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TREATMENT OF NEUROPATHIC PAIN WITH KETAMINE AND ARA 290   Overlapping pathways

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Chapter 1

Introduction

Neuropathic pain

Pain serves as a warning for the body of potential damage from a noxious stimulus and allows for appropriate measures to avoid irreversible damage. Physiological pain is directly correlated to the noxious stimulus, i.e. if the stimulus persists, the pain persists and if the stimulus dissipates, the pain dissipates. Neuropathic pain, however, is a maladaptive response to lesions arising from the nervous system following trauma (e.g. accident, surgical), toxicity (e.g. cisplatin) or systemic disease (e.g. diabetes mellitus, sarcoidosis). This type of pain is often chronic in nature and is no longer correlated to the initial stimulus, i.e. the pain persists after the initial stimulus has dissipated or the pain is experienced in an exaggerated form.[1] These exaggerated pain perceptions include thermal and mechanical allodynia (a non-painful stimulus is perceived as painful, e.g. cold intolerance or the rubbing of clothes) and hyperalgesia (a painful stimulus is perceived as more painful).[2] Regardless of the underlying cause of chronic neuropathic pain, this disease causes great disability in everyday life and often results in the inability to maintain a job or in reduced social participation.[3] In addition, it is a disease that is difficult to treat with conventional pain medication and is often empirically treated with antidepressants and antiepileptics[4] with variable efficacy and often intolerable side effects. The mechanism of the development of neuropathic pain, regardless of the underlying cause, is diverse and includes intertwined and converging pathways such as inflammation, loss of peripheral nerve fibers, N-methyl-D-aspartate (NMDA) receptor upregulation and glia involvement.[5] Targeting either of these targets has provided ample evidence of neuropathic pain relief or disease modification suggesting involvement of one or more of these targets.

Inflammation

The body responds to pathogens or tissue damage with an inflammatory reaction, aimed at preventing infection with a pathogen, or the removal of debris after damage. Tissue damage to a peripheral nerve induces an inflammatory reaction characterized by the release of inflammatory mediators, such as cytokines (e.g. interleukins, tumor necrosis factor α: TNF-α)[6-8] that recruit more immune cells or destroy damaged cells. These cytokines are released by residing and recruited immune cells (e.g. macrophages, T-cells) or support cells (e.g. Schwann cells). Alternatively, many systemic diseases such as diabetes mellitus or sarcoidosis induce local or systemic inflammatory processes. Local inflammatory reactions after nerve damage have their effect in the peripheral nerve by providing an environment of constant noxious

stimuli resulting decreased thresholds for signal transduction (i.e. decreased depolarization thresholds) resulting in enhanced pain signaling. This barrage of signals induces central sensitization in the spinal cord[9], lowering the threshold at which neurons depolarize, causing an altered perception of pain[10]. Alternatively peripheral nervous system inflammation can result in retrograde transport of TNF-α to the central nervous system expanding the inflammatory reaction to the central nervous system, resulting in cytokine release in the dorsal horn[11]. Alternatively the central nervous system can become inflamed either by retrograde transport of cytokines, like TNF-α, or local inflammatory reaction[12] by resident nervous system immune cells (i.e. microglia) or support cells (i.e. astrocytes)[13], which will be discussed in the following paragraphs.

Microglia

Microglia are the resident macrophages of the central nervous system and over the years, this cell type has been correlated to neuropathic pain states arising from various types of lesions[14] and has become an interesting target for pharmacological treatment[15] of neuropathic pain. It has been shown that these cells become activated by various inflammatory cytokines (TNF-α, interleukins)[16] and chemokines (e.g. chemokine (C-C motif) ligand 2: CCL2, also known as macrophage chemoattractant protein 1: MCP-1)[17;18], cytokines and chemokines that are released after nerve damage. Their phenotype changes from resting (i.e. ramified with a small soma) to activated (i.e. amoeboid, retracted rami and a thickened soma)[19]. Additionally, intracellular signaling pathways such as the P38 mitogen activated protein kinase (P38-MAPK)[20], janus kinase-signal transducer and activator of transcription (JAK-STAT)[21] involved in the regulation of transcription factors for, for instance the production of cytokines, become phosphorilyzed increasing cytokine production and release, creating a self-sustained inflammatory process contributing to neuropathic pain.

Astrocytes

Astrocytes are the support cells of the central nervous system and make up for the blood to central nervous system barrier. After peripheral nerve injury, astrocytes are activated in the spinal cord, in response to inflammatory mediators (e.g., TNF-α)[22]. Astrocyte activation may manifest as the phosphorylation of several intracellular signaling pathways and proliferation of these cells (i.e. astrogliosis)[23]. Activation of the intracellular signaling pathways results in the production of inflammatory

cytokines and chemokines (e.g., interleukin-1β: IL-1β and MCP-1)[22]. These mediators can lead to enhanced pain states by acting at both presynaptic sites on primary afferents and post-synaptic sites on dorsal horn neurons causing increased excitation and decreased inhibition of spinal cord nociceptive neurons[24].

Loss of peripheral small nerve fibers

Systemic diseases (diabetes mellitus and sarcoidosis), critical illness neuropathy (due to sepsis or multi organ failure resulting in prolonged intensive care unit stay) as well as pain syndromes such as fibromyalgia are associated with the loss of the small sensory fibers in the epidermis of the skin: small fiber neuropathy (SFN) and subsequent neuropathic pain[25-28]. SFN may result from a continuous inflammatory state of the peripheral nerves innervating the skin with infiltration of immune cells, cytokine production and degeneration of the nerves and thereby contributing to sensory deficits, such as dysesthesia or pain.

The N-methyl-D-aspartate receptor

Glutamate is the central nervous system's major neurotransmitter that acts on the NMDA receptor. This receptor consists of two obligatory NR1 subunits that can be coupled to either two NR2A through D or two NR3A through B subunits to yield a functioning receptor (e.g. $NR1_2/NR2A_2$ configuration). Activation of the NMDA receptor by glutamate results in the removal of the physical magnesium ion block that seals the receptor, resulting in an influx of calcium ions, allowing depolarization of the nerve and signal transduction (reviewed in[29]). In neuropathic pain states, the NMDA receptor becomes upregulated in the dorsal horn, increasing synaptic transmission and contributing to exaggerated pain states such as allodynia and hyperalgesia[30]. The NR2A containing NMDA receptors are ubiquitously distributed throughout the brain and spinal cord, while the NR2B containing NMDA receptors are restricted to areas specific for pain signaling, i.e. laminae I and II of the spinal cord dorsal horn and thalamus[31]. The NR2B receptor subunit has been positively correlated to various pain states, including inflammatory[32] and neuropathic pain[31].

The innate repair receptor

Erythropoietin (EPO) is involved in the genesis of red blood cells. Hypoxia induces stabilization of hypoxia inducible factor 1α (HIF-1α) resulting in the transcription of EPO. EPO, in turn, activates the erythropoietin receptor dimer (EPOR$_2$) present on hematopoietic cells, resulting in increased survival of erythroblasts. Alternatively, erythropoietin possesses anti-inflammatory properties by acting as a natural antagonist of TNF-α through a different receptor configuration: EPOR-β-common-receptor (EPOR-βcR), termed the innate repair receptor (IRR) (reviewed in[33]). This βcR consist of the β chains of the granulocyte-macrophage colony-stimulating factor (GM-CSF)/interleukin 3/interleukin 5 receptors, commonly utilized by type 1 cytokines involved in the innate and acquired immunity (reviewed in[34]). Activation of the IRR by endogenous or recombinant EPO results in attenuation of the immune response, increased survival of tissue, and enhanced regeneration, thus tissue protection and tissue repair[35-38].

Treatment

Currently, chronic neuropathic pain is treated according to the following algorithm with variable results. Current guidelines recommend pharmacological treatment with antidepressants (amitryptiline) followed by antiepileptics (carbamazepine and gabapentin), opioids (tramadol) or topical capsaicine[4]. Treatment with these drugs is often inadequate resulting in insufficient pain relief and is accompanied by side effects that may be severe and intolerable.

Treatment with NMDAR antagonists

Over the past few years, ketamine has gained interest as a pharmacological treatment for chronic neuropathic pain[39]. A relatively short treatment paradigm induces long-term relief of neuropathic pain symptoms in complex regional pain syndrome type 1 patients[40]. The treatment with ketamine, however coincides with undesirable and intolerable side effects, such as nausea, dizziness, anxiety and psychosis. Ketamine is a non-selective NMDAR antagonist, targeting all the NMDAR subtypes, some of which may be involved in the observed side effects. It is unclear, however, if ketamine or its active metabolite norketamine is responsible for the pain relief and/or induced side effects. NMDA receptor antagonists that are specific for the more pain specific NR2B subunit are being developed, one of which is Traxoprodil (CP-101,606) which is devoid of psychomimetic side effects[41] and may be effective in pain relief.
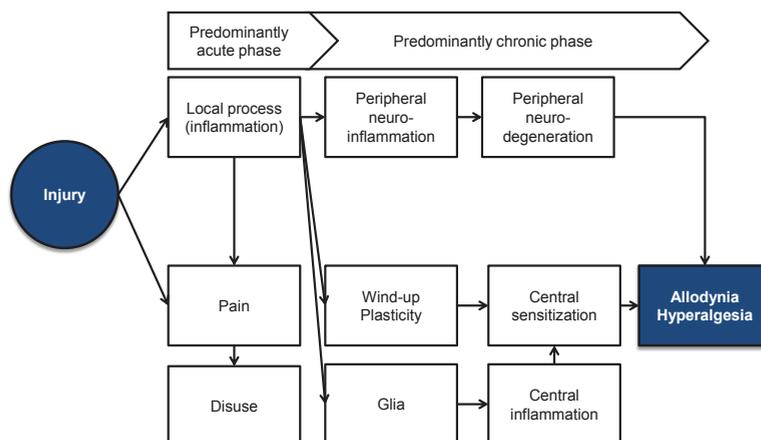
Figure 1: Schematic representation of the current understanding of the development of neuropathic pain.

Rationale for treatment with ARA 290

Inflammation is an important part of the mechanism in the onset and maintenance of neuropathic pain. Counteracting the inflammatory response with EPO has proven to be effective in several types of injury, including injuries to the nervous system. However, EPO induces hematopoiesis as an undesired effect to the tissue protective and regenerative properties. Therefore, derivatives of EPO that are tissue protective but not hematopoietic have been developed[42], one of which is the small helix B peptide ARA 290. This linear 11-amino acid peptide is a representation of the amino acids of EPO interacting with the EPOR and has tissue protective effects equal to EPO, but without hematopoietic effects[43]. Treatment with ARA 290 may be effective in treating or preventing neuropathic pain after nerve injury induced neuropathic pain.

Aims

The experiments described in this thesis were designed to investigate:
- The treatment of neuropathic pain with ARA 290
- The treatment of neuropathic pain with NMDAR antagonists ketamine, norketamine and Traxoprodil
- The overlapping pathways of ketamine and ARA 290 in the treatment of neuropathic pain
- The feasibility of CCM as an objective measure of small fiber neuropathy in sarcoidosis patients with neuropathic pain
- The effect of treatment with ARA 290 on pain and nerve fiber density in patients with sarcoidosis and painful small fiber neuropathy

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Chapter 2

Assessment of allodynia relief by tissue-protective molecules in a rat model of nerve injury induced neuropathic pain

Maarten Swartjes, Marieke Niesters, Albert Dahan

Introduction

Neuropathic pain is a chronic disease with a mechanism that is diverse and not yet completely understood. It is characterized by allodynia (increased sensitivity to a non-painful stimulus) and hyperalgesia (increased sensitivity to a painful stimulus) of either mechanical (touch or pressure) or thermal (cold or heat) origin[1]. These pain states can become disabling to patients resulting in reduced social participation and inability to maintain a job[2]. Up until now, pharmacological (i.e. treatment with opioids, NSAIDS, antidepressants) or non-pharmacological treatment (spinal cord stimulation, physiotherapy) of neuropathic pain with has shown limited efficacy. The mechanism leading to neuropathic pain includes central and peripheral sensitization, neuronal plasticity and neurogenic inflammation. These elements share intrinsic pathways that ultimately lead to altered nociception[3,4]. In animal experiments erythropoietin (EPO) has shown to cross the blood brain barrier and to be neuroprotective[5,6]. Additionally it has shown to be able to alleviate neuropathic pain following nerve injury presumably due to the tissue protective effects of EPO, resulting in increased survival of neuronal cells and reduced inflammation of the nervous system. In 2003, Campana and Meyers showed that treating rats with recombinant human EPO (rhEPO) following L5 spinal nerve crush (SNC) alleviated allodynia and decreased the time to recover from SNC, whereas animals in the vehicle treatment group showed a higher degree of allodynia and a longer time to reach recovery. This effect was supported by the observation that rhEPO prevented apoptosis of dorsal root ganglion (DRG) cells and induction of phosphorylated JAK-2, a molecule when phosphorylated induces apoptosis[7]. In addition to the peripheral effects observed, rhEPO showed a central effect by protecting neurons in the spinal cord in a rat model of neuropathic pain. Following L5 proximal nerve root crush, rhEPO treated animals showed less allodynia when compared to vehicle treated animals which was accompanied by less apoptosis of neurons in both the ventral and dorsal horns of the spinal cord and identification of the EPO receptor (EPOR) and lower levels of TNF-α in spinal cord neurons[8]. A study performed by Keswani et al.[9] assessed the role of the EPOR and showed neuroprotective effects in both in vitro and in vivo models. They showed in vitro that EPO is being produced by neurons and Schwann cells and that the EPOR is being expressed predominantly by neurons and was not restricted to the soma of the neuron. Additionally they showed beneficial effects of EPO in neurotoxicity. In an animal model of nerve damage they showed that EPO mRNA was increased in dorsal root ganglia (DRG) as well as in the sciatic nerve, while the EPOR mRNA was increased solely in the DRG . Additionally, in acrylamide induced neuropathy, EPO protected denervation of the skin, improved motor function in the grip strength test and prevented hyperalgesia in the paw withdrawal test. The role

of EPO and TNF-α in neuropathic pain states was again explored by Campana et al.[10] in a chronic constriction injury model (CCI). They showed in animals with nerve injury that TNF-α was increased in injured nerves proximal to the injury and that rhEPO was able to reduce pain behavior. The induction of TNF-α was counteracted by rhEPO resulting in lower levels of the cytokine. Additionally, Jia et al.[11] showed that treating animals that had received a L5 spinal nerve transection with rhEPO showed decreased mechanical and thermal hyperalgesia with respect to control animals. This coincided with less microglia activation, decreased pro-inflammatory cytokine production (IL-1, 6 and TNF-α), increased anti-inflammatory cytokine production (IL-10) and decreased the expression of NF-κB, a signaling molecule important in pain processing. Both the expression of the cytokines and NF-κB was shown to be dose dependent[12]. Also EPO derivatives devoid of erythropoietic properties show these effects. In a model of neuropathic pain where nucleus pulposus was applied to the DRG of animals EPO and asialo-EPO, an EPO derivative without erythropoietic properties, decreased mechanical allodynia and decreased levels of phospho-P38, a signaling molecule important in pain processing and inflammation, and TNF-α[13]. The EPO-derivative ARA 290, an 11-amino-acid peptide mimicking the 3-dimensional structure of B helix of EPO[14] has shown to be able to prevent the onset of allodynia in animals with nerve injury. In a rat model of neuropathic pain where animals received a spared nerve injury (SNI), a short treatment paradigm resulted in a delay of onset of allodynia, while the same paradigm complemented with a once per week main-tenance treatment prevented the onset of allodynia for the duration of 15 weeks. It was shown that ARA 290 works through the EPOR-β-common-receptor (EPOR-βcR) complex. Mice devoid of the βcR showed no response to ARA 290, whereas wild type mice showed reduced levels of allodynia[15]. EPO and its derivatives show efficacy in neuropathic pain making these molecules promising agents as treatment modalities.

Materials

Induction of the neuropathic pain model: spared nerve injury

1. Female Sprague-Dawley rats, 8 weeks old
2. Ethanol 70% and wipes
3. Absorbing under pad
4. Syringe equipped with a 25G needle containing buprenorphin
5. Vapor anesthetics (Sevoflurane, isoflurane: see Note 1)
6. (Animal) shaver
7. Tape
8. Disinfectant

9. Gauzes
10. Small cotton swabs
11. 5-0 silk sutures
12. 4-0 nylon sutures
13. Standard pattern forceps, straight (Fine Science Tools, Heidelberg, Germany)
14. Metzenbaum scissors, straight 14.5 cm (Fine Science Tools, Heidelberg, Germany)
15. Bonn micro forceps, smooth 7 cm (Fine Science Tools, Heidelberg, Germany)
16. Vannas spring scissors, straight 4 mm blade (Fine Science Tools, Heidelberg, Germany)
17. Student iris scissors, straight 11.5 cm (Fine Science Tools, Heidelberg, Germany)
18. Halsey needle holder, smooth (Fine Science Tools, Heidelberg, Germany)

Assessment of pain: Tactile allodynia

1. Plateau with grid (UGO Basile, Varese, Italy)
2. Perspex cages with lid (UGO Basile, Varese, Italy)
3. Semmes-Weinstein monofilaments (North Coast Medical Inc., San Jose, CA, USA)

Assessment of pain: Cold allodynia

1. Syringe (1 ml)
2. Needle 25G, bent 90°
3. Acetone, analytical grade

Treatment with ARA 290

1. PBS
2. ARA 290 (Araim Pharmaceuticals, Ossining, NY, USA)
3. Syringe, 1 ml equipped with 25G needle

Methods

Induction of the neuropathic pain model: spared nerve injury

1. Sterilize the instruments, for instance with a table top sterilizer.
2. Disinfect the surgical area of the table with 70% ethanol.
3. Place an absorbing under pad on the surgical area and place the surgical tools.
4. Fifteen minutes prior to surgery, administer a single dose of 0.01 to 0.05 mg/kg buprenorphin subcutaneously in the scruff of the neck for the relief of acute post operative pain.
5. To start surgery, induce and maintain anesthesia (6% induction, 3% maintenance in medicinal air mixture).
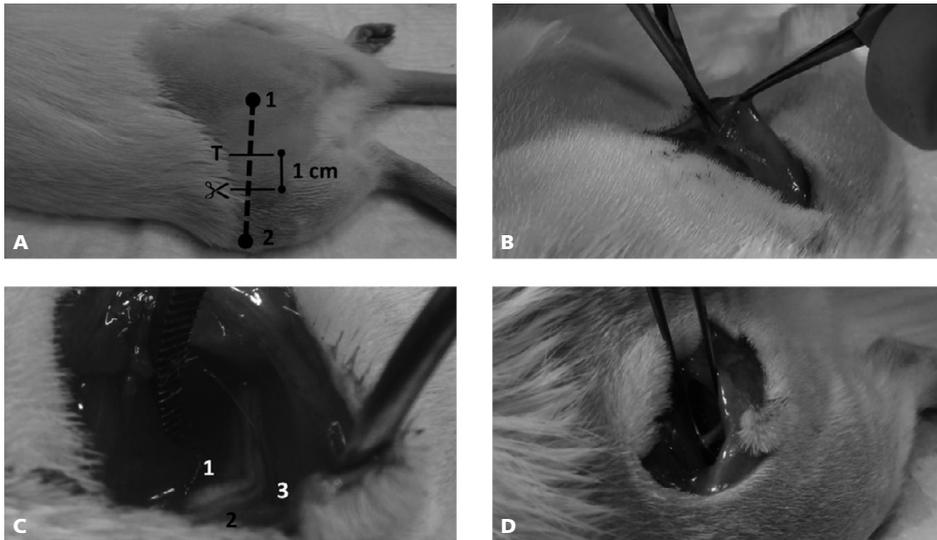
Figure 1: Surgery for induction of the spared nerve injury. A: Superficial landmarks for orientation: 1) Crest of ilium, 2) Patella, T) Site of trifurcation, ✂): Location of first incision. B: Making the incision in between the two heads of the biceps femoris muscle with a micro scissor to enter the site of the location where the trifurcation is being situated. C: Sciatic nerve and trifurcation: 1) Common peroneal nerve, 2) Tibial nerve, 3) Sural nerve. D: Ligation and transection of the common peroneal nerve. Lifting the nerve produces a bridge that allows safe transection of the nerve.

6. Place animal on the stomach and shave the leg that is going to be operated on.
7. Disinfect the shaved hind leg and direct and fixate it with a piece of tape towards yourself.
8. Draw an imaginary line between the patella and the crest of the ilium and locate the center of the line. This is approximately where the trifurcation of the nerve is situated (Figure1A).
9. Lift the skin of the hind leg with the standard pattern forceps.
10. Make a small incision with the Metzenbaum scissors perpendicular to the imaginary line 1 cm distally from where the trifurcation is supposed to be.
11. Insert the Metzenbaum scissors horizontally and closed into the small incision between the skin and the muscle layer and detach the skin from the underlying tissue by opening the scissors and carefully withdrawing it. Repeat this procedure until the skin is sufficiently detached.
12. Make an incision to proximal with a total length of 3-4 cm following the femoral bone.
13. Retract the skin to expose the underlying muscles.
14. Locate the margins of the two heads of the biceps femoris muscle, which is characterized by a white line of adjoining fascia.

15. Carefully lift the medial part of the muscle with the Bonn micro forceps to create a small indentation (Figure 1B) .

16. Carefully cut the fascia with the Vannas spring scissors to detach the muscles. This allows the exposure of the space where the nerves and vessels are situated.

17. Expose the sciatic nerve and its trifurcation carefully by blunt preparation with the standard pattern curved forceps. Insert the forceps in a closed manner and allow it to open in order to make space (see Note 2). Be careful not to touch or stretch the sciatic nerve, its branches or the vessels that are situated in that area.

18. Identify tibial, common peroneal and caudal cutaneous sural nerve (Figure1C). The tibial and common peroneal will be the nerves that are going to be transected. The cutaneous sural nerve will be spared.

19. Carefully free tibial and common peroneal nerve from their surroundings with a cotton swab.

20. Place the curved Moria iris forceps under the tibial nerve and use it to guide a 5-0 suture to pass under the nerve. Ligate the nerve at approximately 1 cm distal from the trifurcation.

21. Repeat the previous step for the common peroneal nerve and ligate with 5-0 suture at approximately 1 cm distal from the trifurcation.

22. Lift the tibial nerve with the curved Moria forceps closed and allow the forceps to open to have the nerve form a bridge of about 4 mm between the two legs of the forceps.

23. Cut the nerve approximately 4 mm from the ligature (see Note 3).

24. Lift the tibial nerve and cut away approximately 3 mm of nerve distal from the suture.

25. Lift the common peroneal nerve with the curved Moria forceps closed and allow the forceps to open to have the nerve form a bridge of about 4 mm between the two legs of the forceps.

26. Cut the nerve approximately 4 mm from the ligature (see Note 4).

27. Lift the common peroneal nerve and cut away approximately 3 mm of nerve distal from the suture.

28. Carefully displace the proximal nerve stumps with a cotton swab.

29. Restore muscle integrity and suture the fascia with 5-0 silk suture

30. Close skin with four 4-0 nylon sutures.

31. Allow animal to awake and monitor for 30-60 minutes under a heating source maintained at 38°C.

32. Transfer the animal to a cage with fresh sawdust, food and water available. Animals can be housed 2 per cage.

Assessment of pain: Tactile allodynia

1. Place the animal in the Perspex cage on the grid and allow to acclimatize for 10-20 minutes.
2. Stimulate the hind paw with the Semmes-Weinstein mono filaments just lateral from the midline. Maintain the filament perpendicular to the paw. Start with the filament that applies the lowest amount of force (1,65). Apply at a rate of 1Hz to a total of 10 stimuli.
3. When a response is observed in the form of an acute withdrawal upon stimulation at any point during stimulation, this is noted and the paw will no further be stimulated with the same filament or with a filament of a higher force.
4. When no response is observed, continue with the next filament. Continue increasing the filament and repeat until the animal responds. This response is noted.
5. Repeat the entire testing sequence to obtain results in duplex.
6. Assessment of pain: Cold allodynia
7. Spray 20 µl of acetone in one fluent application on the plantar surface by using the 1 ml syringe with bent needle.
8. Observe the response of the animal and score according to the scoring table (Table 1).
9. After 2 minutes rest, repeat the sequence to obtain results in duplex.

Table 1: Scoring for cold allodynia.

Response	Score
No response	0
Startle response lasting less than 1 second	1
Clear withdrawal lasting between 1 and 5 seconds	2
Clear withdrawal lasting between 5 and 30 second (with or without licking)	3
Clear withdrawal lasting over 30 seconds (with or without licking and repeated shaking)	4

Treatment with ARA 290

1. Make stock solution of ARA 290 of 1 mg/ml in PBS and store at 4°C.
2. Administer 30 µg/kg ARA 290 or vehicle (PBS) in a total volume of 200 µl intra peritoneally (i.p.) with a 1 ml syringe mounted with a 25G needle (Note 4).

Results

Sixteen animals were given the spared nerve injury as previously described and were randomly allocated to a treatment group. Eight animals received a sham operation.
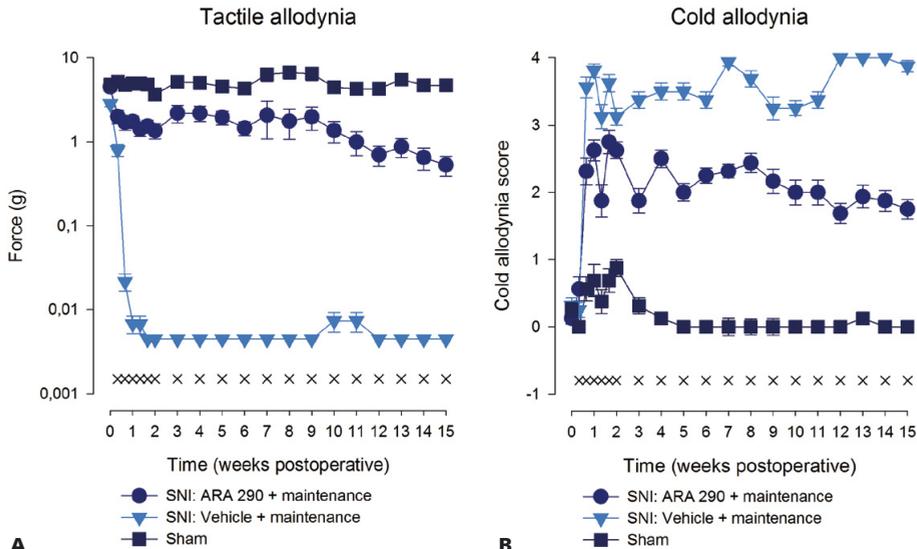
Figure 2: Effect of ARA 290 on the development of neuropathic pain. Treatment with ARA 290 results in the prevention of developing: A: tactile allodynia and B: cold allodynia for a period of 15 weeks. X = treatment with either ARA 290 or vehicle.

In short, animals were anesthetized with sevoflurane (6% induction, 3% maintenance) and the trifurcation of the nerve was exposed. No ligation and transection was performed and the wound was closed in two layers. Twenty-four hours post injury animals received treatment with ARA 290 or vehicle 5 times at 2 day intervals followed by once a week maintenance therapy. Within the first two weeks following nerve injury, vehicle-treated animals showed rapid development of tactile allodynia to the lowest applicable force of 0.004 g. In contrast, i.p. injections with ARA 290 produced long-term relief of tactile allodynia lasting at least 15 weeks (Figure 2A). The allodynic responses differed significantly between treatment groups (repeated measures ANOVA, post hoc Holm-Sidak: $P < 0.001$ versus vehicle-treated animals). Similarly, cold allodynia developed in animals treated with vehicle following nerve lesion with mean scores between 3 and 4 (4 being the maximum score) during the 15 week study period (Figure 2B). Treatment with ARA 290 was associated with significantly less cold allodynia with mean scores between 1.8 and 2.9 (Kruskal-Wallis, post hoc Tukey test: $P < 0.001$ versus vehicle-treated animals).

Notes

1. Anesthesia is induced and maintained with vaporized anesthetic agents (i.e. sevoflurane, isoflurane) rather than ketamine, for ketamine and other NMDA

receptor antagonists, the class of drugs ketamine belongs to, have shown to reduce neuropathic pain in both humans and animals[16,17].

2. Literature describes this procedure to be done by making an incision through the muscle[18]. This induces collateral damage and may cause blood loss. The method described in this chapter has been developed to perform the procedure without any to minimal blood loss.

3. The most important thing to remember while performing the surgery is to maintain a visual on every action in order not to cause additional damage.

4. Treatment is being given after the behavioral tests to minimize influence from stress on behavioral tests due to handling the animals during i.p. administration.

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Chapter 3

ARA 290, a peptide derived from the tertiary structure of erythropoietin, produces long-term relief of neuropathic pain.
An experimental study in rats and β-common-receptor knockout mice

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Introduction

Neuropathic pain is a difficult-to-treat chronic pain disorder. It is characterized by allodynia (increased sensitivity to nonpainful stimuli) and hyperalgesia (increased sensitivity to painful stimuli) to mechanical (i.e., touch, pressure) and/or thermal (cold) stimuli.[1] The mechanisms of neuropathic pain are diverse and not fully understood. Key elements include central and peripheral sensitization, neuronal plasticity, and neurogenic inflammation.[2,3] These elements share intrinsic properties and pathways and ultimate behavioral effects on the perception of painful and nonpainful stimuli. Management of neuropathic pain is characterized by a trial-and-error approach, with interventions including pharmacologic treatment (opioids, antidepressants, antiepileptics, nonsteroidal anti-inflammatory drugs, and their combinations), spinal cord stimulation, and physiotherapy, often with limited success.

The effects of current pharmacologic approaches are limited with respect to efficacy, duration of effect, and the occurrence of often-unacceptable side effects.[1] Recent experimental studies examined the effect of exogenous erythropoietin in painful peripheral neuropathy models.[4-9] The results indicate that exogenous erythropoietin facilitates recovery of sensory and motor functions, including a reduction of allodynia. Erythropoietin possesses generalized tissue-protective and trophic properties that have been demonstrated in various tissues, including neural, cardiovascular, and renal tissues.[10-14] Erythropoietin produces its tissue-protective effects via activation of the erythropoietin receptor (EPOR)-β-common-receptor complex (EPOR-βcR complex), which is locally up-regulated after tissue injury.[11,15] Endogenous erythropoietin, produced in injured tissues, is considered a biologic antagonist of the pro-inflammatory cytokine tumor necrosis factor-α (TNF-α), which is produced by immune cells secondary to their activation after an initial tissue insult.[11] The tissue-protective effects of erythropoietin are distinct from its effects on hematopoiesis. The hematopoietic effect of erythropoietin is mediated through the EPOR homodimer ($EPOR_2$) present on erythrocyte precursor cells.[11] The affinity of erythropoietin for the $EPOR_2$ is 100 times greater than its affinity for the EPOR-βcR complex. Thus, using exogenous erythropoietin for tissue protection requires high circulating plasma concentrations. The use of exogenous erythropoietin has several disadvantages, including the activation of hematopoiesis and an increased risk of cardiovascular complications, including hypertension and thrombosis.[16]

The robust tissue-protective effects of erythropoietin prompted the development of erythropoietin analogs that retain their effect at the EPOR-βcR complex (and consequently their tissue-protective effects) but do not interact with the erythropoietin receptor homodimer (and thus do not cause erythropoiesis and cardiovascular complications). Various erythropoietin analogs have been produced that are

tissue-protective in vivo, including carbamylated erythropoietin and the small helix B surface peptide ARA 290.[11,17,18] ARA 290 is an 11-amino-acid peptide that mimics the tertiary structure of erythropoietin and has been shown to have tissue-protective properties without stimulating hematopoiesis.[11,17]

Because the ability of ARA 290 to treat neuropathic pain after peripheral nerve injury remains unknown, the current study was designed to explore the effect of ARA 290 on behavioral responses after unilateral nerve injury of the sciatic nerve in rats and mice and to determine whether the β-common receptor is involved by using mice lacking the β-common-receptor (βcR knockout or βcR^{-/-} mice) and consequently lacking the EPOR-βcR complex.

Materials and Methods

Animals

The experimental protocol was approved by the Animal Ethics Committee (Dier-ethische Commissie) of the Leiden University Medical Center, Leiden, The Netherlands, and experiments were performed in accordance with the guidelines of the International Association for the Study of Pain.[19] The rats used in this study were 8-week-old female Sprague-Dawley rats (Charles River, Maastricht, The Netherlands) weighing 200–260 g. βcR^{-/-} mice used for the experiments, as described previously, were obtained from Dr. Nimesh Patel, Ph.D. (Kidney Research United Kingdom Career Development Fellow, The William Harvey Research Institute, Centre for Translational Medicine & Therapeutics, London, United Kingdom).[20] Confirmation of βcR^{-/-} was done as described by Robb et al.[20] using Southern blot analysis. Control strain-matched, wild-type mice (C57/BL6) were obtained from Charles River. The mice were 8–12 weeks of age when tested.

Animals were housed two per cage in individually ventilated cages for the duration of the entire experimental period under standard laboratory conditions with water and food ad libitum and a light–dark cycle (12:12 h; lights on 7:00 AM). At the end of the studies, the animals were killed by exsanguination during sevoflurane, 6%, anesthesia.

Surgery

Before surgery, animals were tested for baseline nociceptive thresholds as described below. Twenty-four rats, 16 βcR^{-/-} mice, and 16 wild-type mice were surgically treated to receive an adapted spared nerve injury (SNI).[21] Animals were anesthetized with sevoflurane (6%) induction and maintenance (3%). A small incision was made in the lateral surface of the left hind limb of the animal, exposing the muscles. The

trifurcation of the sciatic nerve was revealed by blunt preparation between the two heads of the biceps femoris muscle. Next, the tibial and common peroneal nerves were tightly ligated with 5–0 silk in rats and 6–0 silk in mice and cut to remove 2–4 mm of the distal nerve. The sural nerve was left intact. To prevent spontaneous nerve reconnection, the transected nerves were displaced. During the surgical procedure, great care was taken not to stretch or touch the sciatic or sural nerves. The wound was closed in two layers with 4–0 silk in rats and 6–0 silk in mice, and a single dose of 0.01 and 0.05 mg/kg buprenorphine was administered in rats and mice, respectively, to relieve postoperative pain. Eight rats, eight βcR$^{-/-}$ mice, and eight wild-type mice received a sham operation. To that end, the animals were anesthetized and the sciatic nerve was exposed as described. After the exposure, no SNI was induced, and the wound was closed in two layers with 4–0 (rats) or 6–0 (mice) silk and a single dose of 0.01 (rats) or 0.05 (mice) mg/kg buprenorphine was administered to relieve postoperative pain. During the surgical procedure, great care was taken not to stretch or touch the exposed nerves.

Study Drugs

ARA 290 (Araim Pharmaceuticals, Ossining, NY) was dissolved in phosphate-buffered saline (PBS) at pH 7.4 to obtain a stock solution of 1 mg/ml. All animals treated with ARA 290 received injections with 30 µg/kg ARA 290 in 200 µl PBS. The peptide was stored at 4 °C between uses. Vehicle treatment consisted of 200 µl PBS at pH 7.4. Both ARA 290 and vehicle were injected intraperitoneally. The ARA 290 dosages used in this study are based on the work of a previous study on the effect of ARA 290 on motor function after sciatic nerve compression injury.[17]

Rat Study Design

The 24 rats that received the SNI were allocated randomly to one of the following treatment groups. Treatment was initiated 24 h after induction of the SNI. Group 1: n = 8; five 30 µg/kg ARA 290 intraperitoneal injections at 2-day intervals, followed by once-a-week maintenance therapy of 30 µg/kg ARA 290. Group 2: n = 8; five vehicle (PBS) intraperitoneal injections at 2-day intervals, followed by once-a-week maintenance therapy of vehicle. Group 3: n = 8; five 30 µg/kg ARA 290 intraperitoneal injections at 2-day intervals, with no maintenance therapy.

Mice Study Design

The 32 mice that received the SNI were randomly allocated to one of the following treatment groups. Treatment was initiated 24 h after induction of the SNI: Groups IA and IB: n = 8 βcR-/- and eight wild-type mice; five 30 µg/kg ARA 290 intraperitoneal injections at 2-day intervals, followed by once-a-week maintenance therapy of intra-

peritoneal injections of 30 µg/kg ARA 290. Group IIA and IIB: n = 8 βcR-/- and eight wild-type mice; five vehicle (PBS) intraperitoneal injections at 2-day intervals, followed by once-a-week maintenance therapy of intraperitoneal injections of vehicle. The follow-up was 4 weeks after surgery.

Measurement of Tactile and Cold Allodynia

Allodynia was assessed before surgery (baseline values) and during follow-up at 1-week intervals on the plantar surfaces of the affected (ipsilateral) and contralateral hind paws. To measure the two types of allodynia, the animals were placed in a see-through box on an increased wire mesh floor. Tactile allodynia was tested first, followed by testing for cold allodynia. Before testing, the animals were allowed to habituate for at least 10 min. When testing coincided with a treatment day, testing was performed before administration of ARA 290 or vehicle. Tactile allodynia was tested with the use of different von Frey hairs (Semmes-Weinstein Monofilaments, North Coast Medical Inc., San Jose, CA) with increasing stiffness (0.004–300 g), causing incremental forces to be exerted on the plantar surface of the affected and contralateral hind paws. The hairs were applied 10 times at intervals of 1–2 s to slightly different loci within the test area. The hind paw that was not surgically treated was tested first. When no response was observed, the ipsilateral hind paw was stimulated in a similar fashion. The force necessary to evoke a pain reflex by a brisk paw withdrawal was recorded, and no additional filaments were applied to the paw that showed a response. The experiment was continued until responses from both the ipsilateral and the contralateral paw were obtained. After a rest period, cold allodynia was tested. Twenty (rats) or 10 (mice) µl acetone was sprayed on the plantar surface of the hind paw, and the response was recorded using the following classification: 0 = no withdrawal, 1 = startle response lasting less than 1 s, 2 = withdrawal lasting between 1 and 5 s, 3 = withdrawal lasting between 5 and 30 s (with or without paw licking), and 4 = withdrawal lasting longer than 30 s (with or without licking and repeated shaking).

Statistical Analysis

A power analysis was based on data from a previous study on the effect of ketamine versus vehicle treatment on tactile allodynia in the rat SNI model.[22] We calculated a group size of at least eight animals was needed to detect a difference between treatments of at least 1 SD between the two groups, with a reliability of 5% and power more than 80%. To analyze the effect of treatment with ARA 290 over time on tactile allodynia, a two-way repeated measures analysis of variance (ANOVA) was used. The tests were followed by a Holm-Sidak test for post hoc comparisons when required. The effect of ARA 290 on cold allodynia was tested with nonparametric tests: Kruskal-

Wallis and post hoc Tukey tests. All statistical analyses were performed with SigmaPlot version 11 (Systat Software Inc., Chicago, IL). Hypothesis testing was two-tailed, with P values<0.05 considered significant. Data are expressed as mean ± SEM.

Results

Effect of ARA 290 Maintenance in the Rat

After SNI, animals that received vehicle treatment showed the rapid development of tactile allodynia with the lowest applicable force of 0.004 g within 2 weeks after surgery. In contrast, intraperitoneal injections of ARA 290 produced long-term relief of tactile allodynia lasting at least 15 weeks (Figure 1A). The allodynic responses differed significantly between treatment groups (main effect: P<0.001; post hoc: ARA 290 vs. vehicle P<0.001, ARA 290 vs. sham P=0.008). In addition to the development of tactile allodynia observed on the ipsilateral side, a decrease of the nociceptive threshold was observed in the contralateral paw (i.e., contralateral allodynia). Contralateral allodynia was greater in vehicle-treated than in ARA 290-treated animals (Figure 1B, main effect: P<0.001; post hoc: ARA 290 vs. vehicle P<0.001, ARA 290 vs.

Figure 1: Effect of ARA 290 treatment during the 15 weeks after spared nerve injury (SNI) surgery on tactile allodynia. A: Ipsilateral paw. B: Contralateral paw. Thirty µg/kg ARA 290 was injected for 5 days at 2-day intervals (first injection within 24 h after surgery), followed by once-a-week maintenance therapy of 30 µg/kg ARA 290. ARA 290 produces significantly less tactile allodynia than does vehicle on ipsilateral (P<0.001) and contralateral paws (P<0.001). All treatments were given via the intraperitoneal route. X=treatment with either ARA 290 or vehicle.
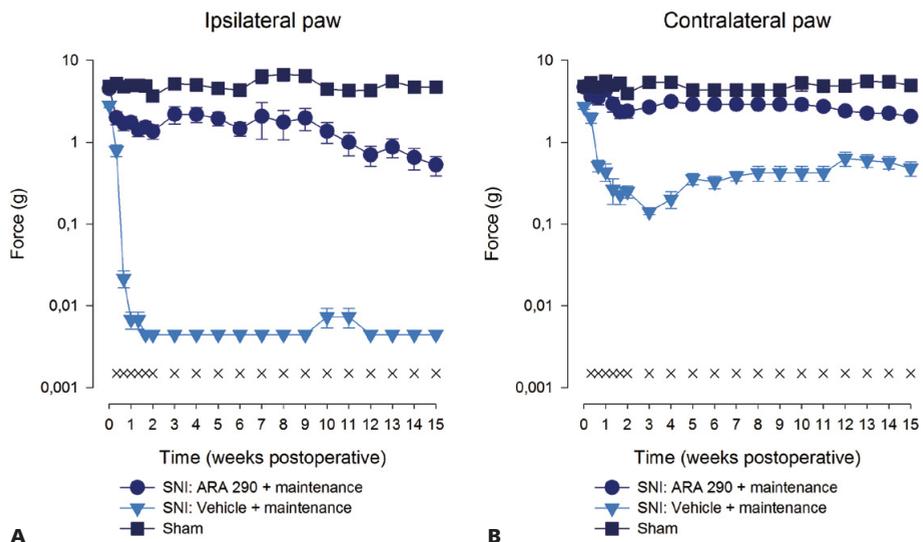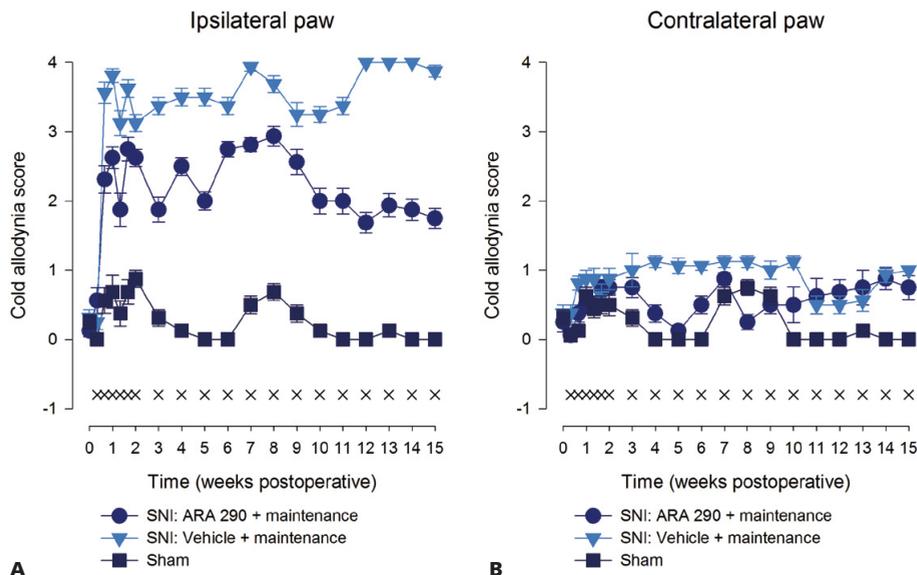
Figure 2: Effect of ARA 290 treatment during the 15 weeks after spared nerve injury (SNI) surgery on cold allodynia. A: Ipsilateral paw. B: Contralateral paw. Thirty µg/kg ARA 290 was injected for 5 days at 2-day intervals (first injection within 24 h after surgery), followed by once-a-week maintenance therapy of 30 µg/kg ARA 290. ARA 290 produces significantly less cold allodynia than does vehicle on ipsilateral (P < 0.001) and contralateral paws (P < 0.001). All treatments were given via the intraperitoneal route. X = treatment with either ARA 290 or vehicle.

sham P < 0.001). Similarly, in animals treated with vehicle, cold allodynia developed rapidly after SNI surgery in the ipsilateral paw, with mean allodynia scores between 3 and 4 (4 is the maximum score) during the 15-week study period. Treatment with ARA 290 was associated with significantly less cold allodynia in the ipsilateral paw, with mean scores between 1.8 and 2.9 (Figure 2A, P < 0.001; compared with vehicle-treated animals by post hoc test). Cold allodynia responses in the contralateral paw averaged to approximately 1 in vehicle-treated animals. A small but significant reduction in cold allodynia was observed during ARA 290 treatment in the contralateral paw (Figure 2B, P < 0.05; compared with vehicle-treated animals by post hoc test).

Effect of 2-week versus Maintenance ARA 290 in the Rat

To assess the effect of early ARA 290 treatment, eight animals received five injections of 30 µg/kg ARA 290 during the initial 2 weeks after SNI surgery and no additional treatment. Animals treated according to this regimen showed a delay in the progression of tactile allodynia for the duration of follow-up but to a lesser extent than that of the group treated with weekly ARA 290 injections (maintenance therapy) (P = 0.018, Figure 3A). Regardless of the therapy received, animals displayed comparable nociceptive thresholds in the contralateral paw (Figure 3B).
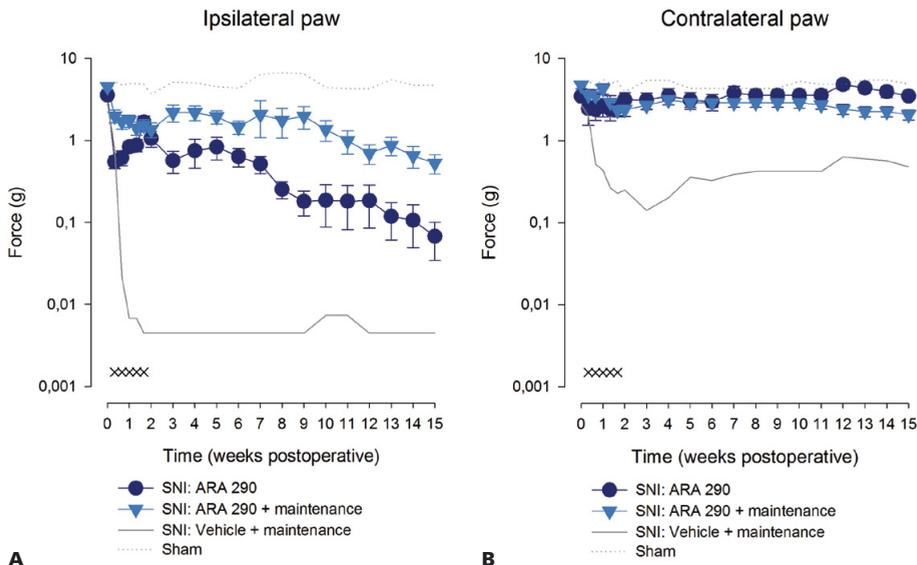
Figure 3: Effect of 2 week therapy versus weekly maintenance therapy on tactile allodynia. A: Ipsilateral paw. B: Contralateral paw. To guide the eye, data from vehicle-treated animals (continuous line) and sham-operated animals (dotted line) are added. Maintenance therapy produced less allodynia than 2-week treatment in the ipsilateral paw (P=0.02), but no difference was observed in the contralateral paw. All treatments were given via the intraperitoneal route. X=treatment with either ARA 290 or vehicle.

Omitting the maintenance therapy resulted in relief of cold allodynia but to a lesser extent than occurred after maintenance therapy (Figure 4A, P<0.001). No difference was observed in the contralateral paw (Figure 4B).

Effect of ARA 290 Maintenance in βcR$^{-/-}$ Mice

A treatment effect on tactile allodynia was observed in both genotypes (P<0.001). ARA 290 had no effect on tactile allodynia in βcR$^{-/-}$ mice (ARA 290 vs. vehicle: P=0.963, post hoc test). One week after SNI surgery, withdrawal of the affected paw occurred at the lowest possible force, 0.004 g, irrespective of treatment with ARA 290 or vehicle (Figure 5).

In contrast, wild-type animals did show an effect of ARA 290 treatment, with withdrawal responses occurring at 0.020 g versus 0.004 g in PBS-treated animals within 2 weeks after surgery (Figure 5, A and B, P=0.027 vs. vehicle-treated mice, post hoc test).

At the contralateral hind paw allodynia was observed that responded to ARA 290 treatment in wild-type animals (P=0.034 vs. vehicle, post hoc test) but not in βcR$^{-/-}$ mice (P=0.941 vs. vehicle, post hoc test) (Figure 5, C and D). In wild-type and βcR$^{-/-}$ animals, cold allodynia developed in the ipsilateral (main effect: P<0.001 in
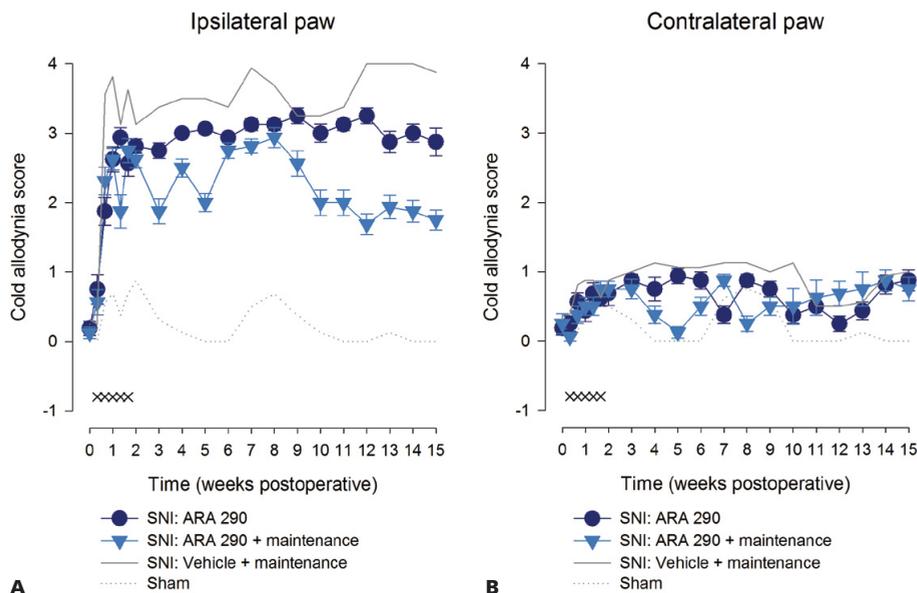
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Figure 4: Effect of 2-week therapy versus weekly maintenance therapy on cold allodynia. A: Ipsilateral paw. B: Contralateral paw. To guide the eye, data from vehicle-treated animals (continuous line) and sham-operated animals (dotted line) are added. Maintenance therapy produced less allodynia than 2-week treatment in the ipsilateral paw (P<0.001), but no difference was observed in the contralateral paw. All treatments were given via the intraperitoneal route. X=treatment with either ARA 290 or vehicle.

both genotypes) but not contralateral hind paw (main effect: P=0.068 in βcR$^{-/-}$ and 0.087 in wild-type mice) (Figure 6). ARA 290 had a significant effect on cold allodynia responses in wild-type (Figure 6A, post hoc: ARA 290 vs. vehicle P<0.05 but not in βcR$^{-/-}$ mice (Figure 6B).

Discussion

The main findings of our studies are: (1) ARA 290 treatment in the 2 weeks after nerve injury produces effective, long-term relief of allodynia in rats; (2) in the same species, ARA 290 therapy was most effective when it was maintained at 1-week intervals; and (3) an effect of ARA 290 on nociceptive withdrawal responses was absent in mice with a homozygous deletion of the β-common-receptor (βcR$^{-/-}$), whereas reduced pain responses were observed in wild-type mice (mice with an intact heterodimer receptor). Our finding of a long-term antiallodynic effect of the ARA 290 peptide is novel and promising, but additional testing in humans is required to predict the effectiveness of ARA 290 in patients with neuropathic pain.

Figure 5: Effect of ARA 290 treatment on tactile allodynia measured in the ipsilateral hind paw (i.e., paw with nerve injury) and contralateral hind paw. A: Effect of ARA 290 therapy in mice with an intact β-common-receptor (wild-type mice), ipsilateral paw. B: Effect of ARA 290 therapy in mice lacking the β-common-receptor (βcR$^{-/-}$ mice), ipsilateral paw. C: Effect of ARA 290 therapy in wild-type mice, contralateral paw. D: Effect of ARA 290 therapy in βcR$^{-/-}$ mice, contralateral paw. ARA 290 caused a relief of allodynia compared with vehicle in wild-type but not βcR$^{-/-}$ animals (wild-type: ipsilateral P=0.027, contralateral P=0.034; βcR$^{-/-}$: ipsilateral P=not significant; contralateral P=not significant). All treatments were given via the intraperitoneal route. X=treatment with either ARA 290 or vehicle.

ARA 290 is a peptide derived from the erythropoietin molecule. In most tissues, including spinal cord and brain, the cytokine erythropoietin is produced in re-
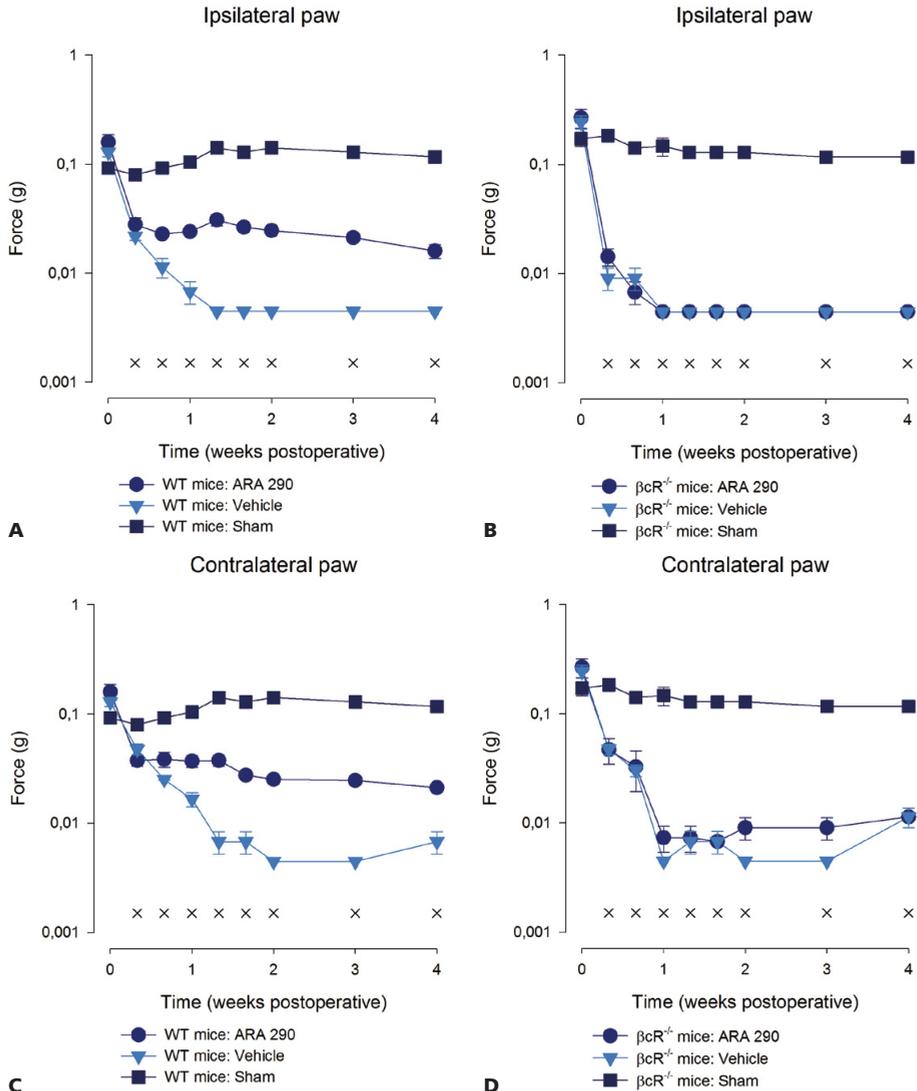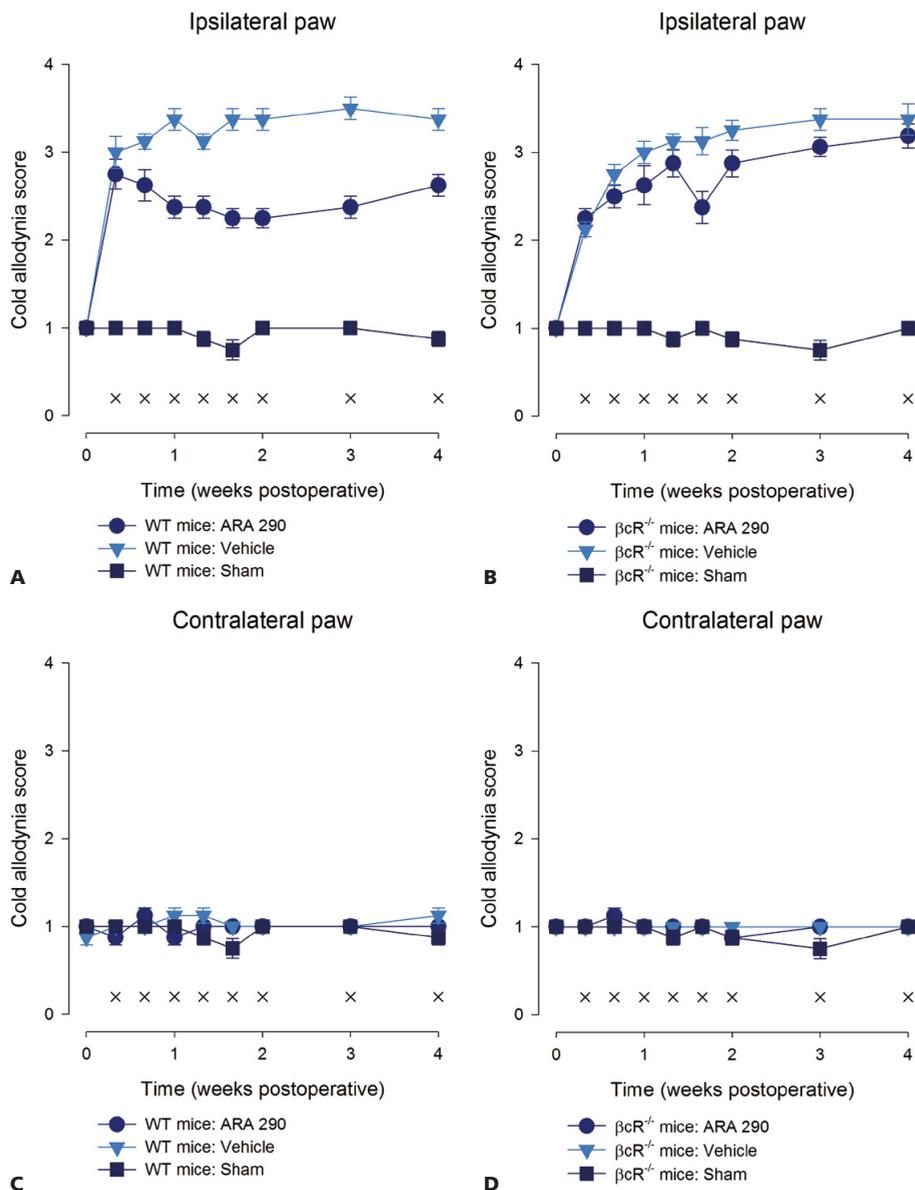
Figure 6: Effect of ARA 290 treatment on cold allodynia measured in the ipsilateral hind paw (i.e., paw with nerve injury) and contralateral hind paw. A: Effect of ARA 290 therapy in mice with an intact β-common-receptor (wild-type mice), ipsilateral paw. B: Effect of ARA 290 therapy in mice lacking the β-common receptor (βcR$^{-/-}$ mice), ipsilateral paw. C: Effect of ARA 290 therapy in wild-type mice, contralateral paw. D: Effect of ARA 290 therapy in βcR$^{-/-}$ mice, contralateral paw. A significant effect was observed in wild-type but not βcR-/- mice at the ipsilateral site only (wild-type: ipsilateral P=0.05, contralateral P=not significant; βcR$^{-/-}$: ipsilateral P=not significant, contralateral P=not significant). All treatments were given via the intraperitoneal route. X=treatment with either ARA 290 or vehicle.

sponse to local injury, counteracting the effects of proinflammatory cytokines.[11,23] Recent animal studies indicate that exogenously administered erythropoietin enhances the process of healing and effectively prevents overt tissue damage after injury.[10-14] For example, Brines et al.[12] showed that systemic administration of recombinant human erythropoietin (rhEPO, 5,000 units/kg) before or as long as 6 h after blunt trauma to the rat brain reduced concussive injury by 50–75%. Similarly, rhEPO reduced the infarct size after carotid artery occlusion in the rat.[12] These local tissue-protective effects are not mediated by the hematopoietic EPOR dimer but through the EPOR-βcR complex, which is locally up-regulated after tissue injury.[11,15,17] To activate this receptor, high local concentrations of erythropoietin are required because the EPOR-βcR complex exhibits a 100-fold lower affinity for erythropoietin than does the hematopoietic EPOR dimer.[11] High local concentrations of exogenously administrated erythropoietin are obtained only after high doses are injected systemically because tissue production of erythropoietin after injury is delayed significantly.[11] The use of high-dose exogenous erythropoietin has several disadvantages, including the activation of hematopoiesis and increased risk of cardiovascular complications (e.g., hypertension, thrombosis). For example, a clinical study on the effect of erythropoietin administration (40,000 units once/week for 4 weeks) to trauma patients admitted to the intensive care unit showed that although mortality was reduced by 50%, there was a 40% increased risk of thrombosis.[16]

Several nonhematopoietic erythropoietin analogues have been developed that selectively activate the EPOR-βcR complex and that have tissue-protective properties, such as carbamylated erythropoietin, asialoerythropoietin, and ARA 290.[11,17,18,24] Several preclinical studies have shown these compounds facilitate wound healing, limit the infarction volume in a stroke model, reduce collateral damage to surrounding tissue adjacent to the injury site in cardiomyopathy, and improve motor function after spinal cord compression.[11,17,24-27]

ARA 290 has been shown to up-regulate EPOR expression in injured tissue.[28] In the current study, we used ARA 290 to assess its effect on nociceptive responses after peripheral nerve injury. ARA 290 caused effective, long-term attenuation of ipsilateral and contralateral tactile and cold allodynia in a SNI model in the rat. The data obtained in βcR$^{-/-}$ mice point toward the β-common-receptor as the site of action of ARA 290 after nerve injury. Our findings are in agreement with previous observations on the effect of exogenous erythropoietin in various models of peripheral nerve injury (including chronic constriction injury, L5 spinal crush injury, and L5 spinal nerve transection).[4-9] In all models, erythropoietin effectively reduced pain behavior coupled with observations of reduced neuroimmune activation related to the anti-TNF activity of erythropoietin. In addition, the site of action of ARA 290 is similar to

that of erythropoietin (i.e., the EPOR-βcR complex) because the erythropoietin effect on motor function after spinal cord injury models is absent in βcR^{-/-} mice.[15]

The neuroanatomical level of the effect of ARA 290 at the β-common-receptor in our experimental pain models remains unknown. We cannot exclude an effect at the (peripheral) site of nerve injury or centrally at spinal or supraspinal sites. However, a complete and prolonged block of the peripheral nerve by use of local anesthetics does not prevent the development of neuropathy, which suggests that central effects are predominant.[29] There is ample evidence that after peripheral nerve injury, as induced in our current study, an innate immune response is triggered in the spinal cord in which proinflammatory cytokines, including TNF-α, are released.[3,5,30-34] This neuroinflammatory response is highly self-amplifying, causing collateral damage to surrounding tissue and leading to sensitization of primary affected and secondary neurons, enhancing allodynia, hyperalgesia, and spontaneous pain. An important issue in this respect is the short half-life of ARA 290 (plasma half-life ≈ 2 min in rats and rabbits).[17] Although this suggests a peripheral rather than a central effect, there is ample evidence that ARA 290 passes the blood–brain barrier. For example, ARA 290 is able to cross the blood–brain barrier to exert its neuroprotective effects in ischemic stroke models and passes the blood–retinal barrier, reducing retinal edema in diabetic animals.[17] Asialoerythropoietin, a nonerythropoietic cytokine with a similarly short plasma half-life of 2 min, passes the blood– brain barrier and appears promptly in the cerebrospinal fluid after intravenous injection and binds to neurons in the hippocampus and cortex in a pattern corresponding to the distribution of the EPOR.[24] Regardless of the location of action of ARA 290, given its short half-life, it is reasonable to assume that ARA 290 initiated a cascade of events involving a series of transduction factors, of which activation of the EPOR-βcR complex is the first step (see also Brines and Cerami[11] Figure 4), that eventually result in the silencing or reduction of the inflammatory response. Evidence from such a sequence of events at central sites may be inferred from previous studies on rhEPO. Jia et al.[8] showed that rhEPO attenuates allodynia and reduces the spinal neuroimmune activation induced by L5 spinal nerve transection with reduced activation of glia cells and reduced production of proinflammatory cytokines (TNF-α, interleukin-1β) and NF-κB activation in the spinal cord. The same group showed that preemptive rhEPO attenuates mechanical and thermal hyperalgesia after L5 spinal nerve transection, as well as the cerebral expression of TNF-α, interleukin-1β, and NF-κB activation.[9] After dorsal root ganglion crush injury, rhEPO reduced local apoptosis and pain behaviors.[6] These data indicate a neuroprotective and anti-inflammatory role of rhEPO at central sites in a variety of neuropathic pain states, causing a significant amelioration of pain behavior. Given the observations in rhEPO-treated animals, the fact that AR290 is an erythropoietin analog acting at the EPOR- βcR complex, and that it is able to pass the blood–brain

barrier, our data may well be explained by an anti-inflammatory and neuroprotective effect of ARA 290 at spinal and possibly supraspinal sites. However, we again stress that a peripheral effect cannot be excluded. A peripheral effect of rhEPO has been observed in an animal model of diabetic neuropathy, where it prevents and reverses intraepidermal neuronal loss,[4] and in chronic constriction injury, rhEPO facilitates the recovery from neuropathic pain and reduces Schwann cell TNF-α expression at the nerve injury site.[5] Despite a large reduction of allodynia maintained during the intensive treatment period, a slow trend toward an increase in pain behavior was observed during the weekly ARA 290 dosing paradigm (Figure 3). This observation could suggest that because of the biologic half-life of ARA 290 of less than 1 week, more frequent dosing could prevent the trend for increased pain. An alternative explanation could be that noninflammatory processes slowly develop to foster proallodynic responses and gain in importance over time or that the inflammatory response becomes more resilient. If true, this suggests that treatment of neuropathic pain caused by nerve injury should be aimed at targeting multiple processes, of which suppression of the immune response is one that requires early (and continuous) treatment. It is not likely that decreasing the interval between nerve injury and the initiation of treatment or using ARA 290 as a preemptive measure results in a more effective relief of neuropathic pain because the EPOR-βcR complex is being up-regulated secondary to tissue damage.[11] Alternatively, more intense treatment during the initial phase (e.g., higher doses or injections at a 1-day interval) may be more effective in neutralizing the initial hit induced by the peripheral nerve injury.

We observed contralateral development of allodynia in mice and rats that was attenuated by ARA 290 treatment (Figures 1 and 5). These findings indicate the presence of neuroinflammation in the spinal cord and dorsal root ganglia at the site opposite from the severed peripheral nerves and suggest the presence of a more generalized inflammatory response in the central nervous system in our SNI animals. Indeed, in unilateral nerve damage, a bilateral increase in TNF-α and activated glia cells in bilateral homo-and heteronymous dorsal root ganglia is observed in a rat model of chronic constriction injury, suggesting a more generalized inflammatory response.[35,36]

In conclusion, our data indicate that the development of allodynia after peripheral nerve injury is effectively prevented for the long term by early treatment with ARA 290. Testing of ARA 290 in patients with chronic pain is required before any conclusions on the effectiveness of ARA 290 in humans may be drawn.

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Chapter 4

ARA 290, a peptide derived from the tertiary structure of erythropoietin, produces long-term relief of neuropathic pain coupled with suppression of the spinal microglia response

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Introduction

Neuropathic pain (NP) is a debilitating condition resulting from lesions of the peripheral or central nervous system with allodynia and hyperalgesia to mechanical or thermal stimuli as main symptoms[1,2]. Treatment of NP is difficult and management of symptoms by pharmacological means (opioids, antidepressants or topical agents such as capsaicin) or non-pharmacological (physiotherapy) is often not adequate. The mechanisms underlying NP are largely driven by peripheral and central inflammation leading to peripheral and central sensitization. Peripherally, macrophages and T-cells are the main contributors to the inflammatory response[3,4]. In the central nervous system astrocytes and microglia play a crucial role in NP states after peripheral nerve injury by showing altered numbers, morphology and activation states[5-11]. There is ample evidence for crosstalk between neurons and glia cells leading to phenomena that underlie allodynia and hyperalgesia[12]. In NP, glia become more abundant and activated as a result of the induced release of proliferative molecules, such as fractalkine (chemokine (C-X3-C motif) ligand 1; CX3CL1) and C-C motif chemoreceptor ligand 2 (CCL2) released by neurons due to increased afferent signaling[13-15], and local release and retrograde transport of TNF-α[16]. These glia cells are involved in driving and maintaining the inflammatory response, especially in the dorsal horn of the spinal cord, by releasing inflammatory mediators, including TNF-α, interleukins 1β and 6 and other signaling molecules for periods that may extend over 2 weeks[17-20]. In addition to the observations in experimental animal studies, a case report of a patient with longstanding complex regional pain syndrome describes increased activation of astrocytes and microglia in the spinal cord after autopsy when compared to patients without a neuropathic pain condition[21]. These observations strongly suggest that astrocytes and microglia serve as potential targets for treatment of neuropathic pain. Indeed, inhibition of activated microglia and astrocytes reduces neuropathic pain symptoms in vivo[22-24].

We recently showed that the neuroprotective synthetic 11-amino acid erythropoietin (EPO) derivative ARA 290 produces effective and long-term pain relief following peripheral nerve damage in the rat[25,26]. ARA 290 produces its effects via activation of the β-common-receptor[25-27]. The β-common-receptor in conjunction with the EPO receptor forms a heterocomplex (designated the innate repair receptor, IRR), which becomes locally up-regulated following tissue injury[28,29]. Its activation initiates a local anti-inflammatory response, inhibition of death signal and anti-apoptosis, thereby preventing overt tissue damage. Additionally, activation of the IRR also promotes tissue repair responses, including neurite outgrowth in the nervous system[30]. In humans we recently showed that chronic ARA 290 administration reduced pain symptoms and

improves functionality in patients with chronic neuropathic pain related to small fiber neuropathy[31]. Various animal studies have shown the tissue-protective effects of ARA 290, all related in part to its anti-inflammatory effects. For example, ARA 290 improves survival following myocardial infarction, reduces organ dysfunction in hemorrhagic shock and suppresses development of atherosclerosis in hyperlipidemic rabbits[32-34].

In this study, we investigated the dose-response effect of ARA 290 on mechanical and thermal allodynia in an experimental rat model of chronic neuropathic pain (using the spared nerve injury model in which two of the three branches of the sciatic nerve are surgically cut). Next, to better understand its mechanism of action, we assessed whether ARA 290 has an anti-inflammatory effect at the level of the spinal cord by visualizing spinal astrocyte and microglia using immunohistochemistry. We hypothesize that ARA 290 reduces the neuroinflammatory response in chronic neuropathic pain.

Methods

Animals

The experimental protocol was approved by the Animal Ethics Committee (Diereth-ische Commissie) of the Leiden University Medical Center, Leiden, The Netherlands and the Animal Care and Use Review Office (ACURO) of the United States Army Medical Department Medical Research and Materiel Command. All experiments were performed in accordance to the guide lines of the International Association for the Study of Pain[35]. Forty-two, eight-week-old, female Sprague-Dawley rats (Charles River, Maastricht, The Netherlands) weighing 200 to 260 grams were used in this study. Animals were housed two per cage in individually ventilated cages for the duration of the entire experimental period under standard laboratory conditions with water and food ad libitum and a 12h-12h light/dark cycle. At the end of the studies the animals were anesthetized and euthanized by exsanguination under 6% sevoflurane anesthesia, perfuse-fixed with 100 ml ice-cold heparinized saline followed by 150 ml 4% paraformaldehyde for tissue extraction.

Neuropathic pain model

Chronic neuropathic pain was induced in 34 rats by spared nerve injury (SNI)[25]. Animals were anesthetized with 6% sevoflurane induction and 3% maintenance. A small incision was made in the lateral surface of the left thigh of the animal, exposing the muscles. The trifurcation of the sciatic nerve was revealed by blunt preparation between the two heads of the biceps femoris muscle. Next, the tibial

and common peroneal nerves were tightly ligated with 5-0 silk in rats and cut to re-move 2-4 mm of the distal nerve. The sural nerve was left intact. In order to prevent spontaneous nerve reconnection, the transected nerves were displaced. During the surgical procedure, great care was taken not to stretch or touch the sciatic or sural nerves. The wound was closed in one layer with 4-0 ethilon and a single dose of 0.01 mg/kg buprenorphine was administered to relieve acute postoperative pain.

Eight animals received a sham operation where the nerve was exposed, but not ligated and transected. The wound was closed in one layer with 4-0 ethilon sutures and a single dose of 0.01 mg/kg buprenorphine was administered for the relief of acute postoperative pain. After surgery, animals were allowed to recover with body temperature maintained at 38°C for 1 h before being transferred to a cage with fresh saw dust.

Treatment

The experimental drug ARA 290 was dissolved in phosphate buffered saline (PBS) to obtain a stock solution, aliquoted and stored at 4°C until use. Prior to injection, the stock solution of was diluted in PBS to yield the desired dose in 200 µl. Following surgery, 34 animals were treated on days 1, 3, 6, 8 and 10 post-surgery. All injections were administered intraperitoneally. Nine of the animals were sacrificed after the 2-week treatment period (Group 1); twenty-five animals were followed for another 18-weeks following treatment and then sacrificed (Group 2). Group 1 animals were randomly allocated to one of the following treatment groups: 0 (=vehicle; PBS), 10, 30 µg/kg (n=3/group). Group 2 animals were randomly allocated to one of the following treatment groups: 0 (=vehicle; PBS), 3, 10, 30, and 60 µg/kg ARA 290 (n=5/group). Three animals in Group 1 and five in Group 2 received sham surgery and were not treated (i.e. sham controls).

Neuropathic pain assay

Allodynia was assessed prior to surgery (baseline values), at days 1, 3, 6, 8 and 10 dur-ing the treatment period, and during follow up from day 14 on at 1-week intervals. The test site was the plantar surface of the injured hind paw. The animals were placed in a transparent cage on an elevated wire mesh floor and were allowed to habituate for at least 10 minutes before testing for mechanical allodynia, followed by thermal allodynia after a short interval to allow recovery from the previous test. When testing coincided with a treatment day, testing was performed prior to admin-istration of ARA 290 or vehicle.

Mechanical allodynia was tested with the use of von Frey hairs (Semmes-Weinstein Monofilaments, North Coast Medical Inc., San Jose, CA) with increasing stiffness

(0.004 – 300 g) causing incremental forces to be exerted on the plantar surface of the injured hind paw. The hairs were applied 10 times at a frequency of 1 Hz to slightly different loci within the test area to avoid sensitization due to repetition. The force necessary to evoke a pain reflex by a brisk paw withdrawal was recorded and no further filaments were applied to the paw that showed a response. All measurements were obtained in duplex with a 1-minute interval between the tests and then averaged.

Cold allodynia was tested by using the acetone test. Twenty microliters of acetone was sprayed on the plantar surface of the hind paw. The response of the animal was recorded using the following classification: 0=no withdrawal, 1=startle response lasting less than 1 s, 2=withdrawal lasting between 1 and 5 s, 3=withdrawal lasting between 5 and 30 s (with or without paw licking) and 4=withdrawal lasting longer than 30 s (with or without licking and repeated shaking). All measurements were obtained in duplex with a 1-min interval between the tests and then averaged.

Immunohistochemistry

After perfuse-fixing the animals, the lumbar spinal cord was extracted and post-fixed in 4% paraformaldehyde for 24 h. After post-fixation, the tissues were cryoprotected for 72 h in 30% sucrose before embedding them in TissueTek (Sakura FineTek, Alphen a/d Rijn, The Netherlands). The extracted lumbar spinal cord was sectioned transversally at a freezing microtome at –20 °C to obtain serially sectioned 20 μm sections. Every 10th section was mounted on a Superfrost+ slide (Menzel Gläser, Braunschweig, Germany) and stored at –80 °C prior to staining. For immunohistological staining, sections for all animals of both time points were stained in one run for each antibody to reduce variability between stainings. The sections were retrieved from the freezer and allowed to thaw before blocking for 1 h with 10% goat serum (Invitrogen, Auckland, New Zealand) with 0.4% Triton X-100 (Sigma-Aldrich, St. Louis, USA). Sections were stained overnight at 4 °C for microglia with 1 μg/ml rabbit-anti-Iba-1 (Wako Chemicals GmbH, Neuss, Germany) or astrocytes with 1:200 rabbit-anti-GFAP (Dako, Heverlee, Belgium) in 3% normal goat serum with 0.4% Triton X-100. After 3 washings in PBS, the slides were incubated for 3 h at room temperature with 1:500 goat-anti-rabbit-Alexa488 (Invitrogen, Eugene, USA) as a secondary antibody in 3% normal goat serum with 0.4% Triton X-100. After incubation, slides were washed 3 times with PBS and Vectashield (Vector Laboratories Inc., Burlingame, USA) as an anti-fading agent was applied. Lastly, the slides were cover slipped and sealed with nail polish. Standardized microphotographs of the dorsal horn were taken with a Leica M5500 fluorescence microscope (Leica Microsystems, Rijswijk, The Netherlands). During photography, the spinal cord segment of the image was determined

with a spinal cord histology atlas on the basis of white matter to grey matter ratio, ventral horn morphology and dorsal horn morphology and documented for classification during analysis[36]. The photomicrographs were analyzed using ImageJ (NIH, Bethesda, MD, USA).

Image analysis

First, images were screened for quality by assessing if the dorsal horn was completely visible, without folds or significant damage. Images that did not meet these criteria were not analyzed (on average 5.3% per group). Next, the remaining 8-bit grey scale images were thresholded using the auto threshold function of ImageJ to create dichromatic images required for analysis of the percentage covered with immunoreactive cells. This function objectively separates signal from noise and no adjustments for background, brightness or contrast were performed. Obtained values were averaged per spinal cord segment for each animal.

Statistics

Allodynia
Behavioral data for effects on tactile and cold allodynia were analyzed by 2-way analysis of variance with post hoc Student-Newman-Keuls comparisons for multiple testing. The effect of dose-dependency was analyzed by calculating the area under the curve with the trapezoid rule and curve fitting the data using a linear function. Log-Rank survival curves were created to determine the duration of allodynia relief by ARA 290 treatment. End-points were defined as reaching the maximum amount of measurable allodynia (reaching the 0.004 g filament or reaching a score of 4 in the acetone test). Holm-Sidak post hoc analysis for multiple comparisons was performed.

Microscopy
Spinal cord microscopy data were analyzed per segment by two-way analysis of variance with post hoc Student-Newman-Keuls comparisons for multiple testing.

All data are presented as mean ± SEM unless otherwise stated. P-values < 0.05 were considered significant.

Results

ARA 290 reduces mechanical and cold allodynia in a dose-dependent manner

Mechanical allodynia

Following SNI, vehicle-treated animals progressively developed mechanical allodynia within 10 days with withdrawal responses to the filament exerting the lowest possible force (0.004 ± 0.0 grams). Sham operated animals showed no decline in response threshold. Regardless of treatment, all SNI groups differed significantly from sham operated animals (P<0.001 for all groups). The two-week treatment with ARA 290 produced a lasting relief of tactile allodynia (Figure 1A, treatment effect P<0.001). Post hoc analysis revealed significant effects for the 30 and 60 µg/kg groups (30 µg/kg: P=0.049 and 60 µg/kg: P<0.001 versus vehicle). In contrast, the lower doses of ARA 290 did not produce significant relief of allodynia (3 µg/kg: P=0.825 and 10 µg/kg: P=0.707 versus vehicle). Comparing efficacy of treatment with ARA 290, a linear dose response relationship was observed with an adjusted R^2 of 0.56 (Figure 1B). Higher doses of ARA 290 resulted in higher AUCs corresponding to animals tolerating stimulation with filaments that exert a greater force and hence less mechanical allodynia. Survival analysis indicates that with higher dosages of ARA 290 relief from allodynia persists for longer time periods (Figure 1C, Log-Rank P<0.001).

Cold allodynia

Following SNI, vehicle-treated animals developed cold allodynia within 7-14 days a mean score of 3.2 ± 0.2 (range 3 to 4). Sham operated animals showed no increase in response. Regardless of treatment, all SNI groups differed significantly from sham-operated animals (P<0.001 for all groups). Animals treated with ARA 290 showed a dose-dependent relief of allodynia (Figure 2A, treatment effect P<0.001). Post hoc analysis showed that at all doses allodynia was significantly less compared to vehicle (P<0.001). A linear ARA 290 dose-response relationship was observed with an adjusted R^2 of 0.78 (Figure 2B). Higher doses of ARA 290 resulted in lower AUCs corresponding to animals responding less vigorously to the application of acetone and hence less thermal allodynia. Survival analysis indicates that a more persisting effect was obtained at higher ARA 290 doses (Figure 2C, Log-Rank P<0.001).

ARA 290 prevents the increase of Iba-1-immunoreactivity in the dorsal horn

In figure 3, representative overviews are given from the spinal cords of animals after 2 weeks of survival that received SNI with vehicle (Figure 3A), SNI with 30 µg/kg ARA 290 (Figure 3B) or sham surgery without treatment (Figure 3C). There was an apparent increased Iba-1-immunoreactivity (Iba-1-IR) on the side of the injury that seemed more pronounced in the 0 µg/kg treated group when compared to the 30

Figure 1: A: Effect of spared nerve injury and treatment with vehicle or different doses of ARA 290 on mechanical allodynia. Animals were sham-operated or received spared nerve injury and 5 doses of vehicle, 3 µg/kg ARA 290, 10 µg/kg ARA 290 (green), 30 µg/kg ARA 290, or 60 µg/kg ARA 290 administered on days 1, 3, 6, 8 and 10 post-surgery. B: Correlation of ARA 290 treatment dose and the relief of mechanical allodynia, calculated by the difference in area under the curves (AUC) of the mechanical allodynia response on day 1 vs. day 140. The adjusted R^2 is 0.56. C: Survival analysis showing the mechanical allodynia-free proportion of animals in time either sham-operated, or receiving spared nerve injury and treated with vehicle or different doses of ARA 290. Log-Rank $P < 0.001$.
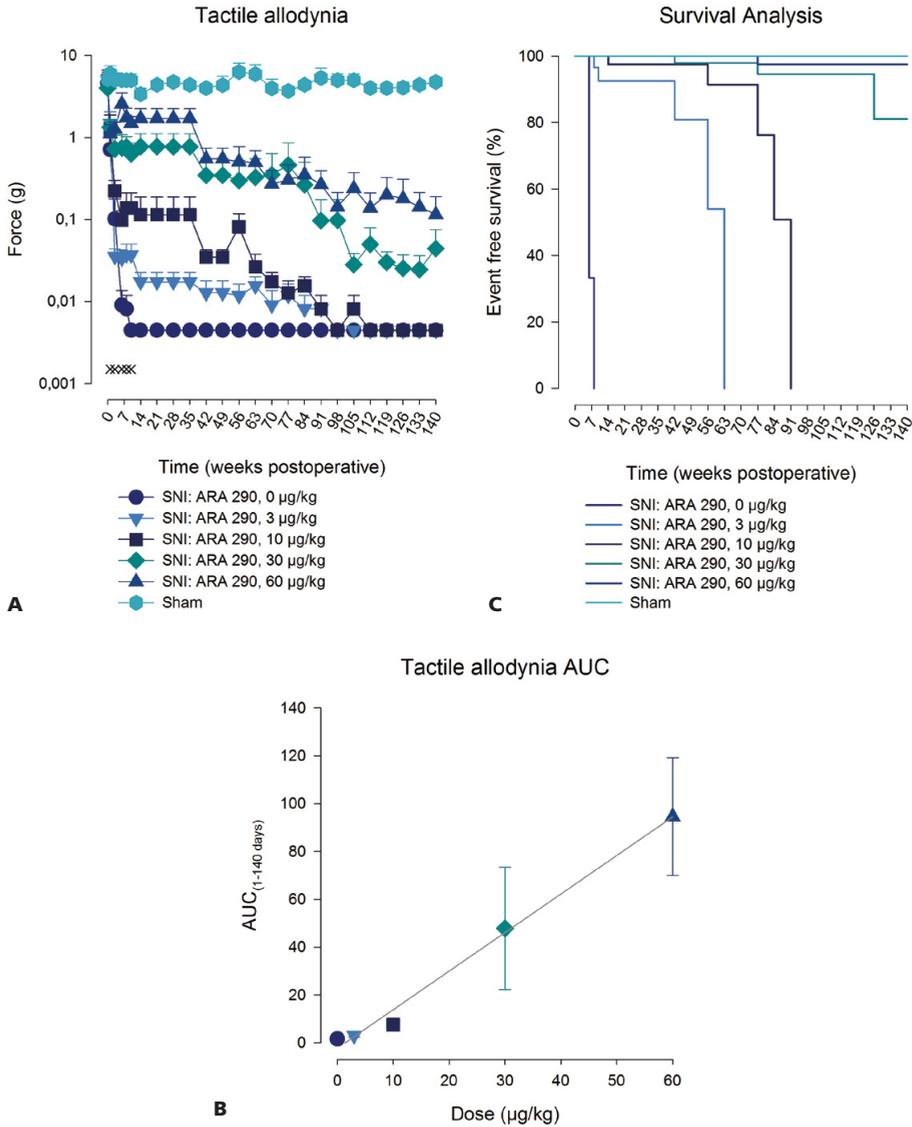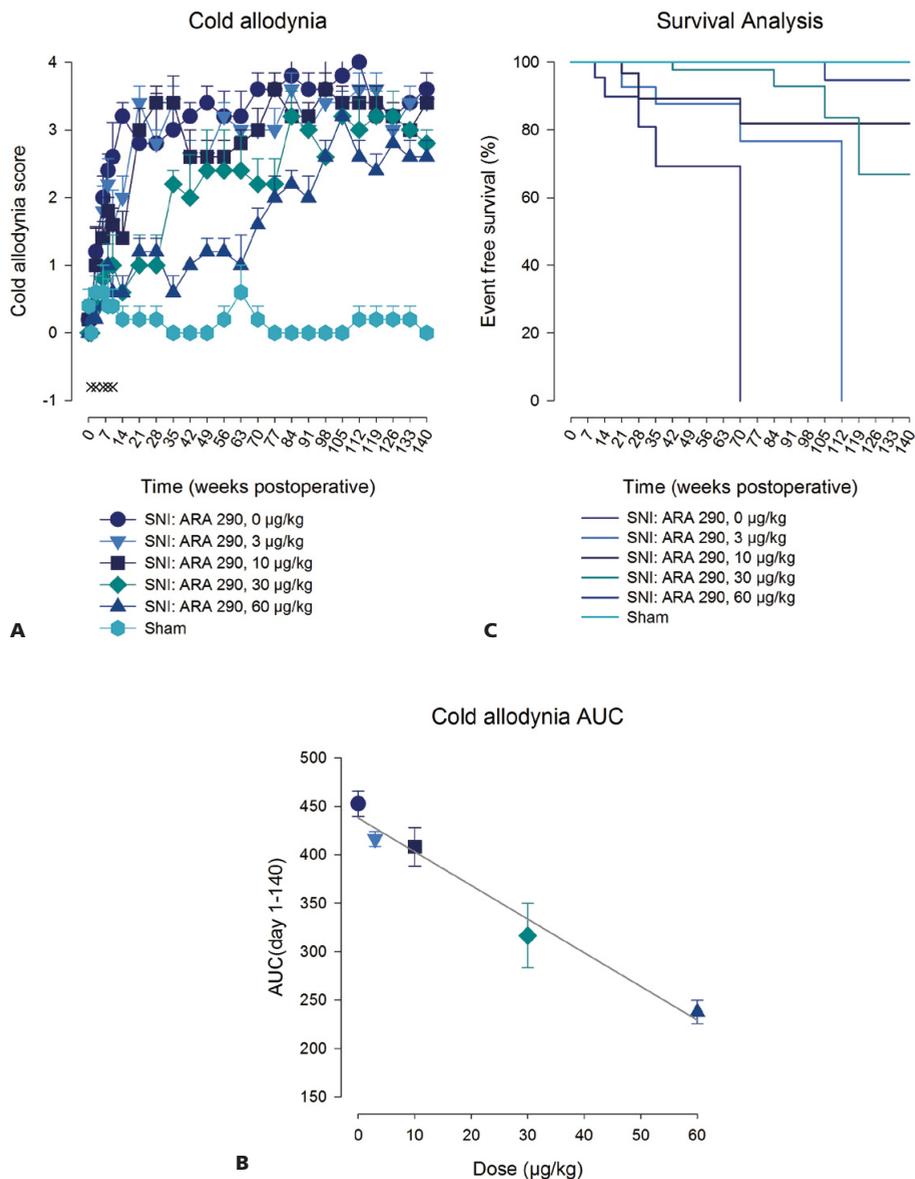
Figure 2: A: Effect of spared nerve injury and treatment with vehicle or different doses of ARA 290 on cold allodynia scores. Animals were sham-operated (grey) or received spared nerve injury and 5 doses of vehicle, 3 µg/kg ARA 290, 10 µg/kg ARA 290, 30 µg/kg ARA 290, or 60 µg/kg ARA 290 administered on days 1, 3, 6, 8 and 10 post-surgery (for each treatment P<0.0001 compared to vehicle). Scoring of cold allodynia is described in the Methods section. B: Correlation of ARA 290 treatment dose and the relief of cold allodynia, calculated by the difference in area under the curves (AUC) of the cold allodynia score on day 1 vs. day 140. The adjusted R^2 is 0.78. C: Survival analysis showing the cold allodynia-free proportion of animals in time either sham-operated, or receiving spared nerve injury and treated with vehicle or different doses of ARA 290. Log-Rank P<0.001.

μg/kg group. The dorsal horns of animals that received SNI with vehicle (Figure 3D), SNI with 30 μg/kg ARA 290 (Figure 3E) or sham surgery without treatment (Figure 3F) showed increased iba-1-IR in the dorsal horn which was more pronounced in vehicle-treated animals. High power magnifications of individual microglia from the dorsal horns of animals that received SNI with 0 μg/kg ARA 290 (Figure 3G), SNI with 30 μg/kg ARA 290 (Figure 3H) or sham surgery without treatment (Figure 3I). Microglia from the vehicle-treated group showed an activated phenotype with an amoeboid shape and retracted rami, whereas microglia from the 30 μg/kg treatment group and sham surgery group showed a resting phenotype with a stretched soma and rami. For further analysis, computerized calculation of the amount of immunoreactivity was performed.

Representative images of recorded photomicrographs of Iba-1-IR in lumbar dorsal horns L1 to L6 of animals at 2 weeks and 20 weeks following SNI surgery and treated with the various ARA 290 doses are given in Figures 4A and B with high power magnifications of microglia cells presented in the inserts. For group 1 (2 weeks post injury), microglia in the L5 segment of the 0 μg/kg treatment group showed an activated phenotype, whereas the microglia in other panels show a resting phenotype. Iba-1-IR was increased in the L5 segment following vehicle treatment only (Figure 5A, $P < 0.05$ versus sham). Irrespective of treatment, no increase in reactivity was observed in any of the other segments. In contrast, in group 2 (20 weeks post injury), Iba-1-IR had spread both cranially and caudally to multiple spinal cord segments in vehicle-treated animals (Figure 4B) with significantly increased Iba-1-IR in segments L2 to L5 ($p < 0.05$ vs. sham). As shown in the inserts of Figure 4B, microglia in the L1-L6 segments of the vehicle and 10 μg/kg treatment groups showed an activated phenotype, whereas the microglia in the 30 μg/kg and sham groups showed a resting phenotype. In Groups 1 and 2, treatment with 30 μg/kg ARA 290 prevented an increase in Iba-1-IR as shown by the absence of Iba-1-IR in all segments (Figures 5A and B; 30 μg/kg: ns vs. sham, $P < 0.05$ vs. vehicle at segments L2 to L4). In Group 2, treatment with 10 μg/kg of ARA 290 did not decrease Iba-1-IR relative to vehicle-treated animals (Figure 5B; ns vs. vehicle, $P < 0.05$ vs. 30 μg/kg at segments L2-L4).

Figure 3 (next page): Representative photomicrographs of Iba-1 immunoreactivity (green) in the L5 spinal cord segment of animals 2 weeks after spared nerve injury (SNI). (A-C) Low power magnifications, (D-F) detailed images of (A-C) as indicated by the white rectangles, and (G-I) high power magnifications of the spinal cord of animals that underwent: A, D and G. SNI and vehicle treatment, B, E and H. SNI and treatment with 30 μg/kg ARA 290, C, F and I. sham surgery without treatment. The left-hand side of the photomicrographs represents the ipsilateral side of the animal, innervating the site of spared nerve injury.
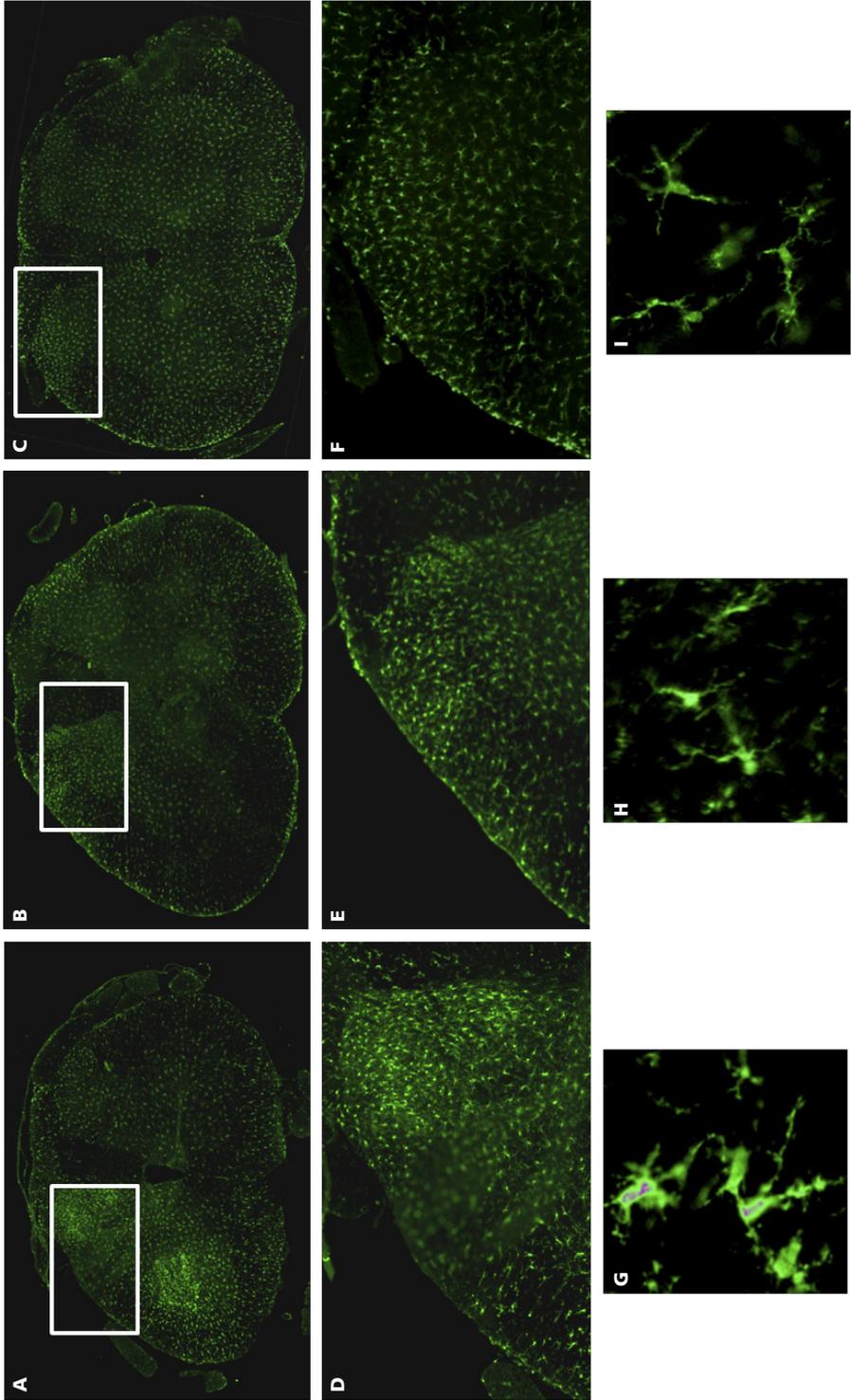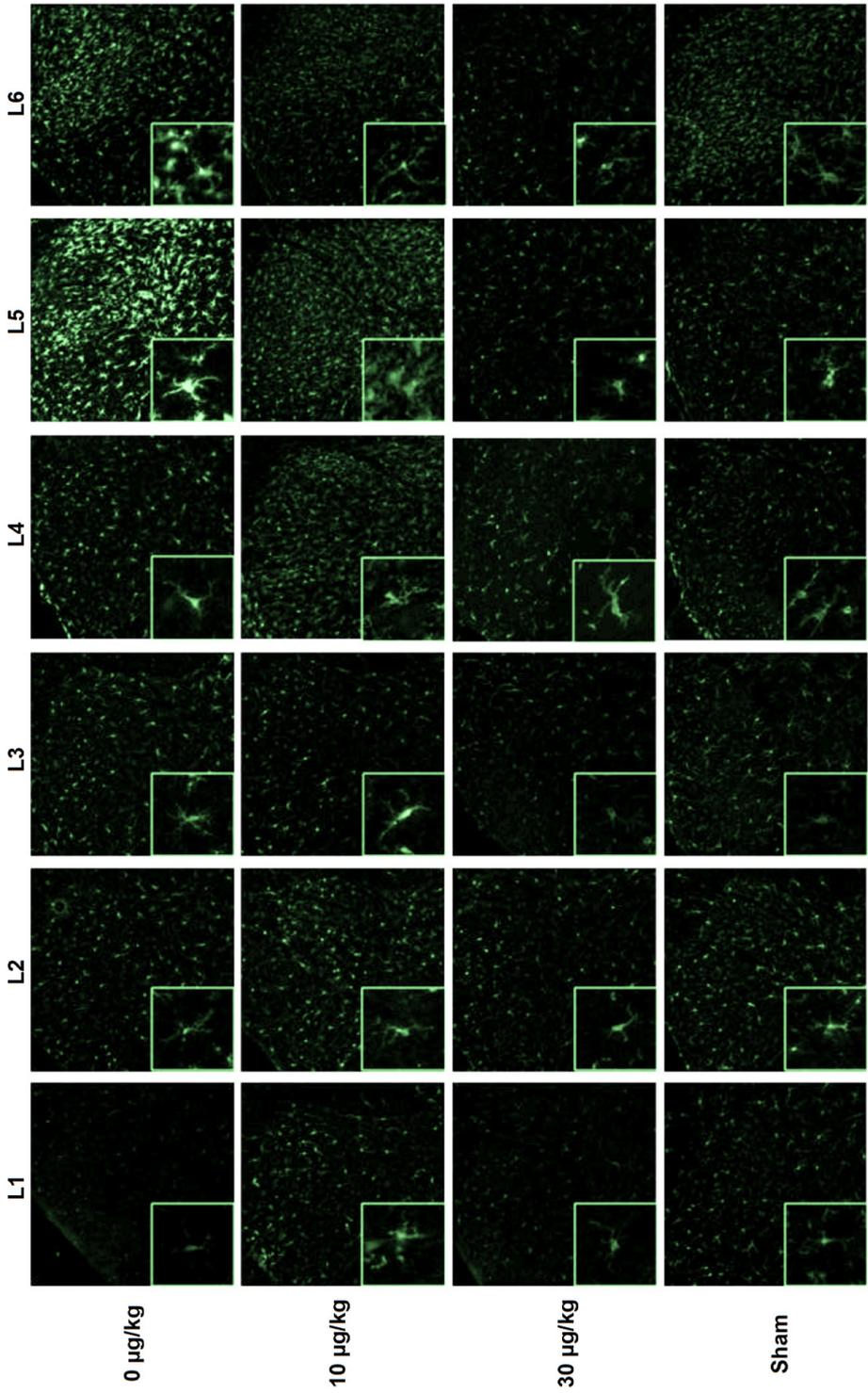
Figure 3.

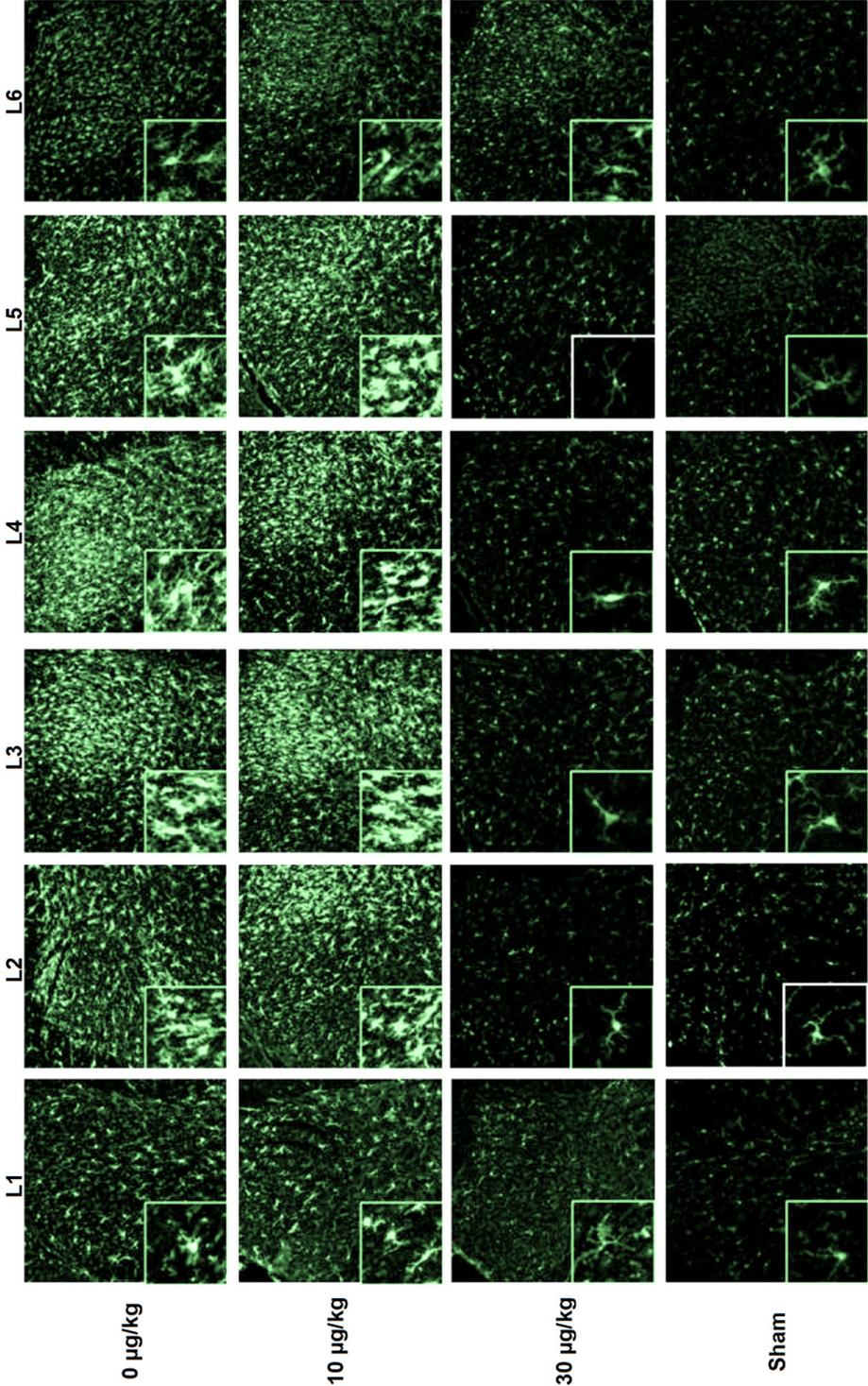**L6** **L5** **L4** **L3** **L2** **L1**

0 µg/kg

10 µg/kg

30 µg/kg

Sham

Figure 4A.

Figure 4B.

Figure 4 (previous pages): A: Representative photomicrographs of Iba-1 immunoreactivity (green) in the lumbar dorsal horns of animals 2 weeks after spared nerve injury (SNI). Animals were either treated with vehicle (upper row), 10 µg/kg ARA 290 (second row) or 30 µg/kg ARA 290 (third row). The bottom row represents sham-operated animals without treatment. In each column, the Iba-1 immunoreactivity signal at different lumbar spinal cord levels (L1-L6) is shown. Inserts show higher magnifications of the photomicrographs.
B: Representative photomicrographs of Iba-1 immunoreactivity (green) in the lumbar dorsal horns of animals 20 weeks after spared nerve injury (SNI). Animals were either treated with vehicle (upper row), 10 µg/kg ARA 290 (second row) or 30 µg/kg ARA 290 (third row). The bottom row represents sham-operated animals without treatment. In each column, the Iba-1 immunoreactivity signal at different lumbar spinal cord levels (L1-L6) is shown. Inserts show higher magnifications of the photomicrographs.

Figure 5: Quantification graphs showing Iba-1 immunoreactivity (percentage of immunostained area) in sections of different lumbar spinal cord levels (L1-L6) from animals 2 weeks (panel A) or 20 weeks (panel B) after sham-operation, spared nerve injury and vehicle-treated, spared nerve injury and treatment with 10 µg/kg ARA 290, or spared nerve injury and treatment with 30 µg/kg ARA 290. A: At 2 weeks post injury, Iba-1-IR was increased in the L5 segment following vehicle treatment only * $P < 0.05$ vs. sham. B: At 20 weeks post injury, vehicle * $P < 0.05$ vs. sham at segments L2-L5) and 10 µg/kg: * $P < 0.05$ vs. sham at segments L2-L4. Data are mean ± SEM.

SNI does not increase GFAP-immunoreactivity in the dorsal horn

SNI did not induce an astrocytic response in vehicle-treated animals relative to sham operated rats for spinal cord segments L1-L6 at either 2 weeks (Figure 6A, Figure 7A) or 20 weeks (Figure 6B, Figure 7B) post injury. No effect of treatment was observed.

L6   L5   L4   L3   L2   L1

0 µg/kg   10 µg/kg   30 µg/kg   Sham

Figure 6A.

L6

L5

L4

L3

L2

L1

0 µg/kg

10 µg/kg

30 µg/kg

Sham
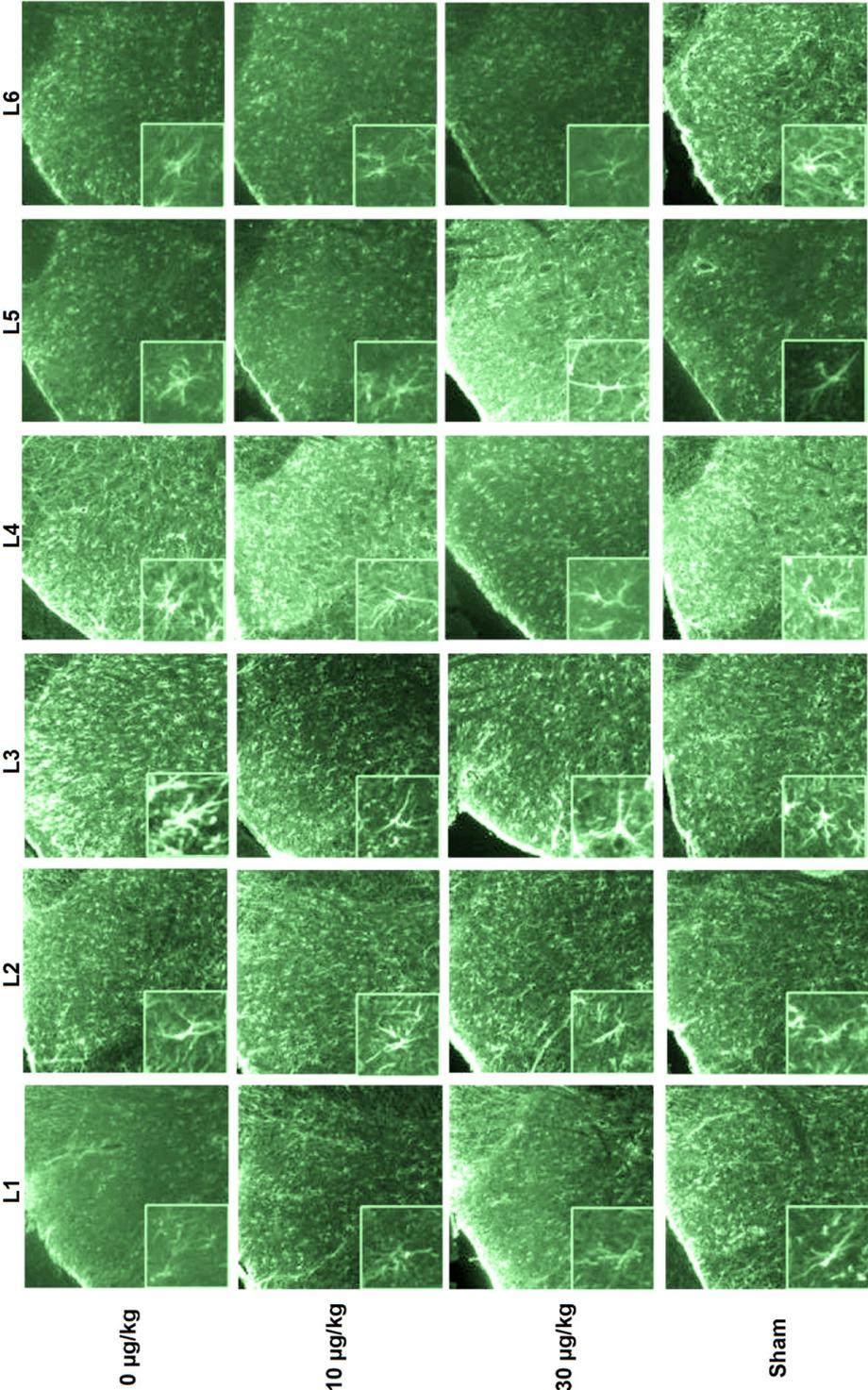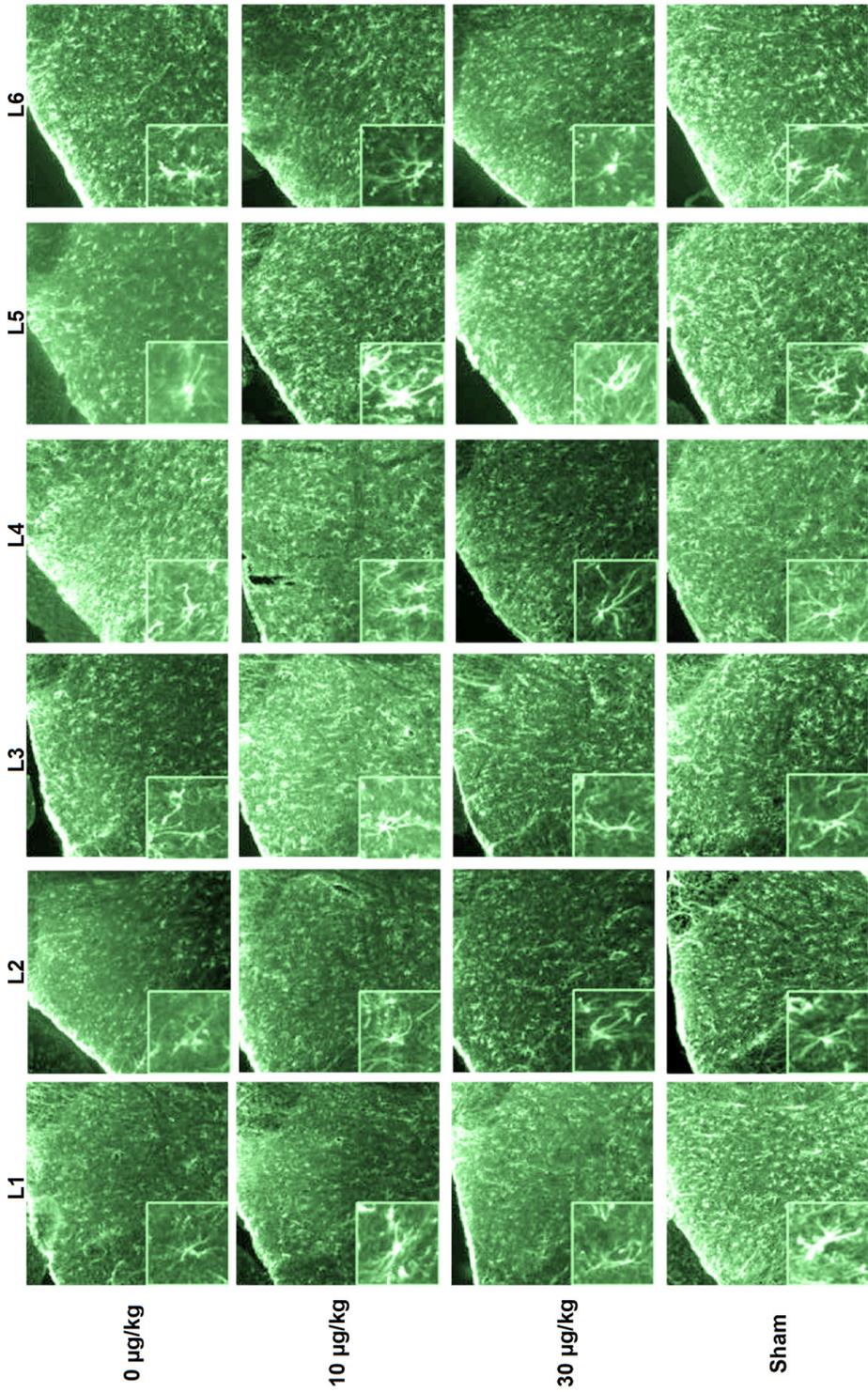
Figure 6B.

Figure 6 (previous pages): A: Representative photomicrographs of GFAP immunoreactivity (green) in the lumbar dorsal horns of animals 2 weeks after spared nerve injury (SNI). Animals were either treated with vehicle (upper row), 10 µg/kg ARA 290 (second row) or 30 µg/kg ARA 290 (third row). The bottom row represents sham-operated animals without treatment. In each column, the GFAP immunoreactivity signal at different lumbar spinal cord levels (L1-L6) is shown. Inserts show higher magnifications of the photomicrographs.

B: Representative photomicrographs of GFAP immunoreactivity (green) in the lumbar dorsal horns of animals 20 weeks after spared nerve injury (SNI). Animals were either treated with vehicle (upper row), 10 µg/kg ARA 290 (second row) or 30 µg/kg ARA 290 (third row). The bottom row represents sham-operated animals without treatment. In each column, the GFAP immunoreactivity signal at different lumbar spinal cord levels (L1-L6) is shown. Inserts show higher magnifications of the photomicrographs.
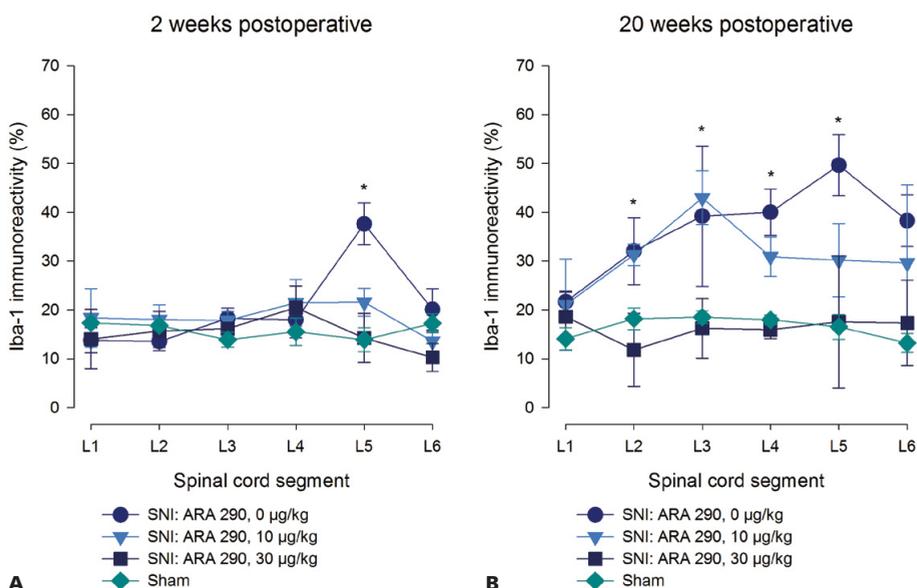

Figure 7. Quantification graphs showing GFAP immunoreactivity (percentage of immunos-tained area) in sections of different lumbar spinal cord levels (L1-L6) from animals 2 weeks (panel A) or 20 weeks (panel B) after sham-operation, spared nerve injury and vehicle-treated, spared nerve injury and treatment with 10 µg/kg ARA 290, or spared nerve injury and treatment with 30 µg/kg ARA 290. Data are mean ± SEM.

Discussion

The main findings of this study are: (1) The spared nerve injury model caused a rapid and long-lasting neuropathy with mechanical and cold allodynia; (2) ARA 290 produced dose-dependent relief of both mechanical and cold allodynia; (3) A spreading microglia response (i.e. Iba-1-IR and phenotype) was apparent from L5 at week 2 following nerve damage to L2-6 at week 20; (4) No effect of nerve injury on the

astrocyte response was observed at weeks 2 and 20 following nerve damage; and (5) ARA 290 suppressed Iba-1R in a dose dependent manner.

Neuropathic pain in animals (due to experimental nerve damage) and humans (due to sarcoidosis or diabetes mellitus type 2) responds well to treatment with ARA 290, in that it produces relief of spontaneous pain (humans) and allodynia (humans and animals)[25,26,31,37]. Studies in mice that lack the β-common-receptor show further that ARA 290 is without behavioral effect (i.e. allodynia is not relieved by ARA 290), implicating this receptor as site of action of ARA 290[25,26].

The β-common-receptor forms a heterocomplex together with EPO receptor and it is believed that this receptor complex, which we designate the innate repair receptor (IRR), is the molecular site of action of both EPO and ARA 290[27,29,38]. Exogenous EPO, similar to ARA 290, reverses allodynia and reduces neuronal apoptosis and proinflammatory cytokine production, neuronal regeneration and the release of anti-inflammatory cytokines[28]. We do not use EPO in our studies as, in contrast to ARA 290, it comes with severe side effects including enhanced hematopoiesis and cardiovascular complications (e.g. hypertension, thrombosis, myocardial infarction). In common with previous studies[25,26], we show here that ARA 290 has effective and prolonged (up to 20 weeks) anti-allodynic effects.

There is ample evidence that peripheral nerve injury results in a strong spinal inflammatory response[17]. For example, we previously showed in mice that surgical damage to the sciatic nerve causes the increase of expression of pro-inflammatory markers including Iba-1 mRNA, GFAP mRNA and CCL2 mRNA, within 7 days following nerve damage[26]. CCL2 plays an important role in the invasion of monocytes from peripheral blood as well as resident macrophages towards the spinal cord lesion site following peripheral nerve damage. In our current study the inflammatory response following SNI was apparent from the increase in Iba-1-IR. The Iba-1-IR response showed a marked expansion from level L5 in week 2 following SNI, to 5 adjoining segments, L2 to L6, at week 20. In addition to the spreading of Iba-1-IR to multiple segments, the intensity of the response also increased over time as shown by a higher degree of Iba-1-IR and phenotypic signs of activation. We are the first to show this spreading inflammatory response in the spared nerve injury model of neuropathic pain. Similar observations were made earlier in experimental models of spinal cord injury and nerve root avulsion[39,40]. Previous reports of glial response following peripheral nerve injury showed that the response area is confined to the spinal cord segments innervated by the damaged nerve[41,42]. However, these responses were measured within a 2-week time frame. This is in agreement with our observation of lack of spreading at week

2. Caudal and cranial expanding inflammation, as observed here, may explain the increase in severity of NP symptoms over time and development of symptoms in areas of the body not innervated by the damaged nerve[43]. Similarly, various experimental reports indicate that the inflammatory responses may spread to contralateral spinal cord areas[8,44]. In this study we did not quantify contralateral inflammation. Our data do suggest that time is an important factor in the spreading of the microglia response.

Iba-1-IR reflects microglia activation in addition to localization and morphology. Our data show increased Iba-1-IR after SNI, which is dose-dependently and long-term reduced by ARA 290 treatment coupled to a dose-dependent and long-term reduction of mechanical and cold allodynia. This long-term effect suggests a disease modulatory effect of ARA 290. We argue that ARA 290 initiates a cascade of events involving several transduction factors of which activation of the IRR is the first step that eventually silences or reduces the inflammatory process[29,36]. Since the activation or recruitment of microglia is largely mediated through the local production of CCL2[45,46], a possible scenario is that ARA 290 reduces the release of CCL2 via activation of the IRR on neuronal and immune cells[26]. However, both at 2 and 20 weeks after SNI and ARA 290 treatment, relief of allodynia was not complete, indicating that the central response to peripheral nerve damage involves multiple systems including neuroinflammation and probably also up-regulation of excitatory pathways and synaptic plastic changes. Of interest is that ARA 290 treatment causes a reduction in NMDA mRNA (subunits NR1, NR2A and NR2B) in SNI animals, suggestive of an additional role, apart from immune-modulation, for ARA 290 in the treatment of neuropathic pain by suppression of excitatory glutamatergic activity[26].

In contrast to a markedly increased Iba-1-IR after SNI, no change in GFAP-IR or astrocytic phenotype (i.e. activation) was observed in animals with an SNI treated with vehicle after 2 and 20 weeks of lesion. This observation stands in contrast with reports describing involvement of astrocytes adjacent to microglia in NP[5,7,10,47,48]. The absence of astrocytosis in our SNI model may be explained by a time-limited astrocyte response (i.e. <2 weeks or between 2-20 weeks). The involvement of astrocytes following peripheral nerve injury reported in the literature varies with some studies showing a relatively short-lived increase in GFAP-IR[7], while others show an increase in GFAP-IR after 14 days that was still present after 150 days[5]. Further studies using more dense observations over time are required to get a reliable indication of the kinetics of the astrocyte response to peripheral nerve damage.

We have argued that the spinal cord is the predominant site of action of ARA 290 following peripheral nerve damage. Indeed, there is ample evidence that peripheral

nerve injury activates an innate immune response activated in the spinal cord[5-11]. Furthermore, a complete block of the peripheral nerve with local anesthetics will not prevent central inflammation following peripheral nerve damage but only delays the development of pain, suggestive of a predominant central effect[49,50]. Still, at this point we cannot exclude an additional peripheral effect of ARA 290. Indeed, EPO specifically reduces axonal TNF-α in Schwann cells after peripheral nerve injury, resulting in attenuation of NP symptoms[51]. Hence, in addition to a central nervous system effect, modulation of the peripheral nerve immune response could also be part of the mechanism of action of ARA 290, but these specific effects remain to be investigated.

Conclusions

In conclusion, in the spared nerve injury model, we show that the erythropoietin-analogue ARA 290 dose-dependently reduces allodynia coupled to suppression of the spinal microglia response, suggestive of a mechanistic link between ARA 290-in-duced suppression of central inflammation and relief of neuropathic pain symptoms.

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Chapter 5

Nonselective and NR2B-selective N-methyl-D-aspartic acid receptor antagonists produce antinociception and long-term relief of allodynia in acute and neuropathic pain

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Introduction

There is ample evidence for the importance of the excitatory N-methyl-D-aspartate (NMDA) glutamate receptor in the development and perseverance of chronic neuropathic pain.[1] NMDA receptors consist of multiple subunits: the obligatory NR1 subunit combines with at least one NR2 subunit.[2,3] Multiple NR2 subunits (A-D) have been identified. The NR1/NR2A NMDA receptor is ubiquitously distributed throughout the brain and spinal cord; NR2Bcontaining NMDA receptors are restricted to specific areas with importance for pain signaling, including dorsal root ganglia, lamina I and II of the dorsal spinal horn, thalamus, hippocampus, and cortex.[4] It is, therefore, not surprising that there is increasing experimental evidence that NR2B-containing receptors are involved in chronic pain responses.[2,5] Both nonselective and NR2B-selective NMDA receptor antagonists induce antinociception in experimental chronic pain models.[6-9] However, an important difference between the two subsets of pharmacologic agents is that, whereas nonselective antagonists cause severe side effects, there are suggestions that NR2B selective agents are devoid of such actions.[10] Extrapolation of animal data on the link between the NMDA receptor and chronic pain has led to the increased popularity of ketamine for the treatment of therapy-resistant chronic noncancer pain, as may be concluded from the increase in the number of case studies and clinical studies on ketamine treatment in neuropathic and chronic, noncancer pain.[11] Currently, proof for the efficacy of ketamine – or any NMDA receptor antagonist in the treatment of neuropathic chronic pain – is, however, limited.[11] Ketamine is effective when used in combination with opioids in the treatment of acute postoperative pain and cancer pain management.[11] Ketamine is currently the most potent NMDA receptor available for use in humans and is nonselective (so far no selective NR2B NMDA receptor antagonists have been registered for pain treatment). Akin to animal observations, ketamine produces serious side effects in humans,[11] including nausea, sedation, and psychedelic effects. Ketamine is rapidly metabolized into another NMDA receptor antagonist, norketamine.[12,13] Some contribution of norketamine to ketamine effects has been observed,[8] but the magnitude of this contribution to ketamine analgesia and side effects remains unknown.

We agree that there is a need for an NMDA receptor antagonist with a clear separation of analgesic effects and side effects, most importantly psychedelic side effects.[8] The antinociceptive properties of three NMDA receptor antagonists – ketamine, norketamine, and Traxoprodil (a highly selective NR2B NMDA receptor antagonist) – were characterized in a model of acute antinociception and a well-established rat model of persistent neuropathic pain, the spared nerve injury (SNI) model.[14] In addition, side effects (stereotypical behavior and activity level) and injured paw func-

tionality were quantified. In contrast with previous studies,[6-9] we used a long-term infusion paradigm (3 h for 5 consecutive days) administered 7 days after peripheral nerve injury. The delay in treatment was chosen to mimic established neuropathic pain states in patients by allowing the development of NMDA receptor sensitization and plasticity. A long-term infusion was used to mimic the observation in humans that prolonged infusion schemes are needed to ensure analgesic effects lasting for weeks rather than hours or days.[11,15]

The aims of the study were to compare ketamine, norketamine, and Traxoprodil with respect to: (1) analgesic behavior in acute and chronic pain paradigms, (2) separation in effect versus side effect, and (3) functionality of the injured paw after nerve injury. We hypothesized that all three NMDA receptors would produce analgesia, with Traxoprodil showing an improved utility index (i.e., analgesia with less side effects than either ketamine or norketamine). We further hypothesized that as a result of the reduction in pain, locomotor function would be improved after treatment with all three drugs in the chronic nerve injury model.

Materials and Methods

Female Sprague-Dawley rats (Charles River Nederland BV, Maastricht, Netherlands), 9 weeks old and weighing approximately 230 g were housed two per cage in individually ventilated cages under standard laboratory conditions with water and food ad libitum and 12 h light/dark cycles (lights on/off at 7:00 AM/PM). After surgery, animals were housed separately. Body weights were determined on the day of testing. All studies were performed during the light phase of the cycle. At the end of the studies, animals were euthanized by exsanguination under 6% sevoflurane anesthesia. The experiments were performed after approval of the protocol by the animal ethics committee of Leiden University (Dier Ethische Commissie, Leiden University Medical Center).

Acute Pain Model

Fifteen rats each received an intravenous cannula in the external jugular vein for drug administration under general anesthesia (6% sevoflurane induction, 3.5% maintenance). The cannula was subcutaneously directed toward the scalp and fixed to the skull. After surgery, animals were allowed to recover for 7 days, after which they were randomly allocated to one of three test groups (5 per group). Group 1 received ketamine (Eurovet Nederland, Bladel, Netherlands) at the following doses: 0, 1.25, 2.5, 5, 7.5 and 10 mg/kg. Group 2 received norketamine (Tocris Bioscience, Bristol, United Kingdom) at the following doses: 0, 2.5, 5, 7.5, 10 and 12.5 mg/kg.

Group 3 received Traxoprodil (Pfizer Inc., New York, NY) at the following doses: 0, 10, 20, 30, 40 mg/kg. Higher doses were not tested because they induced loss of consciousness. Each dose was tested on a separate day. The order of doses was random. Acute antinociception was assessed by applying an infrared thermal stimulus to the plantar surface of the hind paws (Plantar Test, Ugo Basile, Comerio VA, Italy). After a period of adaptation, the test drug was infused (dissolved in 200 μl normal saline) and paw withdrawal times (PWT) were obtained at regular intervals (5, 10, 15, 25, 40, and 55 min after infusion). The intensity of the heat stimulus was set to obtain baseline PWT at approximately 5 s and a cut-off value of 20 s was used to prevent burning of the skin. Each animal received one heat stimulus to each of the hind paws per time point. Measurements were averaged for further analysis.

Stereotypical behaviors and activity levels were scored as an indication of the drug's side effects. Stereotypical behaviors were scored on a scale from 0 to 3, with 0 = normal behavior, 1 = increased explorative (sniffing) behavior, 2 = increased urge to move around the cage, and 3 = inability to hold still with weaving/shaking/twitching of the head and body. Activity level was scored on a scale from 0 to 3, with 0 = normal activity, 1 = mildly impaired activity (i.e., disturbance in paw support), 2 = moderately impaired activity (i.e., a tendency to fall over but able to regain an upright position after falling), and 3 = severely impaired activity (i.e., inability to maintain paw support, falling with an inability to regain the upright position). These scores were adapted from Holtman et al.[8] and possibly relate to psychedelic side effects observed in humans.

Chronic Pain Model

The SNI model was designed according to the model of persistent neuropathic pain by Decosterd and Woolf.[14] In 32 animals, the skin on lateral surface of the thigh was incised under sevoflurane anesthesia (induction 6%, maintenance 3.5%). The sciatic nerve, with its three terminal branches (sural, common peroneal, and tibial nerves), was exposed by blunt preparation. The common peroneal and the tibial nerves were tightly ligated with 5.0 silk sutures and sectioned distal to the ligation, removing 2–4 mm of distal nerve stump. Great care was taken to avoid any contact with or stretching of the intact sural nerve. The nerve stumps were dislocated (more than 1 cm) to prevent regeneration. Muscle and skin were closed in two layers. The SNI model results in early (within 24 h), prolonged (more than 6 months), and robust (all animals are responders) behavioral changes. Next, a venous catheter was placed in the jugular vein for intravenous drug infusion. The catheter was placed subcutaneously at the back of the neck and fixed on the surface of the skull.

After a 1-week rest period, animals were randomly allocated to receive intravenous ketamine, norketamine, Traxoprodil, or vehicle. Eight animals were assigned to each

study group. Randomization was performed in blocks of four; four successively operated animals were randomized into one of the four treatment groups. Intravenous infusions were given continuously for 3 h for 5 consecutive days: 3 mg/kg/h ketamine (9 mg/kg per day for 5 days), 9 mg/kg/h norketamine (27 mg/kg per day for 5 days), or 10 mg/kg/h Traxoprodil (30 mg/kg per day for 5 days). Vehicle was normal saline (0.9% NaCl) given at a rate of 1 ml/h. Ketamine, norketamine, and Traxoprodil doses were based on a pilot study aimed at producing equiefficacy with respect to mechanical antiallodynic effect combined with minimal side effects.

In three other animals, a sham operation was performed. Under sevoflurane anesthesia, the skin on the lateral surface of the thigh and underlying muscle was incised. The sciatic nerve and its three terminal branches were exposed by blunt preparation. No nerve ligation was performed. Subsequently, muscle and skin were closed in two layers. No treatment was given to these animals.

Postoperatively, the animals were given 0.1 mg/kg buprenorphine and monitored for 1 h with body temperature maintained at 38 °C. Thereafter, animals were monitored/ tested weekly for motor functions, behavior changes (autotomy), body weight, and allodynia of the injured paw. The study ended 10 weeks post surgery.

In order to measure mechanical allodynia, the animals were placed in a see-through box on a wire mesh floor. With the use of different von Frey hairs (Semmes-Weinstein monofilaments, North Coast Medical Inc., San Jose, CA) with increasing stiffness (0.004–300 g), incremental forces were exerted on the plantar surface of the affected hind paw. The hairs were applied 10 times at intervals of 2–3 s to slightly different loci within the test area.[7] The force necessary to induce a withdrawal reflex was recorded. Peak allodynia was defined as the minimum force that would trigger a withdrawal response in the first 10 postoperative weeks.

Next, animals were tested for cold allodynia with an acetone spray test. The stimulus was applied by spraying 20 µl acetone on the plantar surface of the affected hind paw. Animal response was scored according to the following classification: 0 = no withdrawal, 1 = startle response lasting less than 1 s, 2 = withdrawal lasting 1–5 s, 3 = withdrawal lasting 5–30 s (with or without paw licking), and 4 = withdrawal lasting longer than 30 s (with or without licking and repeated shaking).

Using a video-based, illuminated footprint analysis system (CatWalk, Noldus Information Technology, Wageningen, Netherlands),[16,17] we measured the following gait and footprint parameters during a short walk of the animals on a glass plate: area of the foot print (surface of paw used during step cycle), stand duration (time spent on paw during step cycle), and maximum intensity (maximum exerted pressure on paw during step cycle). All data were collected for further analysis, which was performed on a blinded data set.

Data and Statistical Analysis

Acute Pain Model

To obtain an estimate of the acute anti-nociceptive potency of the test drugs, PWT data (latencies) were fitted to the following model (adapted from Romberg et al.,[18] and Lötsch et al.[19]):

$$PWT(d) + PWT_B + PWT_0 + 5 \cdot [d/X_5]^\gamma$$

PWT(d) is the peak latency observed after dose d; PWTB, the latency before drug infusion; PWT_0, peak latency observed after saline; X_5, drug dose causing an increase in latency of 5 s (i.e., approximate ED_{50}); and γ, a shape parameter. The model was fitted to the data with the statistical package NONMEM (Nonlinear Mixed Effects Modeling, version VII; ICON Development Solutions, Ellicott City, MD).[20]

For the two measured side effects, typical behavior and activity level, data were analyzed using the following model (adapted from Romberg et al.,[18] and Lötsch et al.[19]):

$$S(d) = S_0 + 1.5 \cdot [d/Y_{1.5}]^\gamma$$

S(d) is the side effect score at dose d; S_0, the score after saline; and $Y_{1.5}$, the drug dose causing an increase in score by 1.5 points. Estimations were performed with NONMEM.[20]

Chronic Pain Model

Power analysis was based on a pilot study of ketamine versus saline treatment effect on mechanical allodynia in the SNI model. Four animals were treated with ketamine, three others with saline. At t=2 weeks (1 week after end of treatment), the mean ± SD force that caused a withdrawal response in ketamine-treated animals was 0.2 ± 0.17 versus 0.02 ± 0.01 g in saline-treated animals. We calculated group sizes of at least eight animals to detect a difference between treatments of at least 1 SD between groups, with a reliability of 5% and a power higher than 80%.

Areas under the curve were calculated from postoperative weeks 1–10 using the trapezoidal rule (AUC1–10). Among treatments, a comparison on AUC, peak effect, and duration of effect was performed using Kruskal-Wallis test and post hoc Tukey tests (hypothesis testing was two-tailed). Duration of effect was defined as the time at which the effect of an individual animal crossed the 95% CI of saline treatment.

The function of the injured paw was expressed as a percentage of the function of the sham animals. Gait analyses were performed in postoperative weeks 1, 3, and 4. Treatment effects were assessed by Kruskal-Wallis and post hoc Dunn's tests (hy-

pothesis testing was two-tailed). The data are expressed as mean ± SEM or median ± 50% quartile range for side effects (typical behavior and level of activity) unless otherwise stated. Statistical analyses were performed with SigmaPlot (version 11; Systat Software, Inc., Chicago, IL). P values less than 0.05 were considered statistically significant.

Results

Acute Pain Model

All three tested NMDA receptor antagonists produced dose-dependent acute anti-nociception (Figure 1). Model parameter estimates are $PWT_B = 5.3 \pm 0.2$ s, $PWT_0 = 1.1 \pm 0.3$ s, ketamine $X_5 = 7.6 \pm 0.9$ mg/kg, norketamine $X_5 = 12.5 \pm 1.3$ mg/kg, and Traxoprodil $X_5 = 37.9 \pm 2.6$ mg/kg. Corresponding values for γ are 0.9 ± 0.2, 1.9 ± 0.5, and 1.7 ± 0.3, respectively.

Stereotypical behavior and activity level scores are given in Figure 2. Both ketamine and norketamine produced dose-dependent side effects. Traxoprodil showed no signs of side effects across the dose range tested. Accordingly, no quantitative analysis was performed on Traxoprodil data. Model parameter estimates were for stereotypical behavior ($S_0 = 0 \pm 0$ [median ± SEM]): ketamine $Y_{1.5} = 3.7 \pm 0.4$ mg/kg and
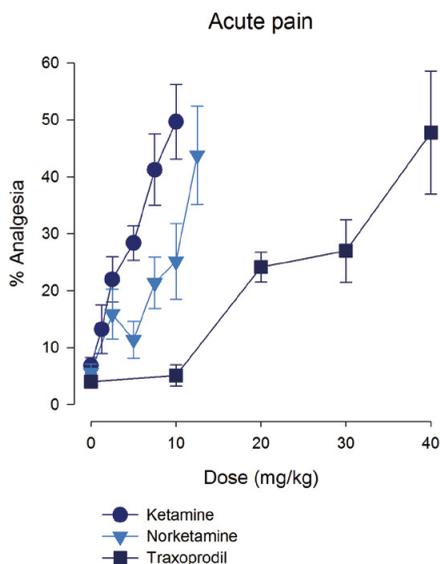

Figure 1: Dose-response relationship of ketamine, norketamine, and Traxoprodil versus paw withdrawal latencies. Percent analgesia was calculated as: (drug latency at dose d - latency after saline)/(cutoff latency - latency after saline). Values are presented as mean ± SEM.
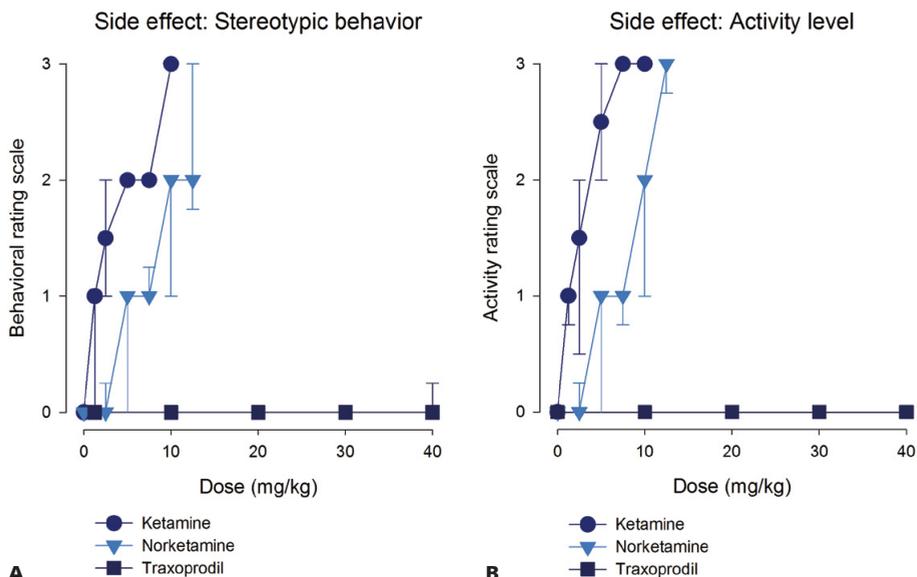
Figure 2: A: Dose vs. Stereotypical behavior. B: Dose vs. level of activity. Displayed for the three test drugs, ketamine, norketamine, and Traxoprodil. Values are presented as mean ± SEM.

norketamine $Y_{1.5} = 9.4 \pm 0.6$ mg/kg, with respective values for γ of 0.6 ± 0.1 and 1.3 ± 0.3. For activity level ($S_0 = 0 \pm 0$): ketamine $Y_{1.5} = 2.8 \pm 0.5$ mg/kg and norketamine $Y_{1.5} = 9.4 \pm 0.4$ mg/kg, with respective values for γ of 0.6 ± 0.1 and 2.1 ± 0.3.

Chronic Pain Model

Although, during treatment, side effects were observed for ketamine and norketamine, but not for Traxoprodil, no side effects were observed in the 8 weeks after treatment. One week after transection of the common peroneal and the tibial nerve, animals displayed overt mechanical allodynia with withdrawal responses induced by 0.004 – 0.02 g filaments versus 8 g in the sham operated group (Figure 3, table 1). Five-day treatment with ketamine, norketamine, and Traxoprodil, but not saline, resulted in alleviation of mechanical allodynia with maximum relief (peak effect) occurring at postoperative week 2 (ketamine and Traxoprodil) and week 3 (ketamine). No difference in efficacy of the three NMDA receptor antagonists on mechanical allodynia, at the doses tested, could be detected.

A significant main effect for treatment on AUC_{1-10} was observed (P<0.001). The AUC_{1-10}s of ketamine, norketamine, and Traxoprodil were significantly larger compared with saline, but did not differ among each other. Similarly, a significant main effect of treatment on peak antiallodynia was present (P<0.001, Table 1). At peak antiallodynia, animals treated with the NMDA receptor antagonists ketamine

Figure 3: The effect of ketamine (A), norketamine (B), and Traxoprodil (C) on mechanical allodynia in the spared nerve injury model of the ipsilateral paw. Squares represent data from placebo-treated animals; circles, animals treated with N-methyl-D-aspartic acid receptor antagonists; triangles, sham-operated animals. X=treatment day. On the y-axis, the force used on injured paw with von Frey filaments to induce withdrawal response. Values are presented as mean ± SEM.

and norketamine responded to a higher force compared with saline-treated rats. However, between treatments, no difference was detected. In contrast, peak antiallodynic effect in the animals treated with Traxoprodil did not differ from peak effect in saline-treated animals. Duration of antiallodynia was similar between

Table 1: Effect of Ketamine, Norketamine, and Traxoprodil on mechanical and cold allodynia

	Ketamine	Norket-amine	Traxoprodil	Saline	Sham	Main effect#
Mechanical Allodynia						
Pre-treatment force (g) 95% c.i.	0.01 ± 0.01 (0.00 – 0.02)	0.02 ± 0.01 (0.01 – 0.03)	0.004 ± 0.00 (0.004 – 0.004)	0.01 ± 0.01 (0.003 – 0.017)	8.7 ± 3.2 (5.1 – 12.3)	P = 0.08
AUC_{1-10} (g.weeks) 95% c.i.	2.7 ± 1.2 * (0.2 – 5.2)	3.5 ± 1.7 * (−0.06 – 7.1)	1.1 ± 0.5* (0.2 – 2.0)	0.04 ± 0.02 (0.03 – 0.05)	-	P < 0.001
Peak anti-allodynic effect (g) 95% c.i.	1.4 ± 0.5 * (0.5 – 2.3)	1.8 ± 1.0 * (−0.3 – 3.9)	0.7 ± 0.3 (0.1 – 1.3)	0.004 ± 0.000 (0.004 – 0.004)	-	P < 0.001
Cold Allodynia						
Pre-treatment score 95% c.i.	2.6 ± 0.3 (2.1 – 3.1)	2.6 ± 0.4 (1.7 – 3.5)	2.5 ± 0.3 (1.8 – 3.2)	3.1 ± 0.3 (2.5 – 3.7)	0.7 ± 0.3 (−0.1 – 1.5)	P = 0.53
AUC_{1-10} (weeks) 95% c.i.	25.4 ± 1.4 * (22.3 – 28.2)	22.1 ± 1.0 * (20.1 – 24.1)	24.3 ± 1.1 * (22.0 – 26.6)	30.1 ± 1.8 (26.5 – 33.7)	-	P < 0.001
Peak anti-allodynic score (95% c.i.)	1.9 ± 0.1 (1.6 – 2.2)	1.3 ± 0.2* (0.8 – 1.8)	2.1 ± 0.2 (1.7 – 2.5)	2.6 ± 0.4 (1.8 – 3.5)	-	P = 0.03

All values are mean ± SEM (95% CI).
# Main effect (P-value), Kruskal-Wallis test. * P< 0.05 vs. saline, post hoc Tukey test.
AUC = area under curve.

treatments and lasted until postoperative weeks 5.6 ± 0.9 (ketamine), 7.1 ± 0.9 (norketamine) and 5.4 ± 0.6 (Traxoprodil). Relative potencies (calculated as dose ratio at which agents had identical AUC_{1-10}) were 1:2 (ketamine:norketamine) and 1:8 (ketamine:Traxoprodil).

Allodynia to cold stimulation occurred in week 1 after nerve injury in all animals (cold allodynia scores before treatment range between 2.5 and 3.1 in animals with SNI vs. 0.7 in sham operated animals, table 1). Ketamine, norketamine, and Traxoprodil induced relief of allodynia with peak antiallodynic effect occurring in week 2 (ketamine and Traxoprodil) and week 3 (norketamine).

A significant main effect for treatment on AUC_{1-10} was observed (Table 1 and Figure 4, P<0.001). The AUC_{1-10}s in animals treated with NMDA receptor antagonists were significantly smaller than the AUC_{1-10} observed in saline-treated animals by 17–27%. No differences in AUC_{1-10}s were observed among the three NMDA treatments. Although a significant main effect was observed for a difference in peak antiallodynic effect (P=0.03), post hoc analysis revealed that only norketamine was different from saline (Table 1). Duration of antiallodynic effect was similar between treatments and lasted until postoperative weeks 4.4 ± 1.1 (ketamine), 5.8 ± 0.7 (norketamine) and

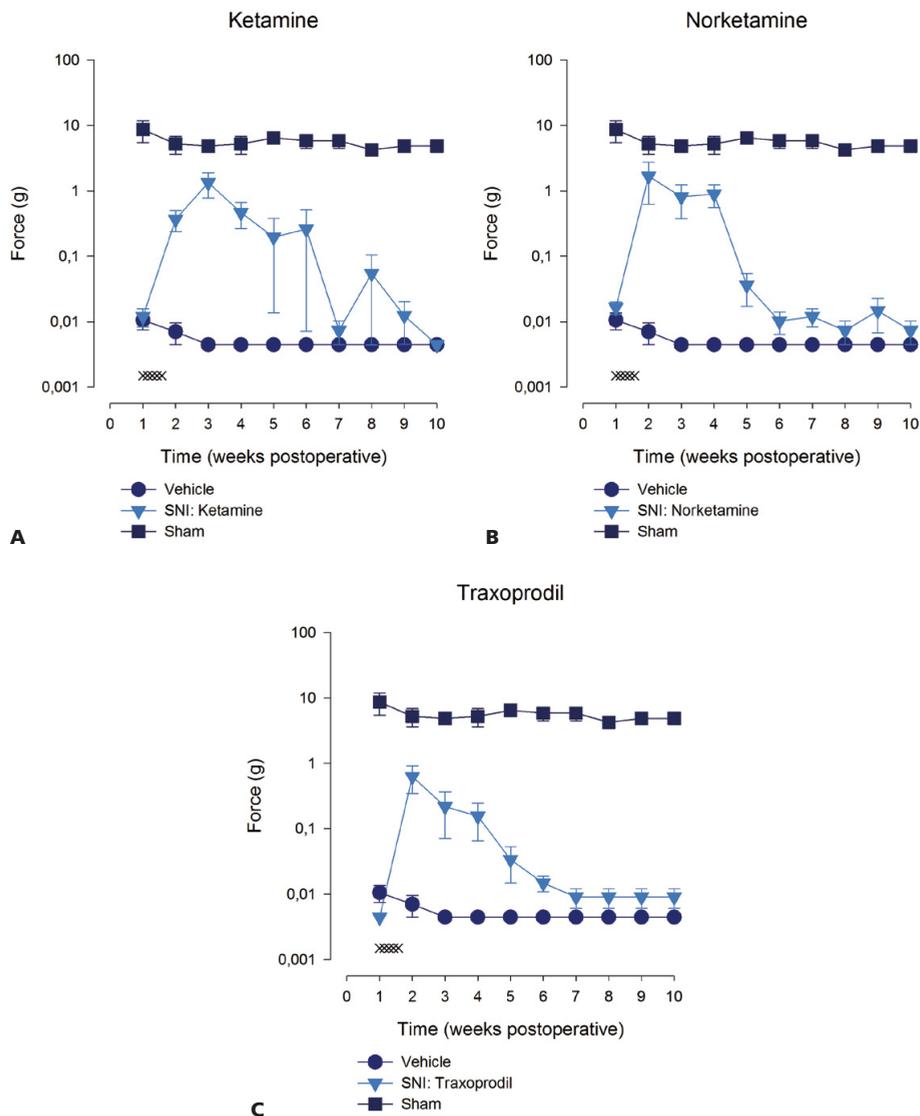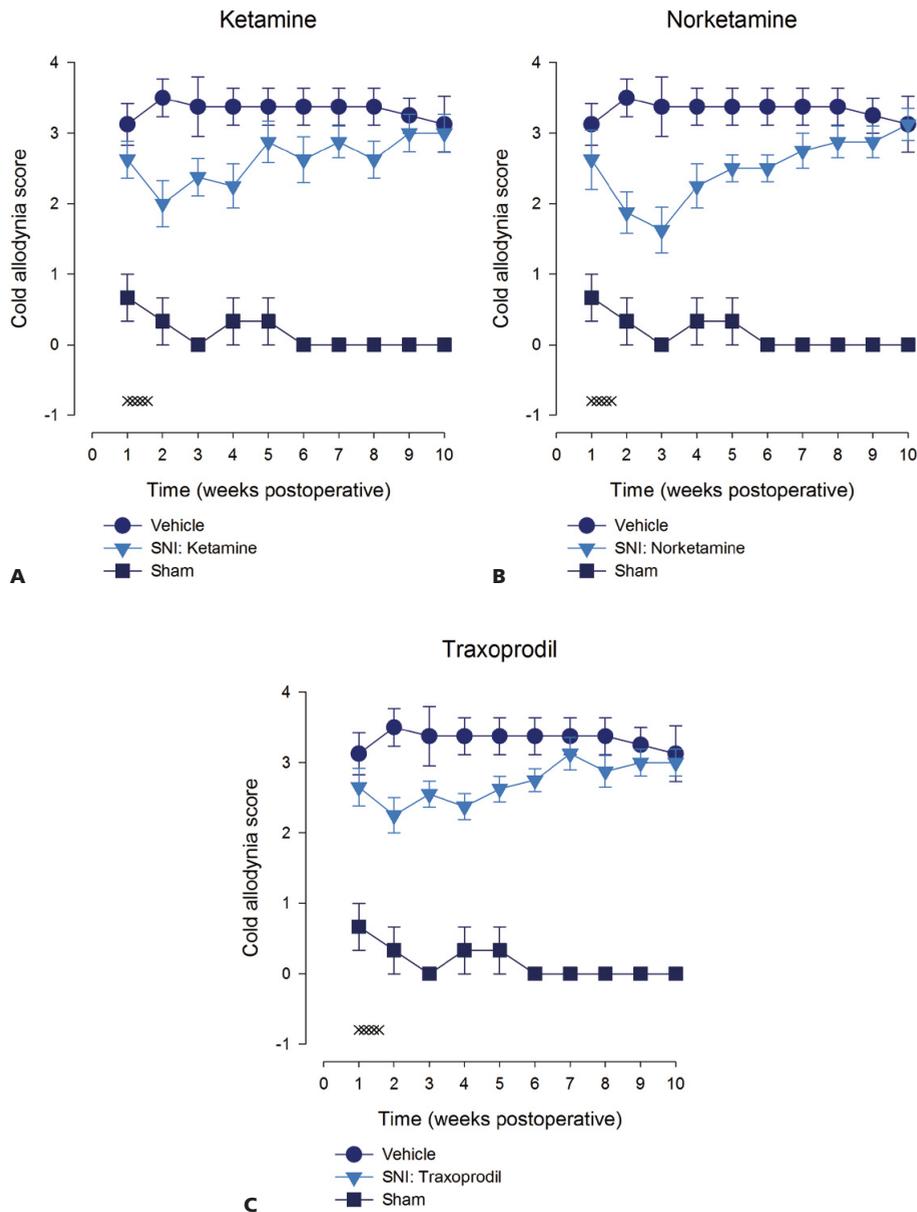**Figure 4:** The effect of ketamine (A), norketamine (B), and Traxoprodil (C) on cold allodynia in the spared nerve injury model of the ipsilateral paw. The y-axis reflects the 4-point scale used to calculate cold-temperature stimulation of the injured paw via acetone spray test: 0 = no withdrawal, 1 = startle response lasting less than 1 s, 2 = withdrawal lasting 1–5 s, 3 = withdrawal lasting 5–30 s (with or without paw licking), and 4 = withdrawal lasting longer than 30 s (with or without licking and repeated shaking). Squares represent data from placebo-treated animals; circles, animals treated with N-methyl-D-aspartic acid receptor antagonists; triangles, sham-operated animals. X = treatment day. Values are presented as mean ± SEM.

4.8 ± 0.6 (Traxoprodil). Relative potencies (calculated as dose/AUC) were 1:3.4 for ketamine:norketamine and ketamine:Traxoprodil.

At postoperative week 1, nerve injured animals displayed severe dysfunction of the affected paw (Figure 5). At week 1, the surface of the injured paw used during a step cycle (area) was 2.6–9.6% (range) of that of the sham operated animals. Time spent on the injured paw during a step cycle (stand) was 19–33%. Pressure on the injured paw during a step cycle (intensity) was 32–37%. In the 2 weeks after treatment, saline-treated animals displayed a further gradual deterioration of gait-related parameters. Significant main effects of treatment were detected for all three tested indices (stand, $P=0.02$; area, $P=0.03$; intensity, $P=0.045$). Post hoc analysis revealed that neither ketamine nor Traxoprodil treatment had a significant effect on any of the measured gait parameters. Only after norketamine treatment improved responses (relative to saline) were observed for all three indices (all $P<0.05$). However, these effects were limited in magnitude and duration of effect (Figure 5).
Peak effect occurred at postoperative week 3, showing an improved value versus saline (area, 17 ± 2% vs. 3.4 ± 0.9%; stand, 52 ± 7% vs. 27 ± 5%; and intensity, 60 ± 4% vs. 33 ± 2%). At week 4, however, gait responses had returned to values observed before treatment (i.e., week 1).

Discussion

Ketamine is the prototypical NMDA receptor antagonist, now in use for nearly 50 years.[21] It is widely applied in the treatment of therapy-resistant chronic pain, as an adjuvant to opioids in the perioperative setting, and in cancer pain management.[11,21] Side effects, occurring at analgesic doses, restrict its use and emphasize the need for an NMDA receptor antagonist with an improved therapeutic index.[8] In this experimental study, we characterized and compared the effects of ketamine with its active metabolite norketamine and the NR2B-selective NMDA receptor antagonist Traxoprodil. Several important observations were made: (1) All three NMDA receptor antagonists were efficacious in a model of acute antinociception and a well-established model of chronic neuropathic pain. (2) Ketamine was most potent in acute and chronic pain models, followed by norketamine and Traxoprodil. (3) Pain relief in the chronic pain model persisted for weeks after treatment termination. (4) Side effects (stereotypical behaviors and loss of activity) were present after treatment with ketamine and norketamine. Although norketamine showed an improved therapeutic index compared with ketamine, side effects occurred over the entire analgesic dose range tested (0 –12.5 mg/kg) for both agents. A clear separation be-
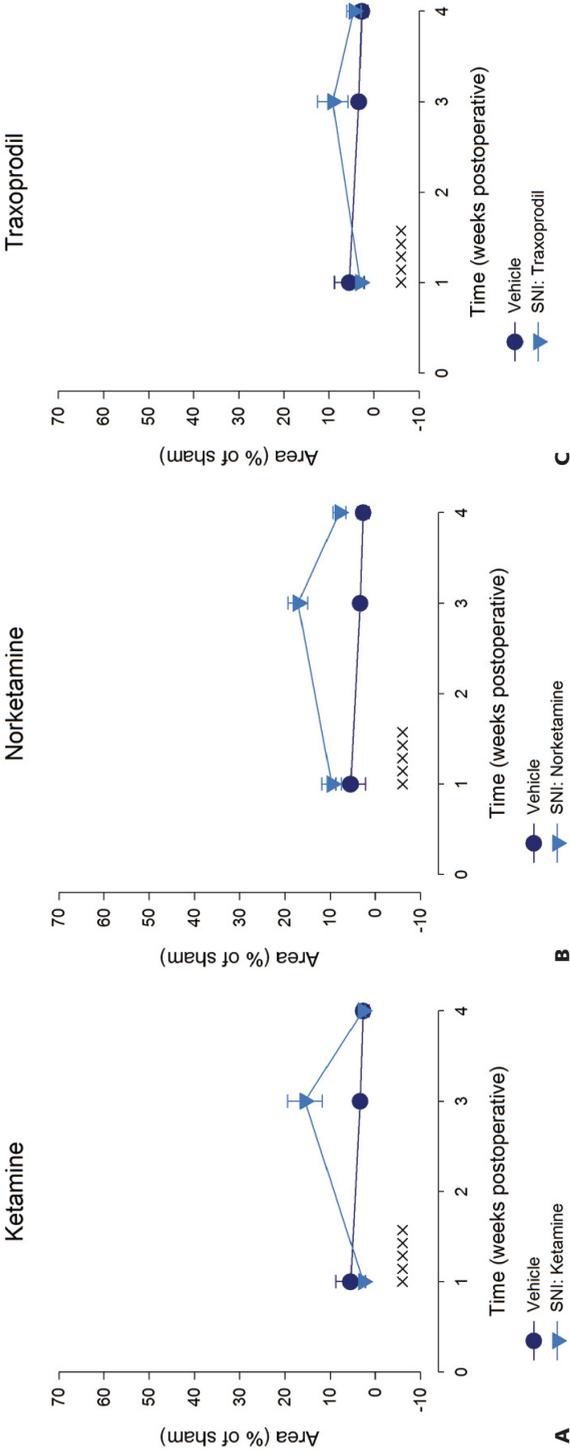
Figure 5: Treatment effect on gait response of ipsilateral paw in first 4 weeks after induction of spared nerve injury. A–C: Effect on area or surface of injured paw used during step cycle.
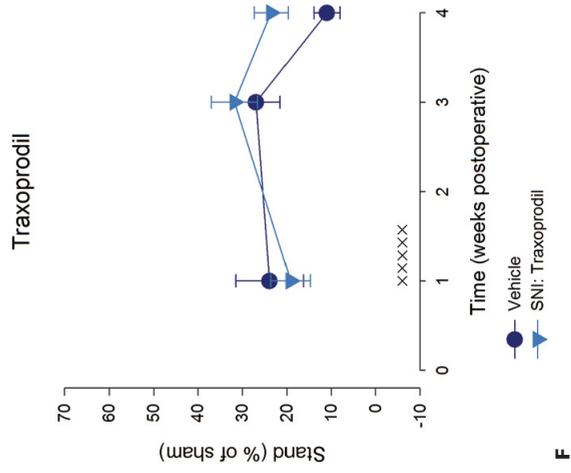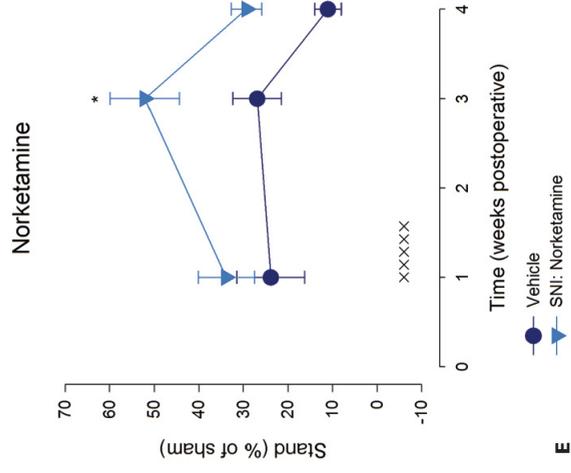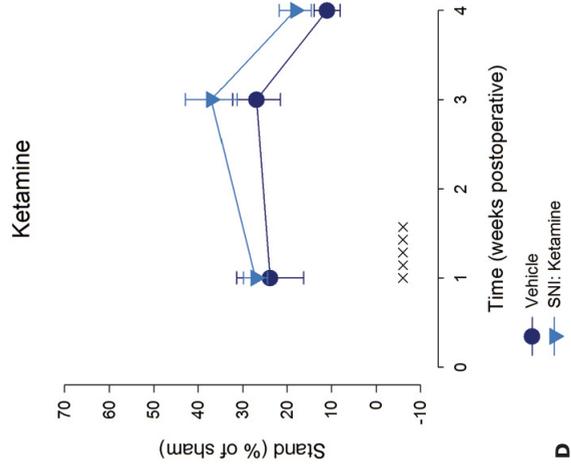
D–F: Effect on stand or time spent on injured paw during step cycle.
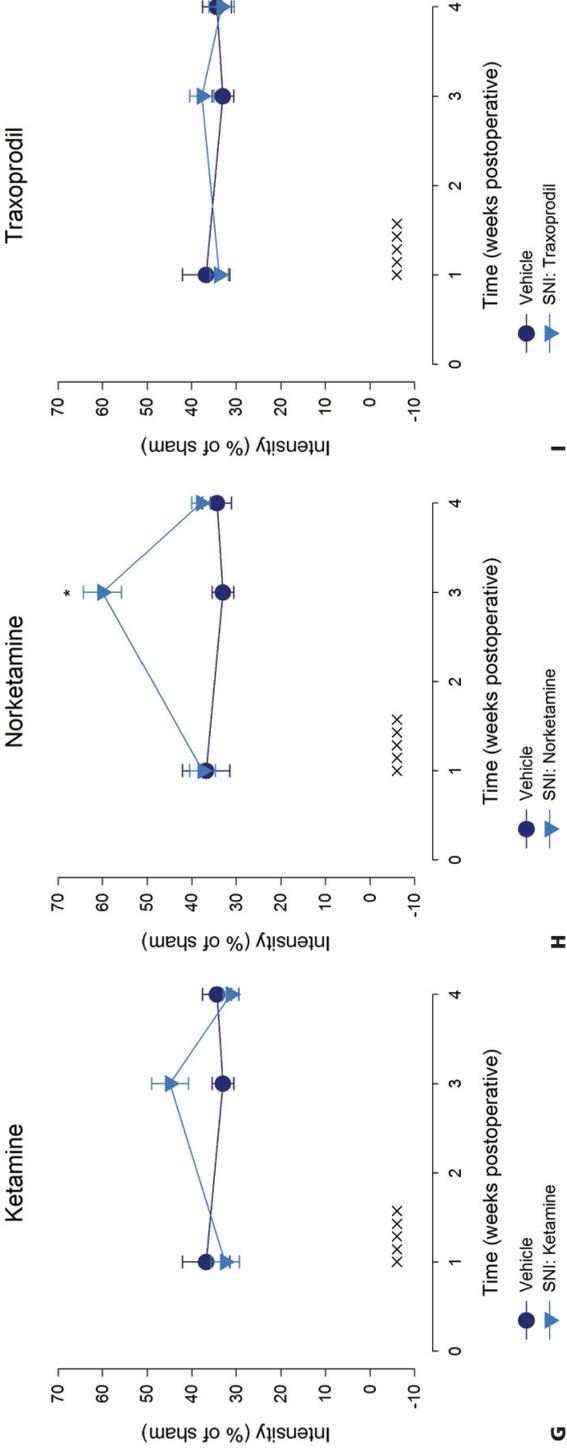
G–I: Effect on intensity or pressure placed on injured paw during step cycle. Data are relative to responses of sham-operated animals. X=treatment day. * P<0.05 versus placebo. Values are presented as mean ± SEM.

tween effect and side effects was present for Traxoprodil with, across the dose range tested (0 – 40 mg/kg), the absence of any signs of agitation and motor dysfunction. (5) After induction of nerve injury, norketamine caused improvement in function of the injured paw during the period of pain relief, although the effect was short-lived and relatively small. In contrast, no improvement was seen after treatment with ketamine or Traxoprodil, despite significant relief of mechanical allodynia.

Potencies for acute pain relief – as determined by parameter X_5, the dose causing an increase in PWT of 5 s (an approximate ED_{50}) – indicate that ketamine was 1.6 times more potent than norketamine and 5 times more potent than Traxoprodil (derived from X_5 ratios). Side effects were present after the administration of ketamine and norketamine. Although norketamine showed an improved therapeutic index (defined as $X_5/Y_{1.5}$, equations 1 and 2) compared with ketamine (agitation, ketamine = 2.1 vs. norketamine = 1.3; motor dysfunction, ketamine = 2.7 vs. norketamine = 1.3), side effects occurred over the entire analgesic dose range for both agents. A clear separation between effect and side effect was present for Traxoprodil with (across the 0-40 mg/kg dose range tested) the absence of any signs of stereotypical behavior and loss of activity.

Our data indicate that Traxoprodil may be clinically useful in the treatment of acute pain, with a superior therapeutic index when compared with ketamine and norketamine. The observation of absence of side effects is in agreement with other studies.[10] With respect to motor function, this finding is likely related to the absence of NMDA receptors containing the NR2B subunit in the cerebellum.[6] The mechanisms by which NMDA receptor antagonists produce acute pain relief have been attributed to activity at non-NMDA receptors, such as the μ-opioid receptor.[22] However, there is evidence for the NR2B-containing NMDA receptors located presynaptically on primary afferent C-fibers in lamina I of the dorsal horn.[6] These receptors may modulate the presynaptic release of substance P and glutamate and consequently modulate acute pain transmission in the spinal cord.[6]

All three NMDA receptor antagonists produced relief of mechanical and cold allodynia in the SNI model. Relative potencies indicate that, for mechanical allodynia, ketamine is twice as potent as norketamine and eight times more potent than Traxoprodil. For cold allodynia, ketamine was 3.4 times more potent than norketamine and Traxoprodil. For none of the three agents did any side effects (agitation, motor dysfunction) occur during the period of testing. We observed that the magnitude and duration of relief of mechanical allodynia was more pronounced than that of cold allodynia (Figure 4). Previously, Qu et al.,[23] showed that pretreatment with ifenprodil, a selective NR2B antagonist, induced relief of mechanical allodynia, but not thermal hyperalgesia, in a spinal nerve ligation model. These findings suggest that the development of different pain expressions or modalities (e.g., mechanical

vs. cold allodynia) is due to activation of different pain pathways after peripheral nerve injury, each with a different (subunit) expression of NMDA receptors and, consequently, distinct sensitivities to different NMDA receptor antagonists.[23] For example, after peripheral nerve injury, sprouting of Aδ-fibers into the superficial layers of the dorsal horn is associated with mechanical allodynia but not thermal hyperalgesia.[23,24]

Several studies on the effect of NMDA receptor antagonists on neuropathic pain test the preemptive effect of treatment or the effect of treatment in the early stage of nerve injury.[6,23,25,26] We performed our infusion 1 week after the induction of nerve injury. This timing was used to induce an established neuropathic pain state. We assumed that 1 week after surgery (the late phase of nerve injury), NMDA receptor sensitization had fully developed with established structural changes in the affected pain pathways (such as up-regulation of NMDA receptors).[9] Our approach mimics the situation in neuropathic pain patients, who are often treated weeks or months after nerve injury has occurred. We infused the test agents for 5 consecutive days. This process was used because, in neuropathic pain patients, long-term or repetitive treatments with NMDA receptor antagonists (rather than short-term infusions) produce long-lasting analgesic effects.[11] For example, we previously showed that a 100-h infusion with S(+)-ketamine produced pain relief that lasted up to 12 weeks in patients with chronic pain as a result of complex regional pain syndrome type 1.[15] In patients with neuropathic pain from spinal cord injury or monoradiculopathy, a relatively short 24-h infusion with Traxoprodil produced pain relief during the infusion period only.[4,5,27] A similar short duration of analgesic effect (8 h) was observed in the rat after an intrathecal injection with ifenprodil given 7 days after dorsal root ganglion compression.[9] We believe that a prolonged analgesic effect is mandatory when treating neuropathic pain patients with intravenous NMDA receptor antagonists to reduce treatment costs and patient discomfort and increase treatment compliance.

We observed long-term relief of mechanical and cold allodynia lasting 3–6 weeks after the initiation of 5-day treatment. Because ketamine exhibits a rapid reduction in ketamine and norketamine plasma concentrations on infusion termination, it is not expected that any active agent was present in the rat during the test phase of our study. Apparently, ketamine initiated a cascade of events (of which the first step is NMDA-receptor desensitization) that caused long-term effective and continuing blockade for central trafficking of pronociceptive signals to the thalamus and cortex.[28,29] In agreement with this theory, Christoph et al.[30] showed that the antiallodynic effect of NMDA receptor antagonists (more than 3 h with ketamine) outlasts the in vivo NMDA receptor antagonism ($t_{1/2}$ = 10–12 min) in rats with chronic nerve constriction injury. In addition, a central reset of central glutamatergic brain circuits involved in pain transmission may play a role.[29] A supraspinal effect of NMDA recep-

tor antagonists is in agreement with studies that point to a role for NR2B NMDA receptors in the forebrain and amygdala in the development and enhancement of neuropathic and inflammatory pain.[31-33] Our findings indicate that long-term relief of neuropathic pain is possible despite a delayed therapy start with all three tested NMDA receptors. More prolonged effects may be feasible by adjusting dosing or duration of treatment, or by repetition of treatment at 4 –5 week intervals.

Injured paw use during locomotion was tested using Cat-Walk automated quantitative gait analysis,[16,17] which has been used previously to quantify tactile allodynia in the rat. Vrinten and Hamers[16] compared gait analysis using von Frey testing in rats with chronic constriction injury of the sciatic nerve. They observed a high degree of correlation between gait parameters and von Frey mechanical allodynia. In contrast, Gabriel et al.[34] were unable to find significant correlations in rats with chronic pain induced by intra-articular λ-carrageenan injection in the knee. Mogil et al.,[35] using a blinded scoring approach, observed differences in dynamic, weight-bearing (gait) changes between sham operated mice and mice with spared nerve (but not chronic constriction injury). However, in the SNI animals, there was a pharmacologic dissociation between mechanical allodynia and gait changes. Morphine, gabapentin, and EMLA cream (2.5% lidocaine ± 2.5% prilocaine) reversed mechanical allodynia but did not affect gait abnormalities of the injured paw. Our data indicate a profound and long-lasting effect of SNI damage on the gait parameters of area, stand, and intensity. In all animals with SNI, we observed reduced use of the affected paw with minimal floor contact during locomotion. We relate the reduced use to the perception of mechanical allodynia during contact of the paw with the surface (weightbearing allodynia).

We are the first to quantify pharmacologic treatment with NMDA receptor antagonists on gait patterns in chronic pain with the CatWalk system. The effect of treatment on gait abnormalities was disappointing. After treatment with norketamine, a significant effect on gait-parameters was observed, but the effects were short-lived (effect in week 3 only, Figure 5) and relatively small. No improvements were observed after treatment with ketamine or Traxoprodil, despite significant relief of mechanical allodynia (measured via von Frey test). The reason for absence of improvement of paw abnormalities (or just a limited effect, as seen with norketamine) may be that weight-bearing allodynia is less sensitive to pharmacologic intervention and requires higher doses. Testing mechanical allodynia with von Frey hairs may be less painful and more sensitive to NMDA receptor antagonism. Alternatively, diminished paw use and gait changes after nerve injury may not reflect (NMDA receptor–related) mechanical allodynia and are therefore not responsive to treatment with NMDA receptor antagonists. Both at the spinal and supraspinal level, gait and pain pathways are distinct. Gait is controlled by spinal networks under the direct influence of

descending pathways originating at the brainstem, which in turn receive afferent information from the cerebellum, basal ganglia, and sensorimotor cortex.[34] It is reasonable to assume that, after the inflicted nerve injury changes occur in these motor pathways (which do not involve NMDA receptor sensitization and up-regulation), a permanent inability to use the paw during locomotion occurs, possibly without spontaneous pain.[35]

The current findings are in agreement with our findings in patients with chronic pain from complex regional pain syndrome type 1.[15,36] After treatment with ketamine, spontaneous pain scores improved significantly. However, there was no improvement of function-related parameters. We argued that pain relief should coincide with improvement of function and use of the affected limb. Our current and previous data do not support this argument. It may well be that improvement of locomotor function and increase in use requires a different treatment approach (e.g., combining pharmacotherapy with physical exercise, physiotherapy, and/or surgical intervention (nerve reconnection or transplantation)).

Conclusion

All three NMDA receptor antagonists caused dose-dependent antinociception in the acute pain model. Likewise, they caused relief of mechanical and cold allodynia for 3–6 weeks after treatment in a chronic neuropathic pain model. In both pain tests, ketamine was most potent, with norketamine 1.5–2 times less potent and Traxoprodil 5–8 times less potent than ketamine. In contrast to nonselective NMDA receptor antagonists, treatment with Traxoprodil caused no side effects. Although all three agents produced long-term relief of mechanical allodynia in nerve-injured animals, improved use of the affected paw during locomotion, as tested by computerized gait analysis, was limited (norketamine) or absent (ketamine and Traxoprodil). These observations make Traxoprodil an attractive alternative to ketamine in the treatment of chronic neuropathic pain. Alternative treatment options are required to induce increase limb (paw) use.

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Chapter 6

Ketamine does not produce relief of neuropathic pain in mice lacking the β-common receptor (CD131)

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Introduction

Neuropathic pain (NP), arising from lesions affecting the somatosensory system[1], is often not adequately treated by current pharmacotherapy, such as antidepressants, opioids, and topical agents (e.g., lidocaine or capsaicin). It is a common feature following trauma or infectious, autoimmune, metabolic, and neurological diseases[2,3], and is often accompanied as well by hyperalgesia. Inflammation arising from injury plays an important role in the development and maintenance of NP and the peripheral and central sensitization phenomena that establish allodynia and hyperalgesia depend upon a variety of neuromodulatory processes[2]. These may include the activation and up-regulation of dorsal horn excitatory glutamatergic N-methyl-D-aspartate receptors (NMDAR), as well as a vigorous inflammatory response within the spinal cord initiated and maintained by microglia and reactive astrocytes as well as the production of TNF-α, interleukins and CCL2 (reviewed in ref.[4]).

Recently, evaluation of preclinical models of NP have shown that erythropoietin (EPO) is locally produced following peripheral nerve injury and functions as an endogenous factor that limits damage and improves nerve function[5,6]. The tissue protective effects are mediated by the EPO receptor-β-common receptor complex[7], rather than the EPO receptor homodimer ($EPOR_2$) involved in erythropoiesis. This isoform, termed the innate repair receptor (IRR), may additionally form functional complexes with other molecules to transduce specific cellular responses (e.g. vascular endothelial growth factor receptor-2 or endothelial nitric oxide synthase, reviewed in ref.[8]). Generally, inflammation and tissue injury induce both the expression of the IRR and the production of local EPO in a characteristic temporal and spatial pattern (reviewed in ref.[9]).

Treatment of NP in animal models with exogenous EPO results in relief of allodynia and hyperalgesia and attenuates a number of detrimental cellular responses, including neuronal apoptosis and pro-inflammatory cytokine production while enhancing beneficial cellular responses, including regeneration and anti-inflammatory cytokine production. Recently, we have shown that the novel EPO-derivative ARA 290, specifically interacting with the IRR and not with the $EPOR_2$ thus rendering it without hematopoietic side effects, is able to persistently relieve NP[10].

Accordingly, recent clinical trials of ARA 290 in patients with neuropathy have shown benefit[11,12]. Similar observations have been made following treatment of patients with NP with ketamine, which has shown to have potent and long-lasting analgesic effects on NP[13-15]. However, ketamine is associated with significant side effects that limit its use outside of closely monitored medical settings. Ketamine acts at multiple receptor systems in addition to antagonism of the NMDAR. Evidence also suggests it also possesses anti-inflammatory activities and inhibits the activation of microglia and astrocytes which play prominent roles in the development of NP[16,17]. The similarity of

action of ARA 290 and ketamine on NP raised the hypothesis that both compounds share a common mechanistic pathway, possibly involving the IRR. To evaluate this possibility, we compared the effects of ketamine and ARA 290 on the expression of the NMDAR subunits, glia cell markers, and the signaling molecule CCL2 in a NP pain model. Further, since receptor gene knock out studies constitute a powerful method to establish roles of specific receptor systems in complex biological responses[18], we also compared the differences in nociception and psychomotor effects, known to depend upon NMDAR, and NP behavior elicited by sciatic nerve injury in wild type mice and to βcR^{-/-} mice that lack responses activated by the IRR.

Materials and Methods

Ethics

The study protocols were approved by the animal ethics committee of the Leiden University Medical Centre and the Animal Care and Use Review Office (ACURO) of the United States Army Medical Department Medical Research and Materiel Command. All experiments were performed according to the guidelines of the International Association for the Study of Pain[19].

Animals

Six to eight week-old female C57Bl/6 mice were purchased from Charles River, Maastricht, The Netherlands. β-common-receptor knockout mice (βcR) were obtained from The William Harvey Research Institute, London, UK. Confirmation of the genotype was done as described by Robb et al. using Southern blot analysis[20]. All animals were housed in groups of 4–5 per individually ventilated cage with water and food available at libitum and a 12 h light-dark cycle (lights on/off at 7AM/7PM).

Drugs

ARA 290 (Araim Pharmaceuticals, New York, USA) and ketamine (Eurovet, Bladel, The Netherlands) were dissolved to yield a 30 μg/kg and 50 mg/kg dose in a 200 ml injection volume and were administered intraperitoneally. All drugs were dissolved in PBS (vehicle).

In vitro Screening Assay

ARA 290 (10 μg) was evaluated in the "High Throughput Profile" of CEREP, Inc. (Poitiers, France) and the N-methyl-D-aspartate binding assay as described at www.cerep.fr. No significant interaction of ARA 290 with any of the screens was observed (data not shown).

QRT-PCR

To establish a profile of the transcriptional changes of the mRNA of specific cytokines and receptors induced by the SNI or and the effect of the investigated drugs on those cytokines, QRT-PCR was performed on tissue of the injured sciatic nerve and spinal cord. Naive mice (n = 5) served as reference for basal mRNA expression levels. Mice that had received SNI, with or without treatment (n = 5/group), were sacrificed 7 days post lesion. The nucleotide sequences of the PCR primers and their fluorogenic probes for the target genes were designed by using the computer program primer express (PE Biosystems) and are included in Table 1. Each fluorescent probe has a reporter dye (FAM for the target RNA and TET for the 18S RNA control) covalently attached at its 5' end and a quencher dye (TAMRA) attached at its 3' end. Before use, the probes were purified in the PolyPak II cartridge (Glen Research, Sterling, VA) following the manufacturer's instructions. RNA was isolated from the sciatic nerve

Table 1: QRT-PCR primers and probes used in this study. Primers and probes used for the quantification of mRNA from NMDA receptor subtypes NR1, NR2A and NR2B (Grin); microglia marker Iba-1 (AIF-1), astrocyte (GFAP) and CCL2; f, Reporter dye1 (FAM:6-carboxyfluorescein); t, Reporter dye 2 (TET:Tetrachloro-6-carboxyfluorescein); q, Quencher dye (TAMRA: 6-carboxytetramethyl-1-rhodamine).

Gene	Type	Sequence (5'–3')
Grin1 (NMDAR NR1)	Forward	GTC CAT CTA CTC TGA CAA GAG
	Reverse	AAA CCA GAC GCT GGA CTG GT
	Probe	f TCC ACC TGA GCT TCC TTC GCA CCGq
Grin2a (NMDAR NR2A)	Forward	ACC TCG CTC TGC TCC AGT TT
	Reverse	GTT GTG GCA GAT GCC CGT AA
	Probe	f CAG TGT CTC AGC TCT TCA TCT CACq
Grin2b (NMDAR NR2B)	Forward	TGG TCT TCT CCA TCA GCA GA
	Reverse	GTT CAT CAC GGA TTG GCG CT
	Probe	f ATC TAC AGC TGT ATC CAC GGA GTA GCq
CCL2	Forward	CTG GAG CAT CCA CGT GTT G
	Reverse	TGG GAT CAT CTT GCT GGT GA
	Probe	f AGC CAG ATG CAG TTA ACG CCC CAC T q
AIF-1 (Iba-1)	Forward	GCA ATT CCT CGA TGA TCC CA
	Reverse	ATG TAC TTC ACC TTG AAG GCT
	Probe	f CAG CAA TGA TGA GGA TCT GCC GTC CAq
GFAP	Forward	CTC AAG AGG AAC ATC GTG GT
	Reverse	TGC TCC TGC TTC GAG TCC TT
	Probe	f TGA CCT CAC CAT CCC GCA TCT CCAq
18S	Forward	AGA AAC GGC TAC CAC ATC CA
	Reverse	CTC GAA AGA GTC CTG TAT TGT
	Probe	tAGG CAG CAG GCG CGC AAA TTA Cq

and spinal cord from each of 5 mice in the experimental groups outlined above with the ABI Prism 6100 Automated Nucleic Acid Workstation according to the manufacturer's protocol. Real-time RT-PCR amplifications were employed as described in ref.[21]. The numbers of copies of the PCR template in the starting sample were calculated by using the sequence detector software incorporated in the ABI Prism 7300 Sequence Detector System. Sense RNAs were synthesized from the standard plasmids by the manufacturer's protocols, using a MAXIscript transcription kit (Ambion). The concentrations of purified sense RNAs were determined as micrograms per optical density unit, and serial dilutions of the sense RNA, using bacterial tRNA as a carrier, were used to generate standard curves. When quantification was relative to an endogenous control, standard curves were prepared for both the target and the endogenous control. We assumed that 18S RNA is present in all tested and control samples of tissue RNA at a constant proportion and normalize the amount of total RNA in our test samples by comparing their 18S RNA fluorescent signal after PCR with that from mouse embryonic stem cell RNA freed from DNA by DNase treatment. Relative mRNA levels are expressed as using 18S RNA as reference.

Acute Antinociception

In uninjured animals (n = 5/treatment group), tail withdrawal latencies (TWL) were recorded to determine the antinociceptive effect of the drugs. The water bath was heated to 47.5 °C which resulted in a baseline response with a threshold of 9–11 seconds. The tail of the mouse was immersed in water and the latency to withdraw the tail was recorded. A cut-off value of 30 s was used to prevent tissue damage. Baselines were recorded prior to injection of the drugs and TWL were recorded 30 and 60 minutes after injection. TWL were obtained in duplicate with an interval of 30 seconds in between measurements and averaged.

Side Effects

Side effects induced by drug treatment (n = 5/treatment group) were assessed in uninjured animals by using a method adapted from ref.[22]. Briefly, animals were observed for 60 min post injection at 5-min intervals. Stereotypic behavior was scores on a 7-point scale as: −3: anesthesia, −2: sedation, −1: drowsiness, 0: normal, 1: moderately increased (increased explorative behavior), 2: increased (increased urge to move around the cage), 3: greatly increased (inability to hold still with weaving, shaking or twitching of the head and body). Activity level was defined as follows: −3: anesthesia, −2: sedation, −1: drowsiness, 0: normal, 1: moderately impaired (disturbances in paw support), 2: impaired (unable to maintain paw support with the ability to regain an upright position after falling over), 3: greatly impaired (inability to regain an upright position after falling over).

Spared Nerve Injury

Mice (n = 8/treatment group per genotype) were anesthetized with isoflurane (4% induction and 2% maintenance) and were operated to receive a spared nerve injury (SNI) as described previously[10]. In short, a lateral incision on the left thigh was made, exposing the muscle. The left sciatic nerve was then exposed by blunt preparation and the tibial and common peroneal nerves were ligated with 6–0 silk sutures, transected and displaced to prohibit any regeneration. Consecutively, muscle integrity was restored and the wound was closed with 5–0 sutures. In case of sham SNI, animals were anesthetized and the sciatic nerve was exposed as described above. After exposure no SNI was induced and the wound was closed. Animals were administered a single s.c. injection of 0.1 mg/kg buprenorphine for the relief of acute post operative pain and were allowed to recover from surgery in a clean cage with body temperature maintained at 38 °C and were observed for 1 h before being transferred back to the cage with fresh sawdust. The animals were followed up for 7 days or 42 days.

Tactile Allodynia

Assessment of tactile allodynia was performed using Semmes-Weinstein monofilaments. Animals were placed in transparent Perspex cages on a grid and allowed to habituate to the experimental environment for 5–10 min. After habituation, filaments were applied to the ipsilateral hind paw by applying 10 stimulations over 10 s. Failure to respond led to progression to the next filament exerting a greater force. Withdrawal of the stimulated paw led to the recording of the force of the corresponding filament. All measurements were done in duplicate with a 30 second interval between measurements and averaged.

Statistical Analysis

All behavioral data was analyzed for a treatment effect by two-way repeated measures analysis of variance (ANOVA) followed by a post hoc Student-Newman-Keuls test for multiple comparisons. QRT-PCR data was analyzed by one-way ANOVA followed by a post hoc Student-Newman-Keuls test for multiple comparisons when distributed normally. In the absence of a normal distribution, as defined by the Shapiro-Wilk criterion, or unequal variance, data was analyzed by a Kruskal-Wallis one-way ANOVA on ranks followed by a Student-Newman-Keuls test for multiple comparisons. P-values < 0.05 were considered significant. Analysis was done with SigmaPlot version 12 (SyStat Software, Inc. Chicago, USA).

Results

Ketamine and ARA 290 Attenuate Neuropathy-related mRNA Changes of the Spinal Cord in a Similar Manner

To evaluate changes in gene expression for potentially relevant receptors and inflammatory molecules in the development of allodynia, animals were sacrificed on day 7 following sciatic nerve injury and real time PCR performed on extracts of the spinal cord. SNI with vehicle treatment moderately changed NMDAR subunit mRNA expression of NR1 (1.27 ± 0.02 fold, $P=0.183$), NR2A (1.83 ± 0.07 fold, $P<0.001$) and NR2B (1.39 ± 0.16 fold, $P=0.101$) when compared to naïve (uninjured and untreated) animals at 7 days post injury (Figs. 1A–C).

Treatment with either drug significantly decreased expression of NR1, NR2A and NR2B mRNA when compared to injured, vehicle treated animals. For NR1 mRNA, treatment resulted in changes in expression of 0.78 ± 0.13 fold ($P=0.042$ versus vehicle) and 0.31 ± 0.05 fold ($P=0.002$ versus vehicle) for ketamine and ARA 290, respectively, with ARA 290 inducing the greater changes in mRNA levels ($P=0.022$ between treatments). For NR2A mRNA, changes in expression of 0.94 ± 0.11 fold ($P<0.001$ versus vehicle) and 0.44 ± 0.08 fold ($P<0.001$ versus vehicle) were observed for ketamine and ARA 290 respectively, with ARA 290 inducing the greater changes in mRNA levels ($P=0.007$ between treatments). The mRNA levels of NR2B after treatment were 1.02 ± 0.11 fold ($P=0.048$ versus vehicle) and 0.45 ± 0.04 fold ($P=0.002$ versus vehicle) for ketamine and ARA 290 respectively, with ARA 290 inducing the greater changes in mRNA levels ($P=0.019$ between treatments). The microglial response to SNI followed by vehicle treatment (mediated by chemokine (C-C motif) ligand 2 (CCL2), also known as macrophage chemotactic protein 1 (MCP-1)) showed a significant 25.53 ± 1.8 fold increase in CCL2 mRNA relative to naïve animals ($P<0.05$), which was attenuated by ketamine (7.26 ± 0.29 fold, $P<0.05$) and ARA 290 (5.27 ± 0.44 fold, $P<0.05$), with ARA 290 inducing a greater change in mRNA levels ($P<0.05$ between treatments, Figure 2A).

SNI followed by vehicle treatment significantly increased the microglia activation marker ionized calcium binding adaptor molecule 1 (Iba-1) mRNA by 4.16 ± 1.06 fold ($P=0.01$ versus naïve). Both treatments significantly decreased Iba-1 mRNA to 1.41 ± 0.27 fold ($P=0.008$ versus vehicle) and 0.96 ± 0.10 fold ($P=0.015$ versus vehicle, Figure 2B) for ketamine and ARA 290 respectively. Ketamine and ARA 290 were equally effective when comparing between treatments ($P=0.839$). The increase in the astrocyte marker glial fibrillary acidic protein (GFAP) mRNA following SNI and vehicle treatment (2.11 ± 0.20 fold, $P<0.001$) was significantly reduced by ketamine (0.99 ± 0.08 fold, $P=0.001$) and ARA 290 (0.51 ± 0.08, $P<0.001$, Figure 2C). Treatment with ARA 290 induced greater changes in mRNA when comparing between treatments ($P=0.039$).
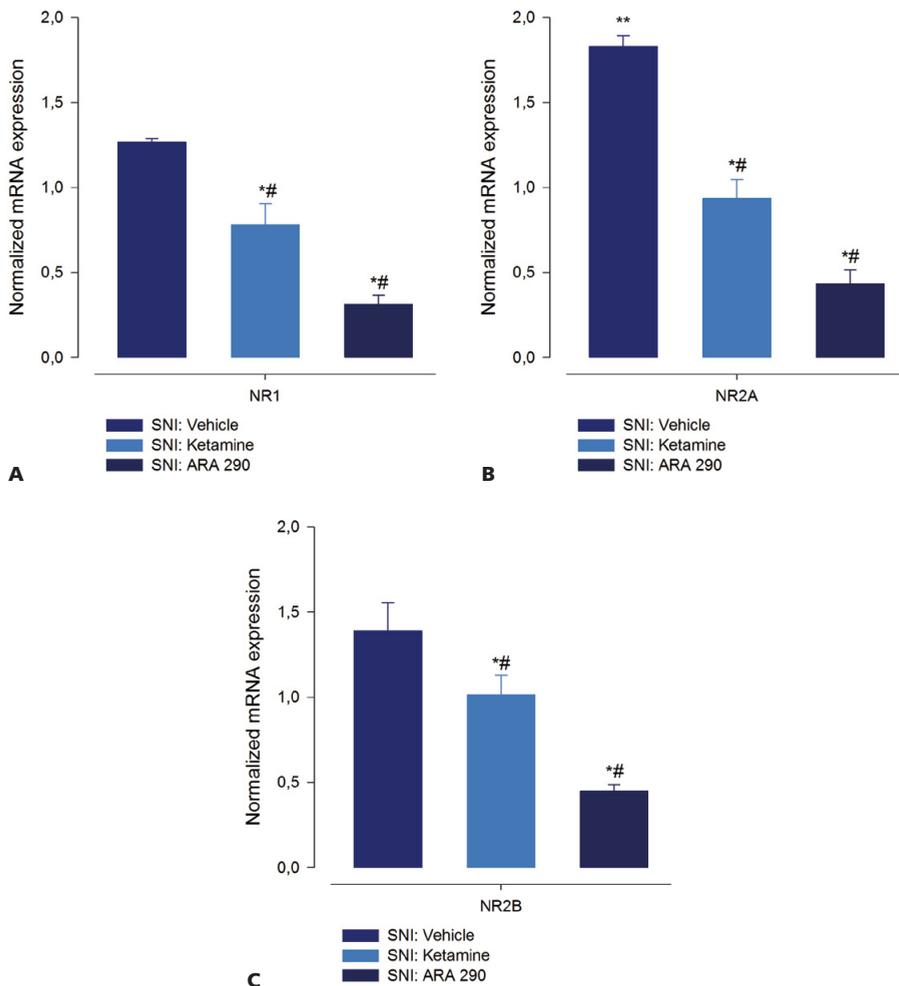
Figure 1: Ketamine and ARA 290 reduce mRNA for NDMA receptor subunits in established neuropathy. Real time PCR data show that NMDA receptor subunits 1 (panel A), 2A (panel B), and 2B (panel C) are all modestly elevated one week following sciatic nerve injury. Administration of ketamine significantly reduces mRNA to baseline levels. In contrast, ARA 290 reduced mRNA for these receptor subunits to substantially below baseline (naïve). * $P < 0.05$ versus vehicle, # $P < 0.05$ between ketamine and ARA 290 treatments, ** $P < 0.05$ versus naïve.

Ketamine and ARA 290 have Divergent Effects on Acute Nociceptive Pain and Behavior

Normal mice exhibited a brisk withdrawal response within 9–11 s after tail immersion in heated water of 47.5°C (Figure 3A). Vehicle treated animals did not show alterations in TWL during the follow up period after injection. Ketamine administration (50 mg/kg intraperitoneally) rapidly induced an acute antinociceptive effect as
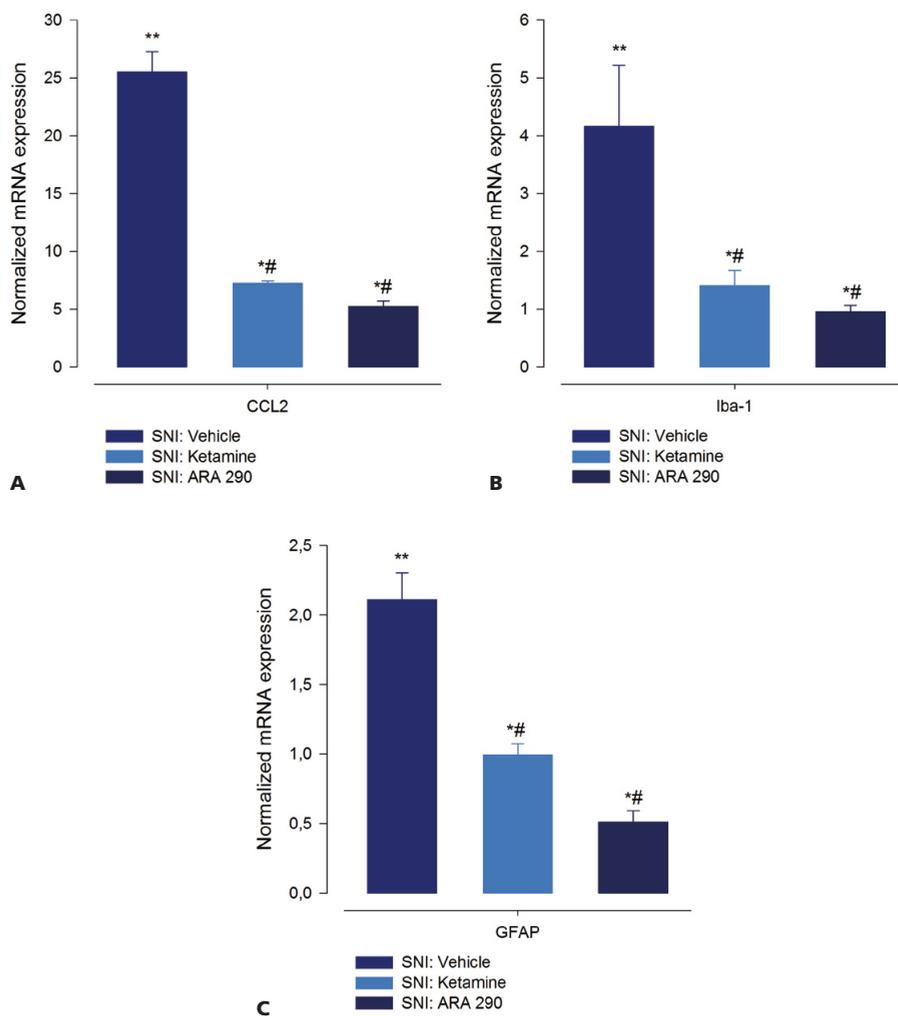
Figure 2: Ketamine and ARA 290 reduce inflammatory mediators in the spinal cord following sciatic nerve injury. One week post surgery, animals showed a marked elevation of CCL2 (panel A), Iba1 (panel B), and GFAP (panel C) compared to naïve controls. Both ketamine and ARA 290 significantly reduced the mRNA levels of these genes to a similar extent. * P<0.05 versus vehicle, # P<0.05 between ketamine and ARA 290 treatments, ** P<0.05 versus naïve.

demonstrated by a marked increase in the withdrawal latency (TWLs of 29.8±0.16 s and 24.85±1.26 s for 30 and 60 min post injection respectively, treatment effect P<0.001 versus vehicle, Figure 3A). In contrast, animals administered ARA 290 (30 µg/kg i.p.) demonstrated no change in latency during the 60 min observation period following administration (treatment effect P=0.977 versus vehicle, Figure 3A). Additionally, ketamine administration was associated with side effects characterized by the induction of stereotypical behavior and changes in locomotor activity. Follow-
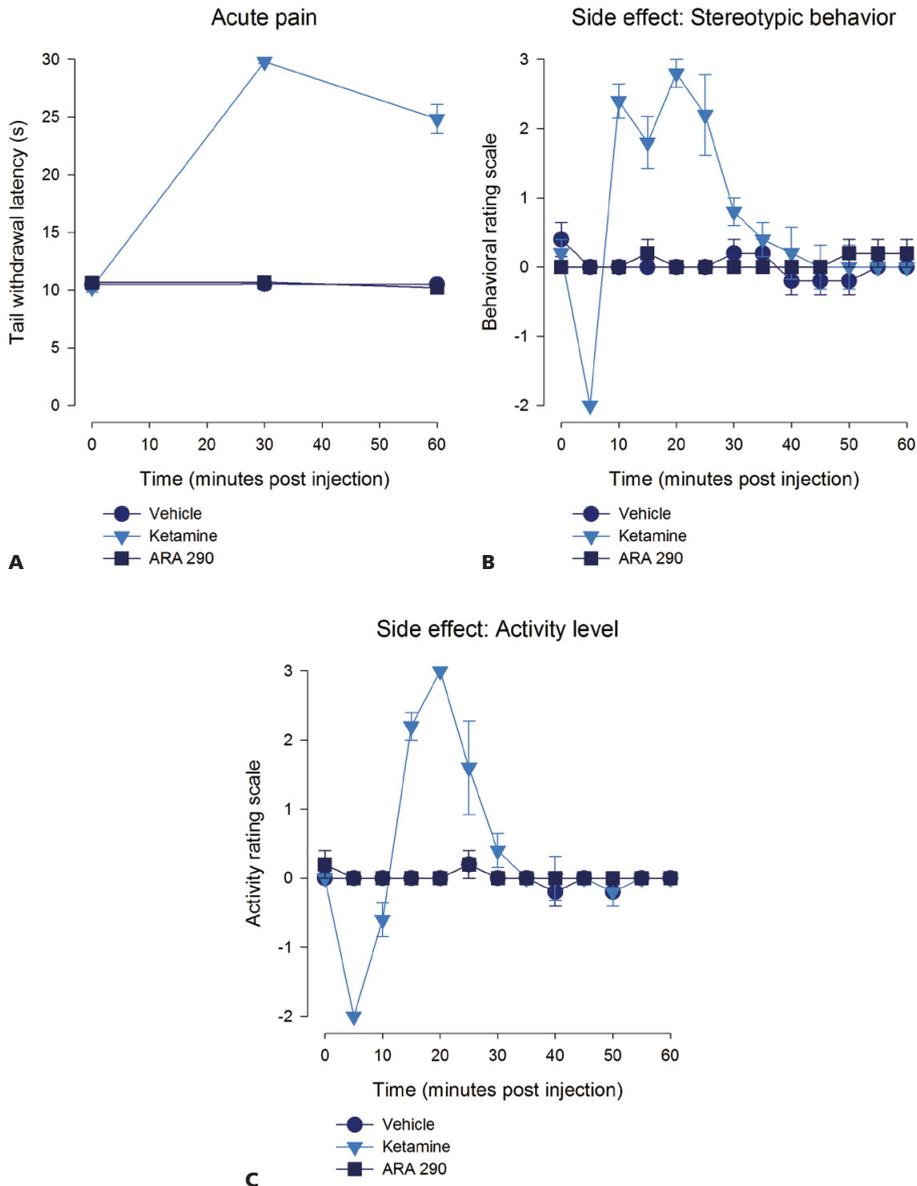
Figure 3: Ketamine and ARA 290 differ in effects on acute nociceptive pain and side effects. A: Ketamine administration increases the latency of tail withdrawal to a thermal stimulus (treatment effect, P<0.001), whereas ARA 290 does not. B: Ketamine treatment had significant biphasic effects on stereotypic behavior: after a period of transient sedation, the animals showed signs of psychomimetic disturbances that lasted for about 20 minutes (treatment effect, P<0.001). ARA 290 did not display these side effects. C: Treatment with ketamine was associated with a biphasic activation of generalized activity (treatment effect, P<0.001) causing an increase in restlessness and explorative behavior after a period of transient sedation.

ing ketamine, administration mice displayed a period of transient sedation which dissipated within 10 min, followed by a longer excitatory state characterized by stereotypical behavior and increased locomotor activity that lasted for 20–25 min before subsiding (treatment effects for stereotypical behavior and activity level, $P<0.001$ and $P<0.001$ versus vehicle respectively, Figs. 3B and C). In contradistinction, no behavioral changes were observed following ARA 290 (treatment effects for stereotypical behavior and activity level, $P=0.549$ and $P=0.346$ versus vehicle respectively, Figs. 3B and C).

Ketamine and ARA 290 do not Effect Allodynia in Mice Lacking the β-common Receptor

Following sciatic nerve surgical transection in which the sural branch is preserved (spared nerve injury; SNI), tactile allodynia developed in both wild type and βcR$^{-/-}$ mice within 24 h as demonstrated by significant decreases in the force required to induce a withdrawal response following plantar stimulation (Figs. 4A and 5A). To evaluate potential effects of ARA 290 and ketamine on the development of allodynia, animals were administered drug or vehicle every other day beginning 24 hours following surgery, and weekly starting in the second week after surgery. Following injury, vehicle treated animals uniformly displayed allodynia, reaching a nadir in the force
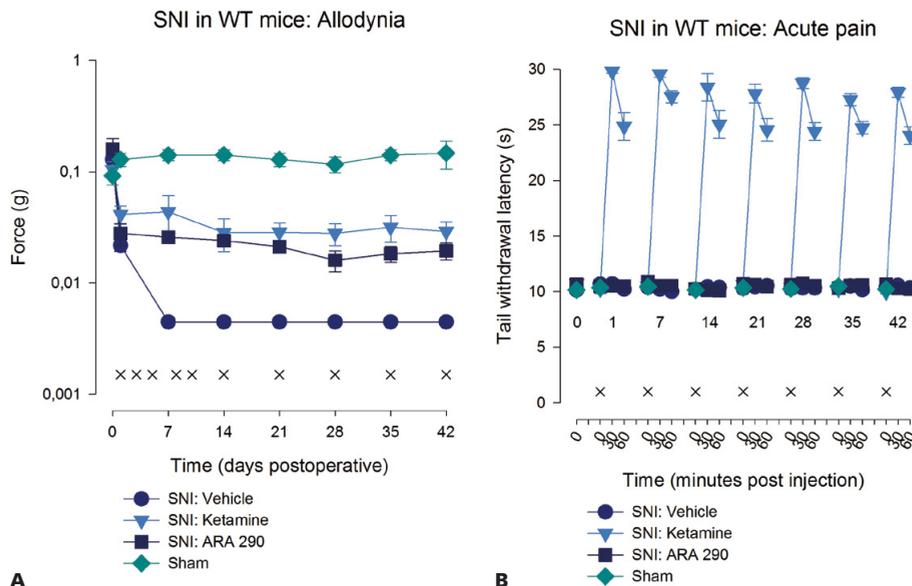
Figure 4: Ketamine and ARA 290 have similar effects on allodynia. A: Treatment with both ketamine and ARA 290 prevented the full development of allodynia (treatment effect, $P=0.049$ and $P=0.03$, respectively). B: The effects of ketamine on acute nociceptive pain remained unchanged over time (treatment effect, $P<0.001$). Digits represent testing days. X = treatment day.
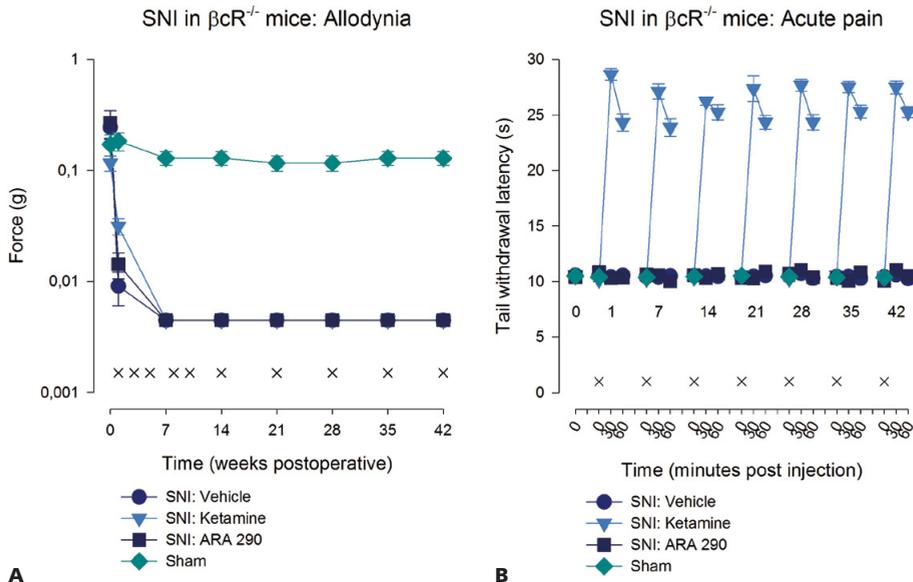
Figure 5: Relief of allodynia depends upon the β-common-receptor (βcR). A: Both ketamine and ARA 290 did not prevent the development of allodynia in βcR$^{-/-}$ mice. B: However, the effect of ketamine on nociceptive pain is unchanged in βcR$^{-/-}$ animals (treatment effect, P<0.001). Digits represent testing days. X=treatment day.

required to induce a withdrawal within 7 days at an applicable force of 0.004±0.0 g. Allodynia was sustained for the duration of the follow up. In C57Bl/6 (wild type; WT) mice, treatment with either ARA 290 (30 μg/kg i.p.) or ketamine (50 mg/kg i.p.) significantly attenuated the development of tactile allodynia during the first week, which was maintained for the duration of the follow up period (treatment effect versus vehicle, P=0.049 and P=0.03 for ketamine and ARA 290 respectively, Figure 4A).

Contrastingly, both ARA 290 and ketamine were ineffective in sciatic nerve transected βcR$^{-/-}$ mice, (treatment effect versus vehicle, P=0.308 and P=0.730 for ketamine and ARA 290, respectively, Figure 5A), although ketamine retained its acute antinociceptive effect (Figs. 4B and 5B) and behavioral effects (data not shown) similar to those observed in WT animals.

Discussion

The results of these experiments show that (1) nerve injury coincides with changes in expression levels of NMDAR subunit mRNA and spinal cord inflammatory mediators (CCL-2, Iba-1 and GFAP) after 7 days of NP, which are blunted by both ketamine and

ARA 290; (2) ketamine induces relief of acute nociceptive pain and behavioral side effects, whereas ARA 290 lacks these acute effects; (3) ketamine and ARA 290 induce relief of allodynia (i.e., analgesia) in a similar manner in wild type mice, but have no effect in mice lacking the βc chain. Contrastingly, βcR^{-/-} status had no effect on the acute antinociceptive or psychomotor effects of ketamine.

As would be predicted if ketamine and ARA 290 shared a common mechanism of action on NP, examination of gene expression in the spinal cord of injured animals shows comparable effects of these two agents on NMDA receptor expression and spinal cord inflammatory marker levels. The development of NP is associated with up-regulation of NMDAR on neurons that are believed to interact with microglia and astrocytes within the dorsal root ganglia and the dorsal horn of the spinal cord ipsilateral to the injured nerve[23]. Surprisingly, seven days following sciatic nerve injury mRNA of the NMDAR subunits NR1, NR2A and NR2B are not markedly elevated above the levels observed in uninjured animals, despite the fact that the maximum measurable amount of allodynia was reached. Similar observations are described in 2 other models of NP, where a significant NMDA receptor upregulation did not occur until after 14 days of NP following spinal cord injury for NR1, NR2A and NR2B[24], or where a reduction of the NMDA receptor at peak levels of allodynia was observed[25]. In our study, ketamine treatment significantly reduces the mRNA of all the NMDA receptor subunits to expression levels comparable to those of naïve animals, in contrast to the slightly elevated levels of untreated controls. In comparison, ARA 290 has a significantly larger suppressive effect and markedly reduced gene expression of the NMDARs below expression levels observed in naïve animals. Dose-response analyses were not performed in this study; therefore it is uncertain whether this difference between ARA 290 and ketamine can be explained by potency or biological factors. In spite of unequal NMDAR mRNA suppression by these agents, the effect of both on allodynia is identical. Taken together the slightly increased expression of the NMDA receptor subunits with respect to the amount of allodynia and the identical effect of both drugs on allodynia further support the possibility that the contribution of NMDAR to allodynia may not be the principal determinant in the development in NP, which has been suggested by the results of an earlier study[26].

Both ketamine and ARA 290 induce an approximately equivalent suppression of microglia, astrocyte and CCL2 mRNA measured within the spinal cord. The activation of spinal cord cells such as microglia and astrocytes is correlated to NP and reducing the numbers or activation states of these cells has shown to be of importance for the reduction of allodynia[27]. Iba1 as a marker for activated microglia and the suppression of its mRNA to baseline levels is consistent with significant attenuation of inflammation within the spinal cord and the subsequent reduction in allodynia. In addition to the reduction of microglia, the observed reduction of astrocytes as

identified by GFAP mRNA may also have contributed to the anti-allodynic effect of both ketamine and ARA 290. Notably, CCL2 is a product of neurons within the effected region of the spinal cord and signals for the accumulation of microglia in NP[28]. Antagonism of the C-C chemokine receptor type 2 (CCR2), the target receptor of CCL2, has shown to decrease allodynia[28,29]. CCL2 signaling and glia cell activation in conjunction with NMDAR up-regulation have been considered to be hallmarks of central sensitization[4], the observed effects of both ARA 290 and ketamine on these effectors is consistent with a reduction of central sensitization and with the observed results of these compounds on NP behavior.

Ketamine and ARA 290 have shown different effects on acute nociception that can be explained by the different receptor targets of the drugs. Specifically, the NMDAR, which mediates glutamate-dependent pain signaling arising from depolarization of the afferent nerve fibers, is antagonized by ketamine, but not by ARA 290. Therefore, blocking NMDAR activity by using 50 mg/kg ketamine results in a profound relief of acute nociceptive pain, whereas treatment with ARA 290 does not. Moreover, treatment with 50 mg/kg ketamine coincides with psychomimetic and locomotor side effects where mice suffer a transient period of sedation, as classified by a re-duced activity level and subsequent explorative (stereotypical) behavior, followed by a hyperactive state. Treatment with ARA 290 did not induce these side effects. These results indicate that ketamine, but not ARA 290, interacts with the NMDAR. In contrast to the data on nociceptive pain, both drugs attenuate the development of allodynia following nerve injury in a similar manner. When administered to WT animals 24 h after lesion, tactile allodynia is persistently decreased in both ketamine and ARA 290 treated animals during the entire follow up period. In addition, the analgesic action of ketamine does not change during SNI status. Conversely, βcR$^{-/-}$ mice with an SNI that were treated with ketamine or ARA 290 did not benefit from treatment and no attenuation of allodynia was observed. However, ketamine was still able to induce acute antinociception in mice lacking the βcR. The βcR requires assembly with other receptor subunits to become a functional signaling unit. As ARA 290 only interacts with the βcR-EPOR heteromer (IRR), it is a distinct possibility that the action of ketamine in this model is also mediated through the IRR. Ketamine possesses strong anti-inflammatory properties and is able to reduce serum TNF-α after sepsis in a murine laparoscopic model, an effect not readily expected from ketamine's action on the NMDA receptor[30]. Furthermore, it has been noted in the SNI model that NMDA receptor blockade by MK 801 did not affect either mechanical or cold allodynia[26].

ARA 290 has a very short plasma half-life ($\approx$2 minutes[31]) while ketamine has a tissue half-life of 10–12 minutes[32,33]. In spite of this, both agents have sustained effects on pain behavior in the spared nerve model. In vivo nerve recording has demonstrated
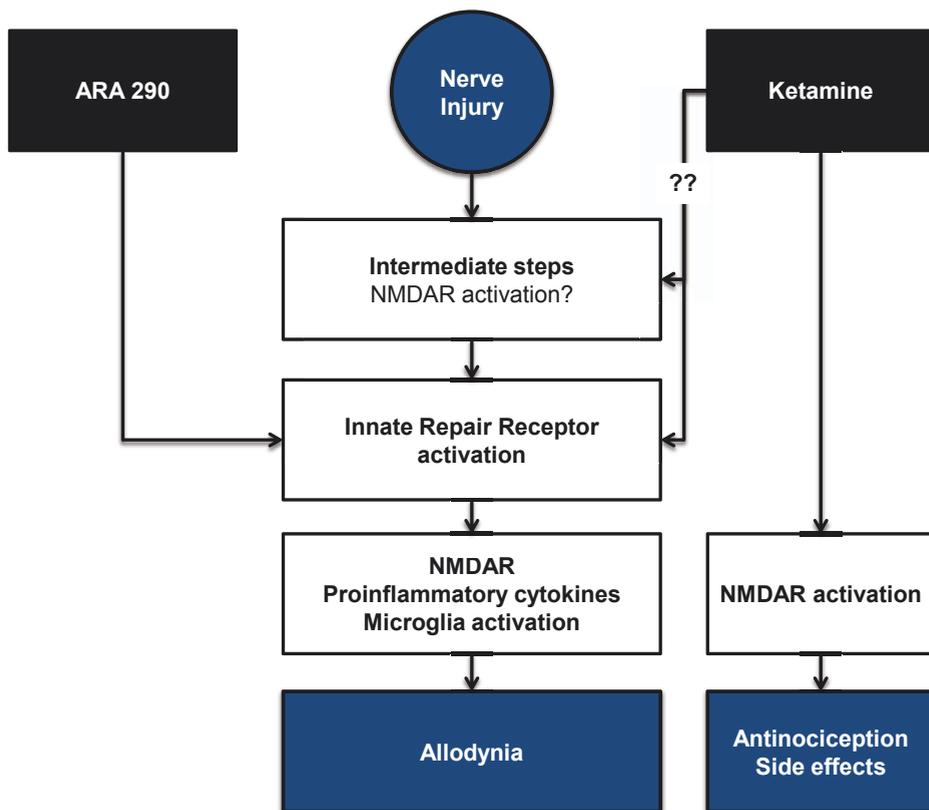
Figure 6: Neuropathic pain involves a pathway that utilizes the Innate Repair Receptor (IRR). Nerve injury results in microglial recruitment, increased expression of NMDAR, and proinflammatory cytokine production, ultimately resulting in allodynia. Activation of the IRR, e.g., by ARA 290, antagonizes this pathway. Ketamine also requires the IRR to reduce allodynia. This may be via a direct interaction with the IRR or alternatively, via modulation of intermediate processes that are upstream of the IRR. Additionally, ketamine interacts with NMDARs that mediate antinociception and psychomotor effects. ARA 290 does not interact with the NMDAR and therefore lacks these additional effects.

that the antiallodynic effects of ketamine are maintained far longer than its NMDAR antagonism[33]. ARA 290 and ketamine administered every 48 hours for the first 5 doses and weekly thereafter prevent the development of allodynia and sustain this (anti-allodynic) state in spite of chronic nerve injury. This observation is consistent with a modulating effect in which brief exposure to these pharmacologic agents activates a molecular switch, to produce long term effects through changes in gene expression. Previously a beneficial effect on pain behavior has been noted for treatment with amitriptyline extending to beyond the elimination half-life and treatment period in the very same model we have employed[34]. This effect is similar to the long-term modulation in the nervous system activity ("plasticity"), which underlies learning,

memory and the development of NP. The observation that ketamine may affect NP behavior by use of a signaling pathway that includes a receptor that is a component of the innate immune response and repair system predicts that it may also affect other functions served by this receptor, such as beneficial effects on inflammation at multiple levels, including the recruitment of immune-competent cells and secretion of pro-inflammatory cytokines and chemokines (reviewed in ref.[35]).

In conclusion, these findings confirm the existence of a pathway in the evolution of NP that involves the IRR (Figure 6).

Ketamine has a distinct effect on acute nociceptive pain, but a different activity in common with ARA 290 on NP via a pathway that requires the βcR. Whether the observed effects depend upon a direct interaction of ketamine with the IRR with require further studies, e.g., receptor binding or NMDAR knockdown experiments. The similar effects of ketamine and ARA 290 on NP in the spared nerve model established through activity of the IRR, could also be true for other models of NP, as well as for other treatments of NP. Although the effects of ketamine on acute pain appear to be pharmacologically driven by interaction by the NMDA receptor, the long-term effects may be described as manipulating a molecular switch, altering downstream gene expression and subsequent detrimental effects. Finally, although ketamine has potent, long lasting anti-neuropathic effects, interaction with NMDARs leads to very significant adverse effects including abuse potential. Utilization of IRR specific ligands, e.g., ARA 290, avoids these undesirable effects and may point a way to improved therapy of neuropathic disease.

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Chapter 7

Corneal nerve density predicts the severity of symptoms in sarcoidosis patients with painful neuropathy

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Innovation

Currently, a definitive diagnosis of small fiber neuropathy (SFN) requires a skin biopsy that demonstrates small nerve fiber loss. However, quantifying IENFD in skin biopsies is an invasive, labor-intensive process that has a low sensitivity for diagnosing SFN and does not correlate with the pain that patients report. Alternatively, CCM is a rapid non-invasive clinical ophthalmic technique for in vivo imaging of corneal nerve fibers. Here we show that CCM is a useful diagnostic tool to evaluate small fiber damage and that corneal nerve fiber density is inversely related to symptoms in patients with sarcoid neuropathy. This technology expands the role of CCM as a surrogate marker for both nerve fiber damage and pain in clinical trials of novel therapeutics in sarcoid and perhaps other small fiber neuropathies.

Introduction

Loss of small, unmyelinated nerve fibers, i.e., small fiber neuropathy (SFN), is an increasingly recognized feature of a wide range of neuropathies[1]. It is a major cause of pain and poor quality of life with an inability to work[2]. SFN is characterized by spontaneous pain, dysesthesiae, paresthesiae, and altered thermal sensory thresholds[3,4]. Additionally loss of post-ganglionic autonomic nerve fibers leads to a wide variety of symptoms including anhidrosis, orthostasis, and a range of other manifestations depending upon the organs affected.

Routine electrodiagnostic studies, such as electromyography and nerve conduction studies, in conjunction with tendon reflexes and strength testing evaluate large nerve fibers. Consequently, these tests remain normal in small fiber neuropathy and pure small fiber damage is not easily evaluated. Based on data from preclinical models, inflammation has been suggested to be a common mechanism for the reduction in small nerve fibers[5] and a recent study has confirmed that pro-inflammatory cytokines are elevated in patients with SFN and pain[6].

Curative therapy for SFN is lacking. Current therapy is directed towards symptomatic pain relief which is generally not satisfactory[1]. Although reduced nerve fiber density as determined by skin biopsy is the hallmark of SFN, sensitivity appears suboptimal in sarcoidosis[7], and to date no study has shown that nerve fiber density obtained by skin biopsy directly relates to patient symptoms, e.g., pain. Hence, no biological marker for pain has yet been established[8,9] and therefore the outcome of clinical trials have been based upon patient-reported outcomes that are highly variable and subjective.

SFN is difficult to diagnose as complaints of pain and autonomic dysfunction are variable and standard electrophysiological testing cannot directly assess the function of the small nerve fibers involved[1]. Additionally, the natural history of SFN is poorly understood and fluctuates over time. The current diagnostic standard for SFN requires the presence of symptoms, a clinical examination consistent with the loss of small nerve fiber function, and a skin biopsy that documents reduced small, unmyelinated and thinly myelinated nerve fibers (Aδ and C)[4]. Because normative values have been derived for the distal leg[10], this site is typically used for diagnosis.

Sarcoidosis is an inflammatory disease that is associated with SFN[2]. The prevalence of SFN in patients with chronic sarcoidosis is not precisely known, but may be as high a ~75%[7,11]. Several questionnaires (the Small Fiber Neuropathy Screening List[12] as well as an autonomic symptom assessment[11]) have been developed to aid in the diagnosis of SFN in patients with sarcoidosis. Although they are useful in screening for patients with SFN, diagnostic confirmation requires a 3 mm skin biopsy and immunohistochemistry to quantify IENFD[13]. Several recent studies have shown that IENFD is reduced in patients with sarcoidosis and neuropathic symptoms[7,11,14].

However, SFN of sarcoidosis has been described as a non-length dependent process that occurs in a "patchy" distribution[2] and therefore it is possible that a biopsy obtained from the distal leg might not reflect the presence of reduced small nerve fibers at other locations. Thus a majority of patients with symptoms of SFN have an ankle IENFD that is not below the 0.05 quantile level of normal that has been suggests as required for a definitive diagnosis of SFN[7,11]. Furthermore, skin biopsy, although well-tolerated with minimal potential adverse effects, is an invasive procedure and sample processing requires a dedicated laboratory for fixation, sectioning, staining, and nerve fiber counting that is time and labor intensive and fraught with significant potential artifacts. Additionally, innervation of the skin is not equally distributed and follow-up biopsies cannot be taken at the exact same location. Hence, skin biopsies are not ideal for following the progression of disease and to assess the potential beneficial effects of therapeutic interventions.

The cornea has the highest density of nerve fibers of any tissue (up to 600 times more than the skin)[15] and therefore any process that targets small nerve fibers may be especially prominent in the eye. Corneal nerve fibers originate from the ophthalmic branch of the trigeminal nerve and distribute radially towards the apex of the cornea parallel to the surface. Corneal innervations consists of predominantly C fibers, i.e., small, unmyelinated fibers that are polymodal nociceptors, that respond to a wide range of mechanical, thermal, and chemical stimuli[15]. For these reasons the corneal nerve fibers may be more reflective of the pain that patients report. Over the last decade, a confocal microscopic technique has been developed to directly, and non-invasively visualize nerve fibers that innervate the cornea, termed corneal

confocal microscopy (CCM)[16]. This technique allows direct visualization of a narrow slice of tissue containing nerve fibers running parallel to the surface. The utility of this methodology has been evaluated in a range of neuropathies including, diabetic neuropathy, Fabry's disease, Charcot-Marie-Tooth disease 1A, and chemotherapy induced neuropathy[17-20]. Corneal nerve fiber number is directly related to the severity of neuropathy derived from a neurological examination that tests both small and large nerve fiber function[19], as well as cooling detection thresholds, axon reflex-mediated neurogenic vasodilatation in response to cutaneous heating by laser Doppler imaging flare technique (LDIFLARE), heart rate variability (HRV)[21] and IENFD[22]. It is currently unknown whether corneal confocal microscopy may aid in identifying nerve fiber loss and severity of pain in patients with sarcoid neuropathy.

Methods

Study Criteria and Patient Population

The results reported here are derived from a study population with chronic sarcoidosis and debilitating symptoms of painful neuropathy (protocol NTR3575 in the International Clinical Trials Registry Platform). After Ethics committee approval and informed consent according to the Declaration of Helsinki, patients were recruited according to the following inclusion criteria:

- Diagnosis of sarcoidosis according to accepted international criteria[23].
- Spontaneous pain level ("pain now" of the Brief Pain Inventory) $\geq 5/10$ or Small fiber neuropathy screening list score (SFNSL) > 37/84.
- Pain defined as distal pain plus one of the following: dysesthesia, burning/painful feet worsening at night, or intolerance of sheets/clothes touching the legs/feet.

Exclusion criteria were:

- Clinically relevant abnormal history of physical and/or mental health.
- A semi recumbent systolic blood pressure of > 150 mmHg and/or diastolic blood pressure of > 90 mmHg at screening.
- History of alcoholism or substance abuse within three years prior to screening.
- Positive pregnancy test.
- Male patients habitually using more than 21 units of alcohol per week and female patients using more than 14 units of alcohol per week.
- Male patient unable/unwilling to use a medically acceptable method of contraception throughout the entire study period. Female patient not using oral contraceptives, or not postmenopausal.
- History of severe allergies, or an anaphylactic reaction or significant intolerability to prescription or non-prescription drugs or food.

Table 1: Patient characteristics

Variable	Value (+ SEM)
Subjects	38
Males/females	20/18
Weight (kg)	82.1+ 2.6
Age	49.5 + 1.5
Height (cm)	177.6 + 1.8
Body Mass Index	25.8 + 0.5
Years since diagnosis of sarcoidosis	8.4 + 1.3
Use of NSAIDs	14
Use of neurological/psychological drugs	11
Use of oral steroids	13
Use of opioids	7
Use of systemic anti-inflammatory drug	10
Prior use anti-TNF therapy	2
High sensitivity C-reactive protein (mg/L)	2.2 + 0.5
Angiotensin converting enzyme (normal 23-67 nmol/min/ml)	49.3 + 5.1
Elevated ACE (number)	11
SFNSL score baseline	43.4 + 2.1
BPI total pain score (range 0-40)	20.6 + 0.9
BPI pain interference (maximum 70)	34.1 + 1.7
6 Minute Walk Test (meters)	473.4 + 15.5
Predicted 6 Minute Walk Test (meters)	692.6 + 9.6

- Vaccination or immunization within the last month.
- Participation in an investigational drug trial in the 3 months prior to study.
- Major surgery within three months prior to screening.
- Donation or loss of blood (> 500 mL) within 3 months prior to screening.

Thirty eight patients (18 females, 20 males) of mean age 49.5 years (range 28-65) satisfying inclusion criteria were evaluated (Table 1). The duration of sarcoidosis was 8.4 ± 1.3 (SEM) years. No patient was using capsaicin topical cream that is known to reduce intraepidermal nerve fiber density. None had serious or progressive lung disease. The mean score of the Brief Pain Inventory Short Form (BPI) was 54.7 (out of 110 total) and the mean SFNSL score was 43.4 (out of 84 total).

Clinical Testing

Quantitative Sensory Testing was accomplished according to the protocol of the German Pain Network[24]. A 6 Minute Walk Test (6 MWT) was performed according to published protocols[25]. Predicted 6 MWT distance for normal individuals as a function of age, gender, and height was calculated using the formula of Troosters et al.[26]

Nerve Fiber Quantification

Skin biopsies (3 mm) were obtained from the proximal thigh (20 cm below the anterior superior iliac spine) and the distal leg (10 cm above the lateral malleolus) and processed following established guidelines[13]. Free floating 50 μm thick sections were cut and stained using rabbit anti-protein gene product 9.5 antibody (Dako Netherlands bv) visualized using a goat anti-rabbit Alexa fluor 488 antibody (Invitrogen, Life Technologies, Grand Island, NY). A minimum of 3 sections selected from the ends and the middle of each biopsy series was evaluated using a Leica M5500 fluorescence microscope (Leica Microsystems, Rijswijk, The Netherlands), magnification 1000x. The nerve fibers were counted manually. Images were recorded with Leica Application Suite, magnification 400x and epidermal lengths were measured using ImageJ (NIH, Bethesda, MD, USA). Normative data of nerve fiber density used for the distal leg was that of Lauria et al.[10] and for the thigh from Umapathi et al.[27]

Corneal confocal microscopy was carried out using the Rostock Cornea Module with the Heidelberg Retina Tomograph III using established methodology[28]. A minimum of 6 images containing nerve fibers (i.e., to be within Bowman's layer) were evaluated using computer software as previously described[29]. Corneal nerve fiber data obtained from Twenty two healthy volunteers (gender (M/F-9/13), age 49.0 ± 2.7, height 167.3 ± 2.3, weight 71.1 ± 3.1, BMI 25.3 ± 0.9) had a mean nerve fiber density = 31.6 ± 6.4 (SD) per mm^2; mean nerve fiber length = 21.7 ± 3.6 mm/mm^2; and mean nerve branch density = 54.6 ± 23.4/mm^2.

Statistics

Statistical analysis was performed using JMP (SAS, Inc, Cary, NC). Stepwise linear regression modeling, analysis of covariance, unpaired t-test, or Mann-Whitney U test were carried out where appropriate.

Results

Almost all patients had a significant reduction in the distance they could walk in 6 minutes as estimated from the normative predictive data generated for older individuals by Troosters et al.[26] which was 693 meters. The mean reduction in expected 6 MWT distance in the sarcoidosis patients was 219 meters (95% confidence interval: 186-253 meters).

Quantitative sensory testing showed that the majority of patients exhibited significant small nerve fiber dysfunction as evidenced by alteration in thermal thresholds (Table 2). The most common abnormality was a decrease by more than 2 SD below the mean of normal volunteers in the cold and warm detection thresholds in ~ 80%

Table 2: Results of quantitative sensory testing. Patients showed functional impairment of both small nerve fibers (Aδ and C) as well as larger sensory nerve fibers (Aβ). Data are expressed as number of patients deviating beyond the 95% confidence interval of a sex- and age-matched normal populations as reported by Rolke et al[24].

Variable	Nerve fibers involved	Change	Number of patients (%)
Cold detection threshold	Aδ & C	Decrease	30 (79)
Warm detection threshold	Aδ & C	decrease	30 (79)
		increase	1 (3)
Thermal sensory limen	Aδ & C	decrease	8 (21)
		increase	2 (5)
Paradoxical heat sensation	Aδ	Decrease	15 (40)
Cold pain threshold	Aδ & C	Increase	3 (8)
Heat pain threshold	C	decrease	4 (11)
		increase	5 (13)
Mechanical detection threshold	Aβ	Decrease	21 (55)
Mechanical pain threshold	Aβ	decrease	15 (40)
		increase	6 (16)
Mechanical pain sensitivity	Aβ + C	decrease	3 (8)
		increase	5 (13)
Dynamic mechanical allodynia	Aβ	Increase	14 (37)
Windup ratio	Aδ & C	Increase	6 (16)
Vibration detection threshold	Aβ	Decrease	35 (92)
Pressure pain threshold	Aδ & C	decrease	4 (11)
		increase	17 (45)

of the patients. Additionally, >90% of the patients showed a decrease in the vibration detection threshold.

Corneal nerve fiber images of patients with sarcoidosis typically showed reduced corneal nerves compared to healthy controls (Figure 1A and B). Quantification showed that the mean corneal nerve fiber density (CNFD; patients: 21.6 fibers/mm^2 ± 5.9 SD versus controls: 31.6 fibers/mm^2 ± 6.4; P<0.0001) and Length (CNFL; patients: 13.2 mm/mm^2 ± 4.0 versus controls; 21.7 mm/mm^2 ± 3.6 P<0.0001) of patients with chronic sarcoidosis were significantly reduced compared to normal controls (Figure 1C and D). In contrast, mean corneal nerve branch density was not significantly different from controls (patients: 51.2/mm^2 ± 30.5 SD versus controls 54.6/mm^2 ± 23.4 SD).

The median intra-epidermal nerve fiber density of the distal leg was significantly reduced compared to age and gender matched normal controls (Figure 2A). The average difference between the normal population age and sex dependent median values and the patient population was 4.7 fibers/mm^2 (P<0.0001; Mann-Whitney Test). Stepwise linear regression modeling determined that age, height, and gender
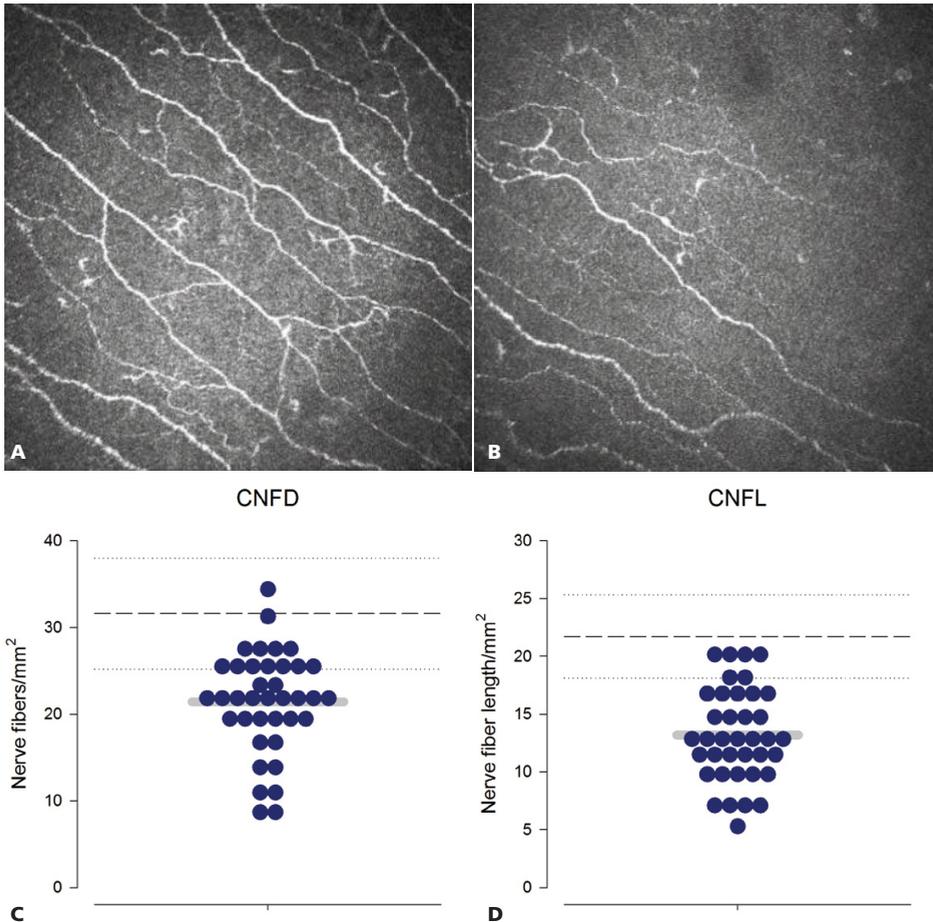
Figure 1: Nerve fibers of the sub-basal layer of the cornea are reduced in number and length in patients with sarcoidosis and symptoms consistent with small fiber neuropathy. A: Confocal images of a typical normal cornea. B: Confocal images cornea of a typical patient with sarcoidosis and neuropathic pain. Comparison illustrates an obvious reduction of nerve fibers in the patient (field of view is 0.4mm by 0.4 mm). These nerves are predominantly small, non-myelinated C fibers. C: Quantification shows that the mean corneal nerve fiber density (CNFD) is reduced in this patient population compared to normal individuals. D: Corneal nerve fiber length (CNFL) is reduced in this patient population compared to normal individuals. The heavy dashed line indicates the mean, the lighter dashed lines indicate 1 SD of a normal population (n = 22). Solid horizontal line indicates the mean value for the sarcoidosis patients. There was no dependence of corneal nerve fiber density or length upon gender, age, or height of either the patients or normal controls. Corneal nerve fiber branching density was not different from controls (not shown).

were covariates of IENFD. In contrast, these variables were not covariates for IENFD of the proximal thigh, for which the density was also reduced approximately 50% compared to normal individuals (mean of patients: 11.0 fibers/mm (confidence interval 9.9-12.0) versus age-matched normal controls[27]: 20.6 fibers/mm (confidence
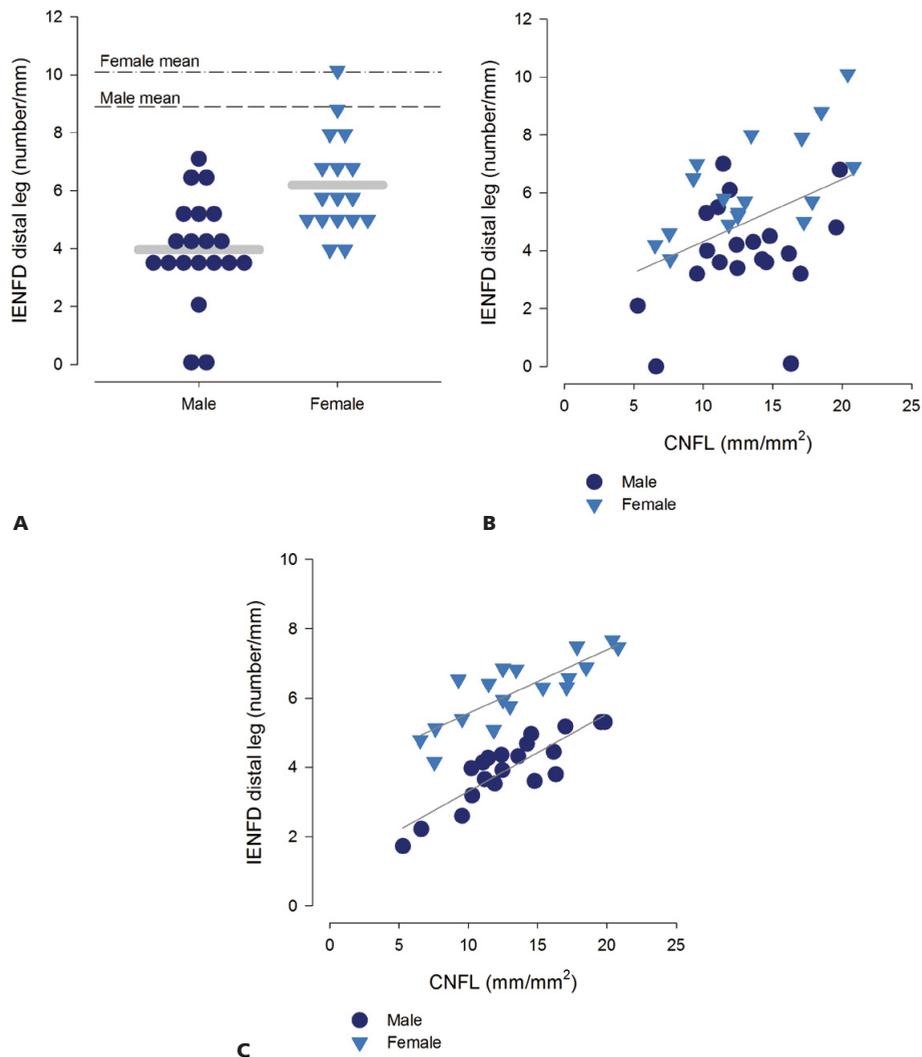
Figure 2: A: The distal leg intraepidermal nerve fiber density of patients with sarcoidosis and symptoms of SFN is reduced compared to normal population. The horizontal lines correspond to the median value of each gender. The dashed line represents the age-dependent median normative value[10]. B: There is a significant linear relationship between CNFL and IENFD of the distal leg (P=0.009). A similar finding was observed for CNFD (data not shown). C: Gender, age, height, and CNFL (or CNFD) are covariates for IENFD of the distal leg. A linear model constructed using these variables provides the relationship between CNFL and IENFD. Here, the least mean squares predicted values of the distal leg IENFD are plotted versus CNFL, showing that the slope of the relationship is the same for female (95% CI: 0.12 to 0.25) and male (95% CI: 0.16 to 0.29) patients.

interval 17.8-23.4)). The mean ratio of IENFD of the proximal thigh to the distal leg was 3.9 ± 1.5 SEM, with one patient equal to 0.9 and the others > 1.0. The patients in this study, therefore, had a peripheral neuropathy of a length-dependent nature.

IENFD of the proximal leg was not significantly correlated to that of the distal leg (Pearson's correlation coefficient=0.20; P=0.22).

However, the IENFD of the distal leg, which is typically employed for diagnosis of SFN, was significantly correlated to CNFL (Figure 2B) and to CNFD (data not shown). Linear regression modeling showed that age and gender were covariates and that a good predictive model incorporating CNFL (Figure 2C) or CNFD (not shown) could be constructed.
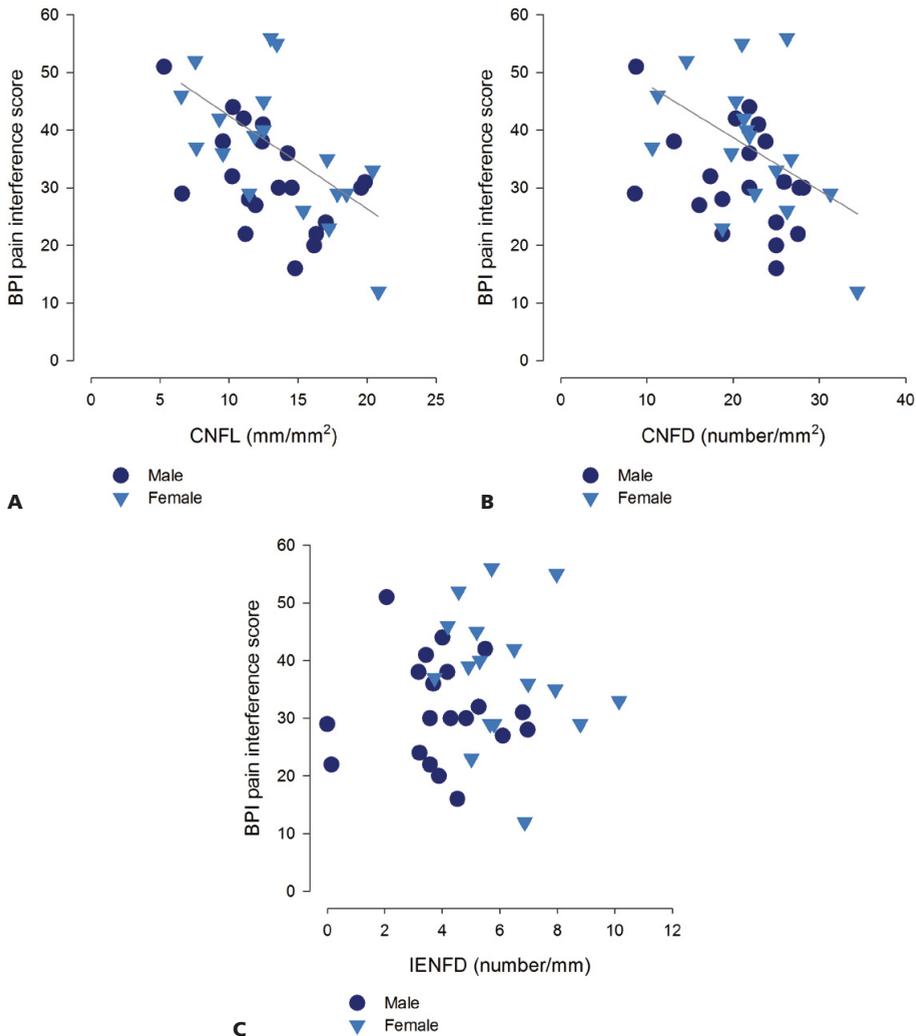

Figure 3: Corneal nerve fiber length and number are correlated with patient related symptoms. A: CNFL is inversely correlated with the Brief Pain Inventory pain interference score (P=0.0005) B: CNFD are inversely correlated with the Brief Pain Inventory pain interference score (P=0.012, respectively). C: In contrast, IENFD has no relationship with the BPI pain interference score.
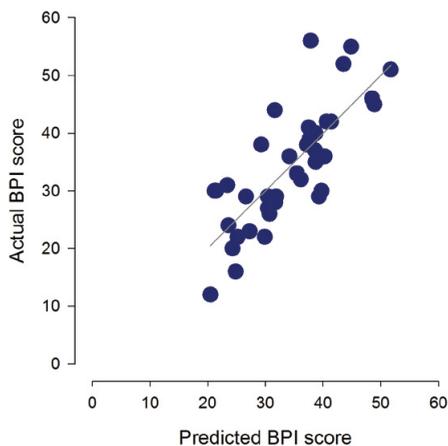
Figure 4: Corneal nerve fiber length can be used to predict the Brief Pain Inventory pain interference score. A linear model constructed with CNFL as the independent variable, with height, weight, and 6 MWT as covariates predicts with high accuracy the BPI interference score (Pearson's correlation coefficient=0.78, P<0.0001).

Additionally, both CNFL (Figure 3A) and CNFD (Figure 3B) were negatively correlated with the pain interference component of the Brief Pain Inventory. Specifically, the relationship between the CNFL and BPI pain interference score for individual patients without controlling for additional variables was described by the linear function BPI pain interference score=−1.38 * CNFL (mm/mm^2) + 52.3 (slope 95% CI: −2.1 to −0.7; Pearson's correlation coefficient of −0.54; P=0.0005) and for CNFD: BPI interference score=−0.71 * CFND + 49.4 (slope 95% CI: −1.3 to −0.2; Pearson's correlation coefficient of 0.4, P=0.012). In contrast, there was no relationship between the BPI pain interference score and IENFD at the ankle (Figure 3C) or proximal thigh (data not shown). A weaker correlation (Pearson's coefficient −0.33; P=0.04) was also noted between CNFL and the "average pain" score of the BPI, whereas no relationship was evident with IENFD (data not shown). No correlation was found between IENFD, CNFL, or CNFD with the "worst", "least", or "now" pain components of the BPI.

Stepwise linear regression analysis including other potential covariates of the BPI pain interference score showed that height, weight, and 6 MWT difference from expected were also inversely related to the pain interference score. Construction of a linear model with CNFL as the dependent variable in addition to weight, height, and 6 MWT deficit accurately predicted BPI pain interference (Figure 4; prediction formula with a slope of 1; Pearson's correlation coefficient=−0.78; P<0.0001).

Discussion

The main findings of this study conducted in a population of sarcoidosis patients having pain consistent with SFN are two-fold: Corneal nerve fiber quantification 1) provides the same information as intra-epidermal nerve fiber densities obtained from the distal leg and can therefore be used for diagnosis and 2) in contrast to IENFD, CCM data is highly predictive of the pain that patients report. Secondary results show that the neuropathy documented by the skin biopsies in this population is of a length-dependent phenotype. The majority of the patients also appear to have involvement of larger nerve fibers based on an elevated vibration detection threshold, and in addition most patients show a significant reduction in functional exercise capacity, as evaluated by the 6 MWT.

Skin biopsies are an invasive procedure with a significant technical threshold of preparation and analysis to overcome. If a follow up biopsy is required, a different region of the skin is examined. Additionally, as previously reported in normal individuals and in patients with sarcoidosis and neuropathy, age and gender are strong covariates of IENFD obtained from the distal leg[7,10,27] and thus potentially complicate interpretation. These problems could potentially be avoided by utilizing CCM-derived corneal nerve fiber data which can be obtained repeatedly from the same central location of the eye.

Notably, the pattern of nerve fiber involvement in our patients was not consistent with a non-length dependent process as reported by some clinicians[2,30]. Here, both biopsy sites on the leg provided equivalent evidence of reduced nerve fiber densities, although the values were not significantly correlated. It should be noted that in diabetes, characterized by an accepted length-dependent neuropathic process, the much shorter corneal nerves also reflect the same pathology as the longer fibers innervating distal extremities[19]. These observations give reassurance that sampling corneal nerve fibers alone is sufficient to yield a diagnosis of reduced numbers of small nerve fiber in the setting of SFN.

There are currently no data available to explain why the corneal nerve fibers are so prominently affected in SFN. One important factor may be the very dense innervation of the cornea predominantly by C fibers. The patients in this study were selected in part by having pain, a primary function of the C fibers, and it is possible that processes that affect these fibers may cause changes more evident against a background of high fiber density, such as in the cornea. Another speculative hypothesis is that since there is no resident blood supply (corneal metabolism relies on diffusion of oxygen from the surrounding tear layer) the small nerve fibers are particularly prone to hypoxic and/or inflammatory injury, similar to what has been proposed to explain the preferential involvement of longer fibers in many neuropathic processes such as diabetes[31].

It is notable that similar to other studies evaluating IENFD, the current study found no relationship between IENFD from thigh and ankle biopsies with the pain that patients reported. In contrast, there was a strong inverse relationship between corneal nerve fiber density and the extent to which the patients reported that pain was interfering with the activities of daily living. Current research has identified a prominent role for inflammation as an inducer of chronic pain states in the central nervous system[32]. Perhaps the central processes of the ophthalmic nerve are more direct participants in a central pain promoting processes, than fibers in the distal extremities that synapse centrally in the spinal cord.

The inverse relationship noted between corneal nerve fiber quantification and the patient reported outcome of pain interference in this study is potentially useful in the clinical assessment of patients reporting pain-related limitations in activities of daily living. The linear model that relates the pain interference score with CNFL and the additional covariates of height, weight, and performance on the 6 MWT allows an objective means by which to corroborate self reported data. Deviations from predicted values would alert a clinician to search for a confounding factor, e.g., pain reports from a malingerer.

Finally, the patients in this study had a clearly reduced functional capacity as indicated by the 6 MWT, in spite of the fact that none of the patients had documented significantly reduced cardiopulmonary status. This finding has recently been observed in patients with chronic sarcoidosis[33]. Although the investigators concluded that poor muscle strength, fatigue, and exercise intolerance were the primary defect, it is also possible that a number also had SFN, as previous studies have shown a high incidence of SFN in the chronic sarcoidosis population[11]. In the present study, the patients were selected for having pain as well, and it is also possible that discomfort contributed significantly to the observed decrease in function.

One limitation of this study is that only patients with pain were evaluated. It is well known that SFN can be present without complaints of pain[3] and it remains to be determined whether the corneal nerve fibers will reflect the neuropathic process in these patients. Additionally, the variable autonomic symptoms that accompany SFN often include xerophthalmia, which has been recently shown to be associated with a decrease in corneal nerve density[34]. In spite of these limitations, we anticipate that the results of this study showing that CCM-derived nerve fiber data reflect with good fidelity that obtained from skin biopsies could apply to other diseases associated with SFN.

Conclusions

Corneal and cutaneous nerve fibers are reduced in the majority of sarcoidosis patients selected for pain and symptoms of SFN. The painful symptoms of SFN are inversely related to corneal nerve fiber density in this population of patients. CCM appears a useful, non-invasive method to quantify nerve density for the diagnosis of SFN. Additionally, CCM may prove to be a non-invasive, repeatable method to follow the natural history of SFN and to test efficacy of therapeutic interventions.

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Chapter 8

ARA 290 improves symptoms in patients with sarcoidosis-associated small nerve fiber loss and increases corneal nerve fiber density

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Introduction

Sarcoidosis is an immune-mediated, inflammatory orphan disease of unclear etiology that can affect virtually any organ of the body[1]. In most individuals diagnosed with sarcoidosis the disease is mild with pulmonary and hilar lymph node involvement that resolves within several years whether treated by immune suppression or not. However, in about one third of patients, sarcoidosis evolves into a chronic, progressive disease[2]. In these refractory cases, therapy has generally consisted of immune suppression which has been associated with a variable response rate[3].

Recently, it has become apparent that a significant proportion of patients with chronic sarcoidosis report symptoms that suggest abnormal function of the small nerve fibers of the sensory and autonomic nervous systems[4-8]. Clinical evaluation by skin biopsy, typically of the distal leg, has shown that many of these patients have a demonstrable reduction in intraepidermal nerve fibers[6,9]. The affected nerves consist of unmyelinated C and lightly myelinated Aδ fibers that comprise the sensory and autonomic peripheral nervous systems. Patients having reduced nerve fiber densities typically complain of pain, numbness, and/or dysesthesia, as well as autonomic symptoms that can be extremely variable depending on the organ affected[5]. The neuropathic symptoms in these patients are frequently severe and therefore are major contributors to the poor quality of life of those afflicted[10].

The etiology of the loss of small nerve fibers in sarcoidosis has not been definitively identified, but one prevalent hypothesis is that nerve fiber dropout is the end result of systemic and/or local inflammation[11]. Neuropathy arising from inflammation can affect both the peripheral nerve endings and the neuronal somata within the dorsal root ganglia of the spinal cord. At the present time, glucocorticoids and other immune suppressants are the principal therapeutic approach to small fiber neuropathy but are often ineffective[8]. In addition to immune modulators as potential disease modifiers, treatment is generally symptomatic, consisting of the analgesics, anti-epileptics, and antidepressants used for other painful neuropathies[12]. Thus, there is a clear need for new therapeutics in sarcoidosis-associated small fiber neuropathy.

ARA 290 is an eleven amino acid peptide derived from the structure of erythropoietin (EPO) that possesses potent tissue protective and tissue repair activities[13]. The actions of ARA 290 are mediated through a receptor consisting of a complex formed by the EPO receptor and beta common receptor subunits[14], termed the innate repair receptor (IRR). In preclinical models of neuropathic pain, ARA 290 has demonstrated beneficial effects that include IRR-dependent prevention of the development of allodynia in a peripheral nerve transection model[15] or in an inflammatory neuritis model[16], as well as attenuation of spinal cord inflammation. Also, EPO, and its non-erythropoietic derivatives, e.g., ARA 290, have been shown to support the regrowth

of intra-epidermal nerve fibers in preclinical models of neuropathy arising from toxins[17] or diabetes[18].

An initial open label study of the effects of three intravenous doses of ARA 290 administered over one week on neuropathic pain of patients with sarcoidosis or diabetes showed a 50% improvement without any safety concerns[19]. The results of a follow up trial of ARA 290[20] administered intravenously three times weekly for 4 weeks to sarcoidosis patients with symptoms of small fiber neuropathy also appeared to be safe and was associated with a significant improvement in the patient reported outcomes of the small fiber neuropathy screening list (SFNSL[21]) and the pain and well-being components of the RAND-36.

Based on these observations, we have conducted the present study to assess the effects of ARA 290 on neuropathic symptoms when given as a daily subcutaneous injection for 28 days. Because of the association of nerve fiber loss with neuropathic symptoms and the potential for ARA 290 to cause nerve fiber regrowth, we hypothesized that ARA 290 administration will improve symptoms and stimulate the regrowth of small nerve fibers. To evaluate this, the nerve fiber densities in the cornea, proximal thigh, and distal leg were assessed. Additionally, cutaneous sensory testing of the face, hand, and foot were determined using quantitative sensory testing, and quality of life assessed with appropriate patient questionnaires. Finally, functional capacity, which is often reduced in chronic sarcoidosis[22], was assessed using the 6 Minute Walk Test.

Methods

Rationale for dose selection

Results of a previous study performed in sarcoidosis patients with painful small fiber neuropathy showed that 2 mg of ARA 290 administered intravenously (IV) three times weekly improved neuropathic symptoms. In the current trial we sought to assess the potential of subcutaneous (SC) dosing, as the IV is not practicable in the outpatient setting. Therefore, a crossover pharmacokinetic study was performed using 10 normal volunteers to compare a 2 mg IV dose that was used in the previous study, to 2, 4, or 6 mg of ARA 290 administered SC[19]. Results of preclinical and in vitro studies have shown that activation of the IRR requires concentrations of ARA $290 \geq \sim 1$ nM (~ 1.3 ng/mL)[14]. Therefore, the area under the curve (AUC) of the pharmacokinetic data was calculated using the trapezoidal rule for the period of time in which the plasma concentrations were > 1.3 ng/mL. The results of this crossover study showed the following median AUCs: 2 mg IV $= 65$ ng/mL*min, 2 mg SC $= 23$ ng/mL*min, 4 mg SC $= 59$ ng/mL*min and 6 mg $= 249$ ng/mL*min, with only the 6 mg

dose differing significantly from the others (P<0.05; Kruskal-Wallis test). Based on these data, the 4 mg SC group was selected for the daily dosing regimen of this trial. Study design.

The trial, entitled "Effects of ARA 290 on the regrowth of epidermal nerve fibers in patients with sarcoidosis", was an investigator-initiated, single site, double blind, placebo-controlled trial carried out at the Leiden University Medical Center after receiving Ethics Committee approval. The trial was registered with the International Clinical Trials Registry (NTR3575) and was assigned EudraCT number 2012-001492-37. All study personnel and patients remained blinded as to the treatments until the end of the follow up period (16 weeks from the beginning of dosing).

The primary outcomes were: 1) change in epidermal or corneal nerve fiber density at day 28 versus baseline; 2) change in cutaneous sensitivity of day 28 versus baseline using Quantitative Sensory Testing; and 3) change in visual acuity or retinal edema, at day 28 versus baseline. Secondary outcomes assessed were: 1) change in the Small Fiber Neuropathy Screening List score at day 35 versus baseline; 2) change in Brief Pain Inventory at day 35 versus baseline; and 3) change in distance walked in the 6 Minute Walk Test at day 28 versus baseline.

Patients who satisfied the international consensus statement for diagnosis of sarcoidosis[23] and had symptoms suggestive of neuropathy were recruited after referral by sarcoidosis specialists. The Consolidated Standards of Reporting Trials (CONSORT) flow chart corresponding to this trial is illustrated in Figure 1. After obtaining informed consent, a total of 38 patients (18 females, 20 males) of mean age 49.5 years (range 28-65) satisfying inclusion criteria were enrolled. These patients had a mean duration since sarcoidosis diagnosis of 8.4 years. The baseline characteristics of these patients with respect to the treatment groups are summarized in Table 1. Although all patients were diagnosed as having sarcoidosis, 2 patients also type 2 diabetes mellitus, a condition known to also be associated with SNFLD[12].

Study inclusion criteria required meeting three thresholds: 1) spontaneous pain level ("pain now" of the Brief Pain Inventory) >5 (scale 0-10); 2) small fiber neuropathy screening list score (SFNSL) >22 (out of 84 possible), or pain <5 and SFNSL >37; and 3) pain defined as distal extremity pain plus one of the following: dysesthesia, burning/painful feet worsening at night, or intolerance of sheets/clothes touching the legs or feet. Additional inclusion criteria were: age between 18 to 65 years (inclusive), a body mass index (BMI) between 18 and 30 kg/m^2 (inclusive), and the ability to read and understand the written consent form, complete study-related procedures, and communicate with the study staff. Exclusion criteria were: abnormal blood pressure, history of alcoholism or illicit drug use, positive pregnancy test, refusal to use acceptable contraception throughout the study period (unless surgically sterilized
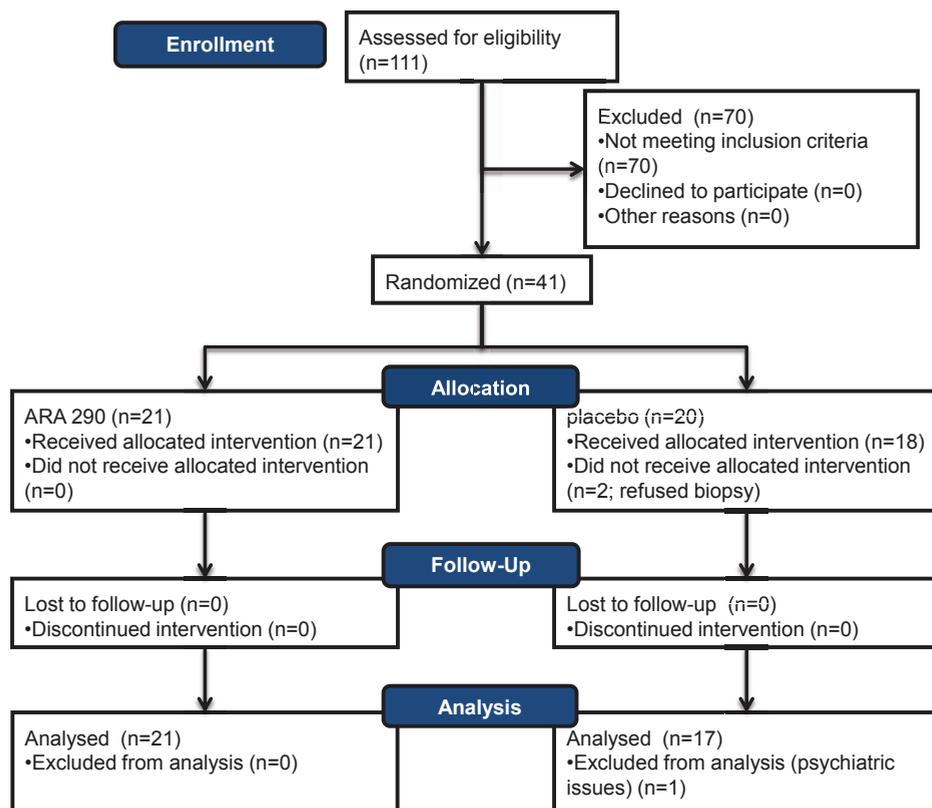
Figure 1: CONSORT flow diagram.

or post-menopausal), vaccination or surgery within the prior 3 months, or use of anti-TNF therapy in the prior 6 months.

Safety was assessed by questioning the patient weekly during ARA 290 administration and throughout the 12 week follow up for the occurrence of adverse events. Additionally, the patients were examined at three occasions during the active treatment phase of the study: baseline, 2 weeks, and 4 weeks at the end of dosing. Additionally, blood was drawn for routine hematology and chemistry at these times points. Finally, serum was obtained for determination of possible anti-ARA 290 antibodies.

Patient Questionnaires

Questionnaires were administered at the screening visit and then weekly during the dosing and follow-up period of 3 months (total 16 weeks). Questionnaire data were also obtained approximately 6 months following the end of the follow up period (i.e., 9 months from end of dosing) to assess durability of any effects. The Brief Pain Inventory Short Form, consisting of pain intensity and pain interference sections, was

Table 1. Baseline Patient Characteristics.

	ARA 290 (n=21)	Placebo (n=17)
Years since diagnosis of sarcoidosis (mean ± SEM)	7.1 ± 1.2	9.9 ± 2.4
Concomitant medical treatment n (%)		
NSAIDS	5 (23.8)	8 (47.1)
Neurological/psychological drugs	5 (23.8)	6 (35.3)
Oral corticosteroids	6 (28.6)	7 (41.2)
Opioids	6 (28.6)	2 (11.8)
Systemic immune suppressants (methotrexate or azathioprine)	7 (33.3)	3 (17.7)
Prior TNF-α antagonist treatment (n=yes)	2 (9.5)	0
SFNSL		
Total score	43.9 ± 2.9	42.8 ± 3.2
Autonomic component	20.6 ± 2.0	20.8 ± 1.5
Pain component	23.3 ± 1.2	22.9 ± 1.2
BPI		
Mean score (Pain now; range 0-10)	5.0 ± 0.4	5.3 ± 0.5
Pain interference (Maximum 70)	32.1 ± 1.9	36.5 ± 2.9
6 Minute Walk		
Test actual (meters)	468 ± 18	479 ± 26
Test predicted (meters)[a]	700 ± 12	683 ± 15
Nerve Fiber Density		
Corneal nerve fiber area (μm^2)	1576 ± 94	1304 ± 104
Normal corneal nerve fiber area[b]	3134 ± 119	
Ankle IENFD (number/mm)	5.3 ± 0.5	4.6 ± 0.4
Normal sex and age adjusted ankle IENFD[c]	9.9 ± 0.3	9.8 ± 0.3
Proximal thigh IENFD (number/mm)	10.8 ± 0.7	11.1 ± 0.9
Normal proximal thigh IENFD[d]	21.1 ± 0.2	21.0 ± 0.1
Laboratory Markers		
High sensitivity C-reactive protein (mg/L)	1.5 ± 0.2	2.9 ± 1.1
Angiotensin converting enzyme[e]	47.4 ± 6.1	53.6 ± 8.0
Number with elevated ACE n (%)	5 (23.8)	6 (35.3)

[a]Predicted 6 minute walk test was calculated using formula from ref[32].
[b]data calculated from[30].
[c]Normal sex-age adjusted ankle IENFD is from ref[26].
[d]Normal proximal thigh IENFD is from ref[27].
[e](normal: 23-67 nmol/min/mL)

administered in the validated Dutch language format. The Small Fiber Neuropathy Screen List (SFNSL) is a questionnaire developed specifically in Dutch patients with sarcoidosis to assess pain and autonomic dysfunction consistent with small nerve fiber loss and damage[21]. In addition to the total score, the questionnaire was divided

into an autonomic component (questions 2-5, 9, 11-16) and a pain component (questions 1, 6-8, 17-21) to assess those dimensions of the patients' neuropathic symptoms.

Quantitative Sensory Testing

Small nerve fiber and large fiber cutaneous sensory function was assessed using Quantitative Sensory Testing of the face, hand, and foot using a Medoc Advanced Medical Systems TSA-II device (Ramat Yishai, Israel), following the published protocol of the German Research Network on Neuropathic Pain[24]. Normative data was

Table 2: Results of baseline quantitative sensory testing. Patients in the ARA 290 and placebo groups showed functional impairment of both small nerve fibers (Aδ and C) as well as larger sensory nerve fibers (Aβ). Data are expressed as number of patients deviating beyond the 95% confidence interval of a sex- and age-matched normal population. Test sites of face, hand, and foot are pooled for calculation of percentages. "Decrease" indicates a loss of function; "Increase" indicates a gain in function compared to a normal population. For example, a decreased CDT means that a patient required a lower temperature stimulus than normal to determine that an object was cold, i.e., a decrease in sensitivity.

Variable	Nerve fibers involved	ARA 290 (n = 21)		Placebo (n = 17)	
		Change	Number of patients (%)	Change	Number of patients (%)
Cold detection threshold	Aδ & C	Decrease	19 (91)	Decrease	11 (65)
Warm detection threshold	Aδ & C	Decrease Increase	17 (81) 1 (5)	Decrease	13 (77)
Thermal sensory limen	Aδ & C	Decrease Increase	4 (19) 2 (10)	Decrease	4 (24)
Paradoxical heat sensation	Aδ	Decrease	8 (38)	Decrease	7 (41)
Cold pain threshold	Aδ & C	Increase	3 (14)	-	0
Heat pain threshold	C	Decrease Increase	3 (14) 5 (24)	Decrease	1 (6)
Mechanical detection threshold	Aβ	Decrease	11 (52)	Decrease	10 (59)
Mechanical pain threshold	Aβ	Decrease Increase	11 (52) 4 (19)	Decrease Increase	4 (24) 2 (12)
Mechanical pain sensitivity	Aβ + C	Decrease Increase	2 (10) 5 (24)	Decrease	1 (6)
Dynamic mechanical allodynia	Aβ	Increase	11 (52)	Increase	3 (18)
Windup ratio	Aβ & C	Increase	4 (19)	Increase	2 (12)
Vibration detection threshold	Aβ	Decrease	20 (95)	Decrease	15 (88)
Pressure pain threshold	Aδ & C	Decrease Increase	3 (14) 10 (48)	Decrease Increase	1 (6) 7 (41)

obtained from Rolke et al.[24]. Baseline data for each patient group is summarized in Table 2. To arrive at the percentages shown in this table, the three regions tested were pooled, i.e., each patient's data was considered abnormal if the results were more than 2 standard deviations from the normative population mean in at least one of the locations evaluated.

Skin Biopsy

Skin biopsies were obtained at baseline and after 28 days from the proximal thigh (20 cm below the anterior superior iliac spine) and the distal leg (10 cm above the lateral malleolus) using a disposable punch biopsy (3 mm) and processed following established guidelines[25]. After fixation of the biopsy specimens, free floating 50 μm thick sections were cut and stained using rabbit anti-protein gene product 9.5 antibody (Dako Netherlands BV, Netherlands) and visualized using a goat anti-rabbit Alexa fluor 488 antibody (Invitrogen, Life Technologies, Grand Island, NY). A minimum of 3 sections selected from each end and the middle of each biopsy specimen were evaluated using a Leica M5500 fluorescence microscope (Leica Microsystems, Rijswijk, The Netherlands), at magnification 1000x. The nerve fibers were counted manually. Images of the sections were recorded using Leica Application Suite, magnification 400x and the length of the epidermal-dermal junction measured using ImageJ (NIH, Bethesda, MD, USA). Sex and age dependent normative data of nerve fiber density used for the distal leg were those of Lauria et al.[26] and for the thigh from Umapathi et al[27]. All measurements and counting was performed by the same individual who was blinded to treatment modality. Technical problems during tissue preparation resulted in the loss of 2 placebo biopsies of the lower leg, one ARA 290 biopsy of the thigh, and three placebo biopsies of the thigh.

Corneal Confocal Microscopy

Corneal nerve fiber density was determined by corneal confocal microscopy carried out using the Rostock Cornea Module with the Heidelberg Retina Tomograph III using established methodology[28]. Briefly, following the application of a topical anesthetic, the sterile objective of the confocal microscope was placed on the apex of the cornea as determined by the characteristic orientation of the nerve fibers in a superior-inferior direction. Using the automatic scan feature of the device, confocal images of graduated depth in the plane of the cornea were acquired. The field of view of each image was 0.4 mm by 0.4 mm. Images containing sensory nerve fibers within the sub-basal layer between Bowman's layer and the basal epithelium were further analyzed. Collected images were subjected to automated analysis employing a custom macro written for FIJI, a public-domain image analysis program, version 1.47e[29]. This macro maps all neurites in the image on the basis of their brightness

and tubeness. The area covered by the mapping is then expressed as a percentage of total image area. For each patient, the ten images with the highest nerve fiber density were averaged to generate a representative sample for that patient for that eye. Since the variation between eyes of different patients was similar to the variation between eyes of individual patients (standard deviation of the mean neurite area between patients=562; standard deviation of the difference between eyes of individual patients=501), each eye was treated as an independent sample. The automated analysis was validated by comparison of 78 randomly selected images in which total neurite length in each image was determined by manually outlining individual neurites. Linear regression analysis showed an excellent goodness of fit (95% confidence interval of the slope: 0.99 – 1.19; R^2=0.76; P<0.0001) between the computer-generated nerve fiber area and the manually measured total nerve fiber length for each image. Both the automated analyses and the manual measurements were performed by a researcher blinded to the treatment modality. The Shapiro-Wilk test showed that at baseline the corneal nerve fiber area data were not distributed normally, therefore non-parametric statistical analysis was performed to determine if a significant treatment effect was observed.

Normative data were calculated from corneal confocal data previously reported[30] obtained from 22 healthy volunteers (M/F: 9/13) age 49 ± 2.7 by determining the mathematical relationship between corneal nerve fiber area and corneal nerve fiber length. The results showed that a normal corneal nerve fiber area is 3134 ± 119 μm^2.

6 Minute Walk Test

The 6 Minute Walk Test (6 MWT), the distance in meters walked in 6 minutes, was conducted following American Thoracic Society guideline[31]. Normal 6 MWT values were calculated using the regression equation developed from data obtained from a healthy, older normal Dutch population by Troosters et al.[32]

Ophthalmologic tests

To assess for possible retinal edema, optical coherence tomography was carried out to quantitate retinal thickness using the Zeiss CIRRUS1 system that includes normative values.

Visual acuity was carried out under standard uniform lighting conditions for patients wearing corrective lenses, if any, using a SLOAN ETDRS chart and scoring system.

Statistical analysis

Statistical analysis was performed using JMP (SAS, Inc, Cary, NC). Parametric and non-parametric tests, linear modeling, and analysis of covariance were carried out where appropriate. P-values<0.05 (two tailed) were considered to be significant.

Results

Safety

No medically significant deviations were noted in the general blood chemistry or hematology assessments. There was no pain or local irritation surrounding the site of the injection into the upper leg or lower abdomen. No serious adverse events were encountered during the dosing period or within the 12 weeks of follow up. Three adverse events judged to be moderate were noted in the placebo group that resolved spontaneously (diarrhea, irritability, and light-headedness). One patient receiving ARA 290 suffered a moderate adverse event consisting of a long term weight loss of 14 kg over several months that stabilized thereafter. Verification of the patient's medical history showed that the weight loss began before entering the study. The etiology of the weight loss was undetermined and persisted after administration of ARA 290 ceased. Multiple, mild adverse events were recorded, all of which spontaneously resolved and none were judged by the investigators as likely to be associated with administration of the study drug. All doses of ARA 290 were administered daily for the full 28 period. One placebo patient suffering from diarrhea discontinued dosing for the last week of the study. No anti-ARA 290 antibodies were detected in any of the post-exposure serum samples.

Primary Endpoints

Nerve Fiber Density
Corneal nerve fibers
The baseline corneal nerve fiber area showed that the patient population exhibited about a 50% reduction compared to normal controls (Figure 2; Table 1). Following 28 days of dosing, the ARA 290 group exhibited a significant increase in the median nerve fiber area over baseline of 14.5%, corresponding to an absolute median increase of 185 μm^2 (P=0.022; Wilcoxon signed rank test). In contrast, the placebo group had a non-significant decrease in median nerve fiber area over baseline of −5.3% and an absolute median decrease of 64 μm^2 (P=0.462). Figure 2C illustrates the corneal nerve density of two normal individuals compared to two ARA 290 patients who showed the best responses.

Intra-epidermal nerve fibers
Similar to the corneal nerve fiber area, at baseline the mean intra-epidermal nerve fiber densities of the proximal and distal leg were significantly reduced by approximately 50% in both treatment groups, compared to the median of age- and sex-matched normal controls (P<0.0001; Table 1). The mean ratio of IENFD of the proximal thigh to the distal leg was 3.9 ± 1.5 SEM, with no patient having a ra-
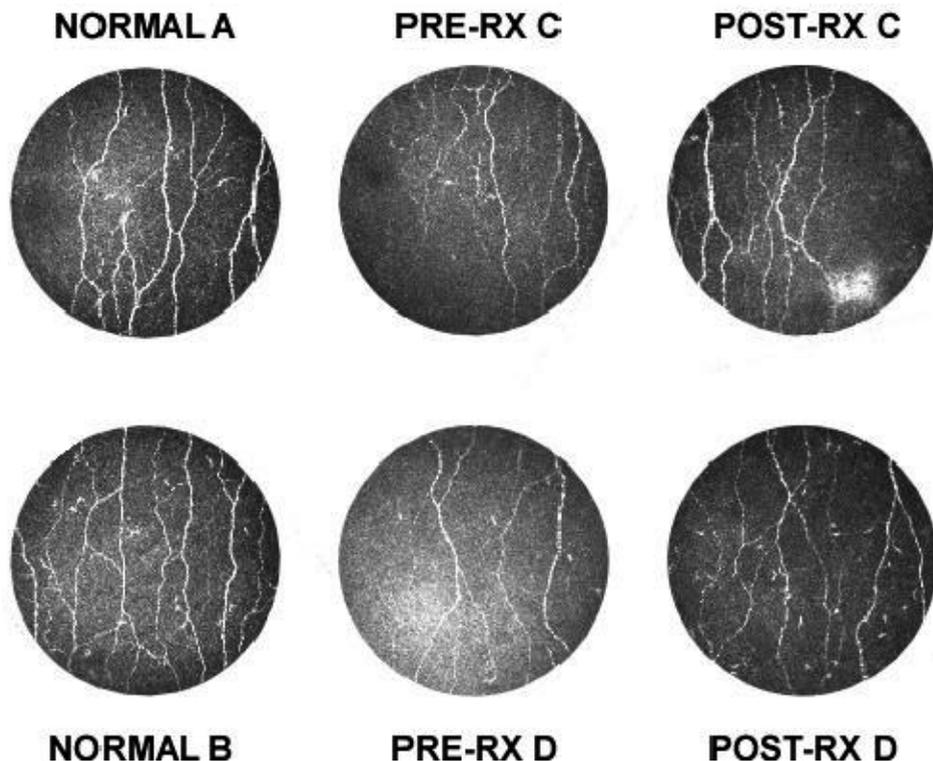
Figure 2: ARA 290 administration is associated with an increase in corneal nerve fiber area. Examples of the distribution and density of corneal nerve fibers obtained via corneal confocal microscopy performed on two normal individuals A and B (Left panels). Examples of corneal nerve density obtained from two sarcoidosis patients show a decreased density at baseline (Middle panel: Pre-RX) and an increase when reimaged after 28 days of ARA 290 administration (Right panel: Post-RX).

tio < 0.9. The patients in this study, therefore, suffered from a peripheral neuropathy characterized by a length-dependent loss of epidermal nerve fibers. IENFD of the proximal leg was not significantly correlated to that of the distal leg (Pearson's correlation coefficient = 0.20; P = 0.22).

Following 28 days of dosing, the ARA 290 group exhibited a mean increase in IENFD in the distal leg of 0.38 ± 0.48 fibers/mm (7.2% of baseline; P = ns), compared to the placebo group with a mean reduction of nerve fiber density of 0.06 ± 0.42 fibers/mm 1.3% of baseline; P = ns). The thigh IENFD at 28 days showed a mean decrease of 0.49 ± 0.53 fibers/mm for the ARA 290 group (–2.3% of baseline; P = ns) and the placebo group had a mean decrease of 1.24 ± 0.88 fibers/mm (–5.7% of baseline; P = ns).

Cutaneous Sensitivity

Baseline QST data showed that as a group the patients with sarcoidosis and painful neuropathy exhibited findings consistent with both small fiber (Aδ and C) and large fiber (Aβ) dysfunction (Table 2). Most patients exhibited a reduced ability to determine cold (CDT; 79% of the study group) or warm temperatures (WDT; 79%), and to detect vibratory stimuli (VDT; 92%). Fifty five percent of the patients also experienced a reduced ability to detect graded mechanical stimuli elicited by von Frey fibers (MDT) or pain caused by graded pin prick (MPT) or to pressure (PPT). A minority of patients in each treatment group also exhibited abnormalities in a variety of the other sensory modalities tested as summarized in Table 2.

Following 28 days of daily dosing, the cold pain threshold (CPT), hot pain threshold (HPT), and the thermal sensory limen (TSL) significantly increased in the ARA 290 group, as illustrated in Figure 3 which summarizes data obtained from the hand testing location. In contrast, there were no changes noted in the placebo group.
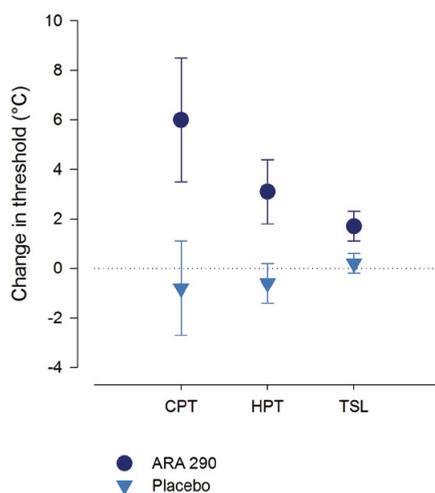

Figure 3: ARA 290 administration increases the threshold for thermal pain and decreases thermal sensitivity in the hand. The cold pain threshold (CPT), heat pain threshold (HPT), and thermal sensitivity limen (TSL) of most patients were within normal limits at baseline (Table 1). Following ARA 290 administration, the mean threshold for determining a painful cold (P = 0.027; paired t test compared to baseline) or hot (P = 0.032) stimulus increased, whereas the placebo group remained unchanged (P = ns). Similarly, the thermal sensory limen (the temperature threshold at which they can discriminate a hot or cold stimulus) increased in the ARA 290 post exposure (P = 0.008). This decreased thermal sensitivity could correspond to reduced symptoms of temperature-induced allodynia. Post ARA 290 treatment, the CPT, HPT, and TSL remained within the normal range. The normative means (in °C) for CPT, HPT, and TSL were 9.7 ± 0.5, 44.8 ± 0.2, and 3.0 ± 0.1 respectively. Similar smaller changes were noted for the face, as well as a non-significant trend for the foot (data not shown).

Although a decreased sensitivity was noted for these sensory modalities, the population means at baseline and after ARA 290 dosing remained within the normal range. Similar, but smaller changes were noted in the face test location, while a non-significant trend was noted at the foot testing site (data not shown). Additionally, the mean cold detection (CDT) and warm detection (WDT) thresholds also decreased (i.e., decreased sensitivity) at the hand and face sites, but the changes were not quite large enough to be statistically significant (data not shown). No changes were observed in any other sensory modality within the QST battery.

Retinal thickness and visual acuity
Baseline average thickness of the macula and central macula, and retinal nerve fibers of both eyes were normal in all patients and did not significantly change over the 28 day observation period (data not shown). Visual acuity at baseline obtained with corrective lenses was normal except for one patient in the ARA 290 group (data not shown). The visual acuity of this patient, and that of all other patients, did not change following ARA 290 exposure.

Secondary Endpoints

Small Fiber Neuropathy Screening List
Baseline scores of the SFNSL developed specifically for sarcoidosis patients showed that the treatment groups were very symptomatic and well-matched with mean baseline values of 43.9 and 42.8 for the ARA 290 and placebo groups respectively (not significantly different; t-test). When evaluated at week 5 (i.e., one week following the end of dosing), the ARA 290 group showed a mean reduction in the SFNSL score of 12.2 ± 1.9 (median of 13.0; ~ 28% reduction from baseline) compared to 3.8 ± 2.1 (median 1.0; ~ 9% reduction from baseline) for placebo (difference between groups: P = 0.005; t test). Construction of proportional responders curves (Figure 4A) showed that the percentage of patients receiving ARA 290 having symptomatic improve-ment in the SFNSL score was greater than the placebo group at each response level. For example, 81% of the ARA 290 patients exhibited at least a 2 point improvement in the SFNSL score, compared to only 47% if patients within the placebo group. This response profile was substantially maintained during 12 week follow up period at which time the mean score reduction from baseline for the ARA 290 group was 9.7 ± 1.8 (median 11.0) and for placebo was 4.1 ± 1.9 (median 3.0; difference between groups: P = 0.037; t-test), in contrast to no significant change for the placebo group. The proportional responder curves at 16 weeks (12 weeks following the end of dos-ing) were similar to that observed immediately following treatment (Figure 4B). Fol-low up at 6 months after the study observation period (i.e., 9 months following the termination of dosing) was possible for 19/21 of the ARA 290 patients and all of the
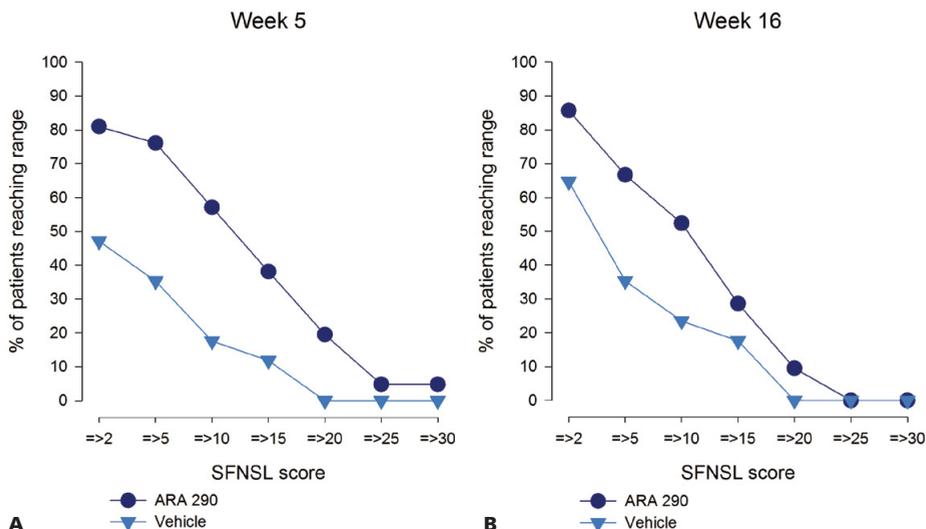
Figure 4: Evaluation of efficacy using the Small Fiber Neuropathy Screening List shows a sustained improvement in the ARA 290 treatment group compared to placebo. A: One week following the end of dosing a larger percentage of patients in the ARA 290 group attained a specified range of score improvement over a broad range of responses. B: This difference was largely maintained at the end of the sixteen weeks of follow-up.

placebo patients and was notable for a mean SFNSL score of 38.1 ± 3.2 SEM versus 43.7 ± 3.2, respectively. This represented a significant improvement over baseline for the ARA 290 group (5.2 ± 1.9) compared to the placebo group (–0.9 ± 2.0; P=0.036). With respect to the autonomic component of the SFNSL, the ARA 290 group demonstrated a significant improvement in the autonomic score when compared to the placebo group with mean improvements of 6.0 ± 1.1 and 1.2 ± 1.3 respectively (P=0.009; t-test). These correspond to a 29% change from baseline for the ARA 290 group compared to a 6% improvement in the placebo group. A significant difference was observed in the pain component with the ARA 290 group having a mean improvement of 6.2 ± 1.1 points (27% of baseline) compared to the placebo group with a mean improvement of 2.6 ± 1.3 points (12% of baseline; P=0.032; t-test).

Brief Pain Inventory
Pain intensity
One week following the last injection (i.e., on day 35), the average Brief Pain Inventory pain intensity score was reduced ~ 9% from baseline in both treatment groups with a mean decrease of –3.4 (out of a maximum of 40). This represented a significant improvement for both the active and placebo arms with respect to baseline (P=0.01; t-test), but with no significant difference between the treatment groups. The individual pain intensity scores were notable for a similar reductions in

"most pain" (−1.2; P=0.003), "average pain" (−1.1; P=0.004), and "pain now" (−1.0; P=0.03), whereas "least pain" did not change from baseline (−0.2; P=0.54).

Pain Interference

In contrast to the mean pain intensity scores that were significantly improved in both groups by week 5, the mean change in the BPI pain interference score differed significantly between the treatment groups by the third week of dosing (P<0.02; Figure 5A). Specifically, while the baseline values of the two groups were not different, the ARA 290 group dropped from a mean score of 32.1 ± 2.3 at baseline to 20.6 ± 2.7 by the week following dosing (a 36% reduction from baseline). This compares to a change in the placebo group from a baseline of 36.5 ± 2.5 to 30.8 ± 3.1 (a 16% change from baseline). Proportional responder analysis (Figure 5B) illustrates that the ARA 290 group exhibited about a 20% greater proportion of responders across the response spectrum up to an improvement total of 20 points. At the time of evaluation 9 months after dosing, 2 patients in the ARA 290 group were lost to follow up. For the remaining patients, the pain interference score did not significantly different from the baseline values. Specifically, the ARA 290 group mean was 30.0 ± 2.2 and the placebo group was 33.9 ± 2.9.

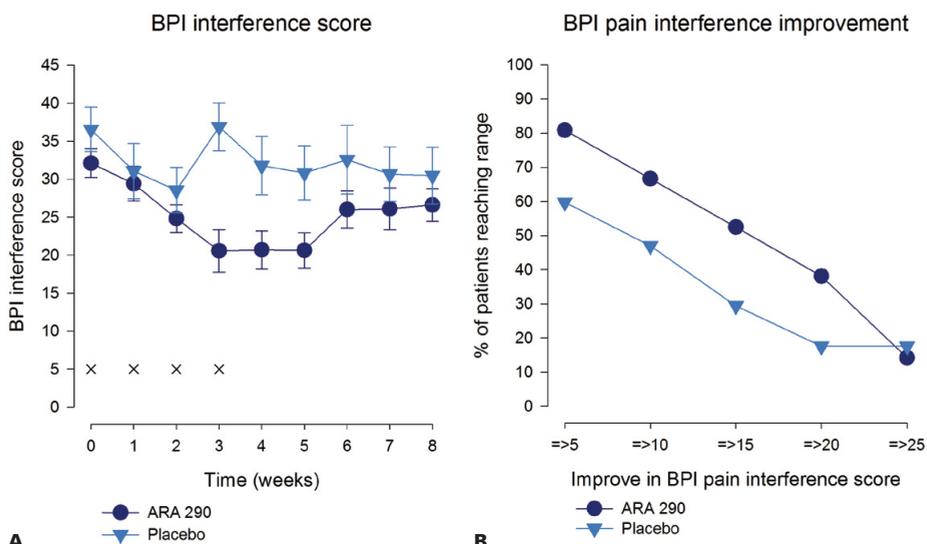**Figure 5:** ARA 290 treatment improves the Brief Pain Inventory pain interference score. A: Weekly pain interference scores significantly decline over the 4 weeks of daily dosing for the ARA 290 compared to the placebo group. X=treatment with either ARA 290 or vehicle. B: A proportional responder display illustrates that the ARA 290 group responded to a larger extent at all levels of improvement.

Six minute walk test (6 MWT)

The 6 MWT is a measure of functional exercise capacity. Both groups had approximately the same baseline 6MWT distance (Table 1), that was significantly less than normal. Using a normative prediction formula for a normal population with the same approximate age spread[32], the patients in this study at baseline exhibited a mean reduction of 219 meters (P<0.0001; 95% confidence interval of −186 to −253 meters) in the actual distance walked in 6 minutes from a predicted value of 693 meters. Following 28 days of daily dosing, the 6 MWT showed that the ARA 290 group increased the distance walked by a mean of 18.7 meters, whereas the placebo group's performance fell by a mean of −15.1 meters (difference between groups: P=0.049; t test). A proportional responder analysis (Figure 6) illustrates that about half of the patients in both treatment groups had an improved their 6 minute walk distance by up to 12 meters. However, for an increase from greater than 25 meters, only 12% in the placebo group improved, compared to 52% of the ARA 290 group. Substantial percentages of the ARA 290 group exhibited even larger increases in the 6 minute walk distance, whereas none of the placebo patients did. A 6 MWT was repeated at 9 months following dosing (2 ARA 290 patients and 1 placebo patient were lost to follow up). The mean change from baseline in the ARA 290 group was 8.3 ± 13.3 meters and for placebo was −12.9 ± 13.9, neither of which constituted a significant change from baseline (P=ns; t test).
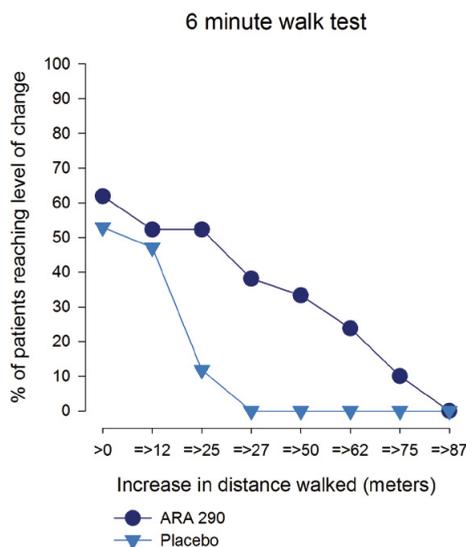

Figure 6: ARA 290 increases the distance patients can walk in 6 minutes. Similar to the results of symptom questionnaires, patients receiving ARA 290 performed better at all levels of response in the 6 Minute Walk Test.

Discussion

Sarcoidosis complicated by small fiber neuropathy is a chronic disease character-ized by the loss of small nerve fibers with associated pain, decreased temperature sensitivity, thermal allodynia, and pronounced autonomic dysfunction that severely degrades quality of life. All patients included in this trial had painful neuropathic symptoms consistent with SFN that were unresponsive to the standard therapies for chronic sarcoidosis that they had received and many continued on immune suppres-sion and symptom-directed therapy throughout the trial.

The principal hypothesis to be tested in this study was whether exposure to ARA 290, a molecule demonstrating tissue protective, anti-inflammatory, and reparative activities in numerous preclinical models, would stimulate nerve fiber regrowth with associated improvements in pain and other sensory symptoms, and autonomic function. To accomplish this, the trial was designed to focus on the assessment of objective endpoints such as small nerve fiber quantification using both skin biopsy and corneal confocal microscopy and to relate these findings to semi-objective sen-sory testing using QST which directly assesses the effects of potential changes in cutaneous innervation. The 6 MWT was also included as a simple semi-objective test that requires the integration of complex sensory stimuli of the lower limbs and good exertion by the patient. Finally, patient reported outcomes were included for sub-jective assessments of pain and the degree to which pain interfered with activities of daily living, as well as symptoms of autonomic dysfunction which could also be potentially related to changes in nerve fiber density.

Baseline nerve fiber data from this study have been analyzed[30] and these show that corneal nerve quantification (density and length) correlates well with the IENFD of the distal, but not the proximal lower limb when adjusted for the covariates of gender and age. Further, at baseline the corneal nerve fiber density (and length) is inversely related to the BPI pain interference score and therefore has relevance for the symptoms that the patients report. Previous work performed in patients with diabetes has also shown a good correspondence between corneal nerve quantifica-tion and nerve fiber counts performed in the distal leg[33], thereby confirming the usefulness of corneal nerve assessment in patients with symptoms of small fiber neuropathy.

The results of nerve fiber assessment following 28 days of dosing show that the corneal nerve fiber density improved significantly in the ARA 290 group when compared to the placebo group at the end of 28 days of dosing. In contrast, no change was observed in the IENFD obtained from the proximal thigh, although a trend was observed for the distal leg biopsy site. Notably, a recent study carried out in a diabetic population has reported positive effects of treatment on corneal nerve

fiber density, although over a longer time scale with no change in the skin biopsy nerve density of the distal extremity[33]. In this study, patients with type 1 diabetes were followed after curative therapy by pancreas transplantation. Twelve months (but not 6 months) after normalization of blood glucose concentrations, a significant increase in corneal nerve fiber density was documented, whereas no changes were observed in the IENFD of the distal leg or in the results of Quantitative Sensory Testing. Additionally, Boyd et al.[34] were able to demonstrate a change in skin biopsy nerve densities following drug administration. These investigators studied type 2 diabetic patients with small fiber neuropathy following 12 weeks of administration of the anti-epileptic drug topiramate and documented an increase in cutaneous nerve fiber length at multiple biopsy sites and in nerve fiber density in the proximal leg. It would be of interest to know what assessment of the corneal nerve fibers would have shown.

Prior study[35] of re-innervation following experimental denervation using capsaicin application to the skin of diabetics with neuropathy or normal individuals has shown that the natural rate of regrowth of sensory nerve fibers is slow in normal individuals and very slow in patients with diabetes. In contrast, regrowth of autonomic fibers is appreciably faster (40-50 days to return to baseline density) than sensory fibers (140-160 days for normalization)[36]. Similar experiments have not been performed on corneal nerve fibers, but the results of a preclinical model shows that rapid regeneration (days to weeks) occurs following mechanical injury[37]. It is possible that the cornea is an especially useful location to evaluate potential nerve regrowth. Corneal confocal microscopy has the benefit that it is a non-invasive technique that can be repeated many times in the same patient and thus is well-suited for longitudinal interventional studies.

As a group, QST showed that the majority of the patients in this study had significantly increased cold, warm, and vibratory detection thresholds. For patients with sensitivity to cold or heat, this could translate into less pain during activities of daily living. Previous study of patients with diabetic neuropathy has reported similar findings in patients that specifically complained of pain[38]. Since thermal sensory function depends upon small fiber function, the admission criterion of neuropathic pain may have specifically selected patients that possess a high degree of fiber loss. This possibility was confirmed by the intra-epidermal and corneal nerve fiber assessments that showed a marked reduction in the mean number of small fibers innervating cutaneous and corneal sites compared to a normal population.

It is currently unclear what sensory changes may be associated with the axon regeneration that occurs during the short time frame of this clinical trial, as the results of few relevant studies have been reported. Clinical studies performed using nerve growth factor (NGF) show that a single injection into normal individuals produces

both mechanical and thermal hypersensitivity at the site of injection which is rapid, reaching a maximum by 21 days and 3 days respectively[39]. Hypersensitivity has been observed at injection site in longer term clinical trials with repeated injections carried out on patients with neuropathy, e.g., diabetic polyneuropathy[40]. As mentioned above, no injection site pain was noted following ARA 290 administration in the current study. The observation of reduced thermal thresholds associated with ARA 290 that occur at several sites examined that were remote to the injection site are suggestive a predominantly central effect in contrast to the peripheral effect previously observed for NGF.

On the basis of preclinical work it also appears that changes in responsiveness may occur within the time frame of the present clinical study. Tanelian and Scott[37] studied a rabbit model in which they produced corneal nerve fiber injury by a small punch biopsy and subsequently used electrophysiological methods to directly determine the behavior of regenerating small nerve fibers to cold stimuli. Their findings document electrophysiological changes that returned to normal by 30 days after injury. If similar changes occur in patients during the early period of regrowth, we would expect to observe changes in thermal thresholds to the extent that axon sprouting has occurred. However, no assessments were carried out during the period of dosing that can provide relevant information. Additionally, the questionnaires administered do not provide information that is helpful in determining thermal sensory thresholds. It will be important to add these assessments in future trials. However, it is highly likely that any changes that might occur in the sensory system as a result of effects of 28 days of dosing with ARA 290 would not have reached a steady state.

The results of this study show that ARA 290 administration to patients with painful small fiber neuropathy is associated with a significant improvement in patient-reported symptoms, compared to patients receiving placebo, without any evident adverse events attributable to the drug. The changes in level of discomfort as assessed by the SFNSL following 4 mg ARA 290 administered daily SC was remarkably similar to what was observed in the previous blinded trial in which 2 mg ARA 290 was administered three times weekly by the IV route[20]. In the prior trial, approximately 80% of the patients in the active arm exhibited some improvement and ~ 40% showed improvement of ~ 50% over baseline. In contrast, while about 45% of the patients in the placebo arm showed some improvement, only ~ 12% showed a 50% improvement. Daily administration of 4 mg ARA 290 administered subcutaneously was well tolerated without any evident adverse effects. Also similar to the previous blinded trial, a large proportion of the change in SFNSL score was attributable to questions that are relevant to autonomic symptoms. Finally, it is remarkable how sustained the response to ARA 290 appears to be. This may reflect the growth of small nerve fibers as the corneal confocal nerve fiber data reveals.

Self-assessment of pain intensity using the BPI showed that similar to the first blinded trial[20], both groups improved equally, indicating a significant placebo effect on this dimension. In contrast, assessment of to what extent the level of pain interfered with activities of daily living, mood, and enjoyment of life showed that patients that received ARA 290 had an immediate reduction in mean score reaching a nadir that was significantly different from placebo by the end of the dosing period. This result suggests that ARA 290 is having a complex activity that extends beyond the sensation of pain to include effects on activities of daily living.

The 6 minute walk test was originally developed to assess functional exercise capacity (i.e., a measure of the ability to engage in physically demanding activities) in patients with chronic cardiopulmonary diseases. Since its introduction, the 6MWT has been used to evaluate functional capacity in a wide range of diseases and in healthy normal individuals[32] and has been used as a means to assess the effects of therapeutic interventions. Studies evaluating patients with chronic sarcoidosis have observed that about 50% of these patients have a markedly impaired baseline 6MWT[22,41]. In the current study, we found that all of the patients had a reduction in expected walk distance, some very severe. The reason for the higher prevalence in this patient population is not clear, but could arise from the fact that the patients were selected for the presence of neuropathic symptoms which involved the feet, which could contribute to a poor performance on a walk test due to sensory deficits and pain.

At the end of dosing, the ARA 290 group had improved a mean of ~19 meters while the placebo group had declined by ~15 meters, about a 4% improvement and 3% decrease of baseline respectively. Although only about half of the patients improved in both groups (Figure 6), the improvement in distance walked in the 6MWT was limited in the placebo group to less than 37 meters, whereas almost one quarter of the ARA 290 patients improved the distance walked by up to 75 meters. A minimally clinically significant difference (MCSD) has not been established for sarcoidosis patients with painful neuropathy, but for patients with cardiopulmonary disease, the MCSD has been determined to be as low as 25 meters[42].

The most prevalent form of SFN occurs in patients with pre-diabetes or diabetes, and in this group retinal edema and visual acuity changes are very common. Additionally, another major clinical manifestation of chronic sarcoidosis is ocular inflammation, especially uveitis that often affects the retina[2]. Alternatively, a recent study has shown that patients with neurosarcoidosis frequently have macular edema even in the absence of ocular symptoms[43]. It was of interest, therefore, to evaluate retinal thickness and visual acuity pre- and post-dosing. At baseline, there were no significant abnormalities observed in the optical coherence tomographic evaluation of either retinal or optic nerve head thickness. Similarly, almost all patients had good

visual acuity at baseline. Therefore, retinal abnormalities and visual acuity impairment do not appear to be a common feature of sarcoidosis complicated by SFN.

The patients included in this trial all had longstanding sarcoidosis with mean time of diagnosis of 8.3 years. They all had failed existing therapy for neuropathy including the use of anti-inflammatory agents (NSAIDs, glucocorticoids, and methotrexate principally), as well as anti-epileptics and antidepressants. About 30% of the patients were using a variety of these drugs during the conduct of this trial. Due to the small numbers of patient studied it is not possible to evaluate synergistic effects with any of these agents. It will be interesting to assess for this possibility in future trials with ARA 290.

The principal limitations of this study are that only patients with pain were studied and these patients did not have known active sarcoid involvement of any other organ. Circulating markers of inflammation were not significantly elevated and presumptive markers of active sarcoidosis, e.g., angiotensinogen converting enzyme levels, where only mildly increased in a minority of patients. Small nerve fiber loss is also well known to occur without associated painful symptoms, e.g., in the prediabetic state[44]. It will be of interest to determine whether corneal nerve fiber density is also abnormal in this patient group.

In conclusion, ARA 290 is the first drug that exhibits the ability to induce small nerve fiber regeneration in the cornea without serious side effects, showing a potential of true disease modification, not just symptom improvement. In addition, this trial design using the combination of objective and subjective endpoints offers insight into correlations with patient reported outcomes, and may provide a blueprint for superior trial design for future pain studies. Most importantly, the results of this study can provide some hope for sarcoidosis patients suffering from small nerve fiber loss and damage that ARA 290 could substantially improve their quality of life.

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Chapter 9

Summary and conclusion

Summary

Neuropathic pain is a disabling disease with a mechanism consisting of several path-ways that ultimately converge in the development and persistence of pain. Hallmark symptoms are tactile and cold allodynia: mechanical and thermal stimuli that are not painful in healthy individuals, but that are perceived as painful in patients. Pharma-cological treatment is often inadequate and coincides with intolerable side effects. New treatments are arising that may be able to target neuropathic pain more ef-ficiently, one of which is the 11-amino acid tissue protective peptide ARA 290. This erythropoietin (EPO) derived peptide is devoid of hematopoietic side effects, such as the formation of erythrocytes, but it has anti-inflammatory properties and promotes cell survival and regeneration of various tissue types, including neuronal tissue. In chapters 2 through 4, we employed a spared nerve injury model (SNI) of chronic neuropathic pain, suitable for evaluating the effect of ARA 290 on behavioral and cellular responses after nerve injury.

In Chapter 2, we elaborated on how to induce the SNI in the rat to generate chronic neuropathic pain and how to quantify tactile and cold allodynia by providing a stepwise and detailed summary on the surgery and the behavioral tests. In this par-ticular procedure we accessed the sciatic nerve (the large nerve running in the thigh, responsible for motor function and sensibility of the hind limbs) by blunt prepara-tion, rather than making an incision through the muscle that covers the nerve as described in the original article of the model, thereby reducing collateral damage. Next, we described how to put this model to use for evaluation of neuropathic pain. The quantification of tactile allodynia was described by using a standardized method of measuring the withdrawal response to stimulation of the hind paw by Semmes-Weinstein monofilaments. Cold allodynia was quantified by assessing the withdrawal response to a spray of acetone on the hind paw. We showed that the SNI model was able to induce long standing neuropathic pain in the rat, making it suitable for evaluating chronic neuropathic pain. Finally, we assessed the effect of ARA 290 on neuropathic pain, of which the original results were published as a part of the research paper discussed in Chapter 3.

In Chapter 3, we assessed the potential of ARA 290 in the relief of allodynia follow-ing spared nerve injury. We showed that a 10 day regimen in which 5 administra-tions of 30 µg/kg ARA 290 were given, followed by a maintenance treatment of once per week, starting at 24 hours post lesion provided a long term relief of both tactile and cold allodynia when compared to vehicle treated animals (treatment effect P<0.001). This effect was superior to treating animals for 10 days without maintenance. Additionally we found that the induction of an unilateral nerve injury resulted in the decrease of the applicable force to the contralateral hind paw as

well, i.e. tactile allodynia. This effect was attenuated by either regimen of ARA 290 (P<0.001). Contra lateral cold allodynia was observed to a small extent. Next, we assessed the effect of ARA 290 in mice devoid of the β-common-receptor (βcR), which is the receptor that couples with the EPO receptor to establish the tissue protective effects of EPO. Mice devoid of the βcR developed both cold and tactile allodynia after SNI and treatment with ARA 290 did not provide relief of their neuropathic pain. ARA 290 produces long-term relief of allodynia because of activation of the βcR. It is argued that relief of neuropathic pain attributable to ARA 290 treatment is related to its anti-inflammatory properties, possibly within the central nervous system. Because ARA 290, in contrast to erythropoietin, is devoid of hematopoietic and cardiovascular side effects, ARA 290 is a promising new drug in the prevention of peripheral nerve injury induced neuropathic pain in humans.

In Chapter 4, we established a dose-response curve for ARA 290 for doses 0, 3, 10, 30 and 60 µg µg/kg. While animals treated with 0 µg/kg ARA 290 showed a rapid increase in tactile allodynia following SNI, this was attenuated by treating with ARA 290 for the doses 30 (P=0.049) and 60 µg/kg (P=0.001), lasting up to 20 weeks postoperative. The reduction of cold allodynia was significant up to 20 weeks postoperative for all tested doses when compared to vehicle (P<0.05). The effect of 0, 10 and 30 µg/kg ARA 290 administered on days 1, 3, 6, 8 and 10 on microgliosis (Iba-1-immunoreactivity) and astrocytosis (GFAP-immunoreactivity) was investigated in animals surviving 2 or 20 weeks following lesion or sham surgery. After 2 weeks of survival, a significant microgliosis was observed in the L5 segment of the spinal cord of animals treated with 0 µg/kg ARA 290 when compared to sham operated (P<0.05), while animals treated with 10 or 30 µg/kg did not show this microgliosis. After 20 weeks of survival, a more widespread and increased microgliosis was ob-served for animals treated with 0 and 10 µg/kg when compared to sham operated animals, indicated by involvement of more spinal cord segments and higher Iba-1-immunoreactivity. Animals treated with 30 µg/kg did not show increased microgliosis when compared (P<0.05). No difference in GFAP-immunoreactivity was observed. The erythropoietin-analogue ARA 290 dose-dependently reduces allodynia and sup-presses microgliosis in the dorsal horn, which is part of the mechanism of action of ARA 290 in producing relief of allodynia following peripheral nerve damage.

The before mentioned effects of ARA 290 closely resemble a more conventional drug that has been on the market for over 50 years and has been widely used as an anesthetic and analgesic for acute pain: ketamine. In subanesthetic doses, this drug has shown to be effective in relieving neuropathic pain with a pharmacodynamic effect that exceeds its pharmacokinetic half life. Treatment with ketamine is accom-panied with psychomimetic side effects, such as psychosis, hallucinations, nausea and vomiting. It is unclear, however if the anti-neuropathic pain effect of ketamine is

contributed to by ketamine itself, or its active metabolite norketamine. Additionally, NMDA receptor antagonists that are devoid of side effects are being developed. In Chapter 5, we evaluated three NMDA receptor antagonists in the treatment of acute and neuropathic pain, as well as the severity of the side effects, or lack thereof.

In Chapter 5, we evaluated the NMDA receptor antagonists ketamine, norketamine and Traxoprodil in a rat model of acute antinociception (paw-withdrawal response to heat at increasing doses of drug), and a model of chronic neuropathic pain (spared nerve injury). Side effects (typical behavior, activity level) were scored and locomotor function of the nerve-injured paw was assessed using computerized gait analysis. In the chronic pain model, treatment was given 7 days following surgery, for 3-h on 5 consecutive days. All three NMDA receptor antagonists caused dose-dependent antinociception in the acute pain model and relief of mechanical and cold allodynia for 3-6 weeks following treatment in the chronic pain model (P<0.001). In both tests, ketamine was most potent with norketamine 1.5-2-times less potent and Traxoprodil 5-8 times less potent than ketamine. Nerve-injury caused the inability to use the affected paw that did not improve after treatment (ketamine and Traxoprodil) or only showed a limited effect (norketamine for all 3 parameters, P<0.05). Traxoprodil but not ketamine or norketamine, showed a clear separation between effect and side effect. The observation that Traxoprodil causes relief of chronic pain outlasting the treatment period with no side effects during treatment makes it an attractive alternative to ketamine in the treatment of chronic neuropathic pain.

Both ARA 290 as the NMDA receptor antagonists ketamine, norketamine and Traxo-prodil prove to be efficient in relieving both tactile and cold allodynia in the SNI model. Additionally, a relatively short treatment paradigm with either type of drugs resulted in a long-term relief of allodynia. In Chapter 6, we compared the effects of ARA 290 and ketamine on spinal cord expressions of NMDA receptor subunits and inflammatory markers. Additionally we assessed the effects on acute and neuro-pathic pain and side effects in similar treatment regimens in the SNI model in both wild-type and $\beta cR^{-/-}$ mice.

In Chapter 6, the overlapping pathways of ARA 290 and ketamine were examined by comparing their effects on the mRNA expression of the NMDA receptor subunits NR1, NR2A and NR2B, inflammatory markers Iba-1 (microglia), GFAP (astrocytes) and chemokine (C-C motif) ligand 2 (CCL-2). We found that that both ketamine and ARA 290 exerted similar effects by significantly decreasing NMDA receptor subunit mRNA expression, as well as that of microglia, astrocytes and CCL-2, all-important con-tributors to the development of neuropathic pain. Although the effects of ketamine and ARA 290 on neuropathic pain and its molecular mediators suggest a common mechanism of action, ARA 290 acts specifically via the innate repair receptor (IRR) involved in tissue protection, and has no affinity for the NMDAR. We speculated

therefore, that the IRR might be critically involved in the action of ketamine on neuropathic pain. To evaluate this, we studied the effects of ketamine and ARA 290 on acute pain, side effects, and allodynia following a spared nerve injury model in mice lacking the β-common receptor (βcR), a structural component of the IRR. Ketamine (50 mg/kg) and ARA 290 (30 μg/kg) produced divergent effects on acute pain: ketamine produced profound antinociception (P<0.001 versus vehicle and ARA 290) accompanied with psychomotor side effects (P<0.001 versus vehicle and ARA 290), but ARA 290 did not, in both normal and βcR$^{-/-}$ mice. In contrast, while both drugs were antiallodynic in wild-type mice (P=0.049 and P=0.03 versus vehicle for ketamine and ARA 290, respectively), they had no effect on neuropathic pain in mice lacking the βcR. Together, these results show that an intact IRR is required for the effective treatment of neuropathic pain with either ketamine or ARA 290, but is not involved in ketamine's analgesic and side effects.

Pain is a subjective outcome that can be measured by numerical rating scales (NRS), or questionnaires that address specific modalities correlated to, for instance, small fiber neuropathy (such as the small fiber neuropathy screening list, SFNSL). Due to this subjectiveness, however, it is not a fully reliable measurement for diagnosing small fiber neuropathy (SFN), due to the inter and intra personal variability. Therefore, small fiber neuropathy is being diagnosed by invasive method of intra-epidermal nerve fiber density evaluated with (fluorescence) microscopy, which is the gold standard for the diagnosis of SFN. The skin, however, is not the only organ that has superficial small nerve fibers. The cornea has a high density of small nerve fibers that can be evaluated by the non-invasive method of corneal confocal microscopy.

In Chapter 7, we showed that corneal confocal microscopy (CCM) is an objective measure for neuropathic pain in sarcoidosis patients with symptoms of SFN that correlates to the symptoms patients report. Pain reported by patients with sarcoidosis was assessed by the brief pain inventory (BPI) and quantified by quantitative sensory testing (QST). The majority (~80%) of sarcoidosis patients showed altered (>2 standard deviations below the mean of healthy individuals) thresholds for all thermal thresholds in QST, indicative of SFN. Currently, a definitive diagnosis of SFN requires a skin biopsy that demonstrates small nerve fiber loss. However, quantifying IENFD in skin biopsies is an invasive, labor-intensive process that has a low sensitivity for diagnosing SFN and does not correlate with the pain that patients report. Alternatively, CCM is a rapid non-invasive clinical ophthalmic technique for in vivo imaging of corneal nerve fibers. CCM revealed that the mean corneal nerve fiber density (CNFD) and corneal nerve fiber length (CNFL) was significantly decreased in sarcoidosis patients when compared to healthy individuals (P<0.0001 for both outcomes). The IENFD was decreased in sarcoidosis patients when compared to healthy controls (P<0.0001). Additionally, we found that both CNFD and CNFL, but

not IENFD, had a negative correlation with the pain interference score from the BPI (P=0.0005 and P=0.012). Finally, a linear model of CNFL as the dependent variable accurately predicted BPI interference (P<0.0001). This technology expands the role of CCM as a surrogate marker for both nerve fiber damage and pain in clinical trials of novel therapeutics in sarcoid and perhaps other small fiber neuropathies.

Finally, in Chapter 8, we evaluated the effect of ARA 290 on nerve fiber loss and corneal nerve fiber density in sarcoidosis patients in a double-blind-randomized clinical study. Small nerve fiber loss and damage (SNFLD) is a frequent complication of sarcoidosis that is associated with autonomic dysfunction and sensory abnormalities, including pain syndromes that severely degrade the quality of life. SNFLD is hypothesized to arise from the effects of immune dysregulation, an essential feature of sarcoidosis, on the peripheral and central nervous systems. Current therapy of sarcoidosis-associated SFNLD consists primarily of immune suppression and symptomatic treatment which, however, is typically unsatisfactory. Here we show that 28 days of daily subcutaneous administration of ARA 290 in a group of patients with documented SNFLD significantly improves neuropathic symptoms. With QST we showed that the thermal sensory thresholds (cold pain threshold, P=0.027 and heat pain threshold, P=0.032) and thermal sensitivity (thermal sensory limen, P=0.008) were increased after treatment with ARA 290, while these parameters were unchanged after placebo treatment. Patient reported symptoms improved for the small fiber neuropathy screening list (SFNSL) that lasted up to 16 weeks after the start of treatment (P=0.037). The brief pain inventory (BPI) also showed improved pain management, but the ARA 290 treatment group did not differ from the placebo treatment group. Notably, the BPI pain interference score differed significantly in the third week of dosing between the ARA 290 treatment group and the placebo group (P=0.02). In addition to improved patient-reported symptom based outcomes, ARA 290 administration was also associated with a significant increase in corneal small nerve fiber density (P=0.022 for ARA 290 versus P=0.462 for placebo), and an increased exercise capacity as assessed by the 6 minute walk test (6MWT) on the final day of dosing (P=0.049). On the basis of these results and of prior studies, ARA 290 is a potential disease modifying agent for treatment of sarcoidosis-associated SNFLD.

Conclusion

The data collected in this thesis show that:
- ARA 290 is effective in relieving neuropathic pain after nerve injury and requires the β-common-receptor

- A part of the mechanism of the relief of neuropathic pain of ARA 290 is through suppression of microglia in the dorsal horn of the spinal cord
- Astrocytes are not crucial for neuropathic pain states at 2 and 20 weeks postoperative
- Ketamine, its active metabolite norketamine and the NR2B selective N-methyl-D-aspartate receptor antagonist Traxoprodil are effective in relieving both acute and neuropathic pain
- The NR2B subunit of the N-methyl-D-aspartate receptor is not involved in the induction of side effects by N-methyl-D-aspartate receptor antagonists
- Ketamine and ARA 290 have overlapping pathways in the relief of neuropathic pain by suppression of spinal cord inflammation
- The β-common-receptor is pivotal in the treatment of neuropathic pain, but not in acute pain
- Sarcoidosis patients have decreased nerve fiber densities in both the epidermis and the cornea
- Corneal confocal microscopy, but not intraepidermal nerve fiber density is related to patient reported symptoms in sarcoidosis patients with small fiber neuropathy
- Treatment of sarcoidosis patients with symptoms of small fiber neuropathy with ARA 290 results in improvement of pain related outcomes
- Treatment of sarcoidosis patients with symptoms of small fiber neuropathy with ARA 290 results in an increased nerve fiber density in the cornea, but not in the epidermis

Chapter 10

Samenvatting en conclusie
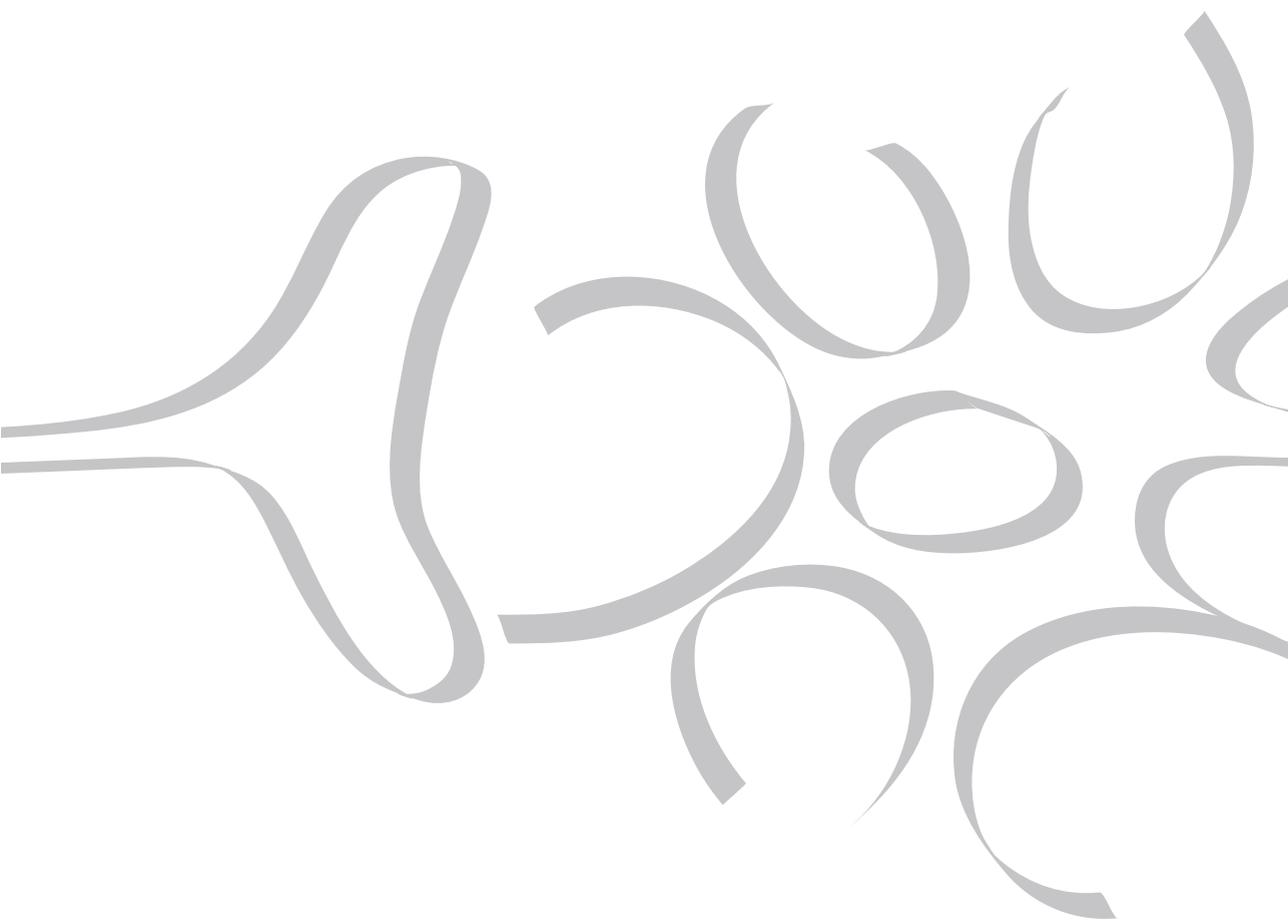
Samenvatting

Neuropathische pijn is een invaliderende ziekte met een mechanisme dat bestaat uit verschillende "pathways", die uiteindelijk convergeren in het ontstaan en onderhouden van pijn. Karakteristieke symptomen zijn tactiele en koude allodynie: mechanische en thermale stimuli die niet pijnlijk zijn in gezonde individuen, maar als pijnlijk worden door patiënten. Farmacologische behandeling is vaak ontoereikend en gaat gepaard met onverdraagbare bijwerkingen. Nieuwe behandelingen worden ontwikkeld die neuropathische pijn op een effectievere manier kunnen bestrijden, waarvan het uit 11 aminozuren bestaande peptide ARA 290 er een is. Dit peptide afgeleid van erythropoietine (EPO) heeft geen hematopoietische bijwerkingen zoals de aanmaak van erytrocyten, maar het heeft wel anti-inflammatoire eigenschappen en het stimuleert celoverleving en regeneratie van verscheidene soorten weefsel, waaronder zenuwweefsel. In hoofdstuk 2 tot en met 4 hebben we het "spared nerve injury" voor chronische neuropathische pijn gebruikt om het effect van ARA 290 te testen op gedragsmatig en cellulair niveau na zenuw schade.

In Hoofdstuk 2 beschreven we hoe het SNI model te induceren in de rat om zo chronische neuropathische pijn te genereren en tactiele en koude allodynie te kunnen kwantificeren door een stapsgewijze gedetailleerde beschrijving te geven van de operatie en de gedragsmatige testen. In deze specifieke procedure benaderden we de nervus ischiadicus (de grote bovenbeenzenuw die zorgt voor de motoriek en het gevoel van de achterpoten) door middel van stompe preparatie in plaats van deze te benaderen door een snede te maken in de spier die deze zenuw bedekt, zoals beschreven staat in het originele artikel dat dit model beschrijft, waardoor bijkomende schade beperkt bleef. Vervolgens beschreven we hoe dit model gebruikt kon worden voor het evalueren van neuropathische pijn. Het kwantificeren van tactiele allodynie werd beschreven met het gebruik van een gestandaardiseerde methode van het meten van de terugtrekrespons bij stimulatie van de achterpoot met Semmes-Weinstein monofilamenten. Koude allodynie werd gekwantificeerd door middel van het meten van de terugtrekrespons ten gevolge van een spray aceton op de achterpoot. We lieten zien dat het mogelijk was door middel van het SNI model om langdurig aanwezige neuropathische te induceren, waardoor dit model geschikt was om chronische neuropathische pijn te vervolgen. Tenslotte onderzochten we het effect van ARA 290 op neuropathische pijn, de resultaten werden gepubliceerd als onderdeel van het onderzoeksartikel beschreven in Hoofdstuk 3.

In Hoofdstuk 3 hebben we de effectiviteit van ARA 290 om allodynie na spared nerve injury the verlichten onderzocht. We vonden dat een doseerschema van 10 dagen waarin 5 toedieningen van 30 µg/kg ARA 290, beginnend 24 uur na de lesie, een langdurige verlichting gaf van zowel tactiele als koude allodynie wanneer dit

werd vergeleken met een placebo behandeling (behandeleffect P<0.001). Dit effect was superieur vergeleken met een 10-daagse behandeling zonder onderhoudsbe-handeling. Daarbij vonden we dat het induceren van een unilaterale zenuwlesie resulteerde in een afname van de uit te oefenen kracht op de contra laterale ach-terpoot (tactiele allodynie). Ook dit effect was verminderd door een behandeling met ARA 290, hetzij met of zonder onderhoudsdosering (P<0.001). Contra laterale koude allodynie werd niet geobserveerd. Vervolgens bestudeerden we het effect van ARA 290 in muizen welke geen β-common-receptor (βcR) hebben, de receptor welke koppelt met de EPO receptor om de weefselbeschermende effecten van EPO te bewerkstelligen. Muizen zonder de βcR ontwikkelden zowel tactiele als koude allodynie na SNI en behandeling met ARA 290 resulteerde niet in de verlichting van neuropathische pijn. ARA 290 bewerkstelligt langdurige verlichting van allodynie door activatie van de βcR. Het verlichtende effect van ARA 290 zou het effect kun-nen zijn van de anti-inflammatoire eigenschappen van dit middel, mogelijk binnen het centrale zenuwstelsel. Omdat ARA 290, in tegenstelling tot EPO, geen hemato-poietische en cardiovasculaire bijwerkingen vertoont, is dit een veelbelovend middel in de behandeling van perifere neuropathische pijn in mensen.

In Hoofdstuk 4 construeerden we een dosis-respons curve voor de doses 0, 3, 10, 30 en 60 μg/kg ARA 290. Dieren behandeld met 0 μg/kg ARA 290 vertoonden een snelle toename van tactiele allodynie door SNI, dat werd verminderd door behandeling met 30 (P=0.049) en 60 μg/kg (P<0.001), durend tot tenminste 20 weken na de operatie. De reductie van koude allodynie was significant tot temminste 20 weken na de operatie voor alle geteste doses (P<0.05) wanneer vergeleken met 0 μg/kg. Het effect van 0, 10 en 30 μg/kg ARA 20- toegediend op dag 1, 3, 6, 8 en 10 op mi-crogliose (Iba-1-immunoreactiviteit) en astrocytose (GFAP-immunoreactiviteit) werd onderzocht in dieren die 2 of 20 weken overleefden na de inductie van de lesie of de sham operatie. Na 2 weken overleving was een significante microgliose zichtbaar in ruggenmerg segment L5 van dieren doe 0 μg/kg ARA 290 ontvingen (P<0.05), terwijl dieren die behandeld werden met 10 en 30 mg/kg deze microgliose niet toonden. Na 20 weken van overleving werd een uitgebreider en toegenomen microgliose gezien in dieren behandeld met 0 en 10 μg/kg ARA 290 vergeleken met sham geopereerde dieren, wat zich openbaarde in een toename van het aantal ruggenmergsegmenten dat microgliose vertoonde en een hogere Iba-1-immunoreactiviteit. Dieren behan-deld met 30 μg/kg ARA 290 vertoonden deze toename van microgliose niet (P<0.05). Er werd geen veranderingen in GFAP-immunoreactiviteit gezien. Het erytropoietine analoog ARA 290 verminderde dosisafhankelijk allodynie en microgliose in de dor-sale hoorn, wat deel is van het werkingsmechanisme van ARA 290 waardoor het verlichting van allodynie na perifere zenuwschade bewerkstelligt.

De eerder genoemde effecten van ARA 290 vertonen een opvallende vergelijking met een meer conventioneel middel dat al meer dan 50 jaar op de markt is en uitgebreid gebruikt is als anestheticum and als analgeticum voor acute pijn: ketamine. In subanesthetische doses is dit middel tevens effectief gebleken in het verlichten van neuropathische pijn met een farmacodynamisch profiel dat zicht uitbreid verder dan de farmacologische halfwaardetijd. De behandeling van ketamine gaat gepaard met psychomimetische bijwerkingen, zoals psychoses, hallucinaties, misselijkheid en braken. Het is onduidelijk echter, of aan het anti-neuropathische pijn effect van ketamine wordt bijgedragen door ketamine zelf, of het actieve metaboliet nor-ketamine. NMDA receptor antagonisten die deze bijwerkingen niet vertonen zijn in ontwikkeling. In Hoofdstuk 5 hebben we 3 NMDA receptor antagonisten vergeleken voor de behandeling van acute en neuropathische pijn, de ernst van de bijwerkingen of de afwezigheid van bijwerkingen.

In Hoofdstuk 5 hebben we de NMDA receptor antagonisten ketamine, norketamine en Traxoprodil onderzocht in een rat model van acute antinociceptie (terugtrek respons van de poot bij hitte stimulatie bij toenemende doses van het geneesmiddel), en een model van chronische neuropathische pijn (spared nerve injury). Bijwerkingen (stereotype gedrag en mate van activiteit) werden gescoord en locomotor functie van de aangedane poot werd onderzocht met behulp van computergestuurde looppatroon analyse. In het chronische pijn model werd de behandeling gestart 7 dagen na de operatie, 3 uur per dag op 5 opeenvolgende dagen. Alle drie de NMDA receptor antagonisten veroorzaakten dosisafhankelijke antinociceptie in het acute pijnmodel en verlichting van tactiele en koude allodynie gedurende 3-6 weken na de behandeling in het chronische pijnmodel (P<0.001). In beide testen was ketamine het meest potent, met norketamine 1,5-2 maal minder potent en Traxoprodil 5-8 maal minder potent dan ketamine. De zenuwlesie veroorzaakte een beperking in het gebruik van de aangedane poot welke niet verbeterde met behandeling (ketamine en Traxoprodil) of slechts een beperkt effect (norketamine voor alle 3 de parameters, P<0.05). Traxoprodil, maar niet ketamine of norketamine toonde een duidelijke scheiding tussen werking en bijwerking. De observatie dat behandeling met Traxoprodil leidt tot een periode van verlichting van chronische pijn die langer duurt dan de behandelingsperiode zelf, zonder bijwerkingen gedurende de behandeling, maakt het een aantrekkelijk alternatief in de behandeling van chronische neuropathische pijn.

Zowel ARA 290 als de NMDA receptor antagonisten ketamine, norketamine en Traxoprodil hebben getoond effectief te zijn in de verlichting van zowel tactiele als koude allodynie in het SNI model. Een relatief korte behandelperiode met beide typen medicatie resulteerde in een langdurige verlichting van allodynie. In Hoofdstuk 6 hebben we de effecten van ARA 290 en ketamine op de expressie van

NMDA receptor subunits en ontstekingsmarkers vergeleken. We vergeleken tevens de effecten op acute en chronische pijn en de bijwerkingen in gelijke behandelings-schema's in het SNI model in zowel wild-type als $\beta cR^{-/-}$ muizen.

In Hoofdstuk 6 onderzochten we de overlappende pathways van ARA 290 and ketamine door de effecten op de mRNA expressie van de NMDA receptor subunits NR1, NR2A en NR2B, ontstekingsmakers Iba-1 (microglia), GFAP (astrocyten) en chemokine (C-C) motif ligand 2 (CCL-2). We vonden dat zowel ketamine als ARA 290 gelijksoortige effecten bewerkstelligden door zowel significant de expressie van mRNA van de NMDA receptor subunits te verminderen als de mRNA expressie van microglia, astrocyten en CCL-2, die allen een belangrijke bijdrage leveren aan de ontwikkeling van neuropathische pijn. Hoewel de effecten van ketamine en ARA 290 op neuropathische pijn en diens moleculaire mediatoren de suggestie wekken van een gezamenlijk mechanisme, werkt ARA 290 specifiek op de "innate repair receptor" (IRR) welke is betrokken bij weefselbescherming en ARA 290 heeft geen interactie met de NMDA receptor. We speculeerden eerder dat de IRR belangrijk zou kunnen zijn in de werking van ketamine op neuropathische pijn. Om dit te onderzoeken hebben we de effecten van ketamine en ARA 290 op acute pijn, bij-werkingen en allodynie in het SNI model in muizen die de β-common-receptor (βcR) missen, een structurele component van de IRR. Ketamine (50 mg/kg) en ARA 290 (30 μg/kg) hadden divergente effecten op acute pijn. Ketamine zorgde voor duidelijke antinociceptie ($P<0.001$ vergeleken met placebo en ARA 290) en psychomotore bijwerkingen ($P<0.001$ vergeleken met placebo en ARA 290), terwijl ARA 290 dit niet had, in zowel normale als $\beta cR^{-/-}$ muizen. In tegenstelling, beide middelen waren effectief in het verlichten van allodynie in wildtype muizen ($P=0.049$ en $P=0.03$ versus placebo voor respectievelijk ketamine en ARA 290), maar waren niet effectief in muizen zonder de βcR. Samengenomen laten deze resultaten zien dat een intacte IRR nodig is voor een effectieve behandeling van neuropathische pijn met zowel ketamine als ARA 290, maar dat deze receptor niet is betrokken in ketamine's anal-getische werking en bijwerkingen.

Pijn is een subjectieve uitkomstmaat die gemeten kan worden met behulp van een numerieke score (numerical rating scale, NRS), of vragenlijsten die specifieke aspecten kunnen meten die gecorreleerd zijn aan, bijvoorbeeld, kleine vezel neu-ropathie (zoals de kleine vezel neuropathie screening lijst, SFNSL). Doordat deze manieren subjectief zijn, is zo een meting niet volledig betrouwbaar om kleine vezel neuropathie (SFN) te diagnosticeren, vanwege de inter- en intrapersoonlijke variabi-liteit. Daarom wordt de diagnose kleine vezel neuropathie gesteld met de invasieve methode van intra-epidermale zenuwvezel dichtheid bepaald met (fluorescentie) microscopie, wat de gouden standaard is voor de diagnose van SFN. De huid is echter niet het enige orgaan met oppervlakkige kleine zenuwvezels. De cornea heeft een

hoge dichtheid van kleine zenuwvezels welke onderzocht kunnen worden met de niet-invasieve methode cornea confocale microscopie.

In Hoofdstuk 7 lieten we zien dat cornea confocale microscopie (CCM) een objectieve maat is voor neuropathische pijn in sarcoidose patiënten met symptomen van SFN dat correleert met de symptomen die de patiënten rapporteren. Pijn gemeld door patiënten werd in onderzocht door middel van de "brief pain inventory" (BPI) en gekwantificeerd door middel van "quantitative sensory testing" (QST). De meerderheid (~80%) van de sarcoidose patiënten vertoonden veranderde (>2 standaard deviaties onder het gemiddelde van gezond individuen) voor alle drempelwaarden van de temperatuur drempelwaarden in de QST dat wijst op SFN. Op dit moment is een huidbiopt nodig om verlies van zenuwvezels aan te tonen om tot een diagnose van SFN te komen. Echter, het kwantificeren van IENFD in huidbiopten is een invasief, arbeidsintensief proces dat een lage sensitiviteit heeft om SFN te diagnosticeren en niet correleert met de symptomen die door de patiënten gerapporteerd worden. Als alternatief is CCM een snelle niet invasieve klinische oogheelkundige techniek voor de in vivo beeldvorming van cornea zenuwvezels. CCM toonde dat de gemiddelde cornea zenuwvezeldichtheid (CNFD) en cornea zenuwvezel lengte (CNFL) significant verminderd waren in sarcoidose patiënten vergeleken met gezonde individuen (P<0.0001 voor beide uitkomstmaten). De IENFD was verminderd in sarcoidose patiënten vergeleken met gezonde individuen (P<0.0001). Daarbij vonden we dat CNFD en CNFL, maar niet IENFD, een negatieve correlatie hadden met de pijn interferentie score van het BPI (P=0.0005 en P=0.012). Tenslotte voorspelde een lineair model met de CNFL als afhankelijke variabele accuraat de BPI pijn interferentie (P<0.0001). Deze technologie vergroot de rol van CCM als een surrogaat marker voor zowel zenuwvezel schade als pijn in klinische studies van nieuwe therapieën in sarcoidose en misschien andere kleine vezel neuropathiën.

Uiteindelijk onderzochten we in Hoofdstuk 8 het effect van ARA 290 op het verlies van zenuwvezels en de zenuwvezeldichtheid in de cornea in sarcoidose patiënten in een dubbelblind gerandomiseerde klinische studie. Kleine zenuwvezel verlies en schade (small nerve fiber loss and damage, SNFLD) is een frequente complicatie van sarcoidose welke is geassocieerd met autonome dysfunctie en sensore afwijkingen, inclusief pijnsyndromen, die een negatieve invloed hebben op de kwaliteit van leven. Van SNFLD wordt gedacht dat dit veroorzaakt door een disregulatie van het immuunsysteem, een belangrijke eigenschap van sarcoidose, die hun weerslag hebben op het centrale en perifere zenuwstelsel. De huidige therapie van sarcoidose-gerelateerde SNFLD bestaat in de eerste plaats uit onderdrukking van het zenuwstelsel en symptomatische behandeling, welke vaak niet tot een bevredigend resultaat leidt. Hier tonen we dat een behandeling van 28 dagen met een dagelijkse dosis van subcutaan ARA 290 significant de neuropathische symptomen verbetert

in patiënten met een vastgestelde SNFLD. Met behulp van QST laten we zien dat de temperatuur gevoelige detectiegrenzen (detectiegrens voor koude pijn, P = 0.027 en detectiegrens voor hittepijn, P = 0.032) en de temperatuurgevoelszin (P = 0.008) significant werden verbeterd na behandeling met ARA 290, terwijl deze parameters niet waren veranderd voor placebo behandeling. Patiënt gemelde symptomen verbeterden voor de small fiber neuropathy screening list (SFNSL) welke aanhielden tot 16 weken na het starten van de behandeling (P = 0.037). De brief pain inventory (BPI) toonde tevens een verbetering van de pijn in de met ARA 290 behandelde groep, maar dit effect verschilde niet van placebo behandelde groep. De BPI pain interference score verschilde significant tijdens de derde week van de dosering tussen de ARA 290 groep en de placebo groep (P = 0.02). In toevoeging tot de verbeterde patiënt gerapporteerde symptoom gebaseerde uitkomstmaten was de behandeling van ARA 290 geassocieerd met een toename van de kleine vezel dichtheid in de cornea (P = 0.022 voor ARA 290 in vergelijking met P = 0.462 voor placebo) en een toegenomen uithoudingsvermogen zoals onderzocht met de 6 minuten looptest (6 minute walk test, 6MWT) op de laatste dag van ARA 290 dosering (P = 0.049). Gebaseerd op deze resultaten en de resultaten van voorgaande studies blijkt dat ARA 290 een potentieel ziekte modificerend medicijn is voor de behandeling van sarcoidose geassocieerde SNFLD.

Conclusie

De data verzameld in dit proefschrift laat zien dat

- ARA 290 effectief is in het verlichten van neuropathische pijn na zenuwschade en dat dit de β-common-receptor vereist
- Een deel van het mechanisme van het verlichten van neuropathische pijn door ARA 290 wordt bewerkstelligd door suppressie van microgliose in de dorsale hoorn van het ruggenmerg
- Astrocyten zijn niet cruciaal voor neuropathische pijn op 2 en 20 weken postoperatief
- Ketamine, de actieve metaboliet norketamine en het NR2B selectieve N-methyl-D-aspartaat receptor antagonist Traxoprodil zijn effectief in het verlichten van acute en neuropathische pijn
- De NR2B subunit van de N-methyl-D-aspartaat receptor is niet betrokken bij de inductie van bijwerkingen door N-methyl-D-aspartaat antagonisten
- Ketamine en ARA 290 hebben overlappende pathways in de verlichting van neuropathische pijn door suppressie van ruggenmerg inflammatie

- De β-common-receptor is essentieel in de behandeling van neuropathische pijn, maar niet voor acute pijn.
- Sarcoidose patiënten hebben verminderde zenuwvezeldichtheid in de epidermis en de cornea
- Cornea confocaal microscopie, maar niet intra epidermale zenuwvezel dichtheid is gerelateerd aan de symptomen die gerapporteerd worden door sarcoidose patiënten met kleine vezel neuropathie.
- Behandeling van saroidose patiënten met symptomen van kleine vezel neuropathie met ARA 290 resulteert in verbeterde uitkomsten gerelateerd aan pijn
- Behandeling van saroidose patiënten met symptomen van kleine vezel neuropathie met ARA 290 vergroot de zenuwvezeldichtheid in de cornea, maar niet in de epidermis

Curriculum Vitae

Maarten Swartjes was born on 7 August 1985 in Nieuwegein. After attending Gymnasium, he started Biopharmaceutical Sciences in 2003 at Leiden University. He obtained his Bachelor's degree in 2006. In that same year, he started Medicine at Leiden University. Being interested in anesthesiology and research, he started in his first year as a student-assistant in clinical research at the department of Anesthesiology. In January 2008, he started working as a student assistant on the preclinical experiments in animal models of neuropathic pain, which allowed him to continue this work as a PhD student in January 2009. He continued to combine his PhD studentship with Medicine until he received his Doctorate's degree in June 2010. After that, he put his study on hold to finish the work described in this thesis. After the completion of the work for this thesis, he re-enrolled in Medicine for the rotations to complete his medical training.

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