Promoter-targeted anti-nociceptive HSV-1 vectors have differential effects on pain based on the neuronal population targeted

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Herpes simplex virus-1 (HSV-1) is a neurotropic virus capable of infecting sensory neurons through their termini at any site on the body. Upon infection, the HSV-1 genome persists in nuclei within sensory ganglia for the life of the host. Replication-defective HSV-1 vectors allow for the efficient transduction of diverse populations of sensory neurons without risk of herpetic disease and therefore represent an ideal gene therapy vehicle for the treatment of peripheral neurological diseases, including chronic pain. To evaluate the potential of HSV-1 vectors to treat pain, a set of replication-defective HSV-1 vectors was generated, driving the expression of an anti-nociceptive product ($GlyR_{IS}$) from neuronal promoters to target expression to distinct neuronal populations. Specifically, the TRPV1 promoter was used to target heat-sensitive nociceptors, and the NF200 promoter was used to target large diameter Aβ-fibers which may be recruited for pain signaling after injury or inflammation. The ubiquitously expressed CMV promoter was used as a control. As expected, when these vectors were used to transduce cultured DRG cells, the neuronal promoters largely expressed in neuronal cells only, while the CMV promoter expressed in neuronal and support cells. For pain studies, vectors were injected under the skin of the right hind footpad in rats. After nine days, baseline thermal and mechanical withdrawal thresholds were taken before and after the application of the ligand ivermectin, activating vector-delivered $GlyR_{IS}$. TRPV1-$GlyR_{IS}$ transduced rats and to a lesser extent CMV-$GlyR_{IS}$ transduced rats showed increased thermal withdrawal thresholds on the transduced side

after ligand administration. Resiniferatoxin (RTX) was then injected to ablate TRPV1+ neurons and induce mechanical allodynia. After 20 days, all RTX-injected rats showed increased thermal withdrawal thresholds with a loss of dependence on vector injection and ligand administration, consistent with TRPV1+ neuron ablation. All RTX-injected rats developed bilateral mechanical allodynia, except for the NF200-GlyR_{IS} transduced rats (Aβ-targeted) which demonstrated decreased mechanical allodynia on the transduced side relative to the contralateral side after ivermectin administration. These differential effects on nociception represent the functional outcome of differentially targeted anti-nociceptive HSV-1 vectors and support the use of promoter-targeting to express transgenes in specific neuronal subpopulations.

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PREFACE

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1.0 INTRODUCTION

Sensory neurons represent the initial site of contact between humans and all the various stimuli of the outside world. If that contact is sufficient to generate an action potential in the neuron, then that stimulus has passed the first hurdle to conscious detection --- all other potential stimuli will go undetected. If that stimulus is noxious, representing the extremes of temperature or the upper extreme of mechanical force, it triggers action potentials through specialized sensory neurons called nociceptors, and may ultimately result in the sensory and emotional experience of pain once processed by the central nervous system (CNS). This acute pain response is adaptive, fostering the conscious avoidance of the source of the noxious stimulus and thereby the avoidance of tissue damage. However, if this pain persists after the noxious stimulus has been removed and the associated tissue damage has resolved, it is considered chronic pain and is no longer adaptive, but pathological. Localized chronic pain (e.g. pain persisting after tissue injury or focal inflammation) requires ongoing input from the peripheral sensory neurons innervating the site of pain. This is evident because administration of lidocaine can be used to block action potential propagation in the sensory neurons, resulting in temporary abatement of chronic pain originating in the peripheral nervous system (PNS) [1]. The sensory neuron therefore represents an accessible target for therapeutic interventions to treat localized chronic pain. Therapy targeting sensory neurons would have the benefit of not affecting the CNS and thereby avoiding the cognitive side effects of systemic opioid therapy. Local anesthetic nerve blocks are not

typically used on a long-term basis due to the risk of overdose, toxicity, and the development of tolerance. Therefore, there is a need to develop novel therapy to quench pain signaling at its peripheral source. The ideal peripheral pain therapy would meet the following criteria: 1- Have an effect restricted to sensory neurons to avoid systemic and motor side effects, 2- Have no potential for toxicity to sensory neurons, and 3- Target the specific population of sensory neurons whose signaling is mediating the pain.

Herpes simplex virus-1 (HSV-1) vectors represent the ideal vehicle for such a therapeutic, because they have the advantage of specifically transducing and persisting in sensory neurons innervating the site of inoculation without spread to the CNS (criterion 1), and have no long-term neurotoxicity or oncogenic potential (criterion 2). Furthermore, replication-defective HSV-1 vectors have been previously used in clinical trials, demonstrating their safety in patients [2, 3]. Therefore, to develop HSV-1 gene therapy for chronic pain, it will be necessary to select an appropriate anti-nociceptive transgene to silence sensory neuron signaling and to target the expression of this product to the sensory neuron population responsible for the maintenance of pain. In this introduction I will review the study of pain, current pain therapy and its limitations, the use of HSV-1 vectors for pain gene therapy, the selection of an anti-nociceptive transgene and the choice of a relevant rodent model for this study.

1.1 PAIN

Pain is defined by the International Association for the Study of Pain as “An unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage” [4]. Chronic pain, while often initiated with nerve damage or inflammation due

to a trauma or infection, is recognized as a pathological process and disease separate from its initiating circumstances [5]. Worldwide, approximately 40% of the population has experienced chronic pain within the last year, with advanced age, female gender, and co-morbid depression or anxiety disorders being associated with higher likelihood of pain [6].

Chronic pain is an umbrella term encompassing a diverse set of pathologies such as pain localized to a peripheral site of injury or inflammation, generalized pain of CNS origin like fibromyalgia, and persistent headache. It could originate from a peripheral or central lesion, or have large contributions from PNS, CNS, and inflammatory mechanisms, as in complex regional pain syndrome [7]. Also, affect and psychological state can have a significant impact on disease severity [8, 9].

Pain originating from a peripheral lesion occurs when primary afferent nociceptors are sensitized, resulting in hyperactive signaling (peripheral sensitization) [9]. This is in part mediated by the recruitment of immune cells and release of inflammatory mediators such as histamine, substance P, bradykinin, NGF, TNFα, prostaglandins & leukotrienes [10]. Primary afferent nociceptors synapse in the dorsal horn of the spinal cord to second order, wide dynamic range neurons. With repetitive signaling from sensitized nociceptors, these neurons likewise become sensitized (central sensitization) by phosphorylation of receptors and ion channels and long-term changes in gene expression [9]. Additionally, microglia in the spinal cord can be activated by chronic nociceptor signaling, causing the release of pro-inflammatory cytokines and further contributing to central sensitization [11]. Peripheral and central sensitization can manifest not only as ongoing or spontaneous pain, but as a painful sensation arising from innocuous stimulation (allodynia) and increased pain in response to noxious stimulation (hyperalgesia) [12].

At the level of the second order neuron, transmission is influenced by inhibitory interneurons using GABA or glycine as their neurotransmitter [13] and inhibition by endogenous opioids [13, 14]. These controls become impaired in the presence of chronic pain and central sensitization [15, 16]. Second order neurons then transduce the signal to the brainstem, thalamus, and from there to higher levels of the brain, including the primary and secondary somatosensory cortices, the insula, and the anterior cingulate cortex. These areas have been termed the "pain matrix" [17] because of their demonstrated activity by functional brain imaging during the experience of pain [18], but may be a more general system for recognition of salient stimuli [19].

While central mechanisms are of great importance to the pathophysiology of pain, this introduction will specifically focus on the peripheral drivers of chronic pain resulting from peripheral injury, inflammation, or infection. This localized pain has the potential to be treated by inhibiting aberrant signaling from primary afferents directly, or by the reduction of peripheral inflammation.

1.1.1 The nociceptor and PNS involvement in chronic pain.

Sensory neurons innervating the skin are composed of a diverse population of fibers, classified based on diameter, myelination, conduction velocity, and neurotransmitter; and further defined by the expression of various sensor ion channels, making each fiber sensitive to a particular set of stimuli. Given the complex sensory milieu that these neurons must contend with, this specialization allows for the discrimination of a broad range of mechanical and thermal stimuli spanning from innocuous-to-painful and hot-to-cold. The neurons responsive to painful stimuli, termed nociceptors, are typically small diameter, unmyelinated, slow-conducting C-fibers and

lightly-myelinated, faster-conducting Aδ fibers [20]. The C-fibers can be broadly classified into two groups which are developmentally responsive to different growth factors and utilize different neurotransmitters. The trkA-positive C-fibers are sensitive to the neurotrophin NGF and utilize peptide neurotransmitters, whereas the c-RET-positive C-fibers are sensitive to GDNF and utilize non-peptide neurotransmitters [21].

The changes in the underlying physiology that result in the development of maladaptive chronic pain are complex and incompletely understood; however, different fiber types are implicated in chronic pain originating from different causes. Specifically, peptidergic C-fibers are implicated in inflammatory pain, whereas non-peptidergic C-fibers and rapidly conducting mechanosensitive Aβ-fibers are implicated in pain signaling following nerve injury [12, 22-24].

TRPV1-positive C-fibers are responsible for the sensing of heat pain [25-27]. This can be experimentally assessed with the thermal withdrawal latency tests. TRPV1-expressing peptidergic C-fibers also play a role in inflammatory hyperalgesia. Furthermore, in the presence of persistent inflammation or following nerve injury, they contribute to progressive mechanical hypersensitivity [28, 29].

1.1.2 Pain therapy and limitations.

Given the complex pathophysiology of chronic pain, a wide variety of drugs can be employed alone or in combination for therapy [8]. Among these are opioids, anticonvulsants, antidepressants, non-steroidal anti-inflammatory drugs (NSAIDs), cannabinoids, NMDA receptor antagonists, local anesthetics, capsaicin, botulinum toxin, and others [30, 31].

For the purpose of this introduction I will focus on the most important traditional pain therapeutics as grouped into four categories: (i) narcotic opioids which signal through the μ-

opioid receptor on second order neurons to reduce pain signaling; (ii) neuromodulatory drugs such as anticonvulsants (gabapentin & pregabalin) and antidepressants (tricyclics & SNRIs); (iii) NSAIDs which function to reduce inflammation that can sensitize primary afferents; and (iv) nerve blocks delivering a sodium channel blocker which prevents action potential propagation, and/or a corticosteroid to drastically reduce inflammation, directly to a neuropathic lesion. Each of these modalities has its own indications and limitations.

Opioids prescribed for pain management are in the morphine class and are agonists of the μ-opioid receptor, which principally inhibit peripheral pain signaling at the level of the second order spinal neuron. Opioids are the strongest systemically delivered pain medications and as such are widely used to treat a large variety of pain conditions [32]. Unfortunately, tolerance develops rapidly to their repeated use, which can lead to dependence, addiction, and withdrawal when discontinued. Paradoxically, opioid use can result in increased pain levels, a phenomenon termed opioid-induced hyperalgesia [33]. Furthermore, as a result of systemic administration, opiates can cause side-effects in a diverse set of organs. Side effects include constipation, nausea, orthostatic hypotension, anti-coagulation, cognitive impairments, and respiratory depression [34], the last of which is potentially fatal in the case of overdose.

Gabapentin and pregabalin are often used as first-line therapy for chronic neuropathic pain. Though first developed as GABA analogues and used as anticonvulsants, they have been widely applied to the treatment of chronic neuropathic pain [35] and for the prophylactic reduction in the development of post-surgical chronic pain [35, 36]. For these applications, their principal mechanism of action is as a ligand of voltage-gated calcium channels by binding to the α2-δ-1 subunit [37]. This subunit is upregulated in DRG and spinal cord after nerve injury [38]

and gabapentin binding results in a subsequent reduction in release of excitatory neurotransmitters in the dorsal horn of the spinal cord [39].

Antidepressants, and specifically tricyclics and selective norepinephrine reuptake inhibitors (SNRIs) are also prescribed as first-line therapy for neuropathic pain [8]. Antidepressants are efficacious for the treatment of chronic pain regardless of the presence of comorbid depression, but would obviously have a dual benefit to depressed patients [40]. This hints at an alternative mechanism of action when used as a pain therapeutic, but this mechanism is not fully understood.

The anticonvulsants and antidepressants are used as first-line pain therapy, but often require the addition of additional drugs to achieve adequate control of severe pain. Additionally, not all patients tolerate the cognitive effects of these drugs.

NSAIDs inhibit cyclooxegenase and the synthesis of prostaglandins and therefore have an anti-inflammatory effect. NSAIDs can be effective in the treatment of mild to moderate pain resulting from inflammation, but are typically not effective in controlling severe pain or pain with a significant neuropathic component. They are therefore most commonly used as adjunct therapy when used in these situations [41].

Nerve blocks [42, 43] have the benefit of being highly effective when properly placed, at the cost of invasiveness – requiring catheter placement into sensitive anatomy. Therefore, they are usually limited to the immediate post-surgical time-frame. Because they deliver therapeutics directly to the site of a neuropathic lesion, they can deliver drugs that would be toxic if taken systemically. A corticosteroid is often used to control inflammation to a much higher degree than can be achieved by the systemic use of NSAIDs. Furthermore, to directly inhibit action

potential propagation, sodium channel blockers can be used, while avoiding dangerous systemic side effects like interference with cardiac muscle contraction.

For pain therapy to work optimally, any comorbid depression or anxiety should be treated in tandem. Furthermore, to address the higher cognitive and affective components of pain, mindfulness meditation, in which the patient attempts to become an observer of her own sensorium and thought processes, has proven to be particularly effective [44, 45]. Hypnosis may also be an effective adjunct pain therapy [46].

Despite the diverse array of available pain therapy, a large proportion of chronic pain sufferers have poorly controlled pain which negatively affects their quality of life [32]. Due to the cognitive and physiological side-effects caused by many traditional systemically-active therapies, many treatment options may prove intolerable. Therefore, the development of novel pain therapies with greater efficacy and fewer side-effects is of critical importance.

1.2 GENE THERAPY FOR PAIN

Given the devastating impact of chronic pain on quality of life and the limitations of conventional pain therapy, there is a desperate need for new, safe, effective and long-term treatments. Pain gene therapy is a relatively new treatment modality. Of the 2142 gene therapy clinical trials to date since 1989, 39 have been indicated for neurological diseases, and of that, two have been indicated for pain [47].

Gene therapy has the potential to deliver one or several gene products directly to the relevant anatomical location [48, 49]. Once delivered, the genes could continuously generate products over the long-term, as demonstrated with HSV-1 vectors [50, 51].

Gene therapy has the potential to overcome each of the limitations of conventional therapy. By shifting the paradigm from small molecules delivered systemically to the targeted expression of anti-nociceptive genes directly to the source of aberrant pain signaling, gene therapy can both avoid systemic side-effects (especially of opioids) and concentrate potent neuromodulatory or anti-inflammatory products directly to their site of action to maximize therapeutic efficacy. For instance, gene therapy can be used to express a potent anti-inflammatory cytokine to control local inflammation without compromising systemic immunity, or an endogenous opioid without engendering tolerance [49, 52]. Alternatively, gene therapy can achieve nerve block without the necessity (and risk of systemic toxicity) of sodium channel blockade and without the risk of neuronal damage by the invasive placement of catheters [53].

1.2.1 HSV-1 vectors.

After inoculation on broken skin or at mucous membranes, HSV-1 undergoes lytic replication in fibroblasts and epithelial cells. As large numbers of virions accumulate at the inoculation site, particles adsorb at sensory nerve termini and infect the neuron. Particles are then transported retrograde to the neuron cell body in dorsal root ganglia (DRG) or trigeminal ganglia (TG) where the viral genome is delivered into the nucleus [54]. In neurons, productive replication is limited and the genome enters a latent state, characterized by minimal transcription of viral genes. During latency, the HSV-1 genome is circularized and heterochromatinized. One non-coding gene product is expressed in a fraction of transduced neurons: the latency associated transcript (LAT). The function of LAT is controversial, generating two stable introns and several microRNA species, but LAT-deleted viruses are impaired in reactivation efficiency [55].

Furthermore, the LAT promoter sequences, active in neurons, have been used to drive long-term expression of transgenes in the CNS [50] and PNS [51].

To generate HSV-1 vectors for gene therapy [56, 57], one common strategy to render HSV-1 replication-defective is to introduce deletions into one or both of the essential immediate early genes ICP4 and ICP27. The resultant recombinant vectors are unable to progress beyond the immediate early stage of gene expression unless grown in cells complementing these deletions [58]. This is often paired with the deletion of the internal repeat region, which prevents isomerization of the genome, as well as eliminating one copy of immediate early genes ICP0 and ICP4 and reducing expression of ICP22. This further reduces viral gene expression without overly compromising vector yield [59].

HSV-1 vectors are currently viewed as a niche vector in the field of gene therapy, accounting for only 3.1% of clinical trials [47]. This is in large part due to practical concerns, with the large size of the HSV genome (~152 kilobase-pairs) making vector engineering a more technically involved process, carried out through homologous recombination in cell culture or through manipulation of the vector propagating as a bacterial artificial chromosome (BAC) in *E. coli* [60]. Furthermore, the larger size results in a greater probability of off-site mutations accumulating through the process of vector engineering. Therefore, HSV-1 vectors are chosen over their more widely used alternatives only for applications where HSV has a significant advantage in transduction efficiency. In other words, HSV-1 vectors are used almost exclusively for the transduction of neurons and have a particular advantage in the transduction of primary afferents, where the use of other vectors typically necessitate the direct injection of high titers directly into sensory ganglia [61].

The large size of HSV-1 vectors, most of which contain large deletions (e.g. the commonly deleted internal repeat is ~15 kilobase-pairs), also significantly increases the carrying capacity. Therefore, HSV-1 vectors can accommodate very large or multiple transgenes. Relevant to this study, the inclusion of large genomic promoter sequence for the purpose of achieving endogenous expression activity is possible with HSV-1 vectors.

1.2.2 Anti-nociceptive transgenes.

Six general strategies have been used to treat pain with HSV vectors: (i) expression of the endogenous opioids, preproenkephalin (PPE) [2, 3, 62-75] & endomorphin [76, 77] for tonic analgesia, (ii) expression of glutamic acid decarboxylase (GAD) to increase GABA-mediated inhibitory tone [2, 78-81], (iii) expression of interleukins to reduce inflammation [82-84] and inversely, of a soluble TNFα receptor to decrease TNF signaling [85], (iv) expression of neurotrophins to promote neuronal survival [86-91], (v) knockdown of pain-induced sodium channel subunit NaV1.7α, and (vi) expression of the inhibitory ion channel GlyR for targeted neuronal silencing [53], as used in this study.

1.2.3 Endogenous opioids: Preproenkephalin A (PPE) & endomorphin.

PPE is the precursor to the endogenous opioids Met- & Leu-enkephalin which signal principally through delta opioid receptors [14]. It was the first transgene tested [92] and the transgene with the most extensive history of use for anti-nocieptive activity in animal models [49]. It is also the only one tested in clinical trials [2, 3] for the treatment of pain with HSV-1 vectors. By expressing PPE in sensory neurons, enkephalins can act directly on nociceptors themselves in an

autocrine or paracrine manner, or be released into the synapse with the second order neurons in the dorsal horn of the spinal cord to limit pain transmission higher up the neuraxis. Extensive studies using HSV-expressed PPE have been conducted on rodent pain models as well as in primates and clinical trials.

In rodents, the expression of PPE from HSV-1 vectors has been effective in reducing pain behavior in models of inflammatory pain, neuropathic pain, visceral pain, and specific disease models.

Inflammatory pain models ---

PPE driven from the cytomegalovirus immediate early promoter (CMV) demonstrated greatly reduced thermal hyperalgesia following capsaicin or DMSO induced hypersensitivity, while baseline thermal responses were unaffected [64]. PPE also reduced pain behavior in the formalin test of inflammatory pain, an effect that could be blocked with opioid receptor antagonist naltrexone [65]. A study on chemically induced polyarthritis demonstrated increased locomotion, lower hyperalgesia, and less bone destruction with the use of the PPE vector [66].

Neuropathic pain models ---

PPE expression was able to reduce open field pain scores from a bone cancer pain model [62]. A study looking to more extensively characterize the effect of PPE in a neuropathic pain model demonstrated that PPE expression prevented pain-induced c-fos expression in the dorsal horn [63]. PPE expression worked synergistically with morphine, lowering the ED_{50} by ten-fold, and the anti-nociceptive effect of PPE was maintained even after tolerance was induced to morphine [63]. To study PPEs effect in a long-term pain model, pertussis toxin was injected intrathecally in mice and PPE expression was able to reverse the induced thermal hyperalgesia

for up to six weeks [67]. Two studies on trigeminal neuropathic pain [69] or myogenic orofacial pain [75] demonstrated lowered pain scores with PPE expression.

Visceral pain models ---

Two studies using a model of bladder hyperactivity and pain demonstrated naloxone-reversible reduced pain behavior following the intrabladder injection of capsaicin [70] or resiniferatoxin [74].

Specific disease models ---

Two studies have assessed PPEs effects on a rat model of pancreatitis [71, 72]. The first demonstrated increased spontaneous behavior, preservation of cellular architecture, and reduced inflammatory infiltrates in the pancreas [71] and the second study demonstrated a normalization of hot-plate sensitivity and prevention of fos induction in the dorsal horn [72]. One study using a model of painful diabetic neuropathy found that the expression of PPE could reduce the induced expression of the NaV1.7 sodium channel in primary afferents and fos induction in the dorsal horn, as well as lowering pain behaviors [68]. Also, the expression of PPE was able to prevent the development of hypersensitivity in a model of post-herpetic neuralgia [93].

A primate study [73] in Macaques demonstrated an anti-hyperalgesic effect of PPE to repeated thermal and chemical stimulation that lasted up to 20 weeks after injection of the HSV-1 vector in the dorsal surface of the foot.

Finally, an HSV-1 vector expressing PPE has been tested in a phase I and ongoing phase II clinical trial [2, 3] for intractable focal pain caused by cancer in terminal patients. The phase I trial demonstrated the safety of this approach, with no serious adverse events reported. It also provided promising preliminary data on efficacy. Subjects receiving the higher vector doses (three patients per group receiving 10^8 or 10^9 pfu) reported lower pain scores throughout the

study. Those patients surviving to the trial's final time-point of 28 days post-injection had pain scores that had risen from their nadir, but had not returned to pre-injection levels [3].

Related to this approach is the expression of a synthetic gene producing the endogenous opioid endomorphin, which signals through μ-opioid receptors. Endomorphin expression reduced pain responses in both neuropathic [76] and inflammatory pain models [77].

Despite the impressive collection of anti-nociceptive data generated using PPE or endomorphin, endogenous opioids were not selected for this study because they can act in a paracrine manner on nearby neurons in the DRG and across the synapse with second order neurons, and therefore are unsuitable for targeted neuronal silencing.

1.2.4 Glutamic acid decarboxylase (GAD).

GAD catalyzes the rate limiting step in GABA synthesis. Its production by HSV-1 vectors serves to increase levels of the inhibitory neurotransmitter GABA to inhibit pain signaling through second order neurons in the spinal cord.

With increased nociceptive input from peripherally sensitized nociceptors, there is a decrease of spinal inhibitory GABAergic tone and reciprocal increase in excitatory glutamatergic tone [94]. GABA is biosynthesized from glutamate by GAD, so by increasing GAD levels, gene therapy can shift the balance from glutamate-mediated excitation to GABA-mediated inhibition. GAD expression reduced pain measures in several neuropathic pain models including spinal cord hemisection [81], spinal nerve ligation [78], lumbar radiculopathy [95], and painful diabetic neuropathy [79] where it also prevented increase in NaV1.7 levels. Furthermore, GAD expression was demonstrated to be more effective than PPE in reducing pain responses in the streptozotocin model of painful diabetic neuropathy [96].

Like PPE, the mechanism of action of GAD expression is through the secretion of a soluble mediator (in this case GABA), and therefore unsuitable for targeted neuronal silencing.

1.2.5 Anti-inflammatory interleukins and the TNFα receptor.

Inflammation can sensitize primary afferents causing increased spontaneous activity and lowered thresholds to generate action potentials, leading to the development of chronic pain. One general strategy to treat inflammatory pain is to express anti-inflammatory cytokines such as IL-4, IL-10, or IL-13 to reduce inflammation, or a soluble TNFα receptor to reduce TNFα-mediated inflammatory signaling. IL-4 [82] and a soluble TNFα receptor [85] expressed from HSV-1 vectors have reduced pain behaviors in a neuropathic pain model. Expression of IL-10 decreased pain behaviors in the formalin test of inflammatory pain [83] and reduced TNFα production from activated microglia *in vitro*.

Use of anti-inflammatory cytokines may be ideal for the reduction of inflammation in inflammatory pain or at the site of a neuropathic lesion in a manner akin to the anti-inflammatory component of a nerve block.

1.2.6 Pro-survival factors: NT-3, NGF, VEGF, & EPO.

For the treatment of diabetic or chemotherapy-induced polyneuropathy, growth factors can be expressed from HSV-1 vectors to serve as pro-survival signals to affected afferents. In these experiments HSV-1 vectors are injected prior to the induction of neuropathy to assess a protective effect. In pyridoxine-induced neuropathy, a protective effect was demonstrated for neurotrophin-3 (NT-3) [97] and nerve growth factor (NGF) [87]. The effect from NGF

expression could be sustained to six months by the use of HSV-1 LAP2 to drive long-term expression [51]. Similarly, expression of NT-3 or NGF protected against cisplatin-induced neuropathy [88].

In the streptozotocin model of painful diabetic neuropathy expression NGF [86], VEGF [98], NT-3 [89], and EPO [90] were protective. The EPO expressing vector, originally using the CMV promoter to drive transgene expression, was subsequently modified to make it doxycycline-inducible [99]; and then finally modified for prolonged, regulated expression using a doxycycline-inducible LAP2 promoter [91].

Use of pro-survival factors may be ideal for the prevention of painful diabetic neuropathy or chemotherapy-induced polyneuropathy. Prophylactic treatment is feasible in both cases as it can be administered prior to chemotherapy or early in the course of diabetes, before the development of neuropathy.

1.2.7 Knockdown of NaV1.7α.

NaV1.7 was strongly implicated in pain by the identification of disease-causing mutations resulting in primary erythermalgia [100, 101], a painful inherited neuropathy, and paroxysmal extreme pain disorder [102]. The pore-forming α-subunit of NaV1.7 is upregulated in the context of streptozotocin-induced painful diabetic neuropathy [103], influencing electrical properties of primary afferents and contributing to peripheral sensitization. Two groups have used HSV-1 vectors to knockdown levels of NaV1.7α [104, 105]. The first expressed an antisense sequence and demonstrated a reduction in inflammatory hyperalgesia [105]. The second used an miRNA approach and demonstrated pain reduction in a model of painful diabetic neuropathy [104].

Knockdown strategies have the potential to be combined with transcriptional or transductional targeting strategies (discussed below) to achieve targeted knockdown of inflammatory or nosogenic products.

1.2.8 Glycine receptor (GlyR) and ivermectin sensitive mutant $GlyR_{IS}$.

The glycine receptor (GlyR) is a ligand-gated chloride channel activated by glycine and expressed in the CNS, but not in the PNS. Its activation in the spinal cord and brainstem results in inhibition of action potential generation. Additionally, the absence of endogenous GlyR in the PNS means that the application of glycine after vector delivery would have an effect limited to vector-delivered channels. For these reasons it was an attractive candidate for peripheral neuronal inhibition.

GlyRs are pentamers composed of two of four possible $GlyR_{\alpha}$ subunits and three $GlyR_{\beta}$ subunits [106]. For transgene purposes, it has been demonstrated that receptors formed as a homopentamer of $GlyR_{\alpha 1}$ are fully functional [107-111]; therefore, this subunit can be expressed from gene therapy vectors as a stand-alone anti-nociceptive transgene.

Early studies of HSV-delivered GlyR in rats demonstrated reduced thermal and operant pain scores in inflammatory pain models and reduced pain scores in a bladder model of visceral pain [53], providing proof of principle for nociceptor silencing using this approach.

This study employs an ivermectin-sensitive variant of GlyR [112], herein referred to as $GlyR_{IS}$, in which two point mutations have been introduced, changing its ligand from glycine (which is not bioavailable if taken orally, and may be present in the PNS milieu) to the FDA-approved [113] anti-helminthic drug ivermectin. This was done to increase the clinical relevance of these vectors by allowing for the future use of orally delivered ivermectin with the intention

that it would specifically activate the vector-delivered $GlyR_{IS}$ channels without off-target effects. However, the study described herein uses local injection of ivermectin, not oral or systemic delivery.

1.2.9 Targeting transduction and transgene expression.

Extensive progress has been made on vector targeting, following the principle of limiting the effect of vectors to the set of cells necessary for the desired therapeutic outcome, and thereby increasing both safety and efficacy. In general, this can be achieved on three non-mutually exclusive levels. The first is the choice of the viral vector, with each species possessing a degree of tropism or evolved preference for certain cell types within the body (in the case of HSV, for sensory neurons). The second is the engineering of receptor-binding proteins on the vector's surface to direct attachment and entry to a desired cell type (transductional targeting). And the third is on the level of gene expression (transcriptional targeting).

Transductional targeting was first achieved by pseudotyping, or the exchange of surface proteins for those of another virus to adopt its entry mechanism. This was classically done with the VSV-G glycoprotein to take advantage of its broad tropism [114]. Transductional targeting has become more sophisticated by generating recombinant viral surface proteins to alter receptor binding. This can be done by directed evolution of a vector with a broad tropism towards specificity for a chosen cell type within its existing repertoire [115-117], by the introduction of point mutations to eliminate receptor binding [118, 119], or the rational engineering of viral surface proteins to replace native receptor-binding domains with novel ones [119].

Transductional targeting has been employed on HSV-1 vectors. To accomplish this, mutations are made in gD, the glycoprotein which binds to HSV-1 receptors HVEM and nectin-1

[120]. By introducing mutations to eliminate HVEM and nectin-1 binding and then introducing the sequence for the variable region of a single chain antibody, HSV-1 entry was redirected to use the epidermal growth factor receptor [119].

Transcriptional targeting, also referred to as promoter-targeting, relies on the use of cell type-specific promoter elements to drive transgene expression in the subpopulation of transduced cells in which the promoter is endogenously active, based on the cellular epigenetic and transcription factor milieu [121]. This strategy is the most flexible, allowing the targeting of a cell type on the basis of the expression of a marker gene of the investigator's choosing. This is particularly useful when targeting expression to neurons, as neuronal populations can be classified by a wide variety of factors, including, fiber size, conduction velocity, neurotransmitter expression, receptor expression, and the presence of electrophysiologically distinct currents based on the presence of certain ion channels [122].

For the development of gene therapy, promoter-targeting has been used to target a diverse range of cell types in tissues throughout the body, including extensive work in neuronal targeting in the CNS [123].

Transcriptional targeting of HSV-1 vectors has focused on the development of oncolytic therapy. The HSV-1 essential gene encoding ICP4 was engineered to target expression to albumin-expressing cells to limit HSV-1 replication to this cell type [124]. More recently, HSV-1 was transcriptionally targeted to replicate in prostate tumors, using a prostate-specific promoter driving the essential gene encoding ICP27, in combination with transductional targeting [125]. Another study characterized eight promoters whose genes were highly expressed in hepatocellular carcinoma (HCC) by microarray and used them to express a marker gene from HSV-1 vectors in HCC *in vitro* and *in vivo* [126].

Finally, to further target gene expression, HSV-1 vectors have also employed miRNA recognition sequences to limit expression in off-target cell types [127].

This study uses the flexibility of transcriptional targeting, allowing for the selection of target neurons based on the expression of a marker gene. We use the TRPV1 promoter to drive $GlyR_{IS}$ expression in heat sensitive nociceptors, and we use the heavy neurofilament (NF200) promoter to drive expression in large diameter Aβ-fibers.

1.3 PAIN MODELS & TESTS OF NOCICEPTION

The definition of pain ("An unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage" [4]) includes an affective component – "unpleasant", "emotional" – and alternatively, relies on a subjective description. This immediately presents two obstacles to the creation of rodent models of pain: the obvious difficulty in assessing a rodent's affect, and the inability of a rodent to provide a description. Given these limitations, the tests used to assess pain in rodents are more precisely tests of nociception, defined as the neural process of encoding stimuli that are damaging or threaten damage to normal tissues.

Noxious stimuli evoke a defensive response in rodents (e.g. a tail flick, paw withdrawal, escape behavior, etc.) and tests are designed to quantify the presence or frequency of responses, or latency until such a response is observed. When a noxious stimulus is used, greater or earlier responses from test animals are defined as hyperalgesia. Allodynia, on the other hand is the state in which stimuli that are normally innocuous are perceived as noxious and elicit a response.

Nociceptive tests can be conducted on naïve animals or on animals that have been genetically, surgically, chemically, or virally manipulated to induce pain.

Tests of nociception have a long history of use and have been the subject of several review articles [128-130]. They can be either tests of evoked responses or measures of ongoing or spontaneous activity (e.g. place preference tests). Tests used to evoke a nociceptive response are typically grouped according to the type of stimulus used: mechanical, thermal, and electrical. Electrical stimulation activates fibers non-specifically and has limited clinical applicability, so I will focus attention on the former two. Mechanical and thermal stimuli are detected through activation of distinct receptors (mechanoreceptors and thermoreceptors) and clinically, mechanical or thermal allodynia or hyperalgesia may result depending on the specific pain syndrome. Ideally, pain experiments should be designed to closely model the clinical pain syndrome being studied and tests of nociception should be used in accordance with the pain outcomes of interest.

Most pain models fall into the categories of surgical injury or chemical irritation [131]. When selecting a pain model, an investigator will likely consider the extent of historical use of the model (i.e. “gold standard” models) allowing the investigator’s study to be compared to a wide variety of other experiments using the same model. Another consideration is the clinical relevance of the model. These are often competing interests, with models such as the formalin test having extensive historical use and yet being highly contrived with limited clinical relevance. Finally the investigator will account for any particular considerations of their experiment that may necessitate the use of one model over another. In this section I will focus on models of superficial pain corresponding to the sensory neurons in which HSV-1 establishes

latency in its viral life cycle; however, HSV-1 vectors have also been used to treat visceral [53] and cancer pain [2, 3].

1.3.1 Pain models.

Surgical pain models involve ligating or transecting nerves at established locations to result in pain localized to the region innervated distal to the nerve injury. Common surgical models of PNS pain in order of most proximal to most distal down the neuraxis (corresponding to greater to more limited pain outcomes) are: (i) spinal nerve ligation (SNL), in which L5 (with or without L6) spinal nerve(s) are tightly ligated [132]; DRG compression by insertion of a metal rod into the L5 intervertebral foramen [133]; chronic constriction injury (CCI) in which loose ligatures are bound around the common sciatic nerve [134]; partial nerve injury in which one third to one half of the sciatic nerve is ligated [135, 136]; and spared nerve injury (SNI), in which the tibial and common peroneal branches of the sciatic nerve are lesioned, sparing the sural nerve [137]. Each of these models results in a well-established pain outcome resulting from the Wallerian degeneration of constricted or ligated nerve fibers.

Disease models attempt to recapitulate a particular human disease in animals and may or may not use the agent of human disease. For instance, the models for chemotherapy-induced polyneuropathy involve administering a large dose of the chemotherapy agent, such as pyridoxine [51, 87, 97] or cisplatin [88]. Similarly, post-herpetic neuralgia, occurring in humans after a VZV viral reactivation event, is induced in rats by the injection of VZV-infected cells [93]. Other disease models use an agent which will damage the correct tissue to simulate the disease. The most widely used model in this category is the use of streptozotocin, a compound toxic to pancreatic β-cells, to model diabetes and its sequella: painful diabetic neuropathy [86,

96, 138]. Another disease model of this type involves the use of Freund's adjuvant to induce polyarthritis in rodents, to model rheumatoid arthritis [66].

Chemical pain models utilize compounds which cause inflammation, damage to neurons, or directly activate ion channels, causing pain. Inflammatory compounds can be injected under the skin of the plantar surface of the hind footpad. The most common compounds used are complete Freund's adjuvant (CFA) [139], zymosan [140], carrageenan [141], and bee venom [142]. Other compounds such as serotonin, bradykinin, prostaglandin E2, substance P and histamine can also be used [143]. Formalin is a commonly used reagent to induce inflammation and directly damage nerve fibers and has the longest history of use [144-146]. Capsaicin can be used to activate TRPV1 channels, causing increased signaling through heat-sensitive nociceptors and lowered thermal withdrawal thresholds [147].

Resiniferatoxin (RTX) irreversibly activates TRPV1 channels resulting in an influx of cytotoxic amounts of calcium and death of the neuron. Therefore early applications of RTX focused on its ability to chemically ablate the TRPV1+ neuronal population. Being that TRPV1 is the receptor for noxious heat, after RTX ablation of TRPV1+ nociceptors thermal tests of nociception show dramatic desensitization to heat as evidenced by increased thermal withdrawal thresholds [148].

Subsequently, it was observed [149] that by 20 days following systemic RTX administration, mechanical thresholds were paradoxically lowered. In other words, animals were experiencing RTX-induced mechanical allodynia. The first study to demonstrate this, also found evidence of inflammation along the length of the nerve, developing over the same time-frame [149]. It is theorized that this inflammation is secondary to the Wallerian degeneration of killed TRPV1+ nerve fibers and sensitizes the remaining large diameter Aβ-fibers.

This study takes advantage of this time-dependent, reciprocal effect (early, sustained increase in thermal thresholds due to TRPV1+ neuron ablation and late decrease in mechanical thresholds due to inflammation), to demonstrate a functional consequence of differentially targeted anti-nociceptive vectors. Specifically, the use of RTX tests the hypotheses that:

1- An anti-nociceptive effect targeted to TRPV1+ neurons will no longer be significantly different from other groups when all TRPV1+ neurons are ablated by RTX, and

2- An anti-nociceptive effect targeted to large, NF200-rich, mechanosensitive fibers will reduce the degree of mechanical allodynia caused by RTX-induced inflammation.

1.3.2 Thermal tests.

The classic thermal tests use latency before a withdrawal response from a noxious thermal stimulus as their metric. Hyperalgesia is therefore indicated by a reduction in withdrawal latency. These tests include the tail flick test (measuring latency until tail flick – a spinal reflex – after submerging in hot water or with exposure to radiant heat) [150], hot plate (latency until paw licking once placed on a 52^{o}C or 55^{o}C plate) [151, 152], cold plate (scoring of nociceptive behaviors over time) [134, 153], and the Hargreaves radiant heat apparatus test in which latency is timed from the application of a radiant heat source to the paw withdrawal reflex, once the rat is acclimated to a heated platform [154]. The Hargreaves test was selected for this study based on its reliable assessment of changes to TRPV1+ neuron function and high reproducibility do to ease of use.

1.3.3 Mechanical tests.

Around the turn of the 20th century, the Austrian-German physiologist Maximilian von Frey developed a methodology for exerting a consistent force to a punctate area of the skin. He utilized hairs of various thicknesses mounted on the ends of handheld applicators – the thicker the hair, the more force it would take to bend [155]. Thereby, the minimal threshold force/hair thickness to elicit a sensation could be assessed. Such implements were eponymously named von Frey hairs, and have kept this name to the present day despite the hair being replaced by the use of nylon monofilaments [156], which exert precisely calibrated gram weights when bent. Using von Frey hairs to determine the force threshold to elicit a withdrawal response from the hind paw of naïve rodents, one can assess whether a test rodent withdraws at a lower force when subjected to a pain model, evidencing mechanical allodynia. A common, efficient procedure to assess withdrawal thresholds and mechanical allodynia with von Frey hairs, termed the "up-down method", uses hairs of increasing force until withdrawal is elicited, then reducing the force and oscillating around the withdrawal point to determine a pattern from which withdrawal thresholds can be calculated [157].

The von Frey test has demonstrated a wide range of thresholds between experimenters, which limits extrapolation of thresholds between labs. However, there is high reproducibility within experiments. This reflects the fact that there are small variations between testing conditions (e.g. angle of application, degree of filament bending, and duration of application), which ultimately requires rigid consistency in protocol by the experimenter to control for these variances. In an intensive review of the causes of inter-experimenter differences in von Frey responses [158], Bove postulated that the ideal mechanical stimulator should apply force along a single axis (bending of filaments causes off-axis forces), via a standardized blunt transducer (not

variably sized cylindrical filaments), applied independent of the relative movements of experimenter and subject, across a continuous range of pressures instead of discrete intervals, and that the experimenter should be blind to the force necessary for withdrawal to avoid bias [158]. Recently, automated von Frey systems have been developed which address some of these concerns. The electronic von Frey developed by Ugo Basile [159] uses a continuous scale, experimenter blind approach and the electronic von Frey developed by Somedic [160] uses an inflexible tip to standardize the transduction surface. Additionally, as Bove commented in his review, many of the conditions of an ideal stimulator are met by another mechanical test, the Randall-Selitto paw pressure test. This study, however, will employ traditional von Frey monofilaments using the quantitative "up-down method" due the lab's technical expertise with this procedure and its extensive historical use [157, 161].

2.0 MATERIALS AND METHODS

2.1 VECTORS

Neuronal promoter-targeted vectors and mCherry-expressing test vectors were generated from the vL vector backbone, which was specifically designed for this experiment. The vL backbone was generated from DBAC [162], which was engineered from the HSV lab strain KOS in which BAC sequences were inserted into the TK locus by homologous recombination in cell culture. Further modifications were introduced by RedE/T mediated homologous recombination in *E. coli* [60]. Deletions were introduced in the genes encoding immediate early proteins ICP4 and ICP27, rendering the vector replication-defective [58, 163]. Further alterations were made from the DBAC vector. First, the internal repeat sequence was deleted, and the promoter for ICP27, which was deleted from its native locus, was reintroduced to drive ICP22, the promoter of which was part of the deleted repeat sequence. The Gateway destination cassette was inserted into the remaining latency locus, replacing the latency promoter elements while maintaining a wild-type copy of ICP0 and the surrounding CTCF chromatin boundary elements [164, 165]. To generate targeted expression vectors, transgenes were cloned into a modified pENTR1A plasmid, containing attL sites for recombination with the attR-containing vector backbone using the Gateway LR Clonase II enzyme mix (Life Technologies).

pENTR1A was modified to delete the CCDB gene and insert the highly-stable αGlobin 3'UTR [166, 167] and SV40 polyadenylation signal. A PmeI linker was inserted into the DraI site, upstream of the ORF insertion site, so that this 8bp restriction site would remain unique after the insertion of the $GlyR_{IS}$ or mCherry ORF. Transgene ORFs were amplified by PCR using a forward primer incorporating a Kozak consensus translation initiation sequence and then inserted into the unique HincII site of this vector. Each of the above elements was confirmed by sequencing. Finally, the promoters were inserted into the unique PmeI site as blunt-end digest fragments from other plasmids of known sequence.

In selecting the promoter sequences for the TRPV1 and NF200 promoters used in this study, we included previously characterized promoter elements. A 1,284 base-pair sequence corresponding to the TRPV1 dual promoter sequence defined by Xue et al. [168] was selected to target expression to heat-sensitive C-fiber nociceptors. For the NF200 promoter, a 970 base-pair sequence surrounding the core promoter elements was purchased from SwitchGear Genomics (Carlsbad, CA) [169], as was the 932 base-pair CGRP promoter [170]. The CMV promoter [171] was isolated from pEGFP-N1 (Clontech) as a 555 base-pair fragment by digestion with AseI and AfeI. The HSV-1 latency promoter region was amplified out of KOS DNA using the primers: gtctgatacccatcctacacc & gtctttgttgaacgacaccg. The sequences used for the CMV promoter, NF200 promoter, TRPV1 promoter, GlyRIS ORF and pENTR1A-derived pEPαGpA multiple cloning site can be found in Appendix A.

The control vector expressing $GlyR_{IS}$ from the CMV promoter was generated from the vH backbone [172], which has served as the basis for previous animal studies [53, 74, 93] and is closely related to the vector used in ongoing clinical trials [2, 3]. vH like vL is a KOS-based vector, deleted for the internal repeat region, ICP4 and ICP27 [58, 163]. Unlike vL, all

modification to vH have been made by homologous recombination in cell culture and as such it contains no BAC sequence. vH also differs in that the promoters for immediate early genes ICP22 and ICP47 have been modified to change them from immediate early to early expression kinetics. Finally, vH differs in the location of the transgene insertion site, with CMV-GlyR_{IS} inserted into both ICP4 loci instead of the LAT locus.

2.1.1 Vector diagnostics and preparation.

Vector clones were subjected to a series of diagnostic tests. First, vector DNA was digested with MfeI to validate genomic integrity. PCR amplification across the transgene insertion site was then carried out and amplicons of the correct size are sent for sequence confirmation. Once the sequence was confirmed, vectors generated in *E. coli* were tested for growth by transfecting (Lipofectamine LTX, Invitrogen) purified vector DNA into 7b cells, a Vero cell line expressing ICP4 and ICP27 to complement vector deletions of these genes [58, 163]. Clones that pass each of these quality control measures were further amplified on 7b cells, in DMEM (with glucose, glutamine and sodium pyruvate, Corning 10-013-CV) supplemented with 2.5% FBS.

The vector was harvested by adding a 5M NaCl PBS solution (Sigma) to the medium to a final concentration of 450mM NaCl and left on a rocker at ambient temperature for 1 hour to dissociate cell-bound virions. Any cells that remained attached to the cell culture flask were scraped and the medium was transferred to 50mL conical tubes and centrifuged for 10 minutes at 2,500 rpm at 4^{o}C. Supernatant was then subjected to filtration using a 0.8µm filter (Nalgene 125-0080) to remove microscopic cellular debris. The filtrate was added to sterile autoclaved 30mL Oak Ridge high speed centrifuge tubes (Nalgene 3114-0030) and centrifuged at 44,000 xg for 45 minutes at 4^{o}C to precipitate virions. Depending on the scale of the preparation, the

supernatant was decanted and additional 30mL volumes of filtrate were added to the same tubes and the centrifuge step repeated. The supernatant was carefully removed by decanting and aspirating the inverted tube and 10mL of PBS was added to the tubes and centrifuged at 44,000 xg for 20 minutes. This fluid was removed and 150uL of PBS was carefully pipetted onto the pellet, which was left to resuspend overnight at 4^{o}C. The next day, sterile glycerol was added to a final concentration of 10% and the mix was gently pipetted up and down until the pellet and glycerol were homogenized. The resultant vector prep was aliquotted into cryovials and sealed into a Styrofoam rack and placed into a -80^{o}C freezer. After at least 24 hours, one of the aliquots was thawed on ice for titration on complementing cells. A series of 10-fold vector dilutions were made in serum free DMEM (Corning 10-013-CV) in duplicate and used to infect monolayers of 7b cells on 48-well plates (BD Falcon 353230). After a two hour incubation at 37^{o}C, 10% methylcellulose in DMEM was added to the wells to limit viral diffusion and the plates were returned to the incubator. Approximately 48 hours later (the time varies with the viral vector backbone being grown), this media was aspirated and a solution of 0.5% (w/v) crystal violet and 0.2% (w/v) sodium acetate solution in 10% ethanol (pH=3.6) was added to each well and allowed to sit for 10 minutes at ambient temperature. The stain was then removed and the plate was washed with water and allowed to dry. Plaques (clearings of infected cells on the stained monolayer) were counted (between 10-100 plaques in the most dilute wells), averaged between duplicates and the concentration of the original aliquot in plaque forming units (pfu) per mL was calculated.

2.2 DRG CELL CULTURE EXPERIMENTS

Sprague-Dawley rat embryos (Charles River, Wilmington, MA) were harvested at day 16 at which point their DRG are just developed to the extent that they are easily removed. Total DRG were combined and digested in a solution of 0.25% trypsin-EDTA (Gibco 25200) in neurobasal media (Gibco 2103) for 1 hour and gently pipetted up and down to dissociate them. Cells were centrifuged out of solution at 1,200 rpm for 3 minutes and washed three times with neurobasal media to remove all traces of trypsin. Finally, the cells were resuspended in neurobasal media warmed to 37^{o}C with 1X B27 supplement (Gibco 17504) and 50ng/mL NGF (Promega G5141). DRG cells were plated at 10^5 cells per well onto coverslips that had been coated in 100ug/mL poly-D-lysine (Sigma P6407) in water for 2 hours at ambient temperature and placed in 24-well plates until completely dry. Cells were incubated at 37^{o}C and 5% CO_2 for 1-2 weeks prior to transductions, with 50% of the media volume replaced every 7 days with fresh media. To limit glial overgrowth, the anti-mitotic FUdR (Sigma F0503) was added to the media at 10uM at the first media change and washed off 24 hours later with fresh media [173].

DRG cells were transduced with $3x10^5$ pfu of vectors, corresponding to an approximate MOI of 3 relative to the number of cell originally plated (glial cell expansion and death from FUdR makes this approximate). Transduction took place over a 1 hour incubation time at 37^{o}C after which the transduction media was exchanged for fresh media. After 48 hours, the cells were imaged for red fluorescence from mCherry expression on a Nikon Eclipse Ti-S/L100 with a Xenon Arc bulb (20110801) at a constant exposure time. Using Metamorph software, composite images were generated overlaying red fluorescent images with bright-field images.

For NF200 immunofluorescence, after the 48 hour incubation, wells were rinsed with PBS and then fixed in 2% buffered formalin solution in PBS for 10 minutes. Three rinses with

PBS were conducted, allowing each PBS rinse to equilibrate for 5 minutes. For blocking, a solution of 5% normal goat serum (Sigma) in PBS was used for 1 hour at ambient temperature. After three rinses, the primary rabbit polyclonal antibody against NF200 (Abcam ab8135) was added at a concentration of 1:1000 in blocking solution and left overnight at 4^{o}C. The following day, antibody solution was rinsed off with PBS x3 and the secondary antibody goat anti-rabbit conjugated to alexafluor-488 (Life technologies A-11008) was added at 1:1000 in blocking solution and incubated for 1 hour at ambient temperature. After a final three rinses with PBS, the cells were imaged as described above and composite images were generated by overlaying mCherry fluorescence, bright-field, and alexafluor-488 green fluorescence images.

2.3 RAT HOUSING & INJECTIONS

Male Sprague-Dawley rats (Charles River, Wilmington, MA) were housed in an animal facility with daily monitoring by an onsite veterinarian and staff and twice weekly cage cleaning. Rats had free access to food pellets and water and were housed in pairs when possible for the duration of the experiments, consistent with the policies of the University of Pittsburgh Institutional Animal Care and Use Committee.

To ensure safe and accurate injection of HSV-1 vectors and RTX (described below) isofluorane anesthesia (Henry Schein Animal Health, Dublin, OH) was used. Isofluorane was selected for its limited duration and rapid recovery time. For this procedure, rats were placed in a sealable container on a porous floor, under which were placed paper towels infused with liquid isofluorane. The container was sealed and rats were monitored and removed at the moment of

unconsciousness at which time they were injected, returned to their cage, and monitored until fully conscious.

2.3.1 Vector injection.

Under isofluorane anesthesia (described above), rats were injected under the skin of the right hind footpad with 10^8 pfu of vector, suspended in 100uL of PBS. Vector was kept on ice between each animal injection and vortexed to maintain homogenization and equal dosing to all rats in a group.

2.3.2 Ivermectin injection.

Due to its proximity to thermal and mechanical threshold measurements, isofluorane anesthesia was not used for ivermectin injections. Instead, rats were allowed to burrow into a folded towel for comfort and held in place. Ivermectin (Sigma I8898) was then injected in 50uL volumes under the skin of the hind footpads bilaterally. Rats were then placed in enclosures for thermal or mechanical threshold measurements (described below) and monitored for a 15 minute acclimation period at minimum, or until they were calm.

2.3.3 RTX injection.

Under isofluorane anesthesia (described above), rats were injected in the left lower quadrant of the peritoneum with 200 ug/kg RTX (Sigma R8756) suspended in normal saline with 10% Tween 80 & 10% ethanol [138, 149]. After injections, pressure was applied to the injection site

and the abdomen was lightly massaged to disperse the RTX solution. Rats were monitored over the next hour for acute side effects and none were observed.

2.4 TESTS OF NOCICEPTION

2.4.1 Hargreaves' test.

Thermal thresholds were assessed with the Hargreaves' test. Rats were placed in a plexiglass enclosure on an elevated glass surface, pre-warmed to 30^{o}C. After a 15 minute acclimation period, a high-intensity light beam was focused centrally on the plantar side of the hind paw, which starts a timer in the apparatus (Hargreaves apparatus, IITC Life Sciences, Woodland Hills, CA). When a withdrawal response was observed, the beam was switched off and the time interval was precisely recorded. The beam intensity was calibrated such that control responses range between 6-8 seconds. At least four measurements, at greater than five minute intervals were made and averaged for the final value. Fold increase over contralateral (uninjected side) was calculated by dividing the ipsilateral average by the contralateral average.

2.4.2 Von Frey filament test.

Mechanical thresholds were assessed with the von Frey filament test using the up-down method [157, 161]. Rats were placed in a plexiglass enclosure on an elevated wire grid (IITC Life Sciences, Woodland Hills, CA). After a 15 minute acclimation period, von Frey filaments (Stoetling, Wood Dale, IL) were applied to the plantar side of the hind paw in increasing

thicknesses corresponding to increasing gram-weight force starting from 10g. Filaments were lightly applied, normal to the hind paw and then pushed in until bent. This force was maintained for a total of ~six seconds. A response was noted if there was a rapid withdrawal, especially with subsequent licking of the footpad. If no response was observed the next thicker filament was then applied. When a withdrawal response was observed, the next thinner filament was then applied. After six observations, the resulting pattern of positive and negative responses were interpreted on a table which converts the finding into gram weight threshold by the Weber–Fechner law. Fold increase over contralateral (uninjected side) was calculated by dividing the ipsilateral average by the contralateral average.

2.5 STATISTICS

For the initial thermal threshold modulation experiment, three-way ANOVA was used to compare variance in the means of groups using vector type (TRPV1-GlyR_{IS}, NF200-GlyR_{IS}, CMV-GlyR_{IS}, or sham), injection side (ipsilateral or contralateral), and ivermectin ligand administration (pre-injection measurement or post-injection measurement) as independent variables. The post-hoc Tukey-Kramer test was used for pair-wise comparisons between groups whose means were determined to be significantly different by ANOVA. In Tukey-Kramer tests, differences in means were compared to critical values of the Studentized range for multiple comparisons with alpha <0.05 (indicated with *) or <0.01 (indicated with **).

For the RTX experiment, three-way ANOVA was used to compare variance in the means of groups using vector type (TRPV1-GlyR_{IS}, NF200-GlyR_{IS}, CMV-GlyR_{IS}, & sham), ivermectin ligand administration (pre-injection measurement or post-injection measurement), and RTX

status (pre-injection or post-injection) as independent variables. The post-hoc Tukey-Kramer test, which is conservative when comparing groups of unequal sample size (one rat in the CMV-$GlyR_{IS}$ group died during the course of the experiment) was used for pair-wise comparisons as described above.

3.0 RESULTS

3.1 VECTOR DESIGN & CHARACTERIZATION IN DRG CELLS

In a typical HSV-1 infection when sensory neurons are infected, the virus enters latency and the genome becomes heterochromatinized and quiescent, with the possible exception of expression from the LAT loci depending on the type of sensory neuron infected [174, 175]. Because the goal of this study is transgene expression in sensory neurons, the genome becoming quiescent impacts vector design in two ways: extensive crippling mutations to the genome beyond the requirement of rendering it replication-defective are unnecessary because the quiescent state will limit viral gene expression; also, it is advantageous to utilize the expression-permissive LAT locus, flanked by chromatin boundary elements [59, 164, 165], for transgene expression. Therefore, the vector was designed with complete deletion of only essential immediate early genes ICP4 and ICP27 [58]. The internal repeat region was also deleted resulting in the vector genome being stabilized by losing the ability to isomerize [54, 59]. Finally, for ease of insertion of a wide variety of transgene constructs, a Gateway destination cassette was inserted into the remaining LAT locus, replacing LAT promoter elements. This cassette contains attachment sites, *att*, evolved in phageλ-*E. coli*, allowing for highly efficient site-specific recombination between the parent vector, vL (Figure 1A) and corresponding *att*-flanked transgene cassettes on plasmids (Figure 1B).

In addition to *att* sites, promoters and the $GlyR_{IS}$ open reading frame, the transgene cassettes utilized the complete consensus Kozak translation initiation sequence, the αglobin 3'UTR for mRNA stability [166, 167], and SV40 polyadenylation sequence (Figure 1B).

For targeting experiments, the sequence for the neuronal TRPV1 promoter was selected based on its previous characterization [168], and the NF200 promoter sequence was obtained commercially (sequences in Appendix A).

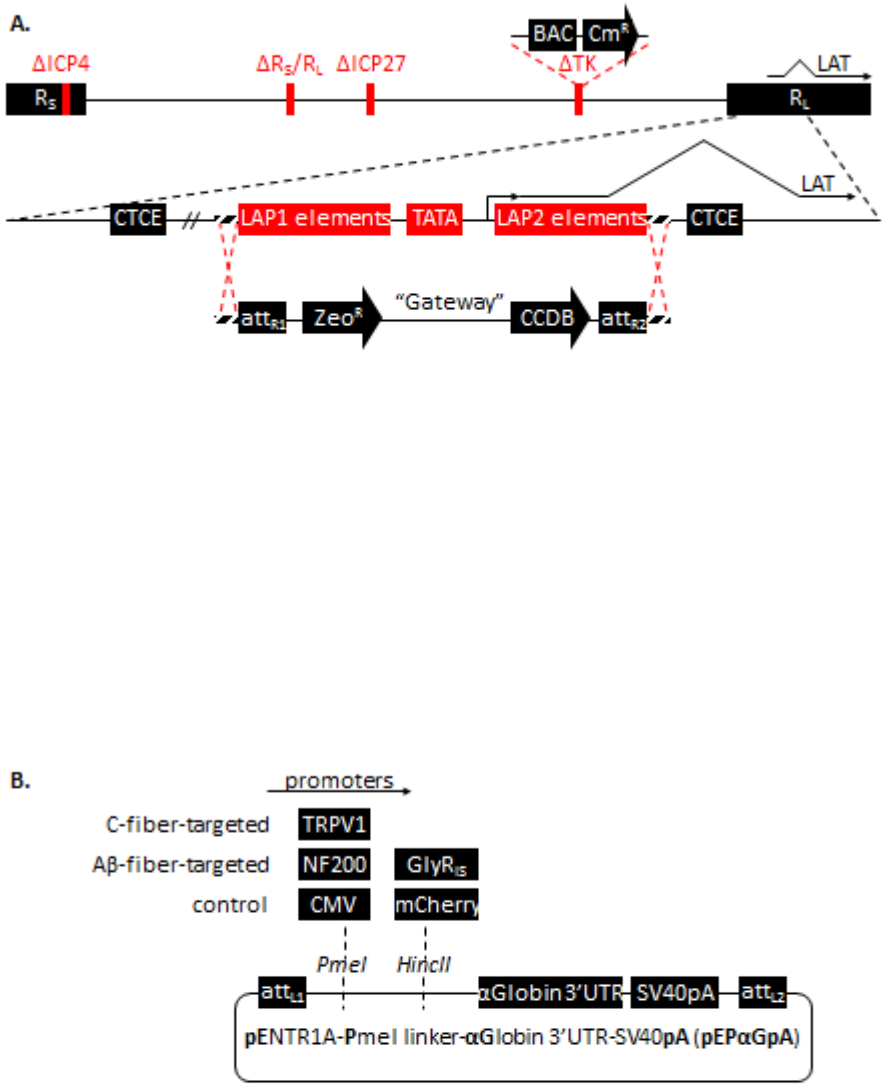

Figure 1. Vector schematic.

(A) The vector backbone vL was generated from a KOS-based replication-defective vector in which BAC sequences were inserted into the TK locus. Deletions were introduced in the internal repeat region and the

genes encoding immediate early proteins ICP4 and ICP27, rendering the vector replication-defective, but preserving immediate early genes ICP22 & ICP47. The Gateway destination cassette was inserted into the remaining latency locus, replacing the latency promoter elements while maintaining a wild-type copy of ICP0 and the surrounding CTCF chromatin boundary elements. To generate targeted expression vectors, transgenes were recombined into the vector backbone via the Gateway cassette.

(B) Transgene constructs inserted into the HSV vL vector backbone. pENTR1A, which contains *attL* sites for site-directed recombination with the *attR*-containing vector backbone was used to generate the transgene constructs used. Between *att* sites, the highly-stable αGlobin 3'UTR and SV40 polyadenylation signal were inserted. Promoter sequences and transgene ORFs incorporating a Kozak consensus translation initiation sequence were inserted into the vector in the combinations shown. These were then recombined into the vL vector backbone to generate the experimental vectors.

For rat behavior experiments the CMV-GlyR_{IS} control vector utilized was generated from the related vH backbone, which was the basis of past rodent pain studies [53, 74, 93] and most closely related to the vector used in clinical trials [2, 3].

3.1.1 Characterization of vectors in cultured embryonic rat DRG cells.

To initially characterize whether the promoters were targeting transgene expression to neurons, a set of vL vectors were generated to express the red fluorescent marker mCherry. Several viral (Figure 2A top panel) and neuronal promoters (Figure 2A bottom panel) were tested for their ability to restrict mCherry expression to neuronal cells within a mixed DRG cell culture of neurons and glial support cells. As controls, the ubiquitously active CMV promoter [171] as well as the HSV-1 LAT promoter elements [176, 177] (re-inserted *in situ*) were used. These were compared to the promoters of neuronal markers TRPV1, expressed in heat-sensitive nociceptors; NF200, expressed in proportion to fiber size and therefore highly expressed in large

diameter Aβ-fibers; and CGRP, one of the definitional C-fiber peptides classifying the neurons expressing it as peptidergic.

DRG cells were transduced and fluorescence was allowed to develop over 48 hours at which point images, displayed as overlays of bright-field and red fluorescence, were taken. The CMV promoter expressed in both neuronal (large round cells) and glial support cells (small linear cells). The LAT promoter as well as all of the neuronal promoters had expression grossly limited to neuronal cells. Of the neuronal promoters tested, the CGRP promoter was striking in its intensity and number of cells expressing it. This is consistent with the fact that the DRG cells had been cultured in media containing NGF, which differentiates neurons into the peptidergic C-fiber lineage. The LAT promoter expressed at the lowest intensity, which can likely be attributed to the fact that downstream enhancer elements, variously referred to as the reactivation critical region [178] or LAT promoter 2 (LAP2) [176, 177], contain three translational start sites which are upstream of and out of frame with the mCherry start codon. In Figure 2B, the NF200 promoter (top panel) and CMV promoter (bottom panel) are compared with the addition of antibody staining of endogenous NF200 displayed in green, further illustrating the neuronal-restricted expression of the NF200-targeted vector compared to the untargeted expression from the CMV promoter.

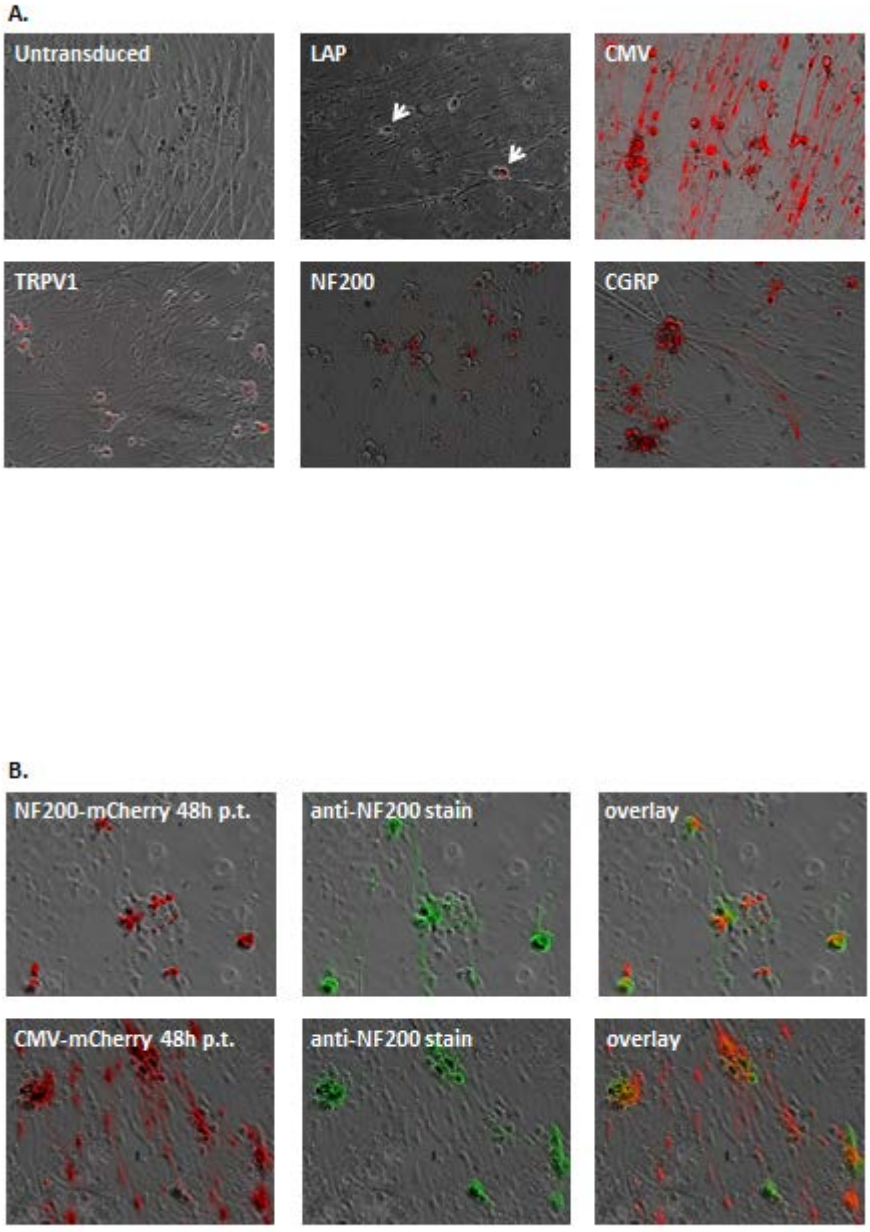

Figure 2. Characterization of transgene expression from vectors in cultured DRG cells.

(A) Expression of mCherry from targeted and control viral vectors in transduced cultured embryonic rat DRG cells, demonstrating gross differences in expression pattern and intensity between promoters.

Dissociated embryonic DRG cells were cultured for 3 weeks in neurobasal media +NGF and then transduced with $3x10^5$pfu of vector (~MOI=3). At day 2 post transduction, cells were imaged for red fluorescence from mCherry. (B) Overlap of vector-derived mCherry expression with the targeted neuronal marker, NF200. At day 2 post transduction, coverslips were fixed and stained with antibodies to NF200. Red fluorescence from vector-expressed mCherry (left column) and NF200 staining in green (center column) was merged to form an overlay (right column). The intensity of red fluorescence from the CMV promoter had to be drastically reduced in the overlay for colocalization to be visible (right, bottom).

3.2 EFFECT OF VECTORS ON NOCICEPTIVE THRESHOLDS IN VIVO

3.2.1 Targeted silencing of TRPV1+ neurons results in elevated thermal withdrawal thresholds.

To first determine whether a functional outcome of differentially targeted expression of $GlyR_{IS}$ could be demonstrated in rats, the heat-sensitive TRPV1 population was selected for modulation as assessed by changes in thermal withdrawal thresholds. The "on-target" TRPV1-targeted (vL) vector and "off-target" NF200-targeted (vL) vector were compared to sham-injected rats to test the hypothesis that only the TRPV1-$GlyR_{IS}$ transduced rats would demonstrate decreased heat sensitivity with increased thermal withdrawal thresholds (Figure 3).

Vectors or PBS were injected under the skin of the right hind footpad of rats (N=5 per group). After eight days, thermal withdrawal thresholds were measured before and after the injection of ivermectin ligand under the skin of the hind footpads. Injections were done bilaterally so that in case the needle injury contributed to hyperalgesia, it would be present on both sides.

Indeed, only the "on-target" TRPV1-GlyR$_{IS}$ transduced rats showed a significantly increased thermal withdrawal threshold after ligand administration. This threshold was also significantly increased from "off-target" NF200-GlyR$_{IS}$ transduced rats and from the rats before ligand administration or the contralateral side after administration.

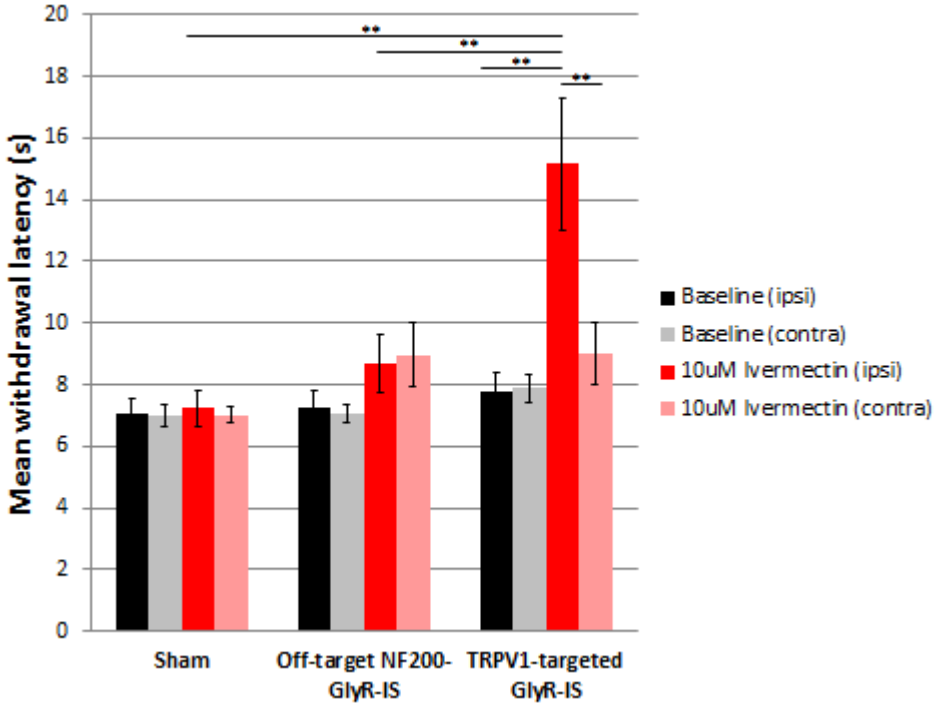

Figure 3. Modulating thermal thresholds with targeted GlyR$_{IS}$ expression.

Paw withdrawal latencies from a radiative heat source (Hargreaves' test) was measured eight days after injection of 10^8pfu of NF200- or TRPV1-targeted GlyR$_{IS}$-expressing HSV vector or 100uL PBS sham injection under the skin of the right hind footpads of male Sprague-Dawley rats (N=5 for each group). After baseline withdrawal latencies were measured, 50uL of 10uM ivermectin were injected into hind footpads bilaterally and after 15 minutes, withdrawal latency measurements were repeated. For each rat tested, at least 4 measurements were taken and averaged. Three-way analysis of variance (ANOVA) using vector, injection side, and ivermectin status was used, followed by a post hoc Tukey-Kramer test. Significance indicated by *p<0.05, **P<0.01.

3.2.2 RTX reveals differential effects of NF200 vs. TRPV1-targeted silencing.

To determine whether targeted expression of an anti-nociceptive product from HSV-1 vectors would demonstrate different functional outcomes based on the neuronal population targeted, RTX was employed. Its reciprocal actions of ablating TRPV1+ heat-sensitive neurons, and later, sensitizing large diameter, mechanosensitive neurons by the inflammatory response to degenerating neurons [149], provide the opportunity to tease out differential effects of TRPV1 vs. NF200-targeted vectors in a single experiment (Figure 4). Repeated from the introduction, this experiment tests the hypotheses that:

1- An anti-nociceptive effect targeted to TRPV1+ neurons will no longer be significantly different from other groups when all TRPV1+ neurons are ablated by RTX, and

2- An anti-nociceptive effect targeted to large, NF200-rich, mechanosensitive fibers will reduce the degree of mechanical allodynia caused by RTX-induced inflammation.

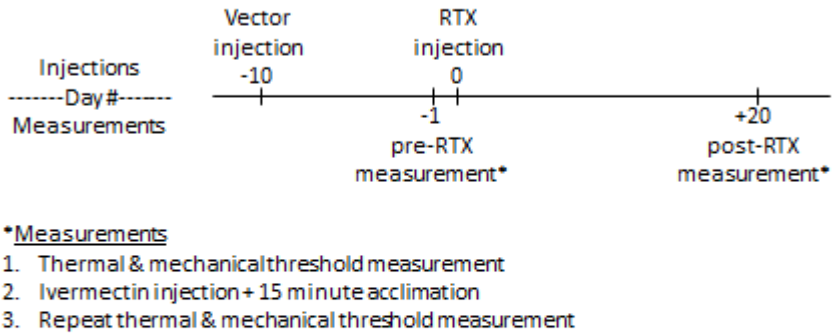

Figure 4. Experimental timeline.

Six rats were assigned to each group based on the vector injected, comparing TRPV1- and NF200-targeted $GlyR_{IS}$ vectors (vL) to CMV-$GlyR_{IS}$ vector (vH) and sham-injected control. Nine days after vector injection (one day prior to RTX injection), the pre-RTX baseline measurement was made. Thermal and mechanical thresholds were assessed before and after ivermectin injection under the skin of the hind footpads bilaterally (the left half of Figures 5 & 6, respectively). This data was represented as fold increase of the transduced side over the contralateral side, as done previously on outbred rats [93] to increase the signal from noise introduced into von Frey measurements [158] repeated after a long hiatus (21-days while the post-RTX mechanical allodynia was being established).

Replicating the results of the earlier study, thermal thresholds were increased in the TRPV1-$GlyR_{IS}$ transduced rats relative to sham and to the rats prior to ligand administration. The CMV-$GlyR_{IS}$ transduced rats also had increased thermal thresholds, though to a lesser extent

than the TRPV1 vector group. By pairwise comparisons, the CMV group was significant only for increased thermal threshold from prior to ligand administration.

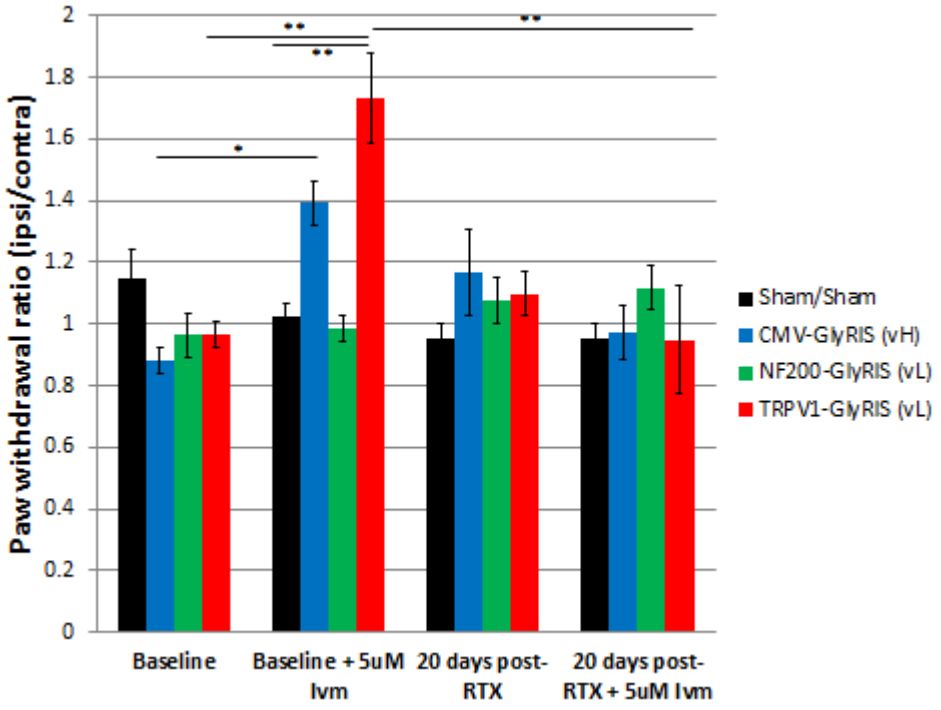

Figure 5. Pre-RTX increase in thermal threshold by TRPV1-targeted vector.

Six rats per group were injected with vectors (10^8pfu) or vehicle under the skin of the right hind footpads. Ten days later, resiniferatoxin (RTX) was injected systemically (200 µg/kg i.p. or vehicle for sham/sham group). Measurements of thermal thresholds using a Hargreaves apparatus were made before and 20 days after RTX injection. For each measurement, thresholds were assessed, ivermectin was injected, and measurements were repeated. Paw withdrawal latencies are the average of at least 4 measurements and presented as fold increase over the uninjected side. Three-way analysis of variance (ANOVA) using vector, IVM status, and RTX status was used, followed by a post hoc Tukey-Kramer test. Significance indicated by *p<0.05, **P<0.01.

RTX was delivered systemically by i.p. injection and rats were allowed to rest for 20 days for mechanical allodynia to develop. After that period, the post-RTX measurement was made (Figures 5 and 6, right half).

Post-RTX ablation of TRPV1+ neurons, the thermal thresholds had equalized between the transduced side & contralateral and between groups as expected (Figure 5).

When mechanical thresholds were assessed, the NF200-GlyR$_{IS}$ transduced group alone demonstrated reduced mechanical allodynia on the transduced side relative to the contralateral side after ligand administration, resulting in an ipsilateral/contralateral ratio which was significantly greater than all other groups, in accordance with the hypothesis (Figure 6).

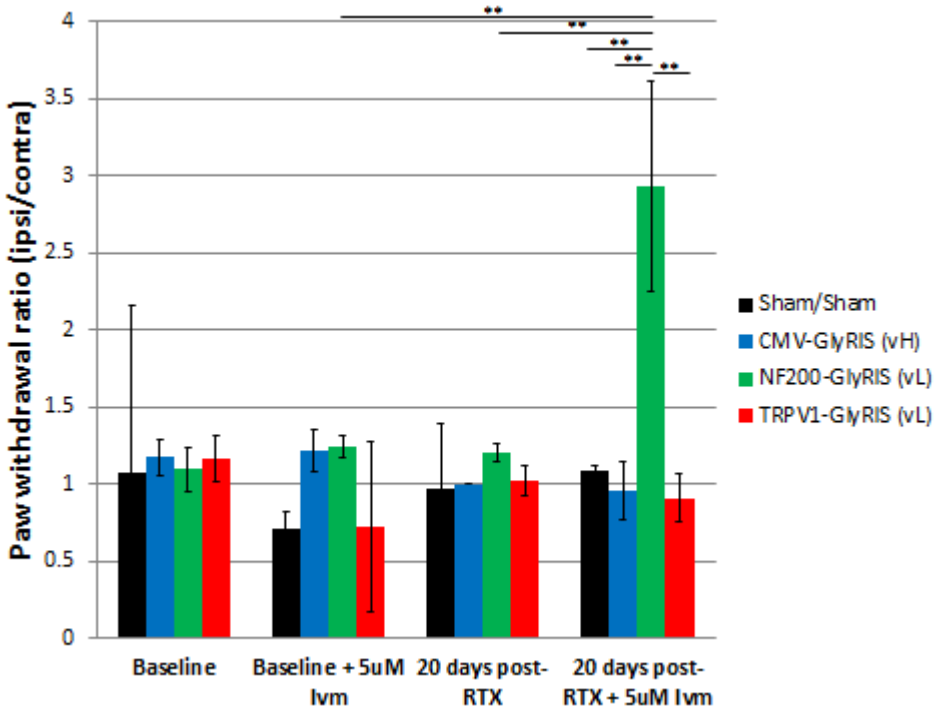

Figure 6. Post-RTX reduction in mechanical allodynia by NF200-targeted vector.

Six rats per group were injected with vectors (10^8pfu) or vehicle under the skin of the right hind footpads. Ten days later, resiniferatoxin (RTX) was injected systemically (200 µg/kg i.p. or vehicle for sham/sham group). Measurements of mechanical thresholds using von Frey filaments and quantified by the up-down

method were made before and 20 days after RTX injection. For each measurement, thresholds were assessed, ivermectin was injected, and measurements were repeated. Paw withdrawal thresholds are presented as fold increase over the uninjected side. Three-way analysis of variance (ANOVA) using vector, IVM status, and RTX status was used, followed by a post hoc Tukey-Kramer test. Significance indicated by *p<0.05, **P<0.01.

4.0 DISCUSSION

The field of gene therapy has grown exponentially over the past decade, gaining back the momentum that was lost with the missteps of the 1990s and early 2000s, including insertional mutagenesis from early retroviral vectors resulting in post-therapy leukemia [179], and poorly conducted trials culminating in the 1999 death of trial subject Jesse Gelsinger [180]. Since then, large strides have been made to improve vector safety. Focus has shifted away from earlier promiscuously integrating retroviral vectors toward viral vectors with minimal or no risk of insertional mutagenesis and low immunogenicity.

The safety and efficacy of gene therapy has seen the greatest improvement with the advancement of gene targeting technology on the transductional [119, 125], transcriptional [124-126], and post-transcriptional level [127].

By using several of these techniques in concert, the contemporary gene therapy investigator has a well-stocked vector targeting toolbox to employ to maximize the likelihood of successful transduction of the target cell type while minimizing unwanted side-effects.

This study was able to demonstrate a functional consequence of promoter-targeting $GlyR_{IS}$ expression. Specifically, TRPV1-targeted vectors were able to elevate baseline thermal thresholds (Figure 3), an effect that was no longer different from controls after RTX-ablation of TRPV1+ neurons (Figure 5); and NF200-targeted vectors were able to reduce mechanical allodynia following RTX insult (Figure 6). While the RTX model is not directly clinically

relevant, it does serve as proof of principle that using neuronal promoters to target the expression of an anti-nociceptive product from HSV-1 vectors to distinct neuronal populations is a viable strategy to achieve differential effects on nociception.

It is noteworthy that the TRPV1-targeted vectors outperformed the CMV vector in pre-RTX thermal threshold elevation and furthermore, the CMV vector had no effect in post-RTX mechanical allodynia reduction (only the NF200-targeted vector had this effect). This could be attributed to some combination of the following factors. Firstly, the vH-derived CMV vector used, though KOS-based and similar in the genes deleted to render it replication-defective [58, 163], was not an identical backbone to the vL-derived neuronal promoter-targeted vectors. The vH backbone was used as a standard of comparison, as it was the basis for several previous therapeutic vectors engineered by the Glorioso lab, used in previous pain studies [53, 74, 93], and very similar to the vector used in clinical trials [2, 3]. The principal difference between vector backbones was the transgene insertion site. Whereas the promoter-targeted vectors utilized the vector's remaining LAT locus to take advantage of its flanking chromatin boundary elements [59, 165], allowing permissive transcription from this site in sensory neurons in which its native product LAT is expressed, the vH-based vector has the CMV-$GlyR_{IS}$ transgene inserted into both ICP4 loci. Therefore, one possible explanation for this vector's lack of effect in reducing post-RTX inflammatory mechanical allodynia may be the heterochromatinization of these loci over the 30 days from vector injection to this measurement. By extension, these loci may have been earlier in their trajectory towards quiescence at the pre-RTX measurement nine days after vector injection, which may account for its lower degree of thermal threshold elevation relative to the TRPV1-targeted vector at the time of that measurement.

A related consideration is that the CMV promoter [171] itself is known to be rendered quiescent over time in vivo, presumably due to cis elements directing its heterochromatinization. If this was the dominant factor in its quiescence, one would expect the CMV promoter to be similarly repressed in other loci and a poor choice for long term expression in animals and patients. This is consistent with the effect of preproenkephalin expression being diminished, but not eliminated over the 7 weeks of initial experiments [64] and over the 28 days of the clinical trial [3].

An alternate, though less probable explanation for the neuronal promoters outperforming the CMV promoter would be some degree of cell-type specific expression from the CMV promoter. The CMV promoter, while conventionally regarded as capable of expressing gene products to high levels in any cell type, could potentially exhibit its own degree of specificity for expression in a subset of neurons. Even if this subset is large, if it excludes critical TRPV1+ or NF200+ neurons necessary for the effects observed with targeted promoters in pre- and post-RTX observations, this could in part account for its diminished or absence effect in this study. This could be evaluated in further targeting studies comparing several different ubiquitously expressing promoters in different loci.

4.1 FUTURE DIRECTIONS

4.1.1 Assessment of targeting efficiency comparing promoters and promoter size.

To lend further support for the targeting method employed in this study, the immediate next step will be to directly assess targeting efficiency. Specifically, I would propose an experiment in

which a short and long size of each promoter is selected to drive the expression of a fluorescent marker (e.g. mCherry). These vectors can be easily generated using the Gateway site-directed recombination system incorporated into the vL parent vector, recombined with the desired plasmid transgene constructs. Once these vectors are generated, hind paw injections can be carried out using the same methodology as in this study. After a 7-30 day interval, DRG would be harvested for tissue sections and immuno-staining for the two targeted neuronal markers (TRPV1 or NF200). Targeting efficiency would be determined as the proportion of total vector-driven fluorescent cells which co-stain for the marker of interest. The expectation would be that for each marker, the CMV promoter would have a low targeting efficiency – expressing non-specifically in most or all transduced neurons. Also, the vector miss-matched for targeting to the immuno-stained population (i.e. sections from rats injected with TRPV1-targeted vector, then assessed for NF200 targeting, or vice versa) would have low targeting efficiency to that cell type, reflecting low overlap of those two markers. Only the vector targeted to the stained marker would be expected to have high targeting efficiency. Finally, it could be determined whether incorporating a larger putative promoter sequence would result in higher targeting efficiency. One of the strengths of the HSV vector platform is a large carrying capacity. Therefore, it would be possible to use up to tens of kilobase-pairs of putative promoter sequence if the greater length was determined to correlate to a higher targeting efficiency.

4.1.2 Generating a suite of promoter-targeted HSV vectors to test hypotheses about nociceptor subpopulation involvement in specific pain models.

Using the vL parent vector designed for this study, there is the potential to easily generate a large suite of differentially-targeted vectors by inserting different neuronal promoters driving $GlyR_{IS}$,

into the vector backbone. Single vectors could be generated to test specific hypotheses or a collection of differentially-targeted anti-nociceptive vectors could be collectively tested on specific pain models. It would be expected that the degree to which a given vector alleviated pain would be in direct proportion to the involvement of the targeted neuronal population in that pain type.

A high yield direction to explore would be to select nociceptor marker genes which could dissect this diverse category of neurons based on different functional products. One principal way in which nociceptors are classified is based on the expression of neuropeptides – substance P (SP) and calcitonin gene related peptide (CGRP) – which define their neurons as peptidergic. Peptidergic nociceptors develop from neuronal precursors stimulated by NGF in embryonic development, whereas non-peptidergic nociceptors switch from NGF to GDNF [21]. Peptidergic nociceptors synapse in lamina I and the outer portion of lamina II of the dorsal horn of the spinal cord, while non-peptidergic neurons synapse in inner lamina II. There are functional consequences to these differences in expression and connectivity [181]. Peptidergic nociceptors have been implicated in inflammatory pain and non-peptidergic nociceptors have been implicated in neuropathic pain [12, 23]. By using the promoters of SP and CGRP, it would be possible to determine the contribution of these two types of peptidergic nociceptors to inflammatory pain models and others. In contrast, non-peptidergic nociceptors express fluoride resistant acid phosphatase (FRAP) [182], neuropeptide responsive GPCRs MrgA & MrgD, and the purinergic receptor $P2X_3$ [183]. By using their promoters, the contribution of subpopulations of non-peptidergic nociceptors to neuropathic pain models and others could be determined.

Another way to distinguish populations of nociceptors is by the expression of distinct ion channel sensors, rendering the neuron sensitive to distinct stimuli. The example from this study

is TRPV1, a channel sensitive to heat, protons, and several biological compounds such as capsaicin and RTX. There are many TRP channels which mediate the detection of various thermal, mechanical, and chemical stimuli [184, 185]. In addition to TRPV1, a couple good candidate genes of this type to use for promoter-targeting would be TRPA1, which is activated by cold and many chemical irritants including formalin [186], and TRPM8 which is activated by a wide array of cold stimuli and menthol [187].

In addition to neuropeptide classification and the presence of specific receptors, there are other markers which correlate to physical differences between neurons. The neurofilaments fall into this category. NF200 which was targeted in this study is the heavy neurofilament (also called NFH). The other peripheral neurofilaments are neurofilaments light & medium (NFL & NFM) and peripherin [188]. The expression of NF200 scales with neuron size and as such it was selected to target large-diameter fibers which tend to be mechanosensitive, non-nociceptive neurons, but which can be recruited to pain signaling following trauma or chemical irritation. Other neurofilament promoters could be used to preferentially express in neurons based on the relative abundance of these structural molecules, allowing for the dissection of primary afferents along a cytostructural axis.

Finally, there are genes products which are induced in certain pain states. TRPV1 for instance has been shown to be differentially upregulated in certain neuronal populations following inflammation [189] or nerve injury [190]. It will be important to determine whether exogenous promoters on viral vectors will follow a similar induction pattern. The identification of pain-inducible promoters and promoter elements may aid in expression when correctly matched to the pain model or syndrome causing its induction. Another promising lead in this direction would be the use of the neuropeptide Y (NPY) promoter. NPY has limited expression

in the PNS under normal conditions, but is highly upregulated following nerve injury [191, 192]. Using this promoter has the potential of adding an additional level of targeting – phenotypic targeting – as it may be nearly quiescent in normal conditions, but provide for the inducible expression of $GlyR_{IS}$ in the pain state.

4.1.3 Advancement to clinical trials.

The ultimate purpose of developing targeted anti-nociceptive vectors is to treat patients with chronic pain. As such, the future direction of greatest importance is to identify one or more vectors with the highest likelihood of treating a known pain syndrome. The next step in identifying such a vector is the testing of vectors on carefully chosen, clinically relevant pain models. One in particular is very appealing: post-herpetic neuralgia (PHN).

PHN is persistent pain after the resolution of an episode of herpes zoster (shingles). Zoster itself represents a reactivation event of latently infected varicella zoster virus (VZV) from its reservoir within sensory ganglia, a tropism it shares with the closely related HSV. This causes replication within the ganglion and subsequent anterograde transport of virions to the corresponding dermatome, where further virus replication can result in a visible lesion. Both zoster and PHN can result in intense allodynia and are the cause of significant reductions in quality of life [193].

As a consequence of replication within sensory ganglia, pain caused by zoster and PHN is localized and originates from a PNS lesion. This makes PHN an attractive target for interventions at the level of the PNS, like anti-nociceptive gene therapy. PHN is an attractive target for HSV anti-nociceptive gene therapy for additional reasons. Firstly, based on the shared tropism of VZV and HSV, both alphaherpesviruses, the insult resulting in chronic neuropathic

pain is in the precise tissue which HSV has evolved to transduce: sensory neurons. In other words, HSV is the ideal delivery vehicle to treat the pain syndrome caused by its sister virus VZV. Secondly, PHN can be recapitulated in a rat model by infecting rats with VZV-infected cells in the same manner as the vector injections in this study [93]. This would mean that using a protocol similar to that used in this study, one or several targeted HSV vectors could be evaluated in a very clinically relevant model – using the causative agent for human disease, not a contrived chemical irritant or surgical injury.

To have the greatest chance to identify an effective targeted vector to reduce PHN pain in a rodent model, a suite of promoter-targeted vectors like the one described in the above section should be tested and compared to constitutively active promoters like CMV or the ubiquitin promoter for the ability to reduce pain behaviors in evoked and spontaneous pain tests using the PHN model. The most effective of these vectors would be a prime candidate for advancement to clinical trials for the treatment of PHN.

The use of an ivermectin-sensitive mutant glycine receptor instead of the wild-type GlyR was intended to facilitate translation of this technology to the clinic. Oral and topical preparations of ivermectin are FDA approved for the treatment of certain helminthic infections [113], which is a strong testament to the safety of this drug. Additional pre-clinical studies will need to be conducted to determine the dose of topical or oral ivermectin necessary to activate vector-delivered $GlyR_{IS}$ channels. The ultimate intent of targeted vectors delivering this channel will be that their activation will be conditional on the use of an ivermectin topical cream, extended release patch, or pill. In this way, pain relief from therapeutic HSV-1 vectors will be able to be controlled and modulated by a treating physician to serve the needs of an individual patient.

4.2 CONCLUDING REMARKS

The flexibility of using neuronal promoters in HSV-1 vectors to achieve targeted silencing of a given population of sensory neurons, defined by the expression of a marker gene, presents the enticing possibility of using a suite of targeted vectors to test hypotheses about which population of sensory neurons mediates a specific pain type. These genetic tools have the potential to increase the resolution of our understanding of the nociceptor subpopulations responsible for the maintenance of chronic pain and may ultimately result in highly targeted pain therapy.

APPENDIX A

TRANSGENE SEQUENCES

A.1 CMV PROMOTER

ATAGTAATCAATTACGGGGTCATTAGTTCATAGCCCATATATGGAGTTCCGCGTTAC

ATAACTTACGGTAAATGGCCCGCCTGGCTGACCGCCCAACGACCCCCGCCCATTGAC

GTCAATAATGACGTATGTTCCCATAGTAACGCCAATAGGGACTTTCCATTGACGTCA

ATGGGTGGAGTATTTACGGTAAACTGCCCACTTGGCAGTACATCAAGTGTATCATAT

GCCAAGTACGCCCCCTATTGACGTCAATGACGGTAAATGGCCCGCCTGGCATTATGC

CCAGTACATGACCTTATGGGACTTTCCTACTTGGCAGTACATCTACGTATTAGTCATC

GCTATTACCATGGTGATGCGGTTTTGGCAGTACATCAATGGGCGTGGATAGCGGTTT

GACTCACGGGGATTTCCAAGTCTCCACCCCATTGACGTCAATGGGAGTTTGTTTTGG

CACCAAAATCAACGGGACTTTCCAAAATGTCGTAACAACTCCGCCCCATTGACGCA

AATGGGCGGTAGGCGTGTACGGTGGGAGGTCTATATAAGC

A.2 NF200 PROMOTER

GTAGGTTCTCTGCCCCTCAAACTCAGCCCAGCTTTCTCCTGCCTGTTCAGGGGACCTT

CTGCCCGCTTCGCTGAGGGTCCGTCCCCTTTACTGGGGCTGGCAGCAGGGTCTCCCA

TCTCCTCTCTCGGGGGCCACTGCAGACTTTTTAGAGAACGCCTTGCCTCCCCCCAAC

CCCACCCATCCGGGGTTCCCTCTCTCCATCCTCTGCAGTGTCTCCCATACCCCCATTC

AGGGTAGCCTTGCTATTCTCCCCAACTCCAGGTCCCCCTTCATCTATTCCGGGGCTGG

CCGCGGAGTTTCCTGAGCGCTCTCCAAGTGGGTCCTCTAGATGTTAGGAGAACACTG

TACCTCCCCCGGTCAGGGGTCTCCTGTCTCCGTTCTATGGAGCGTCCATGCTCCCATT

CAGGACTGCCTTGCTCCCTCCTCTGTTCCGGGGCTGGCTGCACAGTCTCTGCACCCCC

TATCCTGAAAGCCTCTCTTAACTATTTGGAAAGCCTCGTGTCCTGTCTCATACAGGG

ATCCCCTCATCCTAATGACTGCAATCTTCCATTGCTCCATCCCGAGGGCATCCTGCCC

CTATTCCCATCAGGTTTCTCCTTGTCCTCTCCCTGTTTCAAGTCCCCTTTCTTATTCCG

AACACACTCGCAGGCTCTTCCGACGCGCACCCGGGGGTCCTCACTGGCCCACTCCGG

GAGTCCTCTGCCCGCTTCCCCGACCTCGAGGGTCTCCTCTGACGCAGCGTCGATTCC

CCTTCCCTCCTCGGTCCCCTGCCCCGCCCCTCTCACTGCGGCGGAGCCGGTCGGCCG

GGGGGCCGCAGGGGAGGAGGCGGAGAGGGCGGGGCCCTCCTCCCCACCCTCTCACT

GCCAAGGGGTTGGACCCGGCCGCGGCGGCTATAAAAGGGCCGGCGCCCTGGTGCTG

CCGCAGTGCCTCCCGCCCCGTCCCGGCCTCGCGCACCTGCTCAGGCCATGAT

A.3 TRPV1 PROMOTER

CAGCTTTTGTACTTCTCCAGGCGGAGCTGCCGTGGCTGCTCCACTGGAGCAGTGTCT

GAAAAAAAAATAAAAAGGAAAGAAAAGGACATGACTGTTTTTCGGTGCGGTGGAA

GAGAAAGTTTATTGTAGATAAAGGGGGAGCATAGACAGAGGCAGACATGTCTGGGA

GAGCCAGAGTGGTTGTGACCCTGAGCCATATGGAGAGGTGGGGTGAGGGGTGGCAG

AGAGGGATCGAGAGAGGAGAGAGGGGAACCAGATGTAGCAGCCAGGAGGCCAAAG

GTACAAAAGGGGTGGGTAACCAAAATGTCTGGATTATATAAAAAAGAGCCAGAGGT

CAGGCCCACTTTGATATGTTAAATAGGCACCTCAGCCATTTATCCAGGTTTGAAATG

TAATATAATTTACATCCCCCTGGCTTCCTAGAGACCGTTGTTTAGACGGATGACCTCT

GCAGAATGTTTGAGGGTGCAGTCTTGCATGTACTCCCTGGTGGGCTTTCTTGGGCAG

GATCTGGGCAGGAATGGGCTTGTTCTAGTCACCCACTGCGTATGATGGATGAACCCG

CTTCCTAGTAGTTAGGATGGCACTGGGGGAGGCGAGAAATTAGCACACGTAACGTT

TTCTTGTGTTCTATTGTTCACTAAGGGACCCCAGTCAAGCAAGACTGGGCCTTGGAA

GACCTAGAGACCACCAAACCTAATCTCTACCCCGGGTCTGAGTACACAGGGACTCA

GAGTCCCAAAGGGGGCAGGGCCTCCAGACAGGTGGCTCAGAGGTCCCAGTCCTTTG

GAAACATGGCATCTTCAGGACACTGGGCTTTGCATCTCTGGCTGTGACAGTCCTTTA

AGGGAGCTACTCCTCAGACATACAGGAGAGATGGTTTGGAAAGTCCGAGATCCAAA

GCCTGGTTCAGGCTGGACTGGGCTGCAGGCTGCTAAGTGCTCCTCTGCCCTGGCATG

GCTGGGGGTGGGGCATTGGCTGTGGTTCCTGAAAAAGGGCAAAAATGATGGGAAAA

GCTTTGGGATCCTCTGGGAATCGGAGCCGTGGTAACAGCAGCTGCTGCCATTGCTGC

AAATGTTTCCTTGAGTGCCAGAGTATGCCCAGAGCCCATCCCTGCCGTACGCCAGGG

GAGGGGCGAGGACCCTCACAGAGGCAGGGAGGCCGGCCACTCTTACCACACAGCA

GCCTGGCTCTCCCACAAAGGACAGCTCCAAGGCACTTGCTCATTTGGGGTGTAAGGG

CGAATTCTGCAGATATCCATCACACTGGCGGCCGCTCGAGATC

A.4 GLYRIS

GCCGCCACCATGTACAGCTTCAATACTCTTCGACTCTACCTTTGGGAGACCATTGTAT

TCTTCAGCCTTGCTGCTTCTAAGGAGGCTGAAGCTGCTCGCTCCGCACCCAAGCCTA

TGTCACCCTCGGATTTCCTGGATAAGCTAATGGGGAGAACCTCCGGATATGATGCCA

GGATCAGGCCCAATTTTAAAGGTCCCCCAGTGAACGTGAGCTGCAACATTTTCATCA

ACAGCTTTGGTTCCATTGCTGAGACAACCATGGACTATAGGGTCAACATCTTCCTGC

GGCAGCAATGGAACGACCCCCGCCTGGCCTATAATGAATACCCTGACGACTCTCTG

GACCTGGACCCATCCATGCTGGACTCCATCTGGAAACCTGACCTGTTCTTTGCCAAC

GAGAAGGGGGCCCACTTCCATGAGATCACCACAGACAACAAATTGCTAAGGATCTC

CCGGAATGGGAATGTCCTCTACAGCATCAGAATCACCCTGACACTGGCCTGCCCCAT

GGACTTGAAGAATTTCCCCATGGATGTCCAGACATGTATCATGCAACTGGAAAGCTT

TGGATATACGATGAATGACCTCATCTTTGAGTGGCAGGAACAGGGAGCCGTGCAGG

TAGCAGATGGACTAACTCTGCCCCAGTTTATCTTGAAGGAAGAGAAGGACTTGAGA

TACTGCACCAAGCACTACAACACAGGTAAA**GC**CACCTGCATTGAGGCCCGGTTCCA

CCTGGAGCGGCAGATGGGTTACTACCTGATTCAGATGTATATTCCCAGCCTGCTCAT

TGTCATCCTCTCATGGATCTCCTTCTGGATCAACATGGATGCTGCACCTGCTCGTGTG

GGCCTAGGCATCACCACTGTGCTCACCATGACCACCCAGAGCTCCGGCTCTCGAGCA

TCTCTGCCCAAGGTGTCCTATGTGAAAGCCATTGACATTTGGATGG**G**AGTTTGCCTG

CTCTTTGTGTTCTCAGCCCTATTAGAATATGCTGCCGTTAACTTTGTGTCTCGGCAAC

ATAAGGAGCTGCTCCGATTCAGGAGGAAGCGGAGACATCACAAGGAGGATGAAGCT

GGAGAAGGCCGCTTTAACTTCTCTGCCTATGGGATGGGCCCAGCCTGTCTACAGGCC

AAGGATGGCATCTCAGTCAAGGGCGCCAACAACAGTAACACCACCAACCCCCCTCC

TGCACCATCTAAGTCCCCAGAGGAGATGCGAAAACTCTTCATCCAGAGGGCCAAGA

AGATCGACAAAATATCCCGCATTGGCTTCCCCATGGCCTTCCTCATTTTCAACATGTT

CTACTGGATCATCTACAAGATTGTCCGTAGAGAGGACGTCCACAACCAGTGA

Mutations from wild-type GlyR (bold & underlined)

A.5 PEPAGPA MULTIPLE CLONING SITE

CTTAAGCTCGGGCCCCAAATAATGATTTTATTTTGACTGATAGTGACCTGTTCGTTGC

AACAAATTGATAAGCAATGCTTTTTTATAATGCCAACTTTGTACAAAAAAGCAGGCT

TTGG**GTTTAAAC**CCAAAGGAACCAATTCA**GTCGAC**TGGATCC*GCTGGAGCCTCGGT*

AGCCGTTCCTCCTGCCCGCTGGGCCTCCCAACGGGCCCTCCTCCCCTCCTTGCACCGGC

*CCTTCCTGGTCTTTGAATAAAGTCTGAGTGGGCAGC*GCGGCCGCACTCGAGATCCAACT

TGTTTATTGCAGCTTATAATGGTTACAAATAAAGCAATAGCATCACAAATTTCACAA

ATAAAGCATTTTTTTCACTGCATTCTAGTTGTGGTTTGTCCAAACTCATCAATGTATC

TTATCATGTCATCTAGACCCAGCTTTCTTGTACAAAGTTGGCATTATAAGAAAGCAT

TGCTTATCAATTTGTTGCAACGAACAGGTCACTATCAGTCAAAATAAAATCATTATT

TGCCATCCAGCTGCAGCTC

PmeI promoter insertion site (bold & underlined)

HincII ORF insertion site (bold & underlined)

αGlobin 3'UTR (italicized & underlined)

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