ARA290, 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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ABSTRACT

Background: Exogenous erythropoietin inhibits development of allodynia in experimental painful neuropathy because of its antiinflammatory and neuroprotective properties at spinal, supraspinal, and possibly peripheral sites. The authors assess the effect of a nonhematopoietic erythropoietin analog, ARA290, on tactile and cold allodynia in a model of neuropathic pain (spared nerve injury) in rats and mice lacking the β-common receptor ($\beta cR^{-/-}$ mice), a component of the receptor complex mediating tissue protection.

Methods: Twenty-four hours after peripheral nerve injury, rats and mice were injected with ARA290 or vehicle (five 30-μg/kg intraperitoneal injections at 2-day intervals, followed by once/week, n = 8/group). In a separate group of eight rats, ARA290 treatment was restricted to five doses during the initial 2 weeks after surgery.

Results: In rats, irrespective of treatment paradigm, ARA290 produced effective, long-term (as long as 15 weeks) relief of tactile and cold allodynia ($P < 0.001$ *vs.* vehicle-treated animals). ARA290 was effective in wild-type mice,

What We Already Know about This Topic

- Erythropoietin reduces neuropathic pain in animals with nerve injury, but it also has undesirable hematopoietic and cardiovascular side effects.

What This Article Tells Us That Is New

- In rats, the nonhematopoietic erythropoietin analog ARA290 produced sustained inhibition of hypersensitivity after nerve injury in normal mice, suggesting this molecule might be useful in developing a treatment for chronic pain. This effect was mediated through the β common receptor.

producing significant relief of allodynia. In contrast, in $\beta cR^{-/-}$ mice no effect of ARA290 was observed.

Conclusions: ARA290 produces long-term relief of allodynia because of activation of the β-common receptor. It is argued that relief of neuropathic pain attributable to ARA290 treatment is related to its antiinflammatory properties, possibly within the central nervous system. Because ARA290, in contrast to erythropoietin, is devoid of hematopoietic and cardiovascular side effects, ARA290 is a promising new drug in the prevention of peripheral nerve injury-induced neuropathic pain in humans.

N EUROPATHIC 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 antiinflammatory

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 ARA290.[11,17,18] ARA290 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 ARA290 to treat neuropathic pain after peripheral nerve injury remains unknown, the current study was designed to explore the effect of ARA290 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 (Dierethische 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

ARA290 (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 ARA290 received injections with 30 μg/kg ARA290 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 ARA290 and vehicle were injected intraperitoneally. The ARA290 dosages used in this study are based on the work of a previous study on the effect of ARA290 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 ARA290 intraperitoneal injections at 2-day intervals, followed by once-a-week maintenance therapy of 30 μg/kg ARA290.

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 ARA290 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 ARA290 intraperitoneal injections at 2-day intervals, followed by once-a-week maintenance therapy of intraperitoneal injections of 30 μg/kg ARA290.

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 ARA290 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 (0.004–15 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 ARA290 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 ARA290 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 ARA290 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 ARA290 produced long-term relief of tactile allodynia lasting at least 15 weeks (fig. 1A). The allodynic responses differed significantly between treatment groups (main effect: $P < 0.001$; *post hoc*: ARA290 *vs.* vehicle $P < 0.001$, ARA290 *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 ARA290-treated animals (fig. 1B, main effect: $P < 0.001$; *post hoc*: ARA290 *vs.* vehicle $P < 0.001$, ARA290 *vs.* sham $P < 0.001$).

Similarly, in animals treated with vehicle, cold allodynia developed rapidly after SNI surgery in the ipsilateral paw,
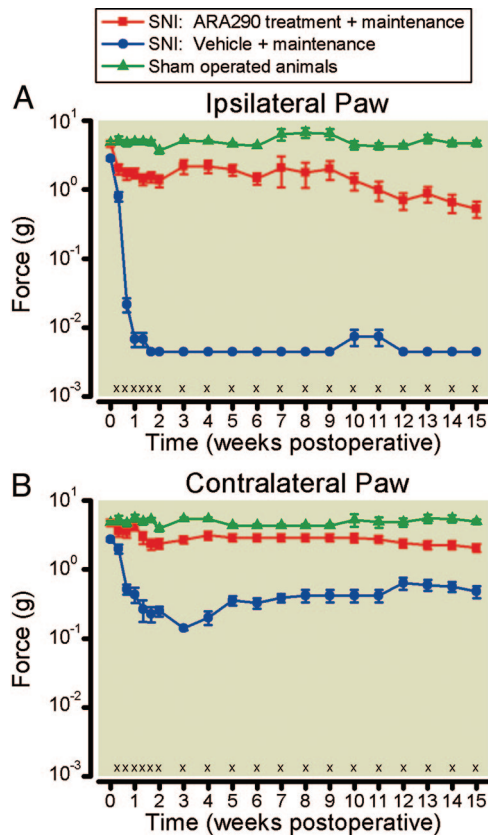
Fig. 1. Effect of ARA290 treatment during the 15 weeks after spared nerve injury (SNI) surgery on tactile allodynia. Ipsilateral paw *(A)*. Contralateral paw *(B)*. Thirty μg/kg ARA290 was injected for 5 days at 2-day intervals (first injection within 24 h after surgery), followed by once-a-week maintenance therapy of 30 μg/kg ARA290. ARA290 treatment *(red squares)*, vehicle treatment *(blue circles)*, sham-operated animals *(green triangles)*. ARA290 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 ARA290 or vehicle.

Fig. 2. Effect of ARA290 maintenance treatment during the 15 weeks after spared nerve injury (SNI) surgery on cold allodynia. Ipsilateral paw *(A)*. Contralateral paw *(B)*. ARA290 treatment *(red squares)*, vehicle treatment *(blue circles)*, sham-operated animals *(green triangles)*. ARA290 produces significantly less cold allodynia than does vehicle on ipsilateral ($P < 0.001$) and contralateral paws ($P = 0.03$). All treatments were given *via* the intraperitoneal route.

with mean allodynia scores between 3 and 4 (4 is the maximum score) during the 15-week study period. Treatment with ARA290 was associated with significantly less cold allodynia in the ipsilateral paw, with mean scores between 1.8 and 2.9 (fig. 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 ARA290 treatment in the contralateral paw (fig. 2B, $P < 0.05$; compared with vehicle-treated animals by *post hoc* test).

Effect of 2-week *versus* Maintenance ARA290 in the Rat

To assess the effect of early ARA290 treatment, eight animals received five injections of 30 μg/kg ARA290 during the initial 2 weeks after SNI surgery and no additional treatment. Animals treated according to this regimen showed a delay in
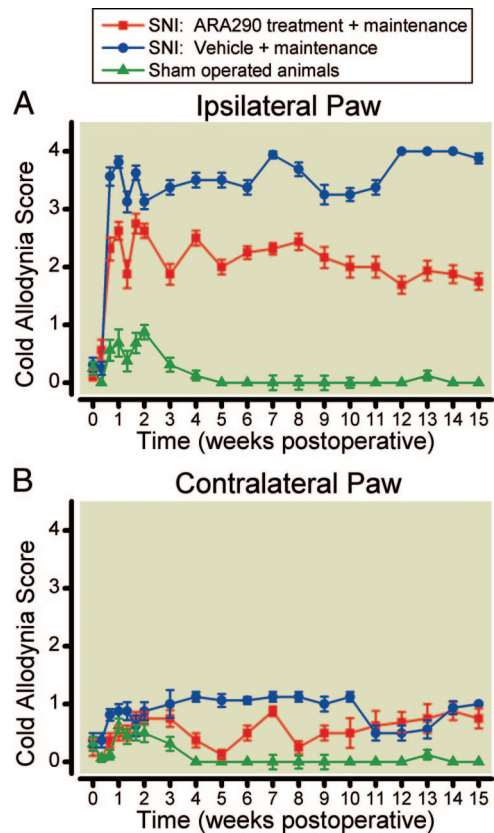
the progression of tactile allodynia for the duration of follow-up but to a lesser extent than that of the group treated with weekly ARA290 injections (maintenance therapy) ($P = 0.018$, fig. 3A). Regardless of the therapy received, animals displayed comparable nociceptive thresholds in the contralateral paw (fig. 3B).

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

Effect of ARA290 Maintenance in βcR$^{-/-}$ Mice

A treatment effect on tactile allodynia was observed in both genotypes ($P < 0.001$). ARA290 had no effect on tactile allodynia in βcR$^{-/-}$ mice (ARA290 *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 ARA290 or vehicle (fig. 5). In contrast, wild-type animals did show an effect of ARA290 treatment, with withdrawal responses occurring at 0.020 g *versus* 0.004 g in PBS-treated animals within 2 weeks after surgery (fig. 5, A and
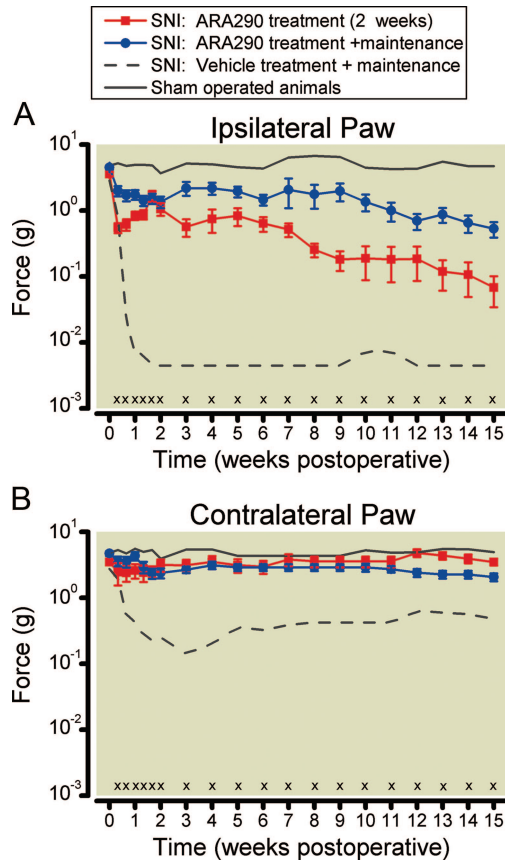
Fig. 3. Effect of 2 week therapy *versus* weekly maintenance therapy on tactile allodynia. Ipsilateral paw *(A)*. Contralateral paw *(B)*. Two-week therapy initiated within 24 h after spare nerve injury (SNI) surgery (30 μg/kg ARA290, injected at 2-day intervals) *(red squares)*. Weekly 30-μg/kg ARA290 intraperitoneal injections in addition to the initial 2-week therapy *(blue circles)*. To guide the eye, data from vehicle-treated animals *(broken line)* and sham-operated animals *(continuous 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 ARA290 (30 μg/kg) or vehicle.

Fig. 4. Effect of 2-week therapy *versus* weekly maintenance therapy on cold allodynia. Ipsilateral paw *(A)*. Contralateral paw *(B)*. Two-week therapy initiated within 24 h after spare nerve injury (SNI) surgery (30 μg/kg ARA290, injected at 2-day interval) *(red squares)*. Weekly 30-μg/kg ARA290 intraperitoneal injections in addition to the initial 2-week therapy *(blue circles)*. To guide the eye, data from vehicle-treated animals *(broken gray line)* and sham-operated animals *(continuous gray 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.

B, P = 0.027 *vs.* vehicle-treated mice, *post hoc* test). At the contralateral hind paw allodynia was observed that responded to ARA290 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) (fig. 5, C and D).

In wild-type and βcR$^{-/-}$ animals, cold allodynia developed in the ipsilateral (main effect: P < 0.001 in both genotypes) but not contralateral hind paw (main effect: P = 0.068 in βcR$^{-/-}$ and 0.087 in wild-type mice) (fig. 6). ARA290 had a significant effect on cold allodynia responses in wild-type (*post hoc*: ARA290 *vs.* vehicle P < 0.05, fig. 5A) but not in βcR$^{-/-}$ mice (fig. 6B).

Discussion

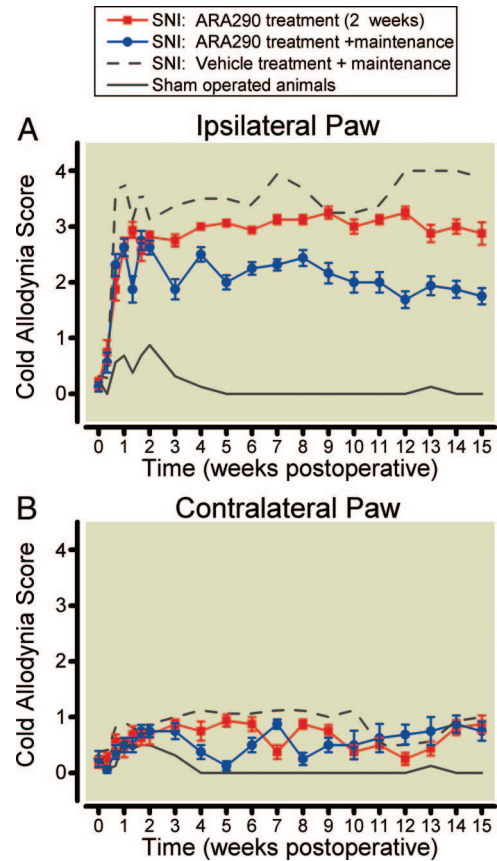The main findings of our studies are: (1) ARA290 treatment in the 2 weeks after nerve injury produces effective, long-term relief of allodynia in rats; (2) in the same species, ARA290 therapy was most effective when it was maintained at 1-week intervals; and (3) an effect of ARA290 on nociceptive withdrawal 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 ARA290 peptide is novel and promising, but additional testing in humans is required to predict the effectiveness of ARA290 in patients with neuropathic pain.

ARA290 is a peptide derived from the erythropoietin molecule. In most tissues, including spinal cord and brain, the cytokine erythropoietin is produced in response to local injury, counteracting the effects of proinflammatory cytokines.[11,23] Recent animal studies indicate that exogenously
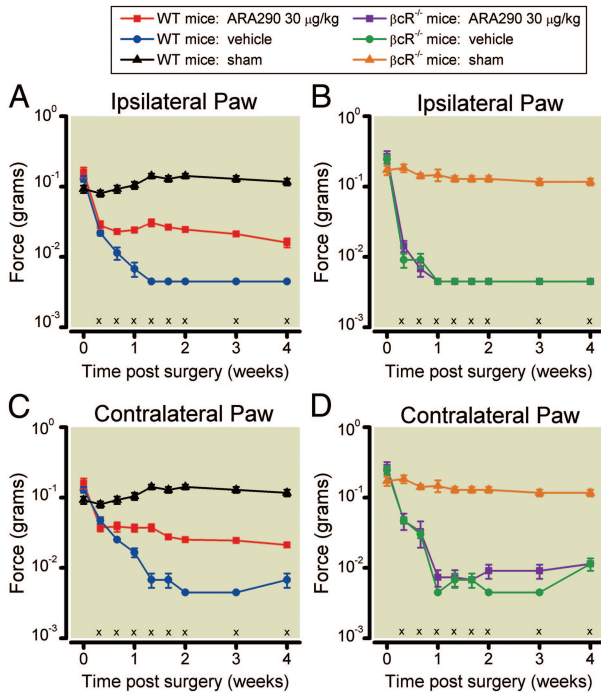
Fig. 5. Effect of ARA290 treatment on tactile allodynia measured in the ipsilateral hind paw (*i.e.*, paw with nerve injury) and contralateral hind paw. Effect of ARA290 therapy in mice with an intact β-common-receptor (wild-type mice), ipsilateral paw (A). Effect of ARA290 therapy in mice lacking the β-common receptor (βcR$^{-/-}$ mice), ipsilateral paw (B). Effect of ARA290 therapy in wild-type mice, contralateral paw (C). Effect of ARA290 therapy in βcR$^{-/-}$ mice, contralateral paw (D). ARA290 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 = moment of treatment with either ARA290 (30 μg/kg) or vehicle.
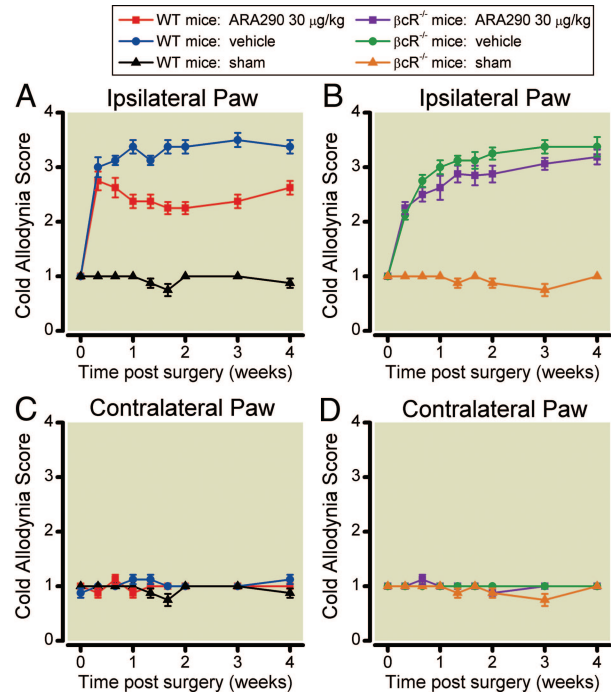
Fig. 6. Effect of ARA290 treatment on cold allodynia measured in the ipsilateral hind paw (*i.e.*, paw with nerve injury) and contralateral hind paw. Effect of ARA290 therapy in mice with an intact β-common receptor (wild-type mice), ipsilateral paw (A). Effect of ARA290 therapy in mice lacking the β-common receptor (βcR$^{-/-}$ mice), ipsilateral paw (B). Effect of ARA290 therapy in wild-type mice, contralateral paw (C). Effect of ARA290 therapy in βcR$^{-/-}$ mice, contralateral paw (D). 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.

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 in-

creased 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 analogs have been developed that selectively activate the EPOR-βcR complex and that have tissue-protective properties, such as carbamylated erythropoietin, asialoerythropoietin, and ARA290.[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] ARA290 has been shown to up-regulate EPOR expression in injured tissue.[28] In the current study, we used ARA290 to assess its effect on nociceptive responses after peripheral nerve injury. ARA290 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 ARA290 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 ARA290 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 ARA290 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 ARA290 (plasma half-life $\approx$ 2 min in rats and rabbits).[17] Although this suggests a peripheral rather than a central effect, there is ample evidence that ARA290 passes the blood–brain barrier. For example, ARA290 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 ARA290, given its short half-life, it is reasonable to assume that ARA290 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] fig. 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 antiinflammatory 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 antiinflammatory and neuroprotective effect of ARA290 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 (fig. 3). This observation could suggest that because of the biologic half-life of ARA290 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 ARA290 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 ARA290 treatment (figs. 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 ARA290. Testing of ARA290 in patients with chronic pain is required before any conclusions on the effectiveness of ARA290 is humans may be drawn.

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