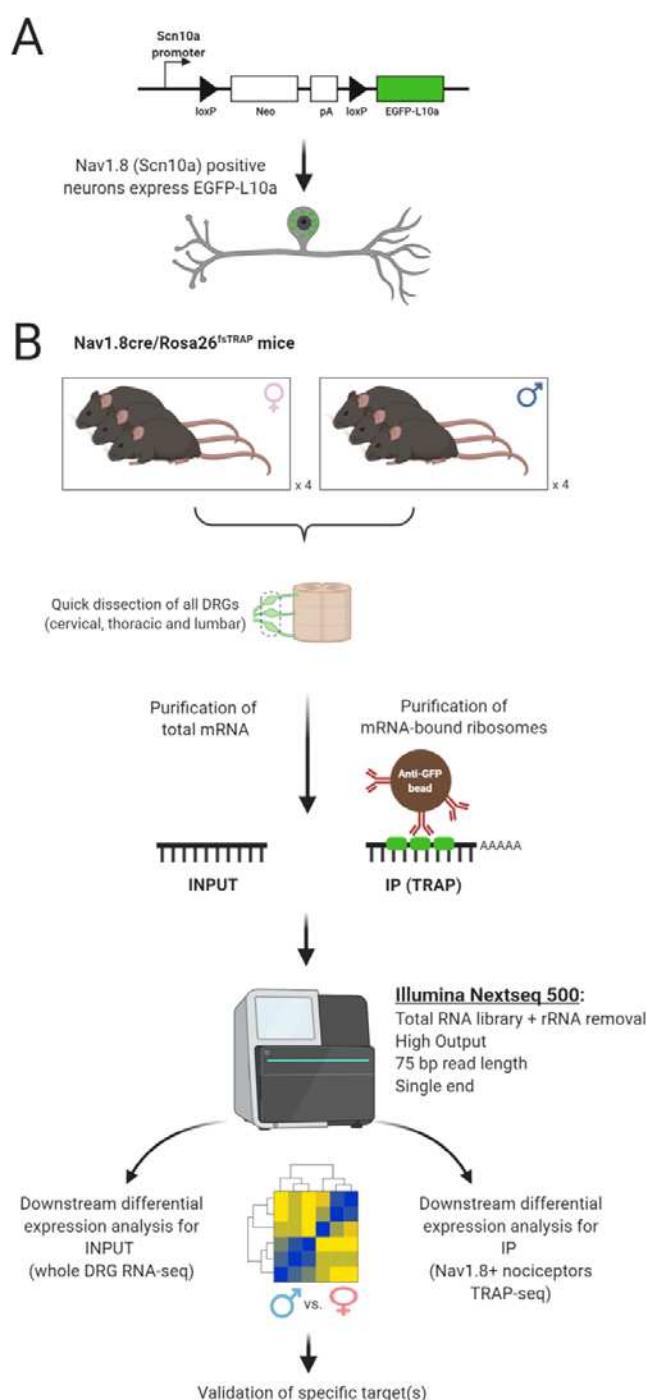
Figure 1: *Outline of workflow for TRAP sequencing to reveal sex differences in nociceptor translatomes.* **A**, eGFP-L10a protein is expressed in Nav1.8-positive nociceptors. **B**, Schematic representation of the methodology shows dissection of all DRGs (cervical, thoracic and lumbar) from Nav1.8cre/Rosa26fsTRAP mice followed by isolation of total RNA (INPUT), and mRNA-bound to the ribosome (IP) using anti-eGFP-coated beads; Samples were sequenced and processed for downstream analysis of differentially expressed genes as shown.
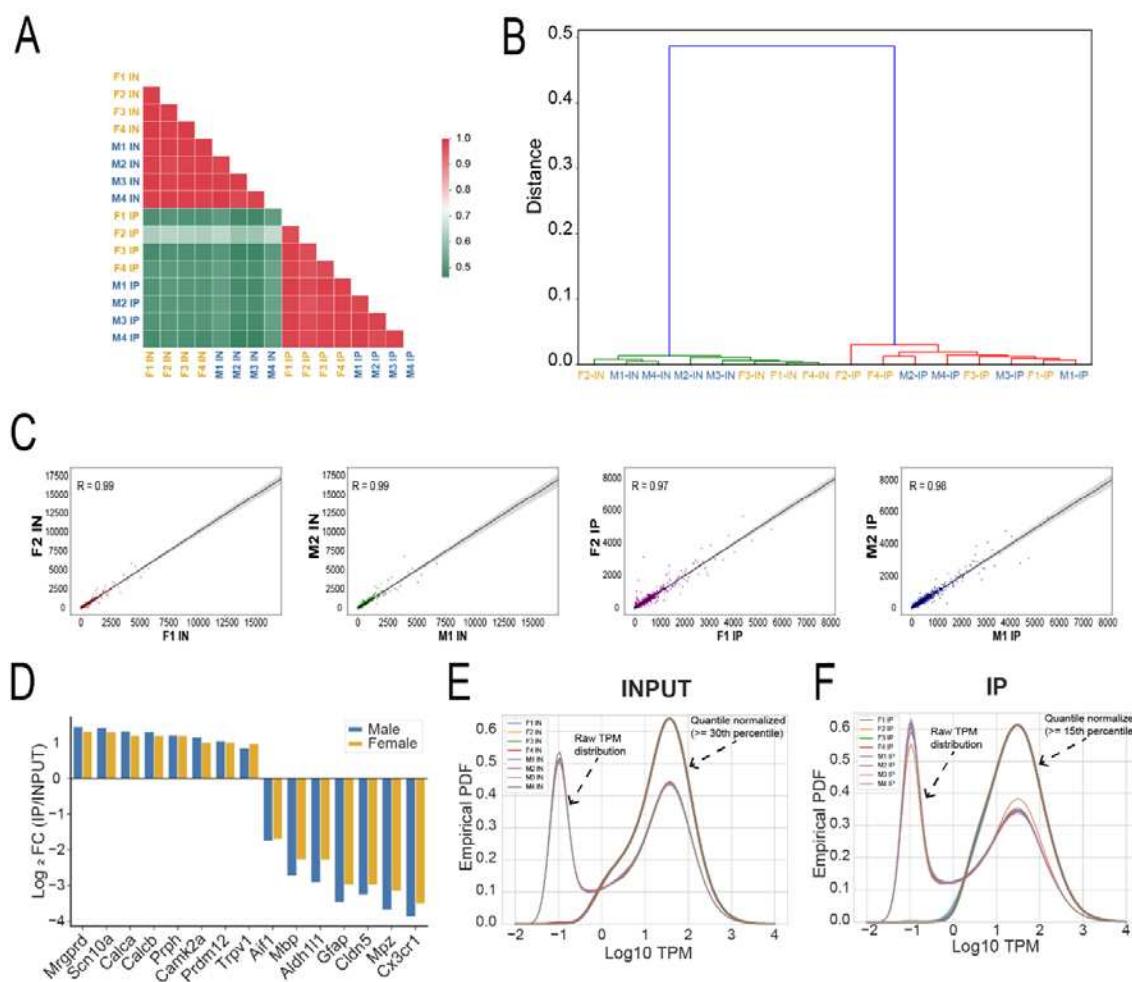
Figure 2: *Nociceptor TRAP sequencing quality control.* **A**, Hierarchical clustering analysis and **B**, Heatmap of the correlation coefficient show clear separation between TRAP-seq and bulk RNA-seq. However, we did not observe a clear distinction between male and female samples. **C**, Linear correlation plots shows high correlation coefficients of gene TPMs within biological replicates for the INPUT and IP fractions (shown for 2 replicates in each sex and assay), suggesting high reproducibility between replicates. **D**, Neuronal markers were enriched in IP fractions, such as *Calca* (encoding CGRP) or *Prph* (peripherin), while glial markers such as *Mbp*, *Mpz*, and *Gfap* were depleted (based on fold changes of median TPMs in each assay). **E, F**, The empirically estimated probability density of the raw TPMs and quantile normalized TPM (qTPM) distributions for the INPUT and IP fractions of all samples are shown. For INPUT samples, TPM distributions are shown for all coding genes and qTPMs shown for systematically transcriptome-expressed genes. For IP samples, TPM distributions were plotted for all coding genes, and qTPMs are shown for systematically translatome-expressed genes.

16
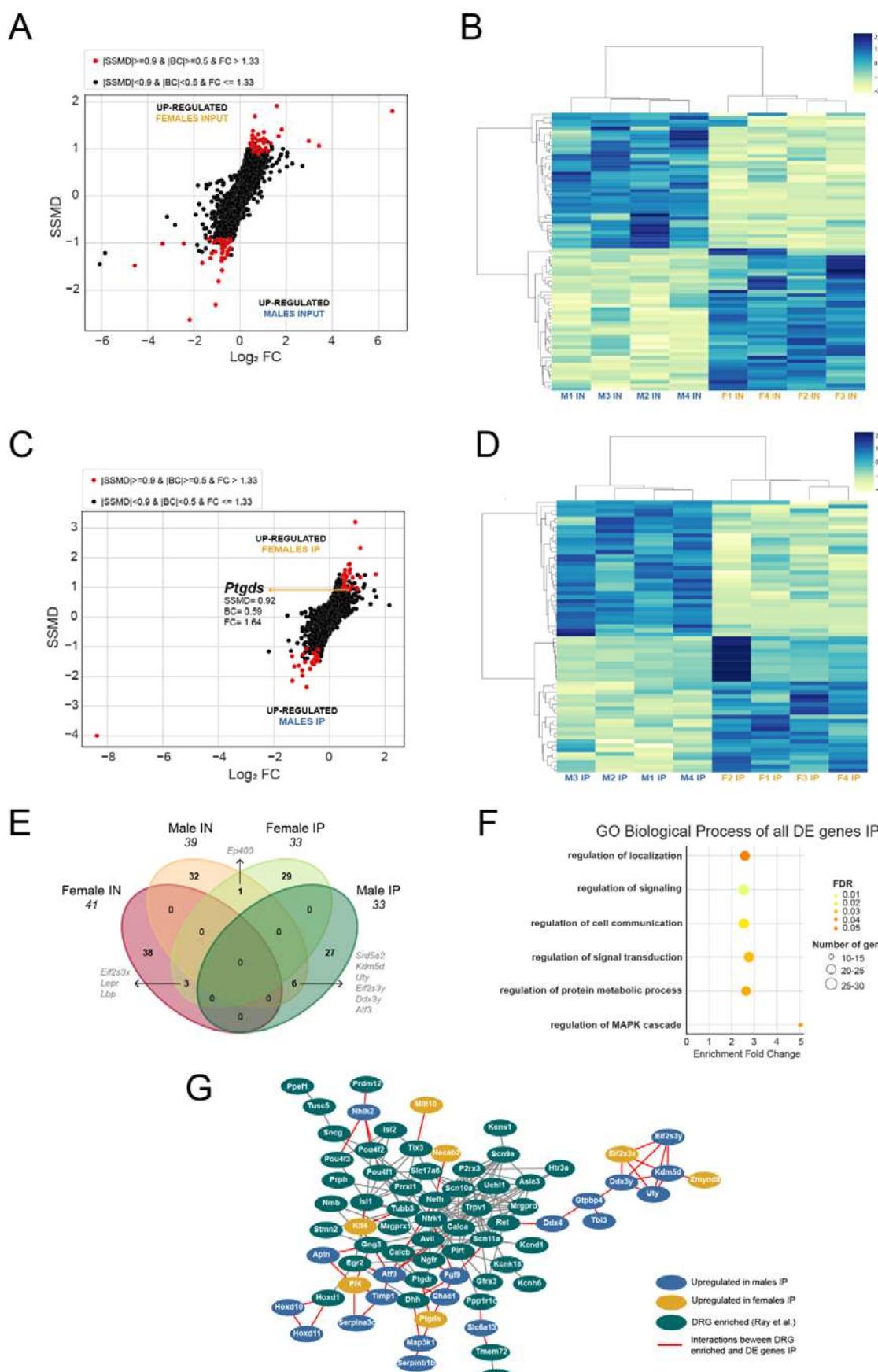
Figure 3: *Differentially translated mRNAs in male and female DRG nociceptors.* **A**, Dual-flashlight plot of INPUT samples showing SSMD and Log_2 fold change values for all autosomal genes on or above the 30th percentile. **B**, Heatmap shows the z-scores of the differentially expressed genes in INPUT (IN) samples. Labels represent sex and biological replicate number. **C**, Dual-flashlight plot of IP samples showing SSMD and Log_2 fold change values for all autosomal genes on or above the 15th percentile. **D**, Heatmap shows the z-scores of the differentially translated mRNAs in IP samples. **E**, Venn diagram comparing the genes identified as differentially expressed. There were few overlaps between INPUT and IP autosomal genes. **F**, GO terms enriched for all genes differentially expressed in IP. **G**, Network of interactions between genes differentially translated between males and females in IP and genes enriched in DRG neurons (Network generated using String database and Cytoscape).
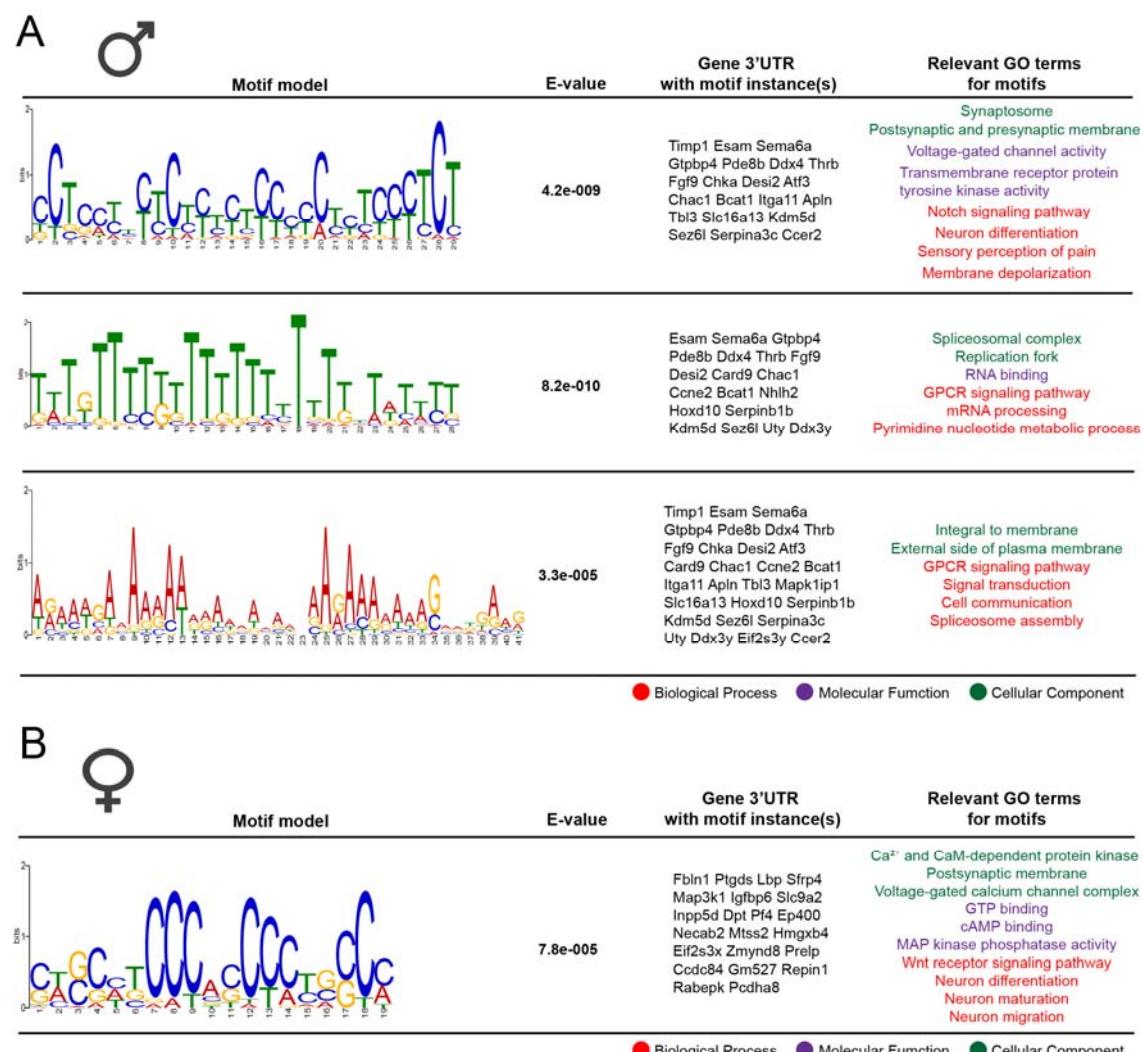
Figure 4: *Enriched motifs identified in the 5' or 3' UTRs of mRNAs differentially translated in males or females.* The motif analysis was conducted on the list of up-regulated mRNAs in both male and female IP fractions. **A**, We found 3 motifs significantly enriched in the 3' UTR of up-regulated male mRNAs compared to the female mRNAs. **B**, We identified one motif

significantly enriched in the 3' UTR of up-regulated female mRNAs compared to the male mRNAs.
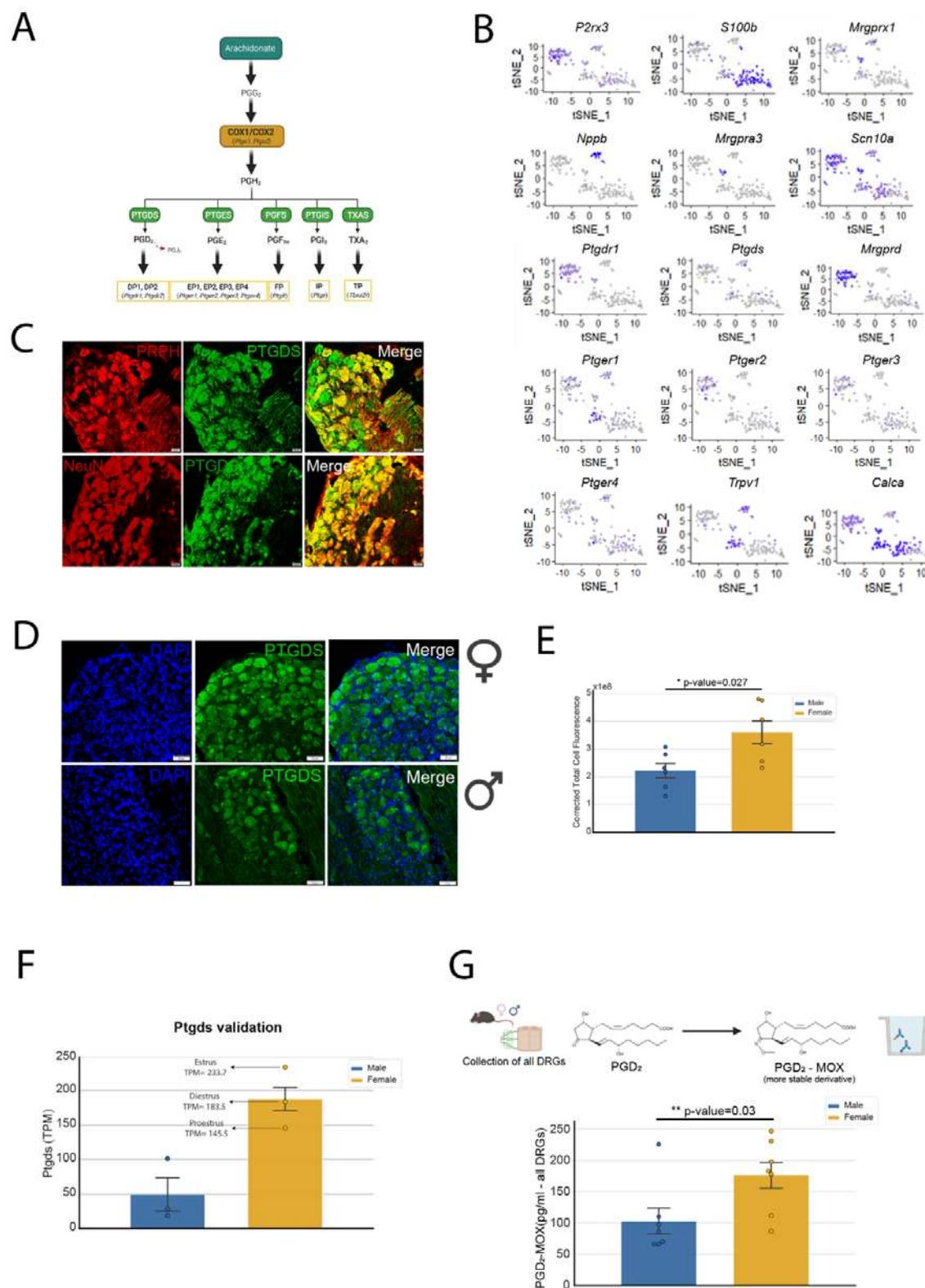
Figure 5: *Ptgds expression is higher in female DRGs and leads to higher production of PGD$_2$.* **A**, Ptgds converts PGH$_2$ to PGD$_2$, which has a very short half-life and is rapidly metabolized to PGJ$_2$. **B**, Single DRG neuron-sequencing shows that *Ptgds* and PGD$_2$ receptor DP1 (*Ptgdr1*) are expressed in neurons in the DRG. *Ptgds* is co-expressed with most neuronal markers, suggesting that it is expressed in all neurons; *Ptgdr1* is mostly expressed in non-peptidergic neurons (co-expressed with *P2rx3*); PGE$_2$ receptors (*Ptger1*, *Ptger2*, *Ptger3*, *Ptger4*) were expressed by most neuronal subtypes. **C**, We confirmed using IHC that Ptgds is expressed in neurons. **D**, **E**, We found that Ptgds has higher expression in female DRG neurons compared to male DRGs, at the protein level (Unpaired t-test, t = 2.584, df = 10, p-value = 0.0272). **F**, In a separate TRAP experiment, we monitored the female estrous cycle and validated that *Ptgds* is higher in females compared to females regardless of estrous cycle. **G**, PGD$_2$-MOX ELISA demonstrated that PGD$_2$ levels are higher in female DRGs (Unpaired t-test, t = 2.381, df = 12, p-value = 0.0347). Panel C scale bar = 20 µm; Panel D scale bar = 50 µm.
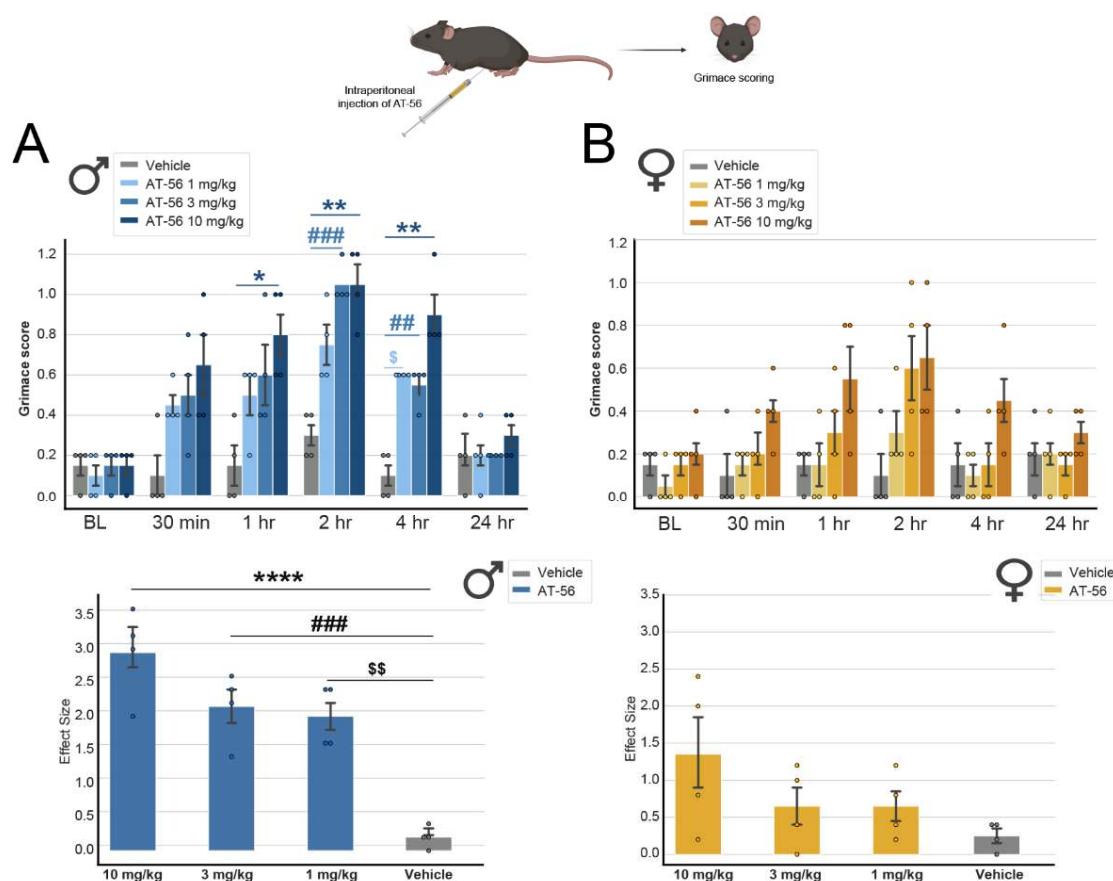
Figure 6: *Inhibition of Ptgds produces robust grimacing behavior in mice that is greater in males.* **A**, Intraperitoneal injection of AT-56, a selective inhibitor of Ptgds, led to grimacing behavior in male mice (Two-way ANOVA, F = 4.279, p-value<0.0001, post-hoc Sidak's, * Vehicle vs. AT-56 10 mg/kg male at 1h, p-value= 0.0314; ** Vehicle vs. AT-56 10 mg/kg male at 2h, p-value= 0.0071; *** Vehicle vs. AT-56 10 mg/kg male at 4h, p-value = 0.0068; ### Vehicle vs. AT-56 3 mg/kg male at 2h, p-value = 0.0004; ## Vehicle vs. AT-56 3 mg/kg male at 4h, p-value = 0.0068; $ Vehicle vs. AT-56 1 mg/kg male at 4h, p-value = 0.0193).

We also calculated the effect size (difference from the baseline) and observed a significant difference for the 3 doses of AT-56 compared to vehicle in males (One way-ANOVA, F=22.00, p-value<0.0001, post-hoc Tukey's; **** 10 mg/kg vs. Vehicle, p-value = <0.0001; **** 10 mg/kg vs. Vehicle p-value = <0.0001; ### 3 mg/kg vs. Vehicle p-value = 0.0006; $$ 1 mg/kg vs. Vehicle p-value= 0.0012). **B**, Grimacing behavior in female mice following AT-56 injection was not different from vehicle (Two-way ANOVA, F = 1.136, p-value = 0.3462). When calculating the effect size (difference from the baseline), we did not observe any significant differences between groups in female mice (Two-way ANOVA, F = 2.109, p-value = 0.1524).
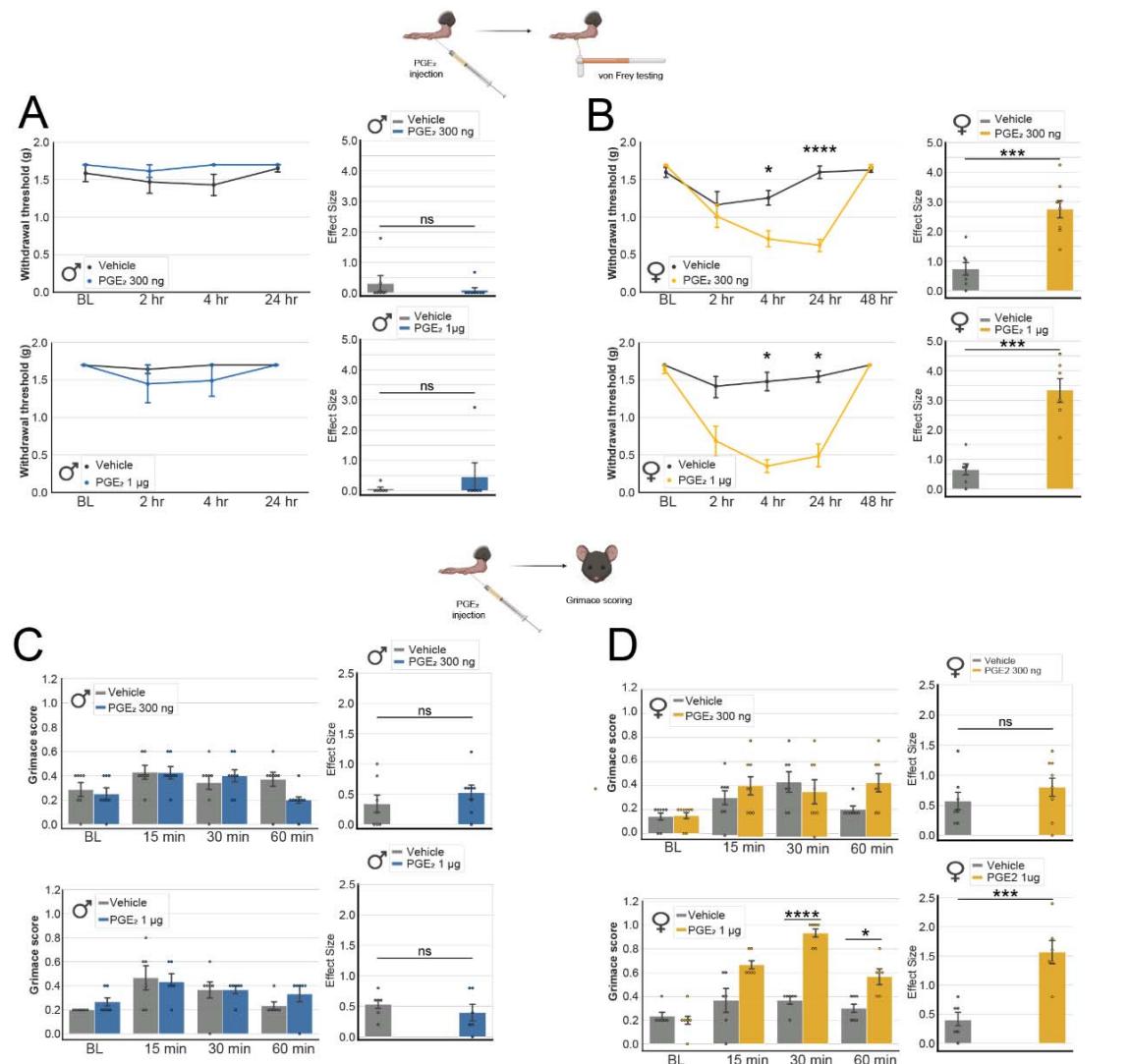
Figure 7: *Intraplantar administration of PGE$_2$ produces greater mechanical allodynia and grimacing in female mice.* **A**, Male mice did not respond to von Frey filaments after injection of 300 ng (Two-way ANOVA RM, F = 1.030, p-value = 0 .3900) or 1 µg (Two-way ANOVA RM, F = 0.7260, p-value = 0.5444) of PGE$_2$. When calculating the effect size we did not observe any statistical significant differences between groups in males (Effect size 300 ng: Unpaired t-test, t = 0.8756, df = 13, p-value = 0.3971; Effect size 1 µg: Unpaired t-test, t = 0.8709, df = 10, p-value = 0.4042). **B**, Female mice showed mechanical allodynia up to 24 hours after injection of both 300 ng of PGE$_2$ (Two-way ANOVA, F = 12.35, p-value <0.0001,

post-hoc Sidak's: * Vehicle - 300ng PGE_2 at 4h, p-value = 0.0161, **** Vehicle - 300ng PGE_2 at 24h, p-value <0.0001) and 1 μg of PGE_2 (Two-way ANOVA RM, F = 10.78, p-value <0.0001, post-hoc Sidak's: *** Vehicle - 1 μg PGE_2 at 4h, p-value = 0.0005, ** Vehicle - 1 μg PGE_2 at 24h, p-value = 0.0026). We also observed an effect size difference between groups in female mice (*** Effect size 300 ng: Unpaired t-test, t = 4.980, df = 13, p-value = 0.0003; *** Effect size 1 μg: Unpaired t-test, t = 5.569, df = 10, p-value = 0.0002). **C**, Male mice did not show any significant grimacing behaviors following administration of 300 ng of PGE_2 (Two-way ANOVA RM, F = 1.996, p-value = 0.1305). At 1 μg of PGE_2 we also did not observe any significant grimacing in male mice (Two-way ANOVA RM, F = 0.5376, p-value = 0.6601). Similarly, we also do not observe any effect size between groups in grimacing of male mice (Effect size 300 ng: Unpaired t-test, t = 0.9225, df = 13, p-value = 0.3731; Effect size 1 μg: Unpaired t-test, t = 0.8305, df = 10, p-value = 0.4257). **D**, Female mice exhibit robust grimacing after 1 μg PGE_2 injection (Two-way ANOVA RM, F = 11.34, p-value <0.0001, post-hoc Sidak's: **** Vehicle - 1ug PGE_2 at 30 min, p-value<0.0001, * Vehicle - 1ug PGE_2 at 60 min, p-value = 0.0257) but not at 300 ng (Two-way ANOVA RM, F = 1.197, p-value = 0.1303). We also observed an effect size in the grimacing scores between 1 μg PGE_2 and vehicle grimacing in female mice (Effect size 300 ng: Unpaired t-test, t = 0.9458, df = 13, p-value = 0.3615; *** Effect size 1 μg: Unpaired t-test, t = 4.669, df = 10, p-value = 0.0009).

A

Scn10a promoter

loxP — Neo — pA — loxP — EGFP-L10a

Nav1.8 (Scn10a) positive neurons express EGFP-L10a

B

Nav1.8cre/Rosa26^hTRAP mice

♀ x 4   ♂ x 4

Quick dissection of all DRGs (cervical, thoracic and lumbar)
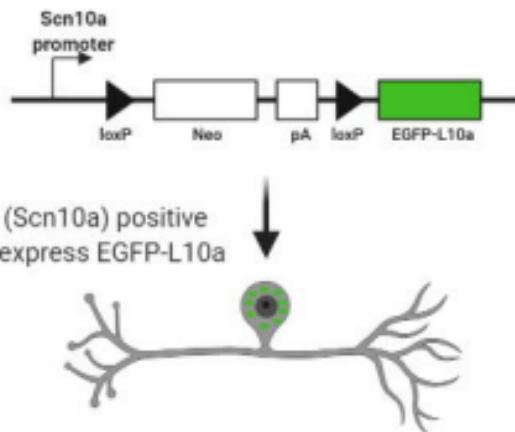
Purification of total mRNA

Purification of mRNA-bound ribosomes

Anti-GFP bead

AAAAA

INPUT

IP (TRAP)

Illumina Nextseq 500:
Total RNA library + rRNA removal
High Output
75 bp read length
Single end

Downstream differential expression analysis for INPUT (whole DRG RNA-seq)

♂ vs. ♀
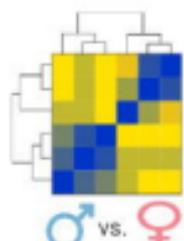
Downstream differential expression analysis for IP (Nav1.8+ nociceptors TRAP-seq)

Validation of specific target(s)

A

Motif model	E-value	Gene 3'UTR with motif instance(s)	Relevant GO terms for motifs
♂	4.2e-009	Timp1 Esam Sema6a Gtpbp4 Pde8b Ddx4 Thrb Fgf9 Chka Desi2 Atf3 Chac1 Bcat1 Itga11 Apln Tbl3 Slc16a13 Kdm5d Sez6l Serpina3c Ccer2	Synaptosome Postsynaptic and presynaptic membrane Voltage-gated channel activity Transmembrane receptor protein tyrosine kinase activity Notch signaling pathway Neuron differentiation Sensory perception of pain Membrane depolarization
	8.2e-010	Esam Sema6a Gtpbp4 Pde8b Ddx4 Thrb Fgf9 Desi2 Card9 Chac1 Ccne2 Bcat1 Nhlh2 Hoxd10 Serpinb1b Kdm5d Sez6l Uty Ddx3y	Spliceosomal complex Replication fork RNA binding GPCR signaling pathway mRNA processing Pyrimidine nucleotide metabolic process
	3.3e-005	Timp1 Esam Sema6a Gtpbp4 Pde8b Ddx4 Thrb Fgf9 Chka Desi2 Atf3 Card9 Chac1 Ccne2 Bcat1 Itga11 Apln Tbl3 Mapk1ip1 Slc16a13 Hoxd10 Serpinb1b Kdm5d Sez6l Serpina3c Uty Ddx3y Elf2s3y Ccer2	Integral to membrane External side of plasma membrane GPCR signaling pathway Signal transduction Cell communication Spliceosome assembly

● Biological Process   ● Molecular Function   ● Cellular Component

B

Motif model	E-value	Gene 3'UTR with motif instance(s)	Relevant GO terms for motifs
♀	7.8e-005	Fbln1 Ptgds Lbp Sfrp4 Map3k1 Igfbp6 Slc9a2 Inpp5d Dpt Pf4 Ep400 Necab2 Mtss2 Hmgxb4 Eif2s3x Zmynd8 Prelp Ccdc84 Gm527 Repin1 Rabepk Pdha8	Ca²⁺ and CaM-dependent protein kinase Postsynaptic membrane Voltage-gated calcium channel complex GTP binding cAMP binding MAP kinase phosphatase activity Wnt receptor signaling pathway Neuron differentiation Neuron maturation Neuron migration

● Biological Process   ● Molecular Function   ● Cellular Component

A

B

P2rx3 *S100b* *Mrgprx1*

Nppb *Mrgpra3* *Scn10a*

Ptgdr1 *Ptgds* *Mrgprd*

Ptger1 *Ptger2* *Ptger3*

Ptger4 *Trpv1* *Calca*

C

PRPH PTGDS Merge

NeuN PTGDS Merge

D

DAPI PTGDS Merge ♀

DAPI PTGDS Merge ♂

E

* p-value=0.027

F

Ptgds validation

G

** p-value=0.03