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**Sex Differences in the Efficacy of Combined IL-10 and IL-10R1 Gene Therapy for
Neuropathic Pain in Mice**

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Abstract

Neuropathic pain is a debilitating condition that affects millions of Americans. Despite decades of research, the disease is refractory to current treatments in a substantial population of patients. Recent advances in the understanding of neuropathic pain have led to the targeting of glial cells to better control the condition. Current clinical therapies do not manage the inflammatory response that originates with glial cells and contributes to this affliction. IL-10 is an anti-inflammatory cytokine that attenuates the inflammatory response that occurs at the neuroimmune interface in neuropathic pain models. IL-10 gene therapy has alleviated neuropathic pain in a number of animal studies. We tested whether gene therapy increasing the expression of IL-10 and IL-10R1 could attenuate allodynia in a chronic constriction injury (CCI) model of neuropathic pain. Our experiments did not produce reversal of neuropathic pain using the combined gene therapy. However, the CCI surgeries did not produce neuropathic pain in the male mice. While the female mice developed neuropathic pain, the expression of plasmid genes could not be definitively confirmed. Therefore, while the present study did not provide convincing evidence that the combined gene therapy could alleviate neuropathic pain, additional experiments should be performed to further investigate the potential of combined IL-10/IL-10R1 gene therapy for combating neuropathic pain and to investigate the sex differences in the biochemical pathways underlying neuropathic pain.

Introduction

Neuropathic pain is a debilitating illness that affects a large portion of the world population. It arises as a direct consequence of a lesion or disease affecting the somatosensory system (Treede et al., 2008). Studies have reported variable numbers for the prevalence of neuropathic pain, ranging from 1.5% to 8% (Taylor, 2006; Torrance et al., 2006; Bouhassira et al., 2008). Regardless of where the prevalence falls within this range, millions of Americans and millions more worldwide are affected. Neuropathic pain is expensive to manage and significantly decreases the quality of life of afflicted patients (Taylor, 2006).

Intrathecal delivery of IL-10 gene therapy has shown promise in alleviating neuropathic pain in an animal model of neuropathic pain in several studies (Milligan et al., 2005a, 2005b, 2006a, 2006b; Ledebuer et al., 2007; Sloane et al., 2009a, 2009b; Soderquist et al., 2010). We hypothesized that overexpressing IL-10R1, part of the IL-10 receptor, could enhance the effectiveness of IL-10 gene therapy. To test this hypothesis, we acquired an AAV9 viral vector that leads to overexpression of the IL-10 receptor (IL-10R1). This technique extends the effective duration of the pain relief (Figure 1). In addition, a recent report suggests that chronic pain develops by different cellular mechanisms in males and females (Sorge et al., 2015). This is of particular concern because the studies of IL-10 gene therapy have been conducted almost exclusively in male animals (Kwilasz et al., 2014), but the majority of chronic pain patients are female (Torrance et al., 2006). My work compared the efficacy of combined IL-10 and IL-10R1 gene therapy in both male and female mice to determine whether over-expression of the IL-10 receptor could create a longer-lasting reversal of allodynia than treatment with IL-10 gene therapy alone.

Aims

1. Compare the effects of combined IL-10 and IL-10R1 gene therapy, as compared to each gene therapy technique in isolation, to determine if the combined therapy creates a longer duration anti-allodynia.
2. Compare the anti-allodynic effects of IL-10 gene therapy techniques in male and female mice.
3. Quantify expression of IL-10, IL-10R1, P2X4, and neuroinflammatory markers at different time points after surgery to determine the effects of gene therapy treatments on mRNA expression.

Background

Neuropathic Pain

Neuropathic pain is a debilitating condition that affects a large portion of the world population. It arises as a direct consequence of a lesion or disease affecting the somatosensory system (Treede et al., 2008). A conservative estimate by Taylor (Taylor, 2006) predicts that the prevalence of neuropathic pain is 1.5%; studies in France and Britain have arrived at higher estimates of 7-8% of the population (Torrance et al., 2006; Bouhassira et al., 2008). Regardless of the exact number, millions of Americans suffer from neuropathic pain, presenting a significant clinical challenge. Despite years of research, current therapeutics do not effectively manage neuropathic pain in a substantial population of patients. Notably, all currently approved therapeutics focus on regulating neuronal processes. However, recent evidence has implicated glial cells, specifically microglia and astrocytes, as the major players in the induction and maintenance of neuropathic pain (Watkins et al., 2001; Watkins and Maier, 2003). Specifically, inflammation has been implicated as a key mechanism underlying the condition, and glial cells

play an important role in generating the inflammatory response (Grace et al., 2014). Taken together, these findings make the inhibiting the inflammatory response produced by glial cells a key target for drug discovery.

Neuroimmune Signaling

The neuroimmune signaling generated by activated glial cells plays a key role in initiating and maintaining neuropathic pain (Grace et al., 2014). Three key cytokines among the myriad of pro-inflammatory substances that are released are tumor necrosis factor (TNF), interleukin-1 (IL-1), and interleukin 6 (IL-6). The neuroimmune interface is extremely complex, with a number of parallel and redundant mechanisms that can enhance pain signals. Two general mechanisms contribute to the neuroimmune enhancement of pain: the enhancement of excitatory synaptic transmissions, and the attenuation of inhibitory synaptic messages (Grace et al., 2014). Within each of these broad categories, multiple distinct pathways exist that can each enhance pain signaling and contribute to neuropathic pain (Grace et al., 2014). Pharmacological substances exist which can inhibit the actions of IL-1 and TNF (Watkins and Maier, 2003, 2004). However, blocking the actions of single pro-inflammatory cytokines is not a viable clinical approach. One issue is that these cytokines perform redundant functions. For example, if IL-1 is pharmacologically inhibited, TNF can perform the same biochemical actions as IL-1 (Watkins and Maier, 2003; Grace et al., 2014). Additionally, the potential effectiveness of inhibiting these molecules is limited by the fact that they perform important protective functions in the neuroimmune interface (Grace et al., 2014). Specifically, both TNF and IL-1 promote neuronal regeneration in different parts of the nervous system (Fontaine et al., 2002; Nadeau et al., 2011). Taken in combination, the presence of redundant mechanisms and the beneficial effects of pro-

inflammatory cytokines in certain situations disqualifies the inhibition of any specific substance as a viable therapeutic method for neuropathic pain. Instead of inhibiting one or more pro-inflammatory cytokines, introducing a cytokine with broad-spectrum anti-inflammatory properties presents an alternate approach (Watkins and Maier, 2003; Grace et al., 2014).

IL-10

A promising candidate for attenuating neuropathic pain is the anti-inflammatory cytokine IL-10 (Watkins and Maier, 2003, 2004). IL-10 performs potent anti-inflammatory actions. Neurons, astrocytes, and microglial cells in the CNS express IL-10. Activation of the IL-10 receptor has been shown to inhibit the release of numerous pro-inflammatory cytokines, including TNF, IL-1 β , and IL-6 (Kwilasz et al., 2014). Additionally, IL-10 receptor activation stimulates the release of factors which mediate pro-inflammatory cytokines, including IL-1 receptor antagonist (Kwilasz et al., 2014). IL-10 exhibits neuroprotective properties through multiple mechanisms. Its inhibition of microglial secretion of pro-inflammatory cytokines increases excitatory amino acid transporter-2 (EAAT2) expression. This, in turn, reduces the buildup of excitatory amino acids and consequently prevents the neurotoxic effects of glutamate buildup (Bachis et al., 2001; Kim et al., 2011). IL-10 also appears to directly promote neuronal survival. Specific subsets of neurons display IL-10 receptors, and the activation of these receptors can promote neuron survival (Boyd et al., 2003). Taken together, its neuroprotective properties and broad-spectrum anti-inflammatory effects make IL-10 a promising candidate for treating neuropathic pain.

IL-10 Gene therapy

IL-10 shows promise as a clinical therapeutic because it has been shown to suppress the production and the actions of all pro-inflammatory cytokines (Moore et al., 2001). However, IL-10 protein has a very short half-life, on the order of 2-3 hours (Milligan et al., 2005b). Injections of IL-10 protein reverse neuropathic pain in the CCI model, but the duration of the effect is similar to the half-life of the protein (Milligan et al., 2005b). As a result, the clinical administration of IL-10 protein would require a chronic indwelling subdural catheter, which makes the therapy unacceptably risky in humans (Watkins and Maier, 2003). However, methods that can enhance expression of IL-10 for longer periods of time could have clinical significance. In order to achieve such an effect, gene therapy techniques have been employed. Some of the first evidence of IL-10's effectiveness came from using adenovirus vectors and adeno-associated viral vectors to increase IL-10 expression (Milligan et al., 2005a, 2005b). These gene therapy techniques stimulated IL-10 production in the meninges, leading to prolonged expression of the protein in the lumbosacral space (Milligan et al., 2005a, 2005b). The analgesic effects of the gene therapy lasted for approximately two weeks, which represented a much longer time period than injection of the IL-10 protein. Subsequent developments included the use of naked plasmid DNA, which produced analgesic effects lasting for several weeks after two closely timed injections (Milligan et al., 2006a). Further improvements were achieved by chemically engineering poly (lactic-co-glycolic-acid) (PLGA) microparticles with encapsulated naked plasmid DNA coding for IL-10. These PLGA microparticles made it possible to produce a long-lasting analgesic effect from a single intrathecal injection (Soderquist et al., 2010). Most recently, Dengler et al. discovered that adding D-mannose to the intrathecal injections increases the potency of gene therapy when naked plasmid is administered by increasing cellular uptake of the plasmid (Dengler et al., 2014).

Using current techniques, IL-10 gene therapy can alleviate neuropathic pain for periods of several weeks. We postulated that the analgesic effect of the gene therapy could be enhanced by overexpressing the receptor for IL-10 to enhance the effects of endogenous IL-10. To test this hypothesis, a gene therapy expressing IL-10R1 was developed. We also hypothesized that combined IL-10 and IL-10R1 gene therapy would extend the duration of the attenuation of neuropathic pain as compared to either gene therapy in isolation.

Sex Differences in Neuroinflammation

Chronic pain affects women at a higher rate than men (Ruau et al., 2012). Historically, the vast majority of pain-related basic science research has been conducted in male rodents. Worries about confounding variables in female animals cause this phenomenon (Mogil and Chanda, 2005). However, studies have shown that, in humans, males and females process pain differently (Zubieta et al., 2002; Mogil et al., 2003). Furthermore, a recent study demonstrated that female mice develop mechanical allodynia without the contributions of microglial cells (Sorge et al., 2015). Specifically, they showed that although similar microgliosis occurs in both sexes, as measured by increased Iba1 expression, inhibition of microglia using minocycline reversed neuropathic pain in male but not female mice (Sorge et al., 2015). Using pharmacological inhibitors of various substances, it was shown that the activity of P2X4, BDNF, and p38 MAPK all play important roles in maintaining neuropathic pain in males but not females (Sorge et al., 2015). This was corroborated by a study that found p38 MAPK inhibitors could reverse neuropathic pain in male but not female mice (Taves et al., 2015). Taken together, these data suggest that microglial activation in and of itself does not necessarily contribute to neuropathic pain in both sexes. Rather, certain biochemical pathways in microglial cells appear

to play a key role in neuropathic pain in male but not female rodents. As such, the efficacy of IL-10/IL-10R1 gene therapy techniques should be tested in both males and females to ensure that they are effective in both sexes. Therefore, we investigated whether female mice displayed the same responses to these gene therapies as male mice by collecting tissue from both sexes and comparing behavioral results.

Methods

Animals

Pathogen-free adult male and female C57BL/6 mice (Jackson, Bar Harbor, ME) were used in all experiments. Mice were housed in rooms that are temperature-controlled (23 +/- 3 °C) and light-controlled (12 hour light/dark cycle; lights on at 0700 h) and provided with standard rodent food and water *ad libitum*. All procedures were approved by the Institutional Animal Care and Use Committee at the University of Colorado at Boulder.

CCI Surgery

Following baseline behavioral assessment, the surgical procedure for chronic constriction of the sciatic nerve was completed as previously described (Bennett and Xie, 1988), but modified for mouse (Ramer et al., 1997; Murphy et al., 1999). Briefly, in isoflurane-(2.5% vol) anesthetized mice, the mid-to-lower back and the dorsal left thigh were shaved and cleaned with 75% ethanol. Using aseptic procedures, the sciatic nerve was carefully isolated, and loosely ligated with 3 segments of 5-0 chromic gut sutures (Ethicon, Somerville, NJ). The overlying muscle was sutured closed with 4-0 sterile silk suture (Ethicon, Somerville, NJ), and animals awoke from anesthesia within approximately 5 minutes.

Plasmid vectors

Plasmid DNA encoding human IL-10 or human IL-10R1 genes in an AAV9 vector was prepared by Virovek (Hayward, CA).

Intrathecal Injection

In mice with intrathecal injections, all drugs were administered via acute i.t. catheter placement. Injections were performed via a lumbar puncture in between L5 and L6. Briefly, an ‘injection catheter’ made from a 27-gauge needle with the plastic hub removed was fitted into polyethylene (PE) 20 tubing, and the needle portion of another 27-gauge needle was inserted at the other end, with the hub of this needle connected to a 10 μ l Hamilton syringe, resembling the ‘injection catheter’ previously described (Milligan et al., 2005a, 2005b), but modified for use in mouse. Gene therapy or vehicle was injected. Each mouse received an injection of an equal volume (8 μ L) of empty viral vector injection (1.2×10^9 vg empty vector), IL-10 vector (6×10^9 vg), IL-10R1 vector (6×10^9 vg), or both gene therapies together (6×10^9 vg IL-10 and 6×10^9 vg IL-10R1). The selected solution was drawn into the injection catheter, and the tip of the 27-gauge needle gently inserted in between L5-L6. During this time, light leg twitching and a tail flick indicated successful i.t. catheter placement. Gene therapy or vehicle was injected during a 5 second interval. Treatment was randomly assigned to animals. Upon completion of injection, the 27-gauge needle was removed. A 100% motor recovery rate was observed from this injection procedure.

Von Frey Behavioral Testing

Baseline responses to light mechanical touch was assessed using the von Frey test after animals were habituated to the testing environment, as described elsewhere (Murphy et al., 1999). Briefly, mice were placed atop 2 mm-thick parallel bars covered with a wire mesh screen with spaces 1 mm apart and habituated for approximately 1 hour per day for 3 days. Mice were unrestrained, and were singly placed under plastic cups. All behavioral testing was performed during the first half of the light cycle in a sound, light, and temperature controlled room. The von Frey test utilizes a series of calibrated monofilaments, (2.83 – 4.31 log stimulus intensity; North Coast Medical, Morgan Hills, CA) applied randomly to the left and right plantar surface of the hindpaw for 3 seconds. Lifting, licking or shaking the paw were be considered a response. For all behavioral testing, threshold assessment was performed in a blinded fashion by J.L.B.

Tissue Collection and PCR

Seven, fourteen, and thirty-five days after i.t. injections, mice were deeply anesthetized with isoflurane (5%), transcardially perfused with 0.9% saline and the left dorsal horn and dorsal root ganglia dissected. cDNA amplification was performed using Quantitect SYBR Green PCR kit (Qiagen, Valenica, CA) in Hard-Shell 96-well, thin-wall PCR plates (Bio-Rad, Hercules, CA) on a CFX96 Real-Time PCR Detection System (Bio-Rad). Each sample was measured in duplicate using the CFX96 Real-Time PCR Detection System (Bio-Rad). Threshold for detection of PCR product was set in the log–linear phase of amplification and the threshold cycle (C_T) was determined for each reaction. The level of the target mRNA was quantified relative to the housekeeping gene (GAPDH) using the DDCT method (Livak and Schmittgen, 2001). GAPDH did not differ significantly between treatments.

Experimental Design – Calibration Tests

Calibration tests were performed to verify the pipetting accuracy and von Frey threshold accuracy of the primary experimenter (J.L.B.). For the pipetting tests, a P20 pipette was used to pipette water onto a high-precision scale (Denver Instrument, Bohemia, NY). Water was added in 5 μL increments from 5 to 20 μL . The mass of the water (in mg) was recorded for each trial. Three measurements were performed at each volume by J.L.B. and an experienced laboratory technician (T.F.).

Another experiment was performed to verify the accuracy of the von Frey testing results obtained by J.L.B. For this verification, two mice received CCI surgeries. Von Frey testing was performed by both J.L.B. and a Research Assistant Professor (P.G.) with extensive experience conducting behavioral testing sixteen days after surgery. The testing was performed individually by both experimenters to ensure blinded results.

Experimental Design – Behavior

Thirty-six mice (24 females and 12 males) were used in the behavioral experiment. Following CCI, the mice were randomly assigned into four different groups of equal sizes ($n=6$ for female mice, $n=3$ for male mice). The mice were handled and habituated as described above. Each mouse was tested three times to determine a baseline pain threshold prior to surgery. The mice then received CCI surgeries. Seven days later the pain threshold was assessed by von Frey testing. That same day, the groups were randomly assigned to one of the four possible treatments and injected intrathecally with viral vector as described above. Following i.t. injection of the viral vector, von Frey testing was performed twice per week on each mouse for 60 days. Males were tested before females and the testing apparatus was thoroughly cleaned with 70% ethanol between each trial to avoid behavioral effects of pheromone exposure.

Experimental Design – Tissue Collection

In total, 192 mice (96 males, 96 females) were used in experiments for tissue collection. These mice followed a similar treatment protocol to that of the behavioral study. Briefly, the mice were handled prior to experimentation. Initially, 12 mice (6 males and 6 females) were euthanized as described above and tissue was collected as described above to provide baseline mRNA levels in naïve animals. Each remaining mouse received CCI surgery. One week later, the mice were assigned to one of five groups, with each group consisting of equal numbers of males and females (n=18 per sex per treatment). One group did not receive any i.t. injection. Each of the remaining four groups received i.t. injection of one of the treatments described above (empty vector, IL-10 gene therapy, IL-10R1 gene therapy, or both gene therapies). Tissue collection was performed at three distinct time points, in addition to the naïve tissue collection at day 0. These time points were day 7, day 14, and day 35. At each time point, tissue was harvested from six mice of each sex in each of the five treatment groups (n=6 of each sex in each treatment group at each timepoint). All tissue was processed as described above and analyzed by quantitative real-time PCR.

Experimental Design – Viral Vector Verification

In order to verify the efficacy of the viral vector, nine mice were used. The mice were divided into three groups (n = 3 per group). All mice received CCI surgery. Seven days later, each group received an intrathecal injection of either empty viral vector, IL-10 gene therapy, or IL-10R1 gene therapy. The mice were sacrificed seven days after receiving the gene therapy, and the left dorsal horn and L4/L5 DRGs were harvested. The tissue was processed by PCR as

described above and expression of hIL-10 and hIL-10R1 were measured.

Primers

All primers spanned exon-intron boundaries to ensure that genomic DNA did not interfere with experimental results. Primers were designed by either Dr. Matthew Frank or Dr. Peter Grace at the University of Colorado – Boulder.

Gene	Sequence
GAPDH (ei)	F: GGAGAAACCTGCCAAGTATG R: GTCATTGAGAGCAATGCCAG
IL-10 (ei) (mouse)	F: GGACTTTAAGGGTACTTGG R: TCACCCAGGGAATTCAAATG
IL-10 (ei) (human)	F: GGACTTTAAGGGTTACCTGG R: AAGAAATCGATGACAGCGCC
IL-10R1 (ei) (mouse)	F: CTAAAGAATGCAACCAAGAG R: TTGGTCACAGTGAAATACTG
IL-10R1 (ei) (human)	F: CCGGGAACTTCACGTTAC R: CACGGTGAAATACTGCCTGG
IL1 β (ei)	F: TGCTGTCGGACCCATATGAG R: ATCCACACTCTCCAGCTGCA
I κ B α (NF- κ BIA) (ei)	F: CACCAACTACAATGGCCACA R: GCTCCTGAGCGTTGACATCA
TNF α (ei)	F: CCCTCACACTCAGATCATCT R: TGTCCTTTGAGATCCATGCCG

Statistics

GraphPad Prism Version 6 (San Diego, CA) was used to calculate statistics and prepare graphs. Statistical analysis differed based on the experiment type. Methods utilized included two-way ANOVA with repeated measures, one-way ANOVA with repeated measures, and paired t-tests. Holm-Sidak *post hoc* tests were used in all analyses that required *post hoc* testing. Specifics regarding the statistical tests are indicated in the results. Data are presented as mean \pm SEM.

Results

Pilot Data

A study performed prior to the initiation of this thesis treated three groups of mice with AAV9 viral vector. One group received IL-10R1 gene therapy alone, the second group received IL-10 gene therapy, and the final group received a combined gene therapy with both IL-10R1 and IL-10 viral vectors. Analysis of the results by two-way ANOVA with repeated measures revealed a significant effect of time and the interaction (time x treatment), but not of treatment (Time: $F_{12, 180} = 14.15$, $P < 0.0001$, treatment: $F_{2, 15} = 2.419$, $P = 0.1228$, time x treatment: $F_{24, 180} = 3.505$, $P < 0.0001$). Following injection, the group that received the combined gene therapy displayed a longer reversal of allodynia than the group that received IL-10R1 gene therapy alone, as indicated by higher withdrawal thresholds at days 37, 42, 45, and 49 (Figure 1). Additionally, although the IL-10 gene therapy data ended at day 45, the mice receiving the combined gene therapy showed significantly less neuropathic pain at that time point (Figure 1). These results motivated the studies in this thesis.

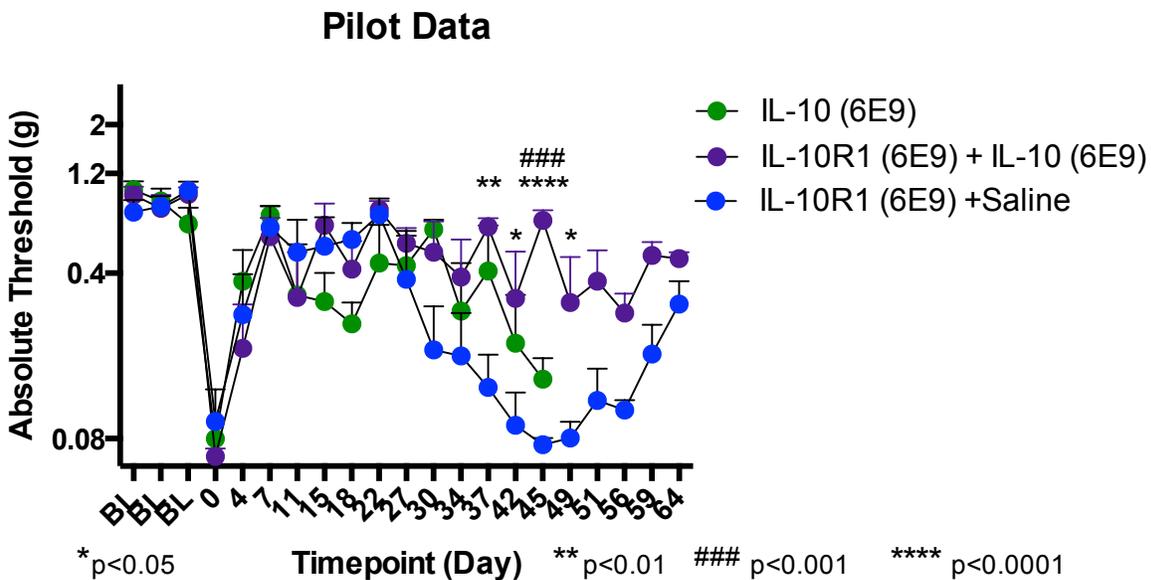


Figure 1. Following CCI surgeries, mice were injected with IL-10 gene therapy alone, IL-10R1 gene therapy alone, or combined IL-10 and IL-10R1 gene therapy (n=6 per group). Mice that received the combined gene therapy displayed a longer-lasting reversal of allodynia than those receiving IL-10R1 gene therapy alone. Withdrawal thresholds for mice receiving the combined treatment were significantly higher than thresholds of mice receiving IL-10R1 alone at days 37, 42, 45, and 49. Additionally, mice receiving the combined gene therapy displayed higher withdrawal thresholds than those receiving IL-10 gene therapy alone at day 45. *: significant difference between IL-10R1 group and combined gene therapy group; #: significant difference between IL-10 group and combined gene therapy group.

Calibration Tests

In order to verify that the pipetting skills of the main experimenter (J.L.B.) were accurate, a pipetting calibration test was performed. J.L.B. pipetted volumes from 5-20 mL onto a high precision scale using a P20 pipette. Another laboratory technician (T.F.) pipetted the same volumes onto the same scale. Paired t-test revealed no significant differences existed between J.L.B. and T.F. ($P > 0.9999$), indicating that J.L.B. pipetted accurately (Figure 2).

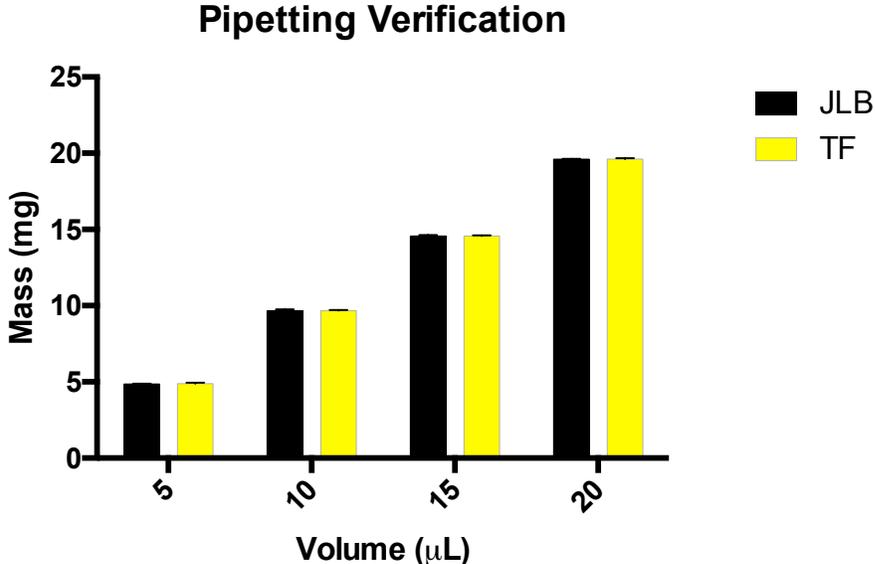


Figure 2. Verification experiment confirmed that the pipetting skills of J.L.B. were accurate when compared to an experience laboratory technician (T.F.). T-tests revealed no significant differences for any volume pipetted (n=3 trials per experimenter per volume, $P > 0.99$).

In order to verify that von Frey withdrawal thresholds were accurately measured by J.L.B., a comparison was conducted between J.L.B and a Research Assistant Professor (P.G.). In order to perform this comparison, two mice received CCI surgeries. Sixteen days later, J.L.B. and P.G. independently tested the withdrawal thresholds of the two mice. The data were compared using a pair t-test, and no significant differences in withdrawal threshold was found between the two experimenters (Figure 3, $P = 0.66$).

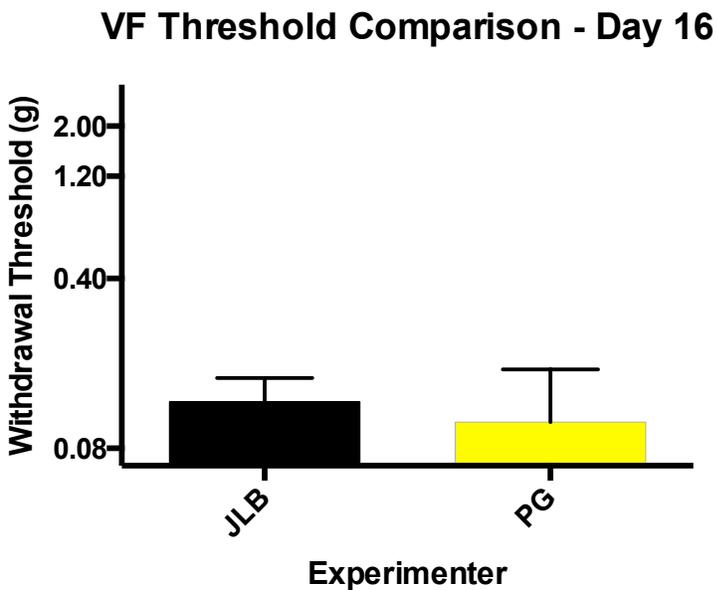


Figure 3. Verification tests confirmed that the behavioral thresholds obtained by J.L.B. were accurate when compared to the thresholds obtained by a Research Assistant Professor (P.G.) with extensive von Frey testing experience ($n=2$ mice tested, $P = 0.66$).

Behavioral Testing

Behavioral testing was performed on both male and female mice. Among the male mice, two-way ANOVA with repeated measures revealed a significant effect of time and treatment, but the effect of the interaction (time x treatment) was not significant (Time: $F_{17, 136} = 4.591$, $P < 0.0001$, treatment: $F_{3, 8} = 4.107$, $P = 0.0489$, time x treatment: $F_{51, 136} = 1.212$, $P = 0.1914$). *Post*

hoc analysis revealed statistically significant differences between groups at days 11, 25, and 32 (Figure 4).

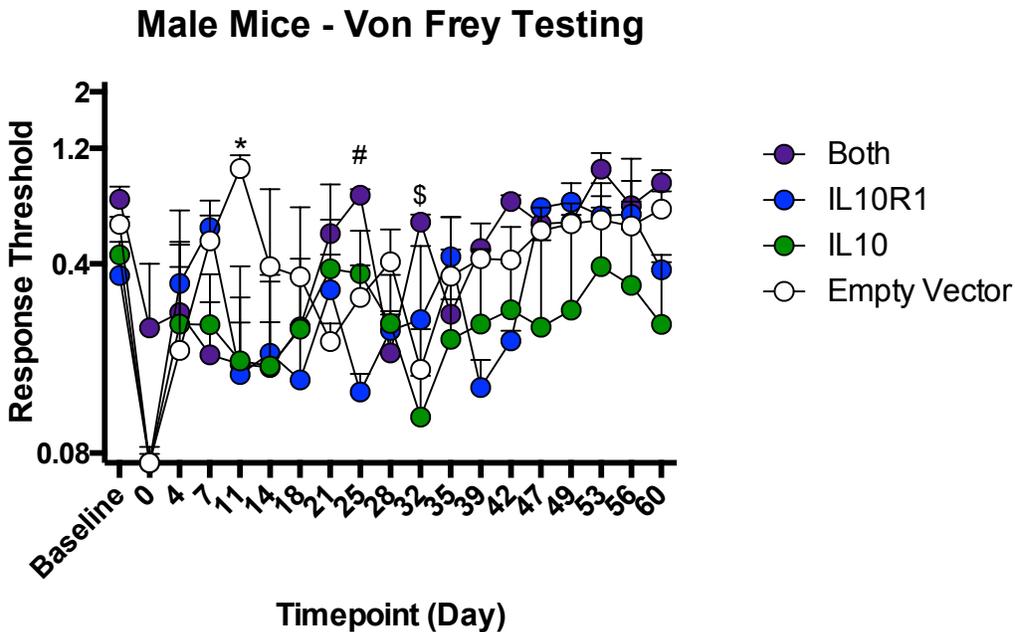


Figure 4. Male mice did not display persistent neuropathic pain. Pain thresholds quickly returned to baseline and fluctuated around baseline for the duration of the study. No robust differences between treatments were observed; however, statistically significant differences existed at days 11, 25, and 32. *: empty vector vs all other treatments, $P < 0.05$, #: IL-10R1 vs combined gene therapy, $P < 0.05$, \$: IL-10 vs combined gene therapy, $P < 0.05$.

Among the female mice, two-way ANOVA with repeated measures revealed a significant effect of time and the interaction (time x treatment), but no significant effect of treatment (time: $F_{17, 340} = 13.42$, $P < 0.0001$, treatment: $F_{3, 20} = 1.153$, $P = 0.3522$, time x treatment: $F_{51, 340} = 1.878$, $P = 0.0006$). *Post hoc* analysis revealed significant differences between groups at days 7, 11, 14, 18, and 21 (Figure 2).

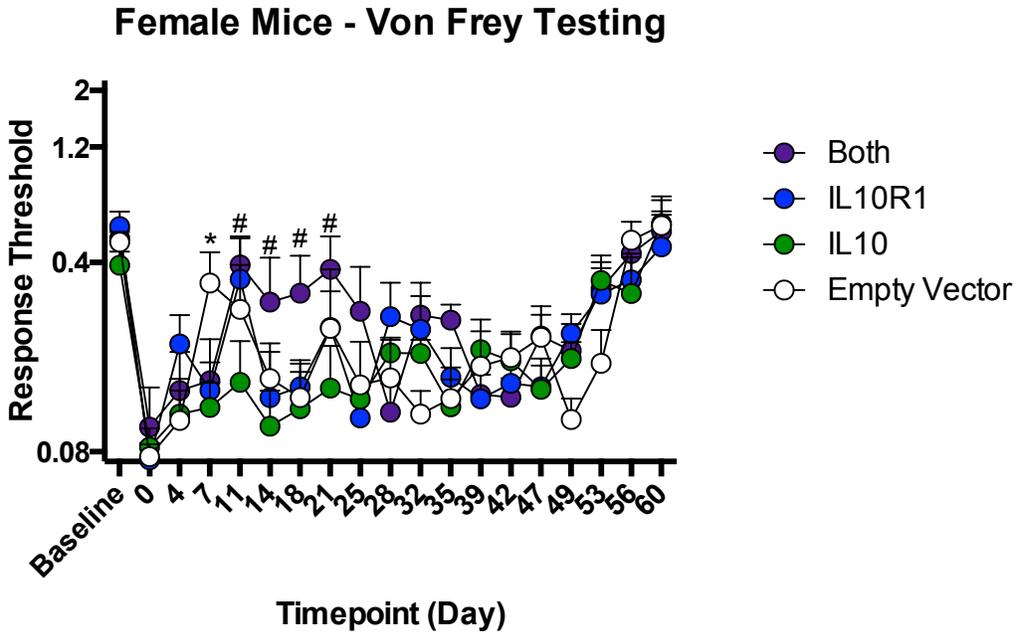


Figure 5. Von Frey testing in females revealed persistent neuropathic pain. Pain thresholds displayed a prolonged decrease to levels expected with neuropathic pain. However, no robust effects of treatment were observed. Statistically significant differences between groups existed at days 7, 11, 14, 18, and 21. *: empty vector vs IL-10, $P < 0.05$, #: IL-10 vs combined gene therapy, $P < 0.05$.

Tissue Collection

NF- κ B

Quantification of nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor alpha ($\text{I}\kappa\text{B}\alpha$) mRNA was used as a proxy for the activation of nuclear factor of kappa light polypeptide gene enhancer in B-cells (NF- κ B). Expression was measured 7, 14, and 35 days post-injection of gene therapy. In order to discern the effects of CCI on the mice, the non-injected CCI group was compared to naïve mice. In the left dorsal horns of male and female mice, two-way ANOVA with repeated measures revealed significant effects of time and sex, but the interaction (time x sex) was not significant (time: $F_{3, 30} = 8.828$, $P = 0.0002$, sex: $F_{1, 10} = 9.868$, $P = 0.0105$, time x sex: $F_{3, 30} = 2.091$, $P = 0.1224$). *Post hoc* analysis revealed a statistically significant difference between the sexes at day 35 (Figure 6A). In the left dorsal root

ganglia (DRG) of male and female mice, two-way ANOVA with repeated measures revealed a significant effect of sex, but the effects of time and the interaction (time x sex) were not significant (time: $F_{3,30} = 0.5410$, $P = 0.6579$, sex: $F_{1,10} = 15.61$, $P = 0.0027$, time x sex: $F_{3,30} = 1.358$, $P = 0.2745$). *Post hoc* analysis revealed a statistically significant difference between the sexes at day 7 (Figure 6B).

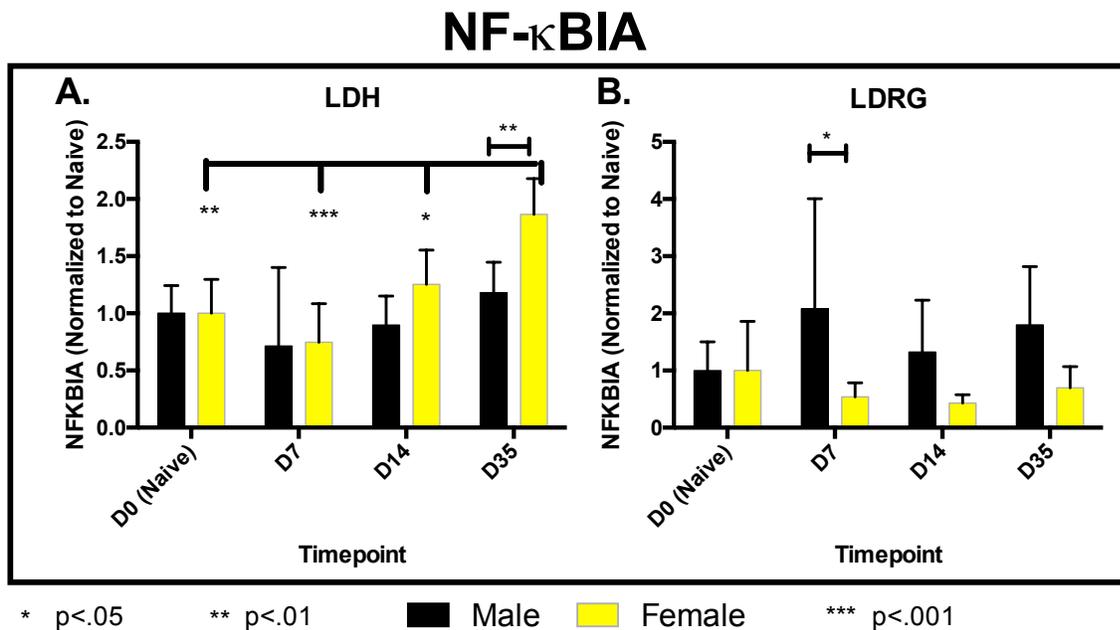


Figure 6. No effects of CCI on NF-κB mRNA expression was observed in males. Significant effects of time ($P = 0.0002$) and sex ($P = 0.0105$) were observed in the dorsal horns, and a significant effect of sex ($P = 0.0027$) was observed in DRG. Significant differences between the sexes were observed in DRGs at day 7 and in dorsal horns at day 35. In the dorsal horn of females, day 35 was significantly different from all other time points (significance vs day 35 indicated in figure).

Tissue was collected for each treatment group at 7, 14, and 35 days post-injection. Data were normalized to naïve mice. In the left dorsal horn of male mice, two-way ANOVA with repeated measures revealed a significant effect of time, but the effects of treatment and the interaction (time x treatment) were not significant (time: $F_{3,75} = 13.27$, $P < 0.0001$, treatment: $F_{4,25} = 0.1723$, $P = 0.9505$, time x treatment: $F_{12,75} = 0.6779$, $P = 0.7672$). *Post hoc* analysis

revealed no significant differences between groups (Figure 7A). In the left DRGs of male mice, two-way ANOVA with repeated measures revealed no significant effects of time, treatment, or the interaction (time x treatment) (time: $F_{3, 75} = 1.526$, $P = 0.2147$, treatment: $F_{4, 25} = 1.574$, $P = 0.2121$, time x treatment: $F_{12, 75} = 0.9188$, $P = 0.5328$). *Post hoc* analysis revealed no significant differences between groups (Figure 7C). In the left dorsal horn of female mice, two-way ANOVA with repeated measures revealed a significant effect of time, but the effects of treatment and the interaction (time x treatment) were not significant (time: $F_{3, 75} = 18.09$, $P < 0.0001$, treatment: $F_{4, 25} = 0.9321$, $P = 0.4613$, time x treatment: $F_{12, 75} = 0.6325$, $P = 0.8080$). *Post hoc* analysis revealed no significant differences between groups (Figure 7B). In the left DRGs of female mice, two-way ANOVA with repeated measures revealed a significant effect of time, but the effects of treatment and the interaction (time x treatment) were not significant (time: $F_{3, 75} = 10.03$, $P < 0.0001$, treatment: $F_{4, 25} = 0.3126$, $P = 0.8668$, time x treatment: $F_{12, 75} = 0.4030$, $P = 0.9582$). *Post hoc* analysis revealed no significant differences between groups (Figure 7D).

NF- κ BIA

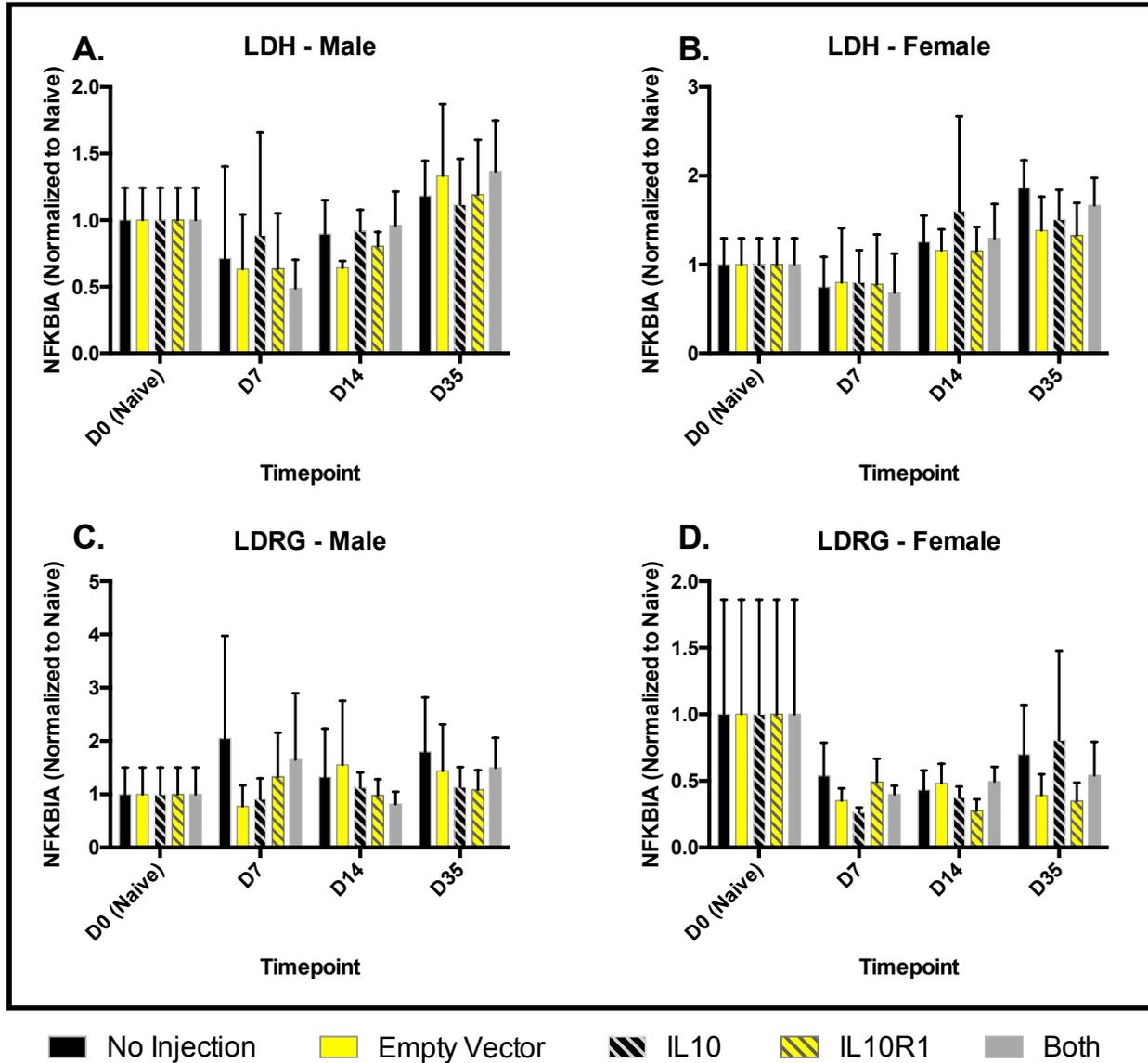


Figure 7. NF- κ B mRNA levels were assessed in each treatment group at 7, 14, and 35 days post-injection to monitor for treatment effects. *Post hoc* tests did not reveal significant differences between treatment groups at any time point. Additionally, no significant differences were seen between any treatment group and mice that did not receive an injection.

P2X4

Quantification of P2X4 mRNA was performed because a sex-difference in P2X4 expression has been reported previously (Sorge et al., 2015). Expression was measured 7, 14,

and 35 days post-injection of gene therapy. In order to discern the effects of CCI on the mice, the non-injected CCI group was compared to naïve mice. In the left dorsal horns of male and female mice, two-way ANOVA with repeated measures revealed significant effects of time and sex, but the interaction (time x sex) was not significant (time: $F_{3,30} = 7.402$, $P = 0.0007$, sex: $F_{1,10} = 13.87$, $P = 0.0039$, time x sex: $F_{3,30} = 2.411$, $P = 0.0864$). *Post hoc* analysis revealed a statistically significant difference between the sexes at day 35 (Figure 8A). In the left DRGs of male and female mice, two-way ANOVA with repeated measures revealed a significant effect of sex, but the effects of time and the interaction (time x sex) were not significant (time: $F_{3,30} = 1.655$, $P = 0.1977$, sex: $F_{1,10} = 13.78$, $P = 0.0040$, time x sex: $F_{3,30} = 0.7950$, $P = 0.5063$). *Post hoc* analysis revealed no statistically significant differences between sexes (Figure 8B).

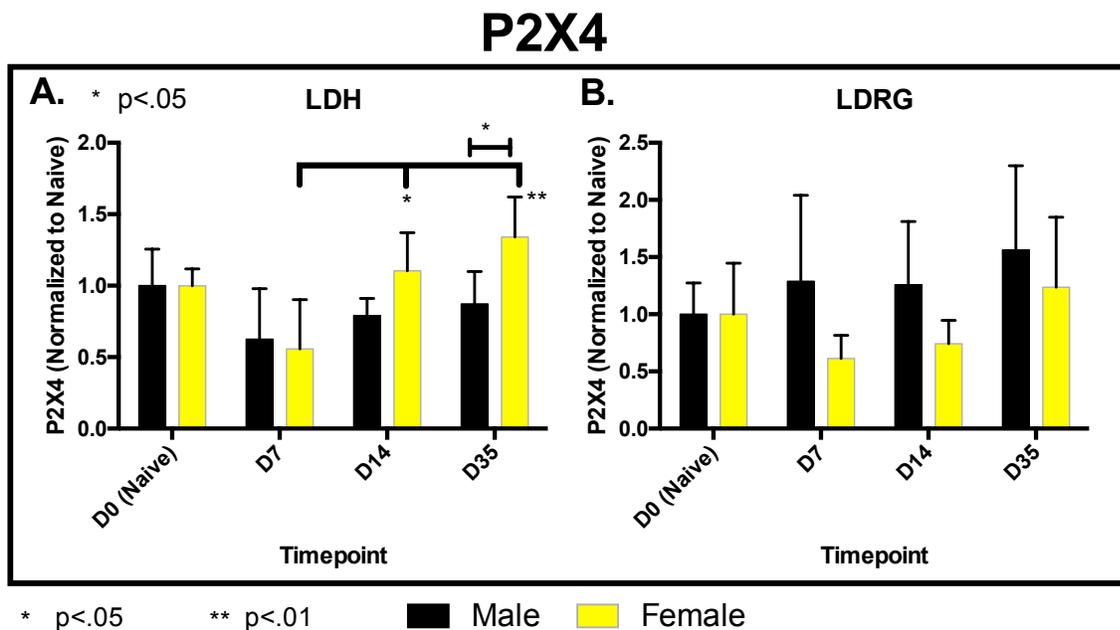


Figure 8. P2X4 mRNA expression was measured in male and female mice to determine the effects of CCI on P2X4 levels. In the left dorsal horn, time ($P=0.0007$) and sex ($P=0.0039$) had significant effects on P2X4 expression, but the interaction (time x sex) did not. In the left dorsal root ganglia, sex had a significant effect on P2X4 expression ($P=0.0040$), but time and the interaction (time x sex) did not. Statistically significant differences in P2X4 expression were observed in the dorsal horn of female mice across time (day 7 vs days 14 and 35). Differences were also observed between male and female mice in the left dorsal horn at day 35.

Data for each of the different treatment groups were compared at each time point. Data were normalized to naïve mice. In the left dorsal horn of male mice, two-way ANOVA with repeated measures revealed a significant effect of time, but the effects of treatment and the interaction (time x treatment) were not significant (time: $F_{3, 75} = 13.19$, $P < 0.0001$, treatment: $F_{4, 25} = 0.6019$, $P = 0.6648$, time x treatment: $F_{12, 75} = 0.5818$, $P = 0.8502$). *Post hoc* analysis revealed no significant differences between groups (Figure 9A). In the left DRGs of male mice, two-way ANOVA with repeated measures revealed a significant effect of time, but the effects of treatment and the interaction (time x treatment) were not significant (time: $F_{3, 75} = 4.939$, $P = 0.0035$, treatment: $F_{4, 25} = 1.546$, $P = 0.2196$, time x treatment: $F_{12, 75} = 1.051$, $P = 0.4134$). *Post hoc* analysis revealed no significant differences between groups (Figure 9C). In the left dorsal horn of female mice, two-way ANOVA with repeated measures revealed a significant effect of time, but the effects of treatment and the interaction (time x treatment) were not significant (time: $F_{3, 75} = 17.20$, $P < 0.0001$, treatment: $F_{4, 25} = 0.6637$, $P = 0.6231$, time x treatment: $F_{12, 75} = 0.2167$, $P = 0.9972$). *Post hoc* analysis revealed no significant differences between groups (Figure 9B). In the left DRGs of female mice, two-way ANOVA with repeated measures revealed a significant effect of time, but the effects of treatment and the interaction (time x treatment) were not significant (time: $F_{3, 75} = 5.642$, $P = 0.0015$, treatment: $F_{4, 25} = 0.8284$, $P = 0.5197$, time x treatment: $F_{12, 75} = 0.6604$, $P = 0.7833$). *Post hoc* analysis revealed no significant differences between groups (Figure 9D).

P2X4

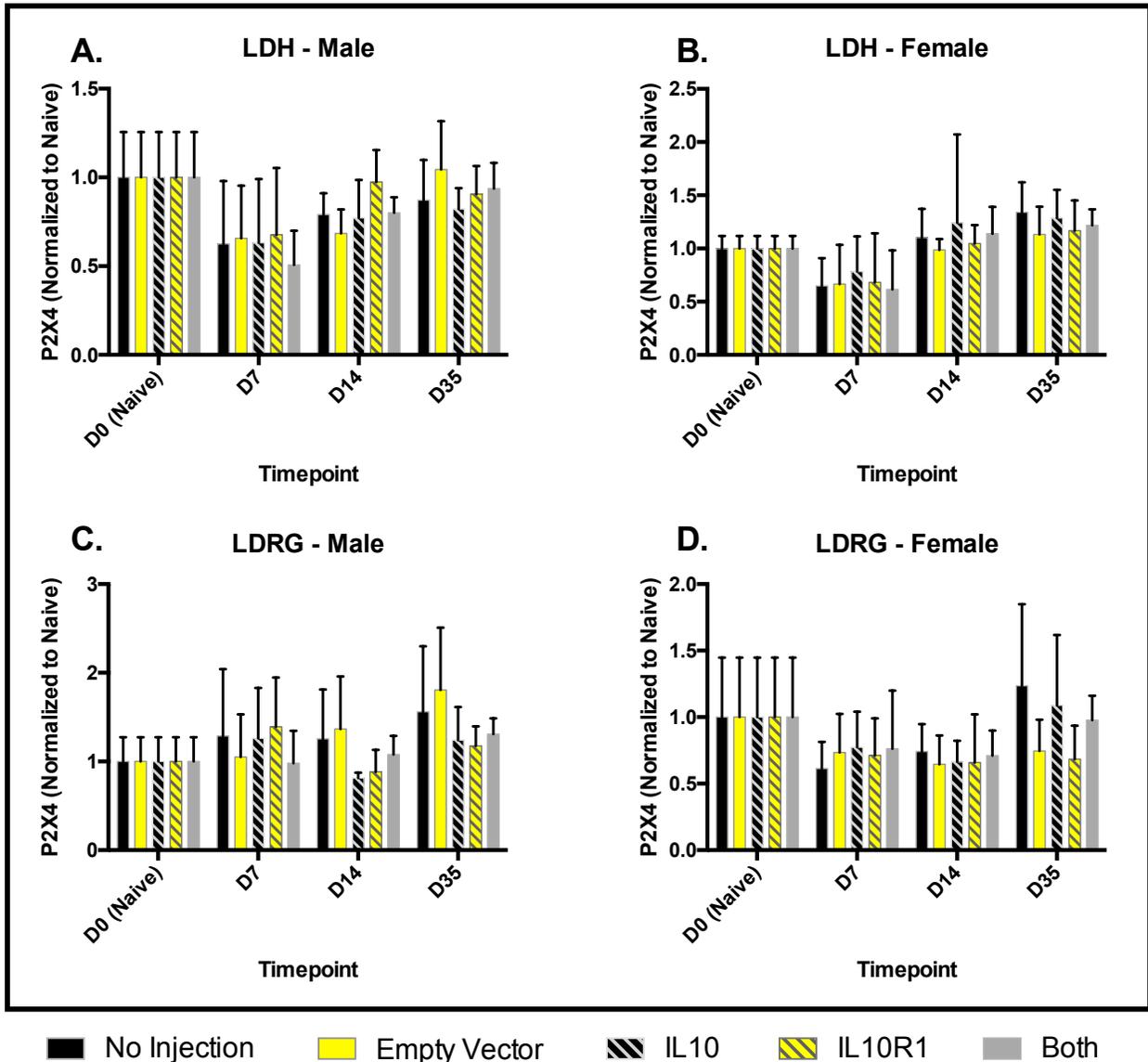


Figure 9. P2X4 mRNA levels were assessed in each treatment group at 7, 14, and 35 days post-injection to monitor for treatment effects. *Post hoc* tests did not reveal significant differences between treatment groups at any time point. Additionally, no significant differences were seen between any treatment group and mice that did not receive an injection.

TNF α

Quantification of *TNF α* mRNA was performed. Expression was measured 7, 14, and 35 days post-injection of gene therapy. In order to discern the effects of CCI on the mice, the non-

injected CCI group was compared to naïve mice. In the left dorsal horns of male and female mice, two-way ANOVA with repeated measures revealed a significant effect of time, but the effects of sex and the interaction (time x sex) were not significant (time: $F_{3,30} = 2.936$, $P = 0.0493$, sex: $F_{1,10} = 0.9546$, $P = 0.3516$, time x sex: $F_{3,30} = 0.8715$, $P = 0.4667$). *Post hoc* analysis revealed no statistically significant differences (Figure 10A). In the left DRGs of male and female mice, two-way ANOVA with repeated measures revealed a significant effect of sex, but the effects of time and the interaction (time x sex) were not significant (time: $F_{3,30} = 1.964$, $P = 0.1407$, sex: $F_{1,10} = 11.97$, $P = 0.0061$, time x sex: $F_{3,30} = 0.6818$, $P = 0.5701$). *Post hoc* analysis revealed no statistically significant differences (Figure 10B).

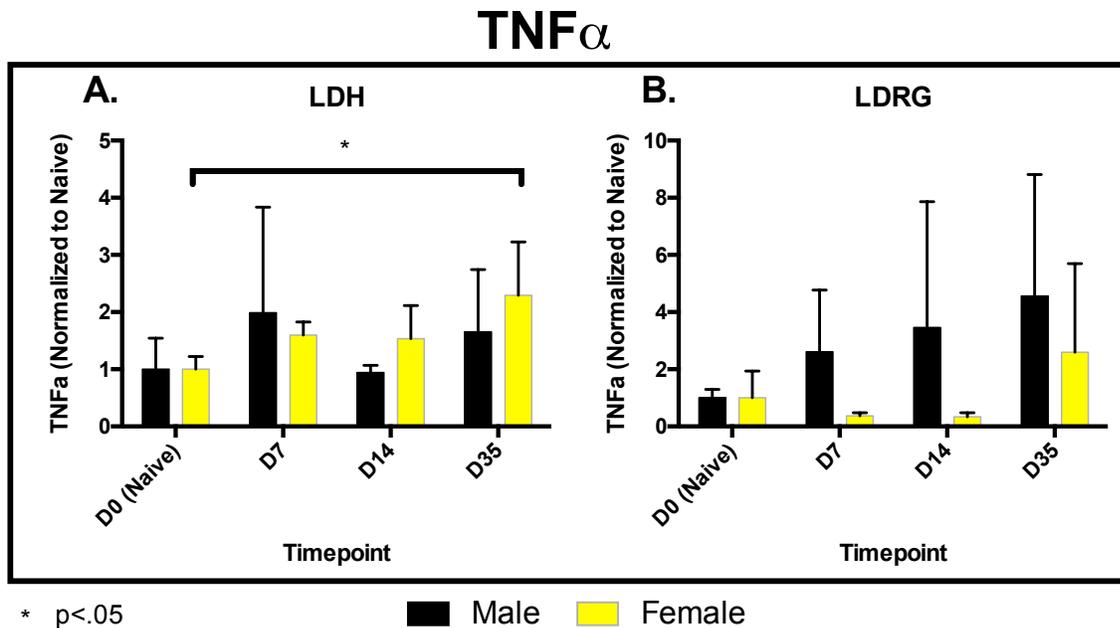


Figure 10. TNF α mRNA expression was measured in male and female mice at 7, 14, and 35 days post-injection to determine the effects of CCI on TNF α levels. In the left dorsal horn, time had a significant effect on TNF α expression ($P = 0.0493$), but sex and the interaction (time x sex) did not. In the left dorsal root ganglia, sex had a significant effect on TNF α expression ($P=0.0061$), but time and the interaction (time x sex) did not. Statistically significant differences in TNF α expression were observed in the dorsal horn of female mice (day 7 vs day 35).

Data from each treatment group were compared at each time point. Data were normalized to naïve mice. In the left dorsal horn of male mice, two-way ANOVA with repeated measures revealed a significant effect of time, but the effects of treatment and the interaction (time x treatment) were not significant (time: $F_{3, 75} = 3.841$, $P = 0.0129$, treatment: $F_{4, 25} = 1.040$, $P = 0.4063$, time x treatment: $F_{12, 75} = 0.5796$, $P = 0.8519$). *Post hoc* analysis revealed no significant differences between groups (Figure 11A). In the left DRGs of male mice, two-way ANOVA with repeated measures revealed a significant effect of time and the interaction (time x treatment), but the effect of treatment was not significant (time: $F_{3, 75} = 6.391$, $P = 0.0006$, treatment: $F_{4, 25} = 1.788$, $P = 0.1629$, time x treatment: $F_{12, 75} = 2.599$, $P = 0.0060$). *Post hoc* analysis revealed significant differences between groups at day 35 (Figure 11C). In the left dorsal horn of female mice, two-way ANOVA with repeated measures revealed a significant effect of time, but the effects of treatment and the interaction were not significant (time: $F_{3, 75} = 13.60$, $P < 0.0001$, treatment: $F_{4, 25} = 1.638$, $P = 0.1960$, time x treatment: $F_{12, 75} = 0.4305$, $P = 0.9463$). *Post hoc* analysis revealed no significant differences between groups (Figure 11B). In the left DRGs of female mice, two-way ANOVA with repeated measures revealed significant effects of time and the interaction (time x treatment), but the effect of treatment was not significant (time: $F_{3, 75} = 4.473$, $P = 0.0061$, treatment: $F_{4, 25} = 1.445$, $P = 0.2485$, time x treatment: $F_{12, 75} = 1.897$, $P = 0.0482$). *Post hoc* analysis revealed significant differences between groups at day 35 ($P < 0.05$) (Figure 11D).

TNF α

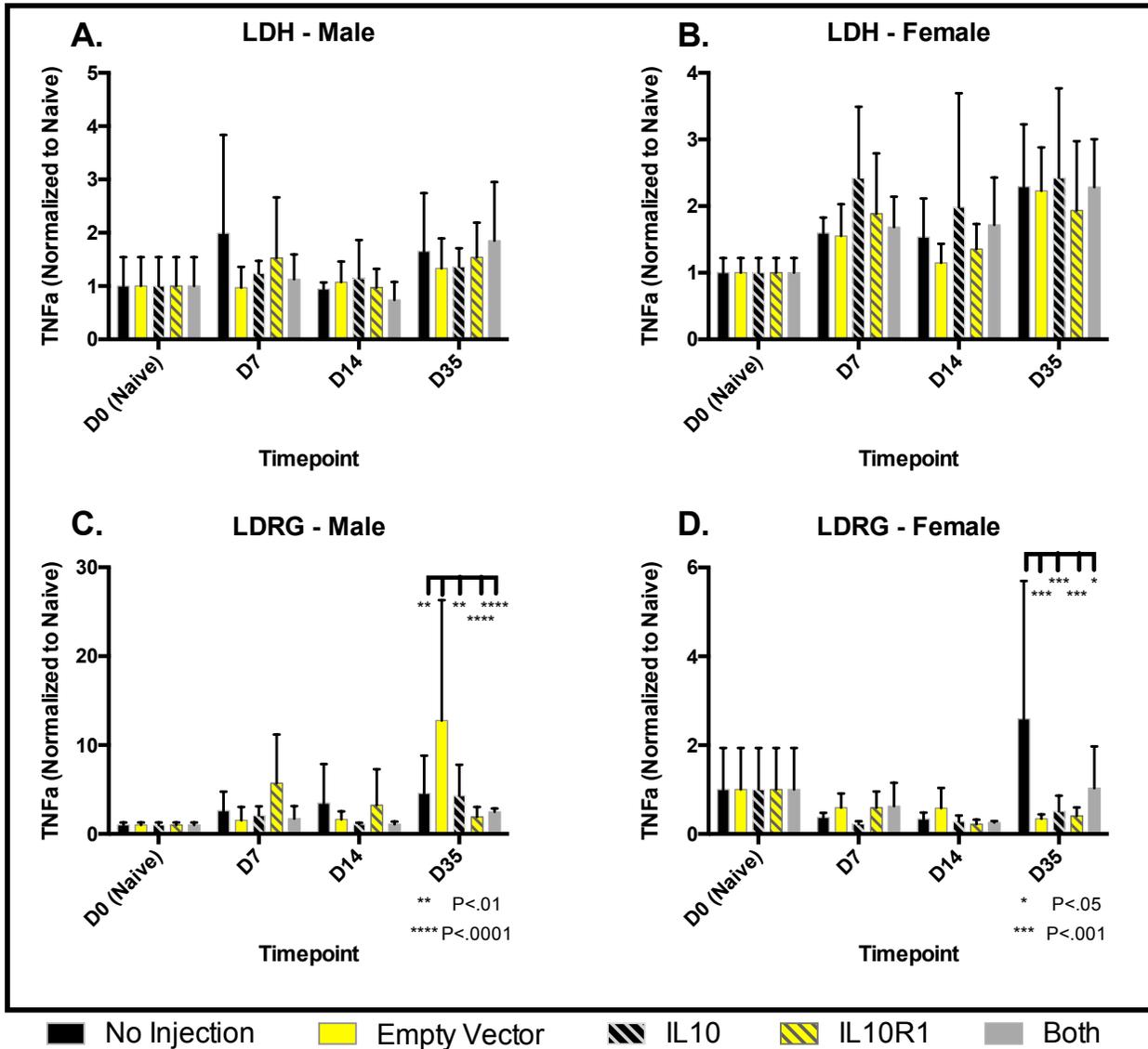


Figure 11. TNF α mRNA levels were assessed in each treatment group at 7, 14, and 35 days post-injection to monitor for treatment effects. *Post hoc* tests did not reveal significant differences between treatment groups at any time point in dorsal horns. Female DRGs displayed significant differences between mice that were not injected and all other groups at day 35. Male DRGs displayed significant differences between mice that received empty virus injection and all other groups at day 35.

IL-1 β

Quantification of IL-1 β mRNA was performed. Expression was measured 7, 14, and 35 days post-injection of gene therapy. In order to discern the effects of CCI on the mice, the non-

injected CCI group was compared to naïve mice. In the left dorsal horns of male and female mice, two-way ANOVA with repeated measures revealed a significant effects of time, sex, and the interaction (time x sex) (time: $F_{3,30} = 5.118$, $P = 0.0056$, sex: $F_{1,10} = 11.79$, $P = 0.0064$, time x sex: $F_{3,30} = 6.386$, $P = 0.0018$). *Post hoc* analysis revealed statistically significant differences between the sexes at days 14 and 35, as well as differences between females at different time points (Figure 12A). In the left DRGs of male and female mice, insufficient data was obtained to perform statistics.

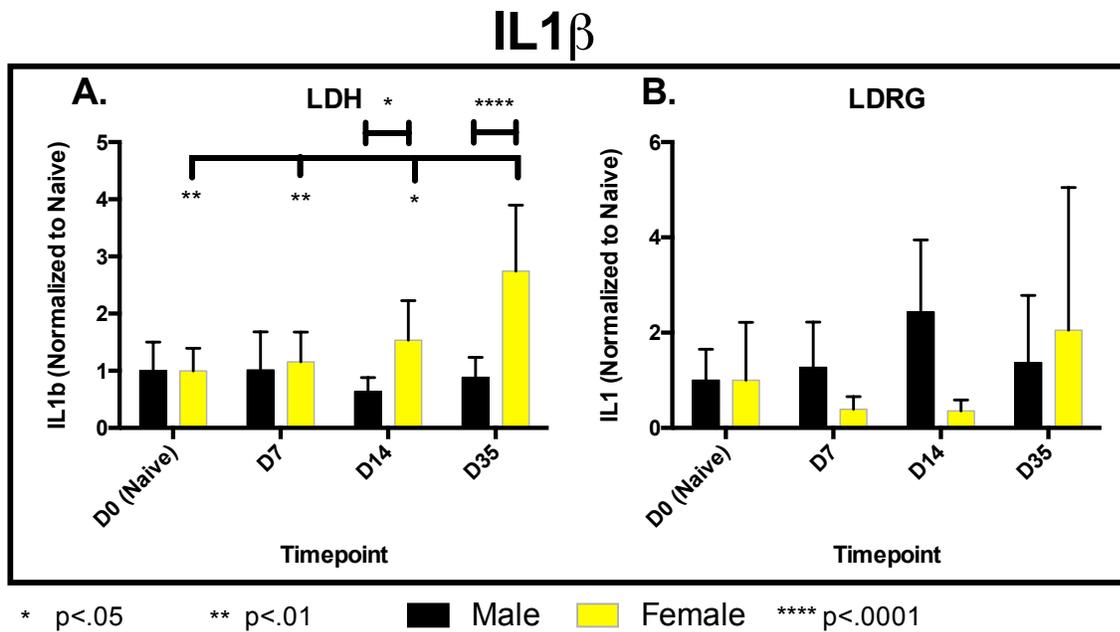


Figure 12. IL-1 β mRNA expression was measured in male and female mice to determine the effects of CCI on IL-1 β levels. In the left dorsal horn, time ($P = 0.0056$), sex ($P = 0.0064$), and the interaction (time x sex) ($P = 0.0018$) all had significant effects on IL-1 β expression. In the left DRGs, insufficient data was obtained to perform statistics. Statistically significant differences in IL-1 β expression over time were observed in the dorsal horn of female mice (day 35 vs all other time points). Differences between the sexes were observed in the dorsal horn at days 14 and 35.

Data from each treatment group were compared at each time point. Data were normalized to naïve mice. In the left dorsal horn of male mice, two-way ANOVA with repeated measures revealed no significant effects of time, treatment, nor the interaction (time x treatment) (time: F_3 ,

$F_{75} = 0.9684$, $P = 0.4122$, treatment: $F_{4, 25} = 1.073$, $P = 0.3906$, time x treatment: $F_{12, 75} = 0.8634$, $P = 0.5865$). *Post hoc* analysis revealed no significant differences between groups (Figure 13A). In the left DRGs of male mice, too many data were lost from inconsistent PCR replicates to perform statistics. In the left dorsal horn of female mice, two-way ANOVA with repeated measures revealed a significant effect of time, but the effects of treatment and the interaction (time x treatment) were not significant (time: $F_{3, 75} = 13.54$, $P < 0.0001$, treatment: $F_{4, 25} = 1.568$, $P = 0.2136$, time x treatment: $F_{12, 75} = 1.877$, $P = 0.0509$). *Post hoc* analysis revealed significant differences between groups at day 14 (Figure 13B). In the left DRGs of female mice, two-way ANOVA with repeated measures revealed no significant effects time, treatment, nor the interaction (time x treatment) (time: $F_{3, 75} = 2.390$, $P = 0.0754$, treatment: $F_{4, 25} = 0.8508$, $P = 0.5067$, time x treatment: $F_{12, 75} = 1.366$, $P = 0.2016$). *Post hoc* analysis revealed significant differences between groups at day 35 (Figure 13D).

IL1 β

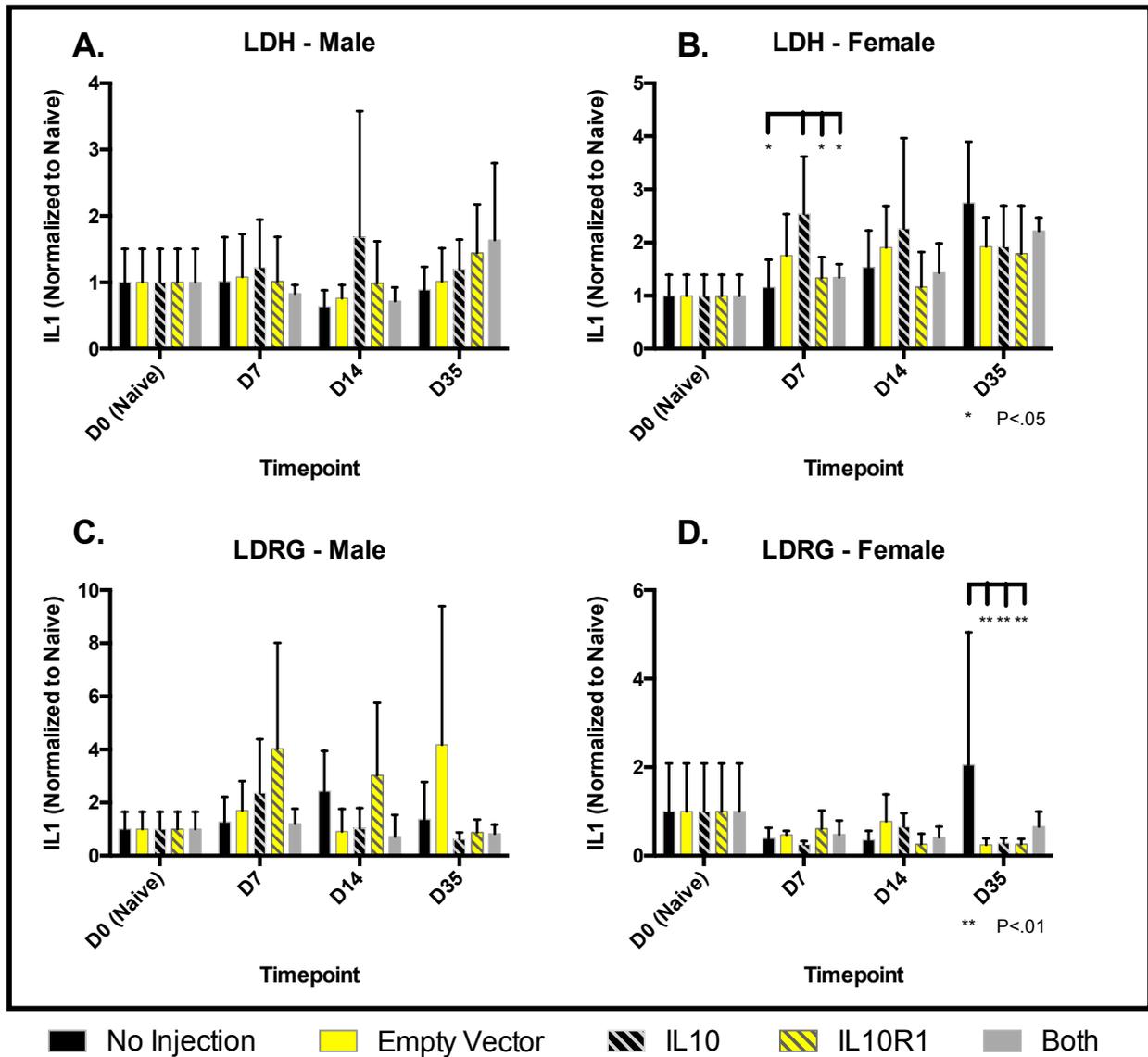


Figure 13. IL-1 β mRNA levels were assessed in each treatment group at 7, 14, and 35 days post-injection to monitor for treatment effects. *Post hoc* tests did not reveal significant differences between treatment groups at any time point in male mice. Female dorsal horns displayed significant differences at day 7 between mice that received IL-10 and mice that received no injection, IL-10R1, or both gene therapies. Female DRGs displayed significant differences at day 35 between mice that received no injection and those which received empty virus, IL-10, or IL-10R1 gene therapy.

IL-10

Mouse IL-10 mRNA expression was measured. However, a large subset of the data had to be excluded due to inconsistent PCR replicates. There was not enough data remaining after excluding inconsistent results to perform statistics.

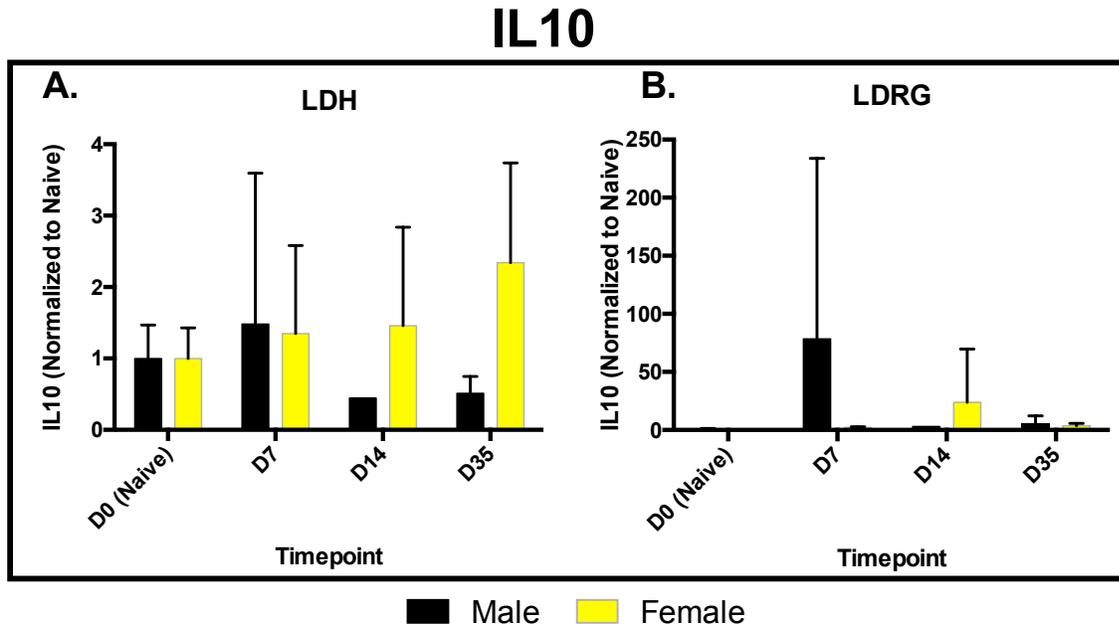


Figure 14. IL-10 mRNA expression was measured in male and female mice to determine the effects of CCI on endogenous IL-10 levels. Insufficient data were obtained to perform statistical analysis.

IL10

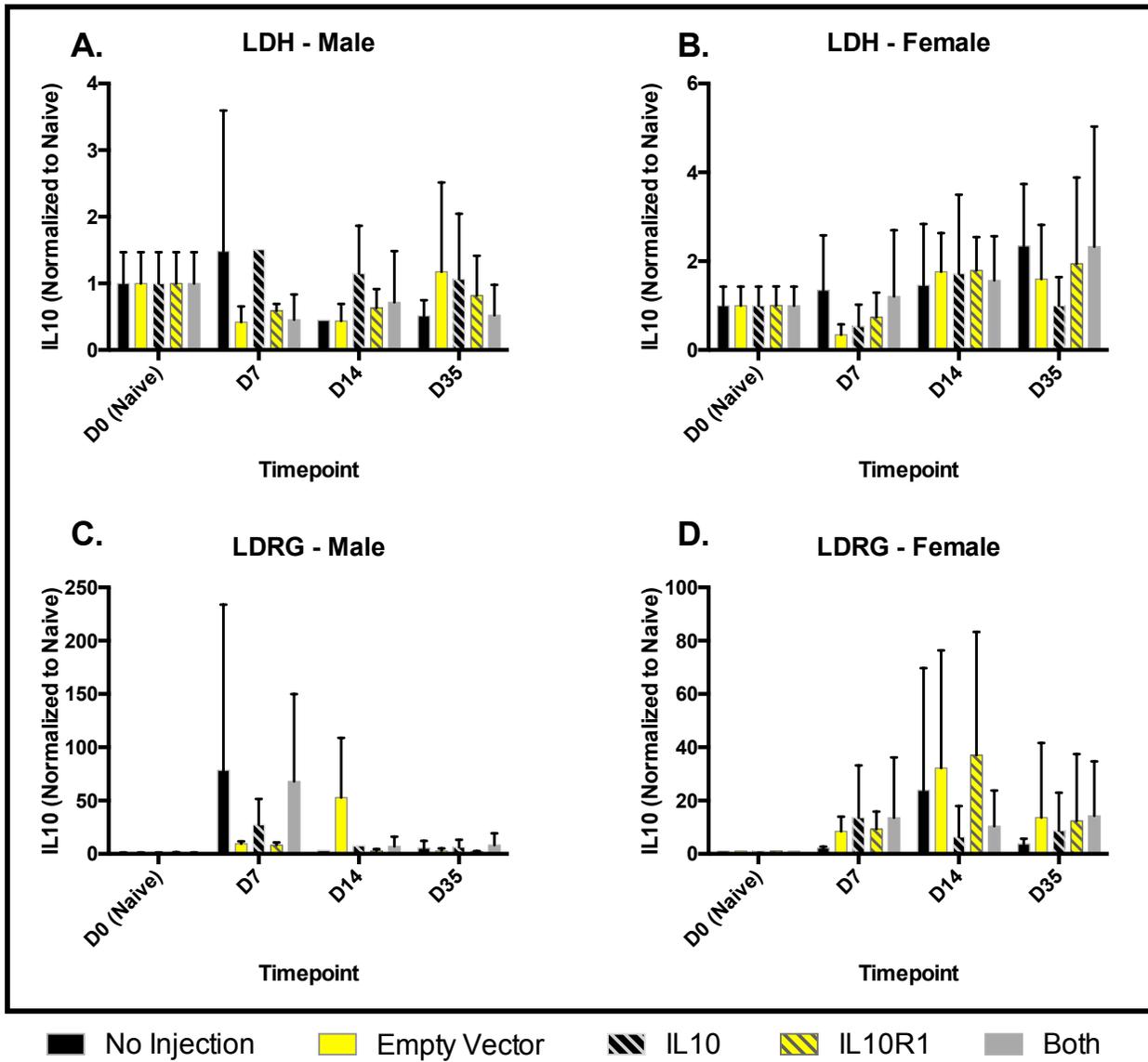


Figure 15. IL-10 mRNA expression was quantified in each sex in each treatment group at 7, 14, and 35 days post-injection. Insufficient data were obtained for statistical analysis.

IL-10R1

Quantification of mouse IL-10R1 mRNA was performed. This experiment measured levels of endogenous IL-10R1, not the IL-10R1 introduced by the gene therapy. Expression was measured 7, 14, and 35 days post-injection of gene therapy. In order to discern the effects of CCI on the mice, the non-injected CCI group was compared to naïve mice. In the left dorsal horns of

male and female mice, two-way ANOVA with repeated measures revealed a significant effect of time, but the effects of sex and the interaction (time x sex) were not significant (time: $F_{3,30} = 6.473$, $P = 0.0016$, sex: $F_{1,10} = 4.590$, $P = 0.0578$, time x sex: $F_{3,30} = 0.7547$, $P = 0.5283$). *Post hoc* analysis revealed no statistically significant differences between the sexes, although differences existed within females across time (Figure 16A). In the left DRGs of male and female mice, two-way ANOVA with repeated measures revealed significant effects of time and sex, but the interaction (time x sex) was not significant (time: $F_{3,30} = 2.928$, $P = 0.0497$, sex: $F_{1,10} = 7.452$, $P = 0.0212$, time x sex: $F_{3,30} = 0.4046$, $P = 0.7507$). *Post hoc* analysis revealed no statistically significant differences between the sexes, although differences existed within female mice across time (Figure 16B).

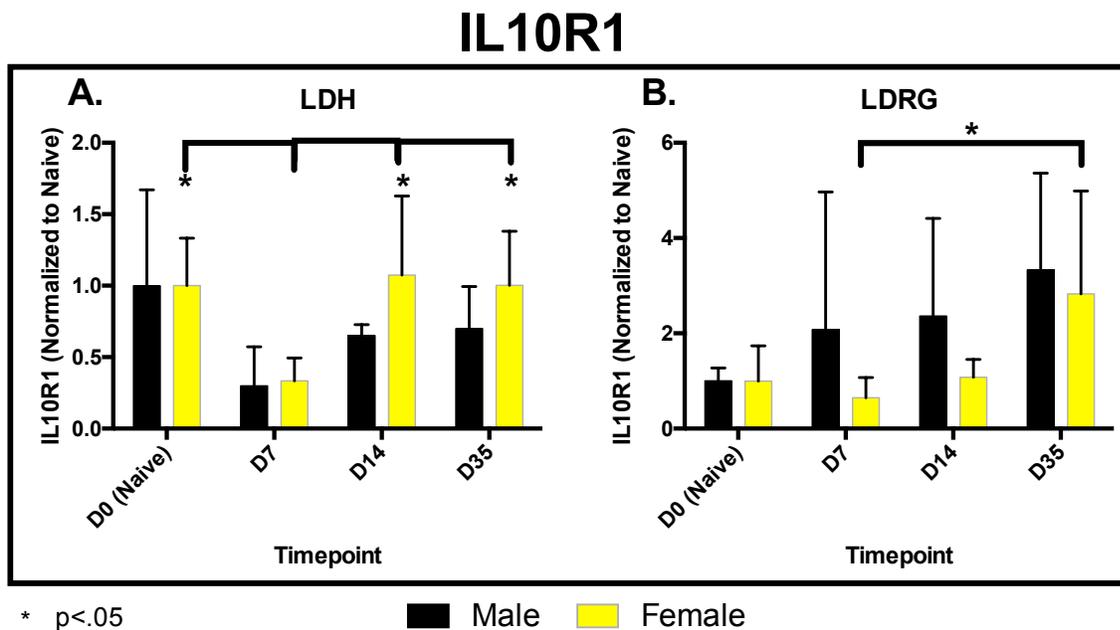


Figure 16. IL-10R1 mRNA expression was measured in male and female mice to determine the effects of CCI on endogenous IL-10R1 levels. In the left dorsal horn, time had a significant effect on IL-10R1 expression ($P = 0.0016$), but sex and the interaction (time x sex) did not. In the left dorsal root ganglia, time ($P = 0.0497$) and sex ($P = 0.0212$) had significant effects on IL-10R1 expression, but the interaction (time x sex) did not. Statistically significant differences in IL-10R1 expression were observed in the dorsal horn of female mice (day 7 vs all other time points) and the dorsal root ganglia on female mice (day 7 vs day 35). *: $P < 0.05$.

Data from each treatment group were compared at each time point. Data were normalized to naïve mice. Variability between PCR replicates in the left dorsal horn of male mice made statistical analysis infeasible. In the left DRGs of male mice, two-way ANOVA with repeated measures revealed a significant effect of time, but the effects of treatment and the interaction (time x treatment) were not significant (time: $F_{3,75} = 9.991$, $P < 0.0001$, treatment: $F_{4,25} = 1.435$, $P = 0.2518$, time x treatment: $F_{12,75} = 0.6145$, $P = 0.8234$). *Post hoc* analysis revealed no statistically significant differences between treatment groups (Figure 17C). In the left dorsal horn of female mice, two-way ANOVA with repeated measures revealed a significant effect of time, but the effects of treatment and the interaction (time x treatment) were not significant (time: $F_{3,75} = 19.54$, $P < 0.0001$, treatment: $F_{4,25} = 0.8943$, $P = 0.4820$, time x treatment: $F_{12,75} = 1.420$, $P = 0.1761$). *Post hoc* analysis revealed significant differences between groups at day 14 (Figure 17B). In the left DRGs of female mice, two-way ANOVA with repeated measures revealed a significant effect of time, but the effects of treatment and the interaction (time x treatment) were not significant (time: $F_{3,75} = 8.246$, $P < 0.0001$, treatment: $F_{4,25} = 2.423$, $P = 0.0748$, time x treatment: $F_{12,75} = 1.469$, $P = 0.1553$). *Post hoc* analysis revealed significant differences between groups at day 35 (Figure 17D).

IL10R1

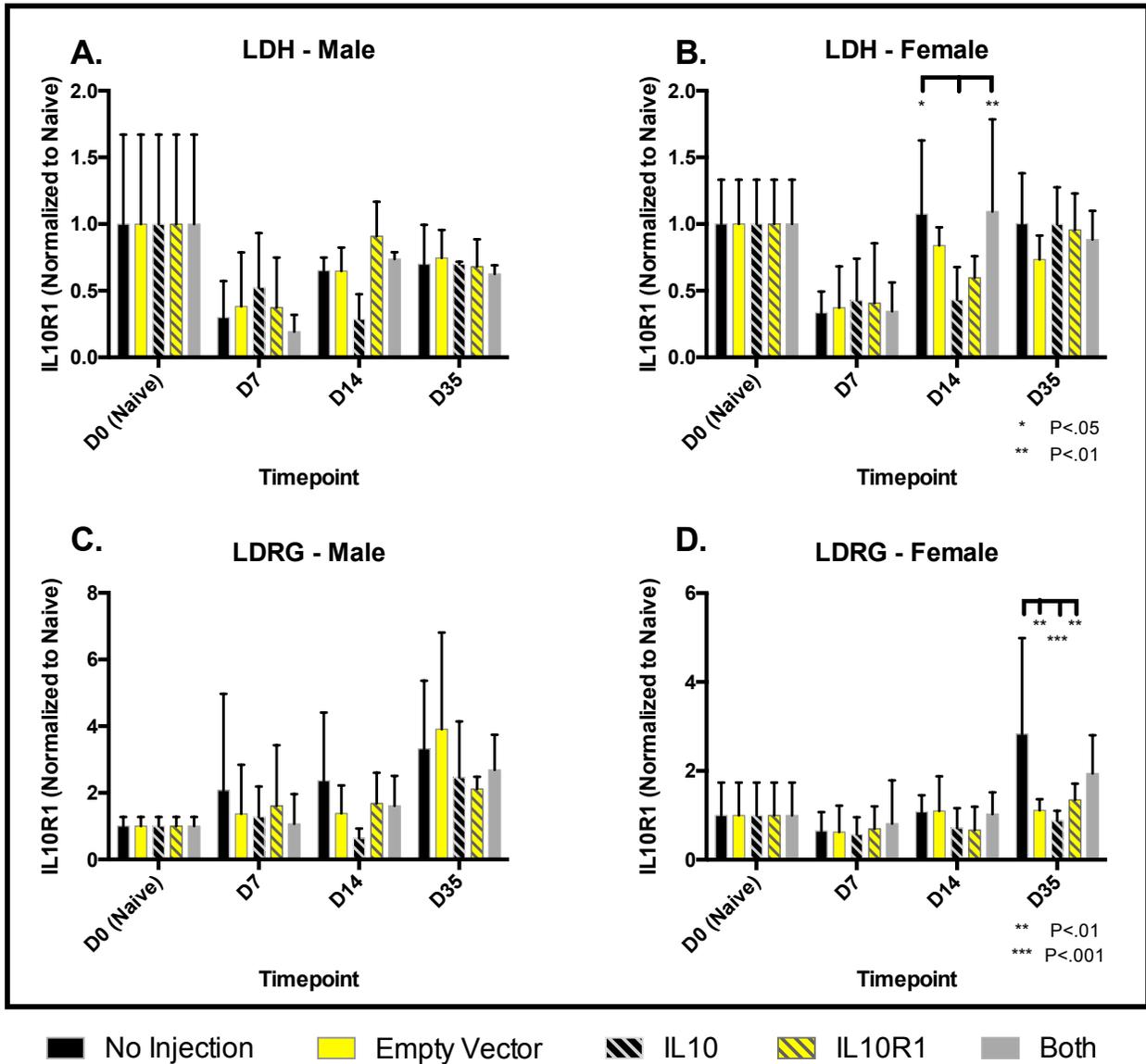


Figure 17. IL-10R1 mRNA levels were assessed in each treatment group at 7, 14, and 35 days post-injection to monitor for treatment effects. *Post hoc* tests did not reveal significant differences between treatment groups at any time point in male mice. Female dorsal horns displayed significant differences at day 14 between mice that received IL-10 and mice that received no injection or both gene therapies. Female DRGs displayed significant differences at day 35 between mice that received no injection and those which received empty virus, IL-10, or IL-10R1 gene therapy.

Groups of mice (n=3 per group) were injected with either empty viral vector, IL-10 gene therapy, or IL-10R1 gene therapy to verify that the viral vector was expressed. Expression was verified using PCR to measure mRNA levels of the genes encoded by each vector. The human IL-10 primers did not specifically amplify the transgene, so expression could not be determined. PCR results showed that human IL-10R1 was expressed in none of the mice injected with empty virus or IL-10. Only one of the three mice which received an injection of IL-10R1 AAV9 vector expressed human IL-10R1 mRNA (Table 1).

Mouse	Gene Therapy Given	IL-10 Expression?	IL-10R1 Expression?
101	Empty Vector	Unknown	No
102	Empty Vector	Unknown	No
103	Empty Vector	Unknown	No
105	IL-10	Unknown	No
106	IL-10	Unknown	No
107	IL-10	Unknown	No
108	IL-10R1	Unknown	No
109	IL-10R1	Unknown	No
110	IL-10R1	Unknown	Yes

Table 1. Mice were injected with the different gene therapies to confirm that the viruses effectively delivered their payloads. None of the mice injected with empty viral vector or IL-10 gene therapy expressed IL-10R1 mRNA in the LDH or LDRG. One of the three mice injected with IL-10R1 gene therapy displayed IL-10R1 in the LDH and LDRG.

Discussion

We hypothesized that combining IL-10 and IL-10R1 gene therapies would extend the duration of anti-allodynia in a mouse model of neuropathic pain. The data did not support this hypothesis. In the male mice, the CCI surgery did not induce persistent neuropathic pain. In the female mice, neuropathic pain was present, but there were no significant treatment effects from the gene therapy. The second hypothesis of this experiment was that male and female mice would respond differently to this gene therapy approach. This hypothesis cannot be accepted nor refuted because the lack of neuropathic pain in the male mice makes such a comparison impossible.

Overall, this study yielded an unfortunate dearth of significant data. Three possible explanations exist for this result: 1) human error in the processing of tissue/behavioral testing, 2) the CCI surgeries did not work, or 3) the treatment did not work.

In order to address the possibility of human data in the experiments, confirmatory tests were conducted verifying the accuracy of the experimenter who extracted RNA, made cDNA, ran PCR, and conducted von Frey testing (J.L.B.). These tests confirmed that the experimenter had accurate pipetting skills (Figure 2). This is further supported by the fact that the experimenter had three years of experience with research that involved large volumes of pipetting, including past experience with extraction, cDNA synthesis, and PCR. Tissue pulls were conducted by J.L.B. as well as laboratory technicians with extensive experience pulling dorsal horns and DRG. Additionally, PCR results showed significant levels of expression of mRNA in each sample (C_T values for GAPDH were 15 or less for the majority of samples, data not shown). The von Frey trials confirmed that J.L.B. arrived at results similar to those obtained by an experienced Research Assistant Professor (Figure 3). While it is impossible to completely rule out human error in any experiment, these results suggest that the level of human error in this experiment were no higher than would be expected in a typical research project.

In order to address the possibility that the CCI surgeries were not effective, the behavioral data were consulted. Both studies demonstrated a significant effect of time ($P < 0.0001$). In the female mice, there is a clear, persistent induction of neuropathic pain. While there were transient reductions in the pain expressed in various treatment groups among the female mice, no treatment displayed a prolonged and statistically significant effect. The implications of this data will be explored in greater length below.

In the male mice, the data did not reveal a persistent induction of neuropathic pain. The data were highly variable, but all treatment groups fluctuated around the baseline during the experimental time course. Based on the lack of a clear induction of neuropathic pain, it appears that the CCI was not effective in the male mice, even though it induced neuropathic pain in female mice.

The lack of a CCI effect in males could be explained by high levels of stress in the mice used in the experiment. Multiple lines of evidence lend credence to this hypothesis. For one, the baseline withdrawal threshold in the male mice was significantly lower in the mice included in the study (Figure 4) than in those in the pilot data (Figure 1) that inspired the experiment. The lower threshold indicates that these mice may have been unusually susceptible to pain prior to the experiment being performed. Another corroborating piece of evidence comes from the measurements of TNF α levels before and after CCI in the mice that did not receive an intrathecal injection (Figure 10). The level of TNF α did not vary significantly between naïve males and those that had received CCI (Figure 10A). This finding is at odds with a robust literature showing an increase in TNF α levels following CCI (DeLeo and Yeziarski, 2001; Okamoto et al., 2001; Watkins et al., 2001; Watkins and Maier, 2004; Ledebøer et al., 2007; Üçeyler et al., 2007; Jancálek et al., 2010). Additionally, the dorsal horns of uninjected male mice that received CCI did not show activation of NF- κ B, which has also been shown to be up-regulated following traumatic nervous system injury (Bethea et al., 1998; DeLeo and Yeziarski, 2001). The mice used in this experiment came from the same animal house as those used in the preliminary experiment. While the animal house was the same, it is possible that an environmental change occurred between the treatment dates, increasing the stress levels in the mice.

In the female mice, a CCI effect was clearly observed. However, no significant differences were observed between treatment groups. Consequently, the possibility that the treatment did not work in the female mice was addressed. The lack of significant differences between treatment groups in the behavioral studies suggest that it is possible that the viral vector was not effective. This could have occurred for a number of reasons. For one, it is possible that the virus was not taken up by cells following injection. However, adeno-associated viral vectors have been successfully used to deliver gene therapies in past experiments (Beutler et al., 2005; Milligan et al., 2005b). Another possibility is that the viral vector somehow degraded during storage.

PCR was used to determine whether the gene therapy successfully induced expression of the gene. Ultimately, although no human IL-10R1 was observed in mice that did not receive the IL-10R1 gene therapy, only one of the three mice injected with the gene therapy displayed measurable levels of human IL-10R1 mRNA. This suggests that the gene therapy was likely ineffective in at least a subset of the mice included in this study. Thus, even if the gene therapy were expressed in some of the mice, the effect would have been diluted by the tissue from the unaffected mice. Therefore, it is not surprising that no significant differences were seen between treatment groups. To further confirm the efficacy of the gene therapy, immunohistochemistry could be performed by injecting a vector expressing the gene therapy linked to a molecule such as enhanced-Green Fluorescent Protein (eGFP). These results would reveal the localization of the gene therapy within the spinal cord, and allow for the identification of the mice that successfully received the therapy. Alternately, the mice could be split into two groups. Each group could then be probed for half of the genes of interest, leaving enough cDNA remaining to

verify the expression of the transgene in each mouse. Then, the data from any mice in which the gene therapy was not effective could be excluded, preventing the dilution of the data.

The female mice that received successful CCI surgeries did not display an immediate increase in TNF α , IL-1 β , and NF- κ B levels. The levels of these pro-inflammatory markers did indeed increase; however, the increase was not statistically significant until day 35. Neuropathic pain, on the other hand, was induced within days following the CCI, and it persisted for weeks thereafter. Thus, neuropathic pain developed without a contribution from these pro-inflammatory cytokines. This supports the assertion that the pathways supporting the development of neuropathic pain might differ between male and female mice. While pro-inflammatory cytokines have been strongly implicated in the development of neuropathic pain, almost all of these studies were conducted in male animals (Kwilasz et al., 2014). The literature on neuropathic pain in female mice is, consequently, much less robust. However, recent studies reported that female mice develop neuropathic pain through a pathway independent from microglial activation (Sorge et al., 2015; Taves et al., 2015). This alternate pathway could lead to the development of neuropathic pain in the female mice without increasing levels of TNF α , IL-1 β , and NF- κ B. The role of pro-inflammatory cytokines in neuropathic pain in female mice remains to be explored (Taves et al., 2015). However, the increased expression of these cytokines at day 35 indicates that the commonly described pathways might play a role in the maintenance of neuropathic pain in female mice.

Another limitation could have affected the results of these studies. This regards the drainage of cerebrospinal fluid (CSF) from the central nervous system. CSF transports waste products around and out of the nervous system (Zenker et al., 1994). Mononuclear cells in the CSF have been demonstrated to contain mRNA for TNF α , IL-10 (Monteyne et al., 1997), and

IL-6 (Navikas et al., 1996). CSF can drain through a number of locations throughout the brain, including the arachnoid villi and the choroid plexus. However, CSF also drains out of the spinal cord and into the lymphatic system near nerve roots (Zenker et al., 1994). Given that the DRGs sit along these nerve roots, CSF cells will pass near the DRGs as they are excreted. Therefore, it is possible that variable amounts of CSF waste could have been collected with each DRG. Since this CSF contains mRNA for several of the cytokines that were probed in this study, inadvertent collection of this waste could have substantially increased the variability of the results. Indeed, high levels of variability are observed in the data obtained from DRG samples.

The behavioral tests did not reveal significant treatment effects of the gene therapy. However, statistical analysis did reveal significant effects of time on both behavioral groups, and the data indicate that the CCI surgeries were effective at inducing neuropathic pain in female mice. Additionally, both studies contained a few data points at which *post hoc* testing revealed significant differences between groups. However, this study compared four treatment groups at 18 different time points. As a result, a total of 108 comparisons were performed between groups. The threshold for statistical significance in this study was set at $\alpha = 0.05$, meaning there was a 1/20 chance that a significant result would be found by chance alone. As a result, in a series with 108 comparisons, five to six significant differences would be expected due to chance. In the study of male mice, five significant differences were discovered; in the female cohort, the analysis also revealed five significant differences. The number of differences encountered in the study was almost exactly that would have been found by chance alone; therefore, these results likely represented random variation and not a treatment effect.

This supposition is supported by the fact that several of the differences occurred between treatment groups in the absence of a difference between treated and untreated mice. If the

treatment had an effect, a significant difference would be expected between the mice that did not receive a treatment and those that did. Only three of the ten statistically significant differences in this study occurred between a treatment group and the untreated group. In these three cases, the untreated group actually had a higher withdrawal threshold than the treatment group, which is the opposite effect from that which is expected based on the literature. Therefore, the statistically significant differences observed in this behavioral study should be considered artifacts of random chance, not meaningful experimental results.

Taken together, the above evidence highlights the important distinction between statistical and biological significance of results. For example, the expression of several of the genes displayed a highly significant effect of time when analyzed by two-way ANOVA with repeated measures. However, post-hoc testing did not reveal significant differences between the levels of expression at many of these time points. Even though the results carried statistical significance, the biological importance of these results was minimal. Therefore, it is always important to distinguish between statistical and biological significance when conducting research.

While no significant results were found in this experiment, the potential of combined IL-10/IL-10R1 gene therapy for neuropathic pain is not annulled by these results. Notably, the pilot data indicated that the combined gene therapy prolonged anti-allodynic effects when compared to IL-10 gene therapy alone. IL-10 gene therapy has been shown to be effective in a number of studies (Milligan et al., 2005a, 2005b, 2006b; Ledebøer et al., 2007; Sloane et al., 2009b; Soderquist et al., 2010; Dengler et al., 2014), including studies which used adeno-associated viral vector to deliver the gene therapy (Milligan et al., 2005b). In this study, the IL-10 gene therapy did not have any effect. It is possible that the serotype of the viral vector was the reason for this,

as this study used AAV9 while a past study used an AAV2 vector (Milligan et al., 2005b). However, it is unlikely that a difference in the serotype of the viral vector would render it ineffective, especially considering the effect shown in the pilot data. It is more likely, therefore, that the specific vector used in this study was not effective. The lack of expression of the transgene in two out of three mice injected with the vector lends support to this hypothesis. In future studies, this combination gene therapy could be delivered through different methods. Possible candidates include naked plasmid DNA (Milligan et al., 2006a), naked plasmid DNA encapsulated in PLGA microparticles (Soderquist et al., 2010), or naked plasmid DNA injected with D-mannose (Dengler et al., 2014).

In future work, it would also be worthwhile to study the localization of the IL-10R1 introduced by the gene therapy. In past experiments, the gene therapy has primarily led to expression of the transgene within the meninges (Milligan et al., 2005b). However, microglia reside within the CNS (Vilhardt, 2005). Since microglia play an important role in secreting the pro-inflammatory cytokines that induce chronic pain (Watkins and Maier, 2003), these microglial cells constitute a potential target for a gene therapy designed to increase IL-10R1 expression. Alternately, the gene therapy could also be effective if it increases the expression of IL-10R1 in other cells with secrete pro-inflammatory cytokines. The meninges contain macrophages (Braun et al., 1993; Ledebuer et al., 2007), and the overexpression of IL-10R1 in these cells could decrease the release of pro-inflammatory cytokines. Therefore, future studies should determine which cells express the IL-10R1 introduced by gene therapy.

Assuming the gene therapy successfully targets microglia or macrophages, evidence from *in vitro* studies supports a potential contribution of endocytosis of IL-10R1 in the development of neuropathic pain. IL-10R1 shows a rapid, marked decrease in its expression (as measured by

mRNA levels) following exposure to LPS (Ledebøer et al., 2002). Additionally, exposure to high doses of IL-10 decreases expression of IL-10R1 when measured 24 hours after the administration of IL-10 (Ledebøer et al., 2002). This study only investigated the levels of IL-10R1 expression for 48 hours following exposure to either LPS or IL-10. However, the evidence presented in the study provides support for the hypothesis that exposure to IL-10 in a neuropathic pain model might induce endocytosis of IL-10R1. Additional *in vitro* studies that monitor IL-10R1 levels for a longer period of time could further elucidate the trends in the expression of the receptor following exposure to various substrates. If IL-10R1 is indeed down regulated in cells following peripheral nerve injury, the IL-10R1 gene therapy could counteract this phenomenon to reduce the severity of neuropathic pain.

The data collected in this study support the hypothesis that IL-10R1 may be endocytosed following CCI. IL-10R1 levels showed a statistically significant decrease compared to naïve mice 7 days after gene therapy injection in female mice (Figure 16). Additionally, male mice displayed a trend toward lower IL-10R1 levels 7 days after injection as compared to naïve (not statistically significant). While these results are not robust, they provide evidence that this hypothesis warrants further investigation.

Taken together, the promising results of the pilot study and the *in vitro* evidence showing endocytosis of IL-10R1 in response to glial activators and IL-10 suggest that combined IL-10 and IL-10R1 gene therapy should continue to be investigated as a potential therapy for neuropathic pain. While the studies performed for this thesis did not yield convincing evidence that this approach is effective, they did not provide conclusive evidence that it does not work. Additionally, the study encountered obstacles as described above. Therefore, future studies, as

described earlier in this discussion, should be performed to continue to investigate the potential of this combined gene therapy for attenuating neuropathic pain.

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