Chapter 2

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

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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 maintenance 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).

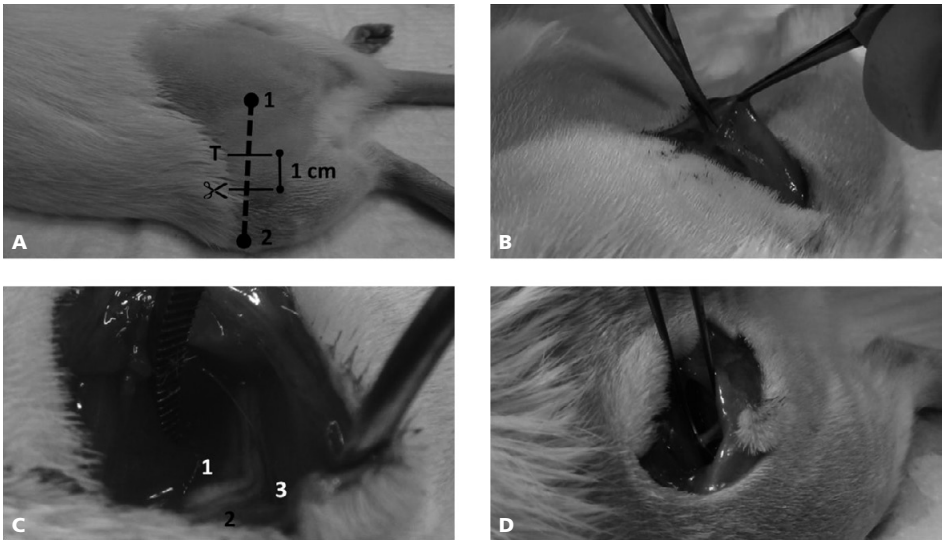

Figure 1: Surgery for induction of the spared nerve injury. A: Superficial landmarks for orientation: 1) Crest of ilium, 2) Patella, T) Site of trifurcation, ✂): Location of first incision. B: Making the incision in between the two heads of the biceps femoris muscle with a micro scissor to enter the 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.

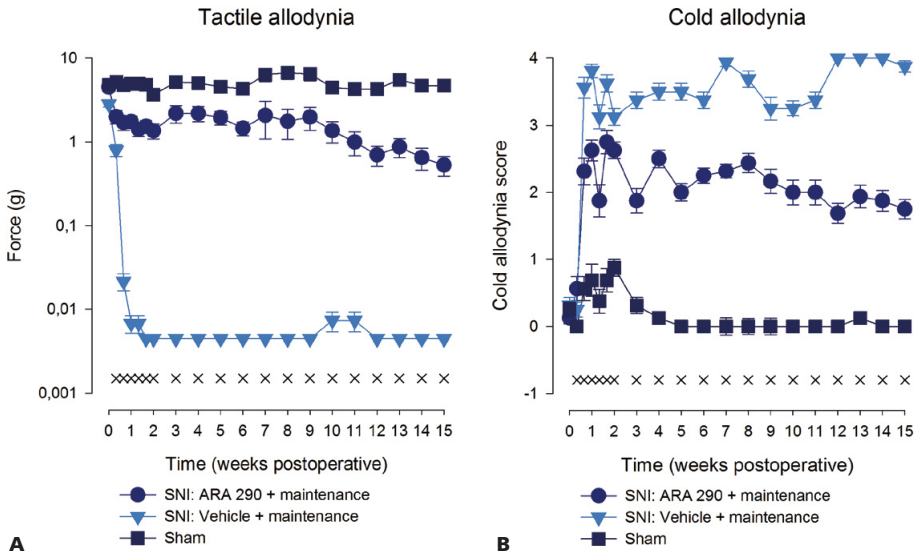

Figure 2: Effect of ARA 290 on the development of neuropathic pain. Treatment with ARA 290 results in the prevention of developing: A: tactile allodynia and B: cold allodynia for a period of 15 weeks. X = treatment with either ARA 290 or vehicle.

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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