INTRODUCTION

Neuropathic pain is a complex and debilitating disorder. Treatments are often ineffective, which may reflect our limited understanding of the underlying mechanisms. Much of our current knowledge has been gained through the use of animal models, such as the chronic constriction injury, spinal nerve ligation and spared nerve injury models. These models, which are all associated with significant nerve injury, produce pain behaviors that are consistent with clinical signs of allodynia and hyperalgesia (Bennett and Xie, 1988; Kim and Chung, 1992; Decosterd and Woolf, 2000). In the clinic however, many patients with the symptoms of neuropathic pain do not appear to have signs of nerve injury on routine clinical testing. These patients may be diagnosed with conditions such as complex regional pain syndrome type 1, non-specific arm pain (also known as repetitive strain injury) or back pain. Studies on the neuritis model, a model of localized peripheral nerve inflammation, suggest that symptoms in these patients may be due to inflammation and not frank nerve injury (Elia et al., 1999; Bove et al., 2003; Dilley et al., 2005). In the neuritis model, animals develop signs of mechanical allodynia and heat hyperalgesia in the absence of axonal degeneration or demyelination (Elia et al., 1999; Chacur et al., 2001; Bove et al., 2003). These behavioral changes are rapid and short-lived, and begin to resolve at 1 week. On electrophysiological examination, intact nociceptive (C-fiber) axons develop ongoing (spontaneous) activity and axonal mechanical sensitivity at the inflamed site (Bove et al., 2003; Dilley et al., 2005).

A novel modulator of inflammation that may be beneficial in the treatment of the symptoms of neuropathic pain is the agent erythropoietin (EPO). EPO is typically associated with hematopoiesis but has also been shown to be tissue protective in other tissues, which include the nervous system (Brines et al., 2000; Villa et al., 2003). Both EPO and its receptors are expressed within the peripheral nervous system and, following nerve injury, EPO is upregulated in Schwann cells (Campana and Myers, 2003). Binding of EPO to receptors on injured neurons can prevent axonal degeneration (Keswani et al., 2004). The neuroprotective activity of EPO in the peripheral nervous system has led to several studies that have examined the systemic administration of recombinant human EPO as a potential treatment for neuropathic pain. These studies have shown that the administration of human recombinant EPO is effective in...
reversing nerve injury-induced pain behaviors, such as mechanical allodynia and heat hyperalgesia (Campana and Myers, 2003; Keswani et al., 2004; Campana et al., 2006; Jia et al., 2009a,b). EPO is reported to perform this function by preventing neuronal apoptosis and axonal degeneration (Sirén et al., 2001; Campana and Myers, 2003; Keswani et al., 2004; Campana et al., 2006) or by reducing the production of cytokines (Villa et al., 2003; Campana et al., 2006; Jia et al., 2009a). Proinflammatory cytokines are reputed to play a significant role in the development of neuropathic pain. For example, tumor necrosis factor-α (TNF-α) can cause primary sensory neurons to develop ongoing (spontaneous) activity (Sorkin et al., 1997; Leem and Bove, 2002; Schafers et al., 2003; Richards et al., 2011), which may drive central mechanisms that lead to the symptoms of allodynia (LaMotte et al., 1991; Gracey et al., 1992; Campbell and Meyer, 2006; Woolf, 2011).

There are a number of issues with administering EPO for the treatment of neuropathic pain. Activation of erythropoiesis by EPO increases the hematocrit. This effect, along with platelet activation and raised blood pressure, will increase the risk of thrombosis (Corwin et al., 2007). More recently an EPO analog, ARA290, has been developed that mimics the neuroprotective activities of EPO without stimulating hematopoiesis (Brines et al., 2008). This 11-amino acid peptide has been shown to provide long-term relief of nerve injury-induced mechanical and cold allodynia in the spared nerve injury model via activation of the tissue-protective receptor (the EPO receptor-β-common-receptor complex) (Swartjes et al., 2011). The present study has expanded on this previous investigation by focusing on the potential beneficial effects of ARA290 on pain behavior in the neuritis model, which, in contrast to the spared nerve injury model, lacks gross nerve pathology. Specifically, it has examined the effects of ARA290 administration on the development of mechanical allodynia and heat hyperalgesia. A previous study from our laboratory has inferred a role for the cytokine TNF-α and chemokine (C—C motif) ligand 2 (CCL2) in the maintenance of neuritis-induced ongoing activity (Richards et al., 2011). Therefore, the effects of ARA290 on the levels of mRNA for TNF-α and CCL2 were also examined at the peak of the neuritis (day 4) and at a later time point (day 11) when most behavioral effects have resolved.

**EXPERIMENTAL PROCEDURES**

**Animals and surgery**

Experiments were carried out in strict accordance with the UK Animals (Scientific Procedures) Act (1986). A total of 58 adult male Sprague Dawley rats (240–355 g; Harlam, UK) were used in this study.

Forty-six animals underwent neuritis surgery as previously described (Elalav et al., 1999; Bove et al., 2003; Dilley et al., 2005). Animals were anesthetized and maintained on isoflurane (1.75%) in oxygen. The left sciatic nerve was exposed at the mid-thigh by blunt dissection through the biceps femoris muscle, and a 7–8 mm length carefully separated from adjacent connective tissue. The nerve was loosely wrapped in a strip (approximately 3 mm × 3 mm × 10 mm) of sterile Gelfoam (Spongostan; Ferrosoan, Denmark) saturated with approximately 150 μl Complete Freund’s adjuvant (Sigma, Dorset, UK; diluted 1:1 using sterile saline). The muscle and skin were closed using 4/0 monofilament sutures (Vicryl; Ethicon, West Lothian, UK) and the animals were allowed to recover for 24 h.

**Behavioral testing**

*Mechanical allodynia.* Mechanical allodynia was tested by applying von Frey hairs of increasing stiffness (Ugo Basile, Varese, Italy) to the glabrous skin on the plantar surface of the foot. The current protocol was modified from previously published methods (Tai and Bennett, 1994).

The test apparatus consisted of Perspex animal enclosures that were raised on a metal-perforated floor. Animals were habituated to the apparatus for 1 h on three consecutive days before the start of the testing period. Behavioral testing was always performed at the same time on each day. On the day of testing, animals were acclimated for 15 min to allow for exploration and major grooming activities to cease. Each von Frey hair was presented perpendicular to the plantar surface of the foot, with sufficient force to cause slight buckling against the paw, and held for 5 s. A positive response was noted if the paw was sharply withdrawn. Each hair was applied a maximum of five times at 10-s intervals or until two consecutive positive responses were recorded. The minimum size von Frey hair that produced two consecutive responses was designated the withdrawal threshold. Testing commenced using a 4-g von Frey hair, which avoided unnecessary repeated application of finer von Frey hairs. If the animal failed to respond, von Frey hairs of increasing stiffness (6, 9, 12 and 15 g) were applied with an interval of 1 min between hairs until two consecutive responses with the same hair were recorded. If there were no responses by the final testing of the 15-g filament, a threshold of 15 g was assigned. If the animal responded to the 4-g von Frey hair, the finest von Frey hair (0.4 g) was applied followed by hairs of ascending stiffness (0.7, 1.2, 1.5 and 2 g). Each repeat test was applied to a different area of the glabrous skin. Both ipsilateral and contralateral sides were tested. The side to be tested first was randomized and the investigator waited 5 min before starting the second side. Three pre-surgery withdrawal thresholds were established on three separate days prior to neuritis surgery and averaged as the baseline value. Withdrawal thresholds were further determined on days 1, 2, 3, 4 and 7 post-surgery.

*Heat hyperalgesia.* Heat-hyperalgesia was tested using the Hargreaves method, as described elsewhere (Hargreaves et al., 1988). The apparatus consisted of Perspex animal enclosures that were raised on an elevated glass platform (Ugo Basile, Italy). Similar to the mechanical allodynia protocol, animals were habituated to the apparatus for 1 h on three consecutive days before the start of the testing period. Behavioral testing was always performed at the same time on each day. On each test day, animals were acclimated for 15 min prior to testing. A calibrated movable radiant heat source beneath the floor was aimed at the mid-plantar hind paw. Onset of the stimulus activated a timer that automatically produced two consecutive responses was designated the withdrawal threshold. Testing commenced using a 4-g von Frey hair, which avoided unnecessary repeated application of finer von Frey hairs. If the animal failed to respond, von Frey hairs of increasing stiffness (6, 9, 12 and 15 g) were applied with an interval of 1 min between hairs until two consecutive responses with the same hair were recorded. If there were no responses by the final testing of the 15-g filament, a threshold of 15 g was assigned. If the animal responded to the 4-g von Frey hair, the finest von Frey hair (0.4 g) was applied followed by hairs of ascending stiffness (0.7, 1.2, 1.5 and 2 g). Each repeat test was applied to a different area of the glabrous skin. Both ipsilateral and contralateral sides were tested. The side to be tested first was randomized and the investigator waited 5 min before starting the second side. Three pre-surgery withdrawal thresholds were established on three separate days prior to neuritis surgery and averaged as the baseline value. Withdrawal thresholds were further determined on days 1, 2, 3, 4 and 7 post-surgery.
respond faster than the first (unpublished observations, K. Pulman & A. Dilley; refer to Fig. 2A and B for the mean ipsilateral and contralateral baseline (day 0) values). Three pre-surgery withdrawal latencies were established on three separate days prior to neuritis surgery and averaged as the baseline value. Withdrawal latencies were further determined on days 1, 2, 3, 4, and 7 post-surgery.

Heat hyperalgesia and mechanical allodynia were tested on the same animals by the same investigator (KP). Mechanical allodynia testing was performed after a 60-min period following the examination of heat hyperalgesia. All behavioral testing was performed blind with respect to treatment.

Study design

Neuritis animals were randomly assigned to either a 30 \( (n = 11) \) or 120 \( \mu \text{g/kg} \) ARA290 \( (n = 9) \) treatment group or to a respective vehicle-treated control group \( (\text{Group A: } n = 11) \), which were tested alongside the 30 \( \mu \text{g/kg} \) ARA290 group, or Group B \( (n = 9) \), which were tested alongside the 120 \( \mu \text{g/kg} \) ARA290 group. Behavioral experiments were generally performed in groups of six (three ARA290-treated and three vehicle-treated animals). Behavior was also tested on an additional six unoperated animals that did not receive any drug.

Drug administration

Animals were administered either 30 or 120 \( \mu \text{g/kg} \) ARA290 (Araim Pharmaceuticals, Ossining, NY, USA) diluted in 200 \( \mu \text{l} \) of 0.9\% saline at pH 7.4. Vehicle-treated animals were administered 200 \( \mu \text{l} \) of 0.9\% saline. Both ARA290 and vehicle were injected intraperitoneally immediately post-behavioral testing at 1, 2, 3, and 4 days following surgery. The 30-\( \mu \text{g/kg} \) dose of ARA290 used in this study was based on the work of a previous study (Swartjes et al., 2011).

Measurements of CCL2 and TNF-\( \alpha \) mRNA

Six unoperated (day 0) and 32 neuritis-treated animals were killed with an overdose of sodium pentobarbital. The neuritis animals were culled on day 1 \( (n = 6) \), 4 \( (n = 12) \); six 120 \( \mu \text{g/kg} \) ARA290-treated and six vehicle-treated) and 11 days \( (n = 14) \); seven 120-\( \mu \text{g/kg} \) ARA290-treated and seven vehicle-treated) post neuritis induction. On day 1 (i.e. 24 h post-surgery), the animals were culled prior to any treatment (i.e. ARA290 or vehicle). The Gelfoam, as well as a 5–6 mm length of sciatic nerve, was removed from the neuritis site for measurements of TNF-\( \alpha \) and CCL2 mRNA. A similar length of sciatic nerve was also removed from the contralateral side and from unoperated animals. All tissue was frozen at –80 \(^{\circ}\)C prior to processing.

Tissues were homogenized in TRIzol Reagent (Invitrogen, Life Technologies, Paisley, UK) using a T88 Ultra-Turrax (IKA, Staufen, Germany), and total RNA was extracted following the manufacturer’s instructions (Invitrogen). RNA quality and concentration were determined using a NanoDrop ND-1000 (NanoDrop Technologies, Wilmington, DE, USA). Reverse transcription and real-time quantitative polymerase chain reaction (qPCR) were carried out as previously reported (Mengozzi et al., 2012). Briefly, 250 ng of total RNA was retrotranscribed, using M-MLV Reverse Transcriptase (Invitrogen), in a total volume of 25 \( \mu \text{l} \). Two microliters of the resulting complementary DNA were subjected to qPCR using TaqMan gene expression assays (Applied Biosystems, Life Technologies, Paisley, UK). Relative gene expression was quantified using the comparative threshold cycle method, according to Applied Biosystems guidelines. Results were normalized to glyceraldehyde 3-phosphate dehydrogenase expression (housekeeping gene) and expressed as arbitrary units, representing gene expression versus a calibrator sample.

For this study, cDNA obtained from rat ischemic cortex, which is known to express TNF-\( \alpha \) and CCL2 (Mengozzi et al., 2012), was used as the calibrator sample.

Statistics

All data were tested for normality using Shapiro–Wilk tests to determine appropriate statistical analysis. The behavioral data are presented as the mean withdrawal and latency thresholds, as well as percent change from the baseline (i.e. normalized) for comparisons between treatments. For both mechanical allodynia and hyperalgesia data, comparisons of time points within groups and comparisons between treatments \( (30, 120 \mu \text{g/kg ARA290 and vehicle-treated groups}) \) were performed using one-way and two-way repeated measures analysis of variance (ANOVA) respectively. Comparisons between treatments at individual time points were performed using one-way ANOVA. ANOVA tests were followed by Bonferroni \( t \) tests for post hoc pair-wise comparisons. Differences in the area under the curve (AUC) were examined using unpaired \( t \) tests. For both CCL2 and TNF-\( \alpha \) mRNA levels, comparisons between time points were analyzed using one-way ANOVA followed by a Bonferroni \( t \) test for post hoc pair-wise comparison. Comparisons between the 120 \( \mu \text{g/kg} \) ARA290 and vehicle-treated groups at individual time points and between day 4 and 11 in ARA290 groups were analyzed using unpaired \( t \) tests. \( p < 0.05 \) was considered significant. Data were expressed as means ± SEM.

RESULTS

Behavioral testing

Mechanical allodynia. The mechanical allodynia results are summarized in Fig. 1. A preliminary analysis of the vehicle data showed no significant difference between the two vehicle-treated groups \( (\text{Group A: } n = 11; \text{ Group B: } n = 9; p = 0.57, \text{ repeated measures ANOVA}) \), and therefore the data were combined.

The average ipsilateral baseline threshold value was 12.91 ± 0.25 g \( (n = 46) \). Following the induction of neuritis, animals in the vehicle-treated group \( (n = 20) \) developed signs of mechanical allodynia (i.e. a decrease in von Frey threshold) on the ipsilateral plantar foot that reached a maximum reduction in von Frey threshold \( (\text{mean} = 7.45 ± 0.60 \text{ g}) \) on day 4 of testing \( (\text{Fig. 1A}) \). The mean percent decrease in threshold on day 4 was 41.6 ± 5.0\% and the most responsive animals were sensitive to the 2-g von Frey hair. A partial reversal of mechanical allodynia was evident on day 7 of testing. In the ARA290-treated groups there was a reduction in withdrawal threshold post-surgery, which was not significant at any time point \( (\text{Fig. 1A}) \). In the unoperated group, there were no signs of mechanical allodynia on the ipsilateral side. Signs of mechanical allodynia were also absent from all groups on the contralateral side (Fig. 1B).

From the ipsilateral normalized data, treatment with ARA290 significantly reduced the development of neuritis-induced mechanical allodynia compared to the vehicle-treated group \( (p < 0.05, \text{ repeated measures ANOVA; Fig. 1C}) \). Comparing individual time points, the drug effects were most pronounced on day 4 of testing, where there was a significant reduction of mechanical allodynia in both the 30 and 120 \( \mu \text{g/kg} \) ARA290 groups compared to the vehicle-treated group (Fig. 1C). When
the AUCs were examined, there was a significant difference between vehicle-treated (mean = \(-176 \pm 21.1\%\) days) and both drug groups (mean = \(-68.9 \pm 35.4\%\) days) for 30 µg/kg and \(-98.4 \pm 21.2\%\) days) for 120 µg/kg; Fig. 1D). There was no significant difference in the AUC for the 30 µg/kg compared to the 120 µg/kg ARA290-treated groups (\(p = 0.51\), unpaired t test).

Heat hyperalgesia. The heat hyperalgesia results are summarized in Fig. 2. A preliminary analysis of the vehicle data showed no significant difference between the two vehicle-treated groups and therefore the data were combined (\(p = 0.72\) repeated measures ANOVA).

The average baseline value was 15.16 ± 0.41 s (\(n = 46\)). Following induction of the neuritis, animals in the vehicle-treated group (\(n = 20\)) developed signs of heat hyperalgesia on the ipsilateral plantar foot that reached a maximum reduction in withdrawal latency (mean = 11.14 ± 1.01 s) on day 3 of testing (Fig. 2A). The mean percent decrease in withdrawal latency on day 3 was 28.2 ± 5.7%. Recovery from hyperalgesia was evident on day 7 of testing. In the ARA290-treated groups there was a reduction in withdrawal latency post-surgery, which was significant on day 2 in the 120 µg/kg ARA290 group (Fig. 2A). In the unoperated group, there were no signs of heat hyperalgesia on the ipsilateral side. On the contralateral side, there was no significant change in withdrawal latency at any individual time point in all groups, except for the 120 µg/kg ARA290 group, where there was a significant reduction on day 3 (Fig. 2B).

From the ipsilateral normalized data, there was no significant difference in withdrawal latencies between the vehicle and ARA290-treated neuritis groups (\(p > 0.20\), repeated measures ANOVA; Fig. 2C).
However, from the graph it would appear on day 3 that there was a transient recovery in withdrawal latency in both drug groups compared to the vehicle-treated group, although this effect was not significant \( (p = 0.10, \text{ one-way ANOVA; Fig. 2C}) \). When the AUCs were examined, there was no significant difference in the AUC between the vehicle and drug-treated groups \( (p > 0.47, \text{ unpaired } t \text{ tests}) \). Drug and vehicle injections were carried out immediately after behavioral testing on days 1, 2, 3, and 4. In (A and B) \( *p < 0.05 \) compared to the baseline for the vehicle and \( \dagger p < 0.05 \) compared to the baseline for the 120 μg/kg ARA290-treated groups \( (\text{repeated measures ANOVA followed by Bonferroni post hoc } t \text{ tests}) \).

**Cytokine mRNA levels**

CCL2. The CCL2 mRNA results are summarized in Fig. 3. In the vehicle-treated group, the level of CCL2 mRNA in the sciatic nerve at the treatment site was significantly raised on day 1 \( (\text{mean} = 190.4 \pm 34.4 \text{ arbitrary units}) \) compared to unoperated \( (\text{day 0}) \) nerve \( (\text{mean} = 4.0 \pm 0.6 \text{ arbitrary units}; \text{Fig. 3}) \). Mean levels had decreased by 85% on day 4 and a further 56% on day 11. In the Gelfoam that surrounded the nerve, CCL2 mRNA levels were significantly raised on day 1 \( (\text{mean} = 203.6 \pm 21.9 \text{ arbitrary units}) \) and day 4 \( (\text{mean} = 146.2 \pm 21.7 \text{ arbitrary units}) \) compared to day 11 \( (\text{mean} = 36.8 \pm 6.7 \text{ arbitrary units}; \text{Fig. 3}) \). The levels of CCL2 mRNA in the contralateral nerve were negligible at each time point \( (p = 0.61, \text{ one-way ANOVA}) \).

There was no significant difference in the levels of CCL2 mRNA in the nerve at the neuritis site or Gelfoam of animals treated with 120-μg/kg ARA290 compared to the vehicle-treated neuritis group at day 4 or 11 \( (p > 0.24; \text{ unpaired } t \text{ tests}; \text{Fig. 3}) \).

TNF-α. The TNF-α mRNA results are summarized in Fig. 4. In the vehicle-treated group, there was an increase in the level of TNF-α mRNA in the sciatic nerve at the treatment site post neuritis \( (\text{mean} = 190.4 \pm 34.4 \text{ arbitrary units}) \) compared to unoperated \( (\text{day 0}) \) nerve \( (\text{mean} = 4.0 \pm 0.6 \text{ arbitrary units}; \text{Fig. 4}) \). Mean levels had decreased by 85% on day 4 and a further 56% on day 11. In the Gelfoam that surrounded the nerve, TNF-α mRNA levels were significantly raised on day 1 \( (\text{mean} = 203.6 \pm 21.9 \text{ arbitrary units}) \) and day 4 \( (\text{mean} = 146.2 \pm 21.7 \text{ arbitrary units}) \) compared to day 11 \( (\text{mean} = 36.8 \pm 6.7 \text{ arbitrary units}; \text{Fig. 4}) \). The levels of TNF-α mRNA in the contralateral nerve were negligible at each time point \( (p = 0.61, \text{ one-way ANOVA}) \).

There was no significant difference in the levels of TNF-α mRNA in the nerve at the neuritis site or Gelfoam of animals treated with 120-μg/kg ARA290 compared to the vehicle-treated neuritis group at day 4 or 11 \( (p > 0.24; \text{ unpaired } t \text{ tests}; \text{Fig. 4}) \).
1 = 5.5 ± 1.5 arbitrary units) compared to unoperated (day 0) nerve (mean = 1.3 ± 0.2 arbitrary units), although not significant (Fig. 4). The highest levels of TNF-α mRNA were recorded in the Gelfoam on day 1 (mean = 19.3 ± 4.6 arbitrary units). These levels were significantly decreased in the Gelfoam by day 4.
(mean = 7.9 ± 1.5 arbitrary units) and remained at a similar level on day 11 (mean = 8.2 ± 1.6 arbitrary units; Fig. 3). The levels of TNF-α mRNA in the contralateral nerve remained low on each test day (p = 0.31, one-way ANOVA).

At 4 and 11 days post-neuritis, there was no significant difference in the levels of TNF-α mRNA in the nerve at the neuritis site or Gelfoam of animals treated with 120 μg/kg ARA290 compared to the vehicle-treated group (p > 0.38; unpaired t tests; Fig. 4).

**DISCUSSION**

The present study examined the therapeutic effects of the non-hematopoietic EPO analog ARA290 on pain behavior in the neuritis model. Whereas previous studies have examined the therapeutic effects of ARA290 and EPO on pain models that are associated with frank nerve injury, the present study has focused on a nerve inflammation model that produces negligible gross nerve pathology (Eliav et al., 2001; Bove et al., 2003; Dilley et al., 2005). The results from this study demonstrated a clear inhibition of neuritis-induced mechanical allodynia following treatment with ARA290, which suggests that EPO analogs may provide useful alternative therapies in patients with symptoms of neuropathic pain who do not have clinical signs of nerve injury.

Vehicle-treated neuritis animals developed signs of mechanical allodynia and heat hyperalgesia that were comparable to previous studies (Eliav et al., 1999; Bove et al., 2003). Both mechanical allodynia and heat hyperalgesia peaked early, reaching a maximum effect on day 4 and 3 of testing, respectively. Since animals were not tested on days 5 and 6, it was not possible to determine whether day 4 was in fact the peak for mechanical allodynia. However based on previous reports (Eliav et al., 1999; Bove et al., 2003), day 4 is likely to represent the maximum effect. Importantly, the behavioral changes following neuritis are brief with signs of recovery by 7 days, which contrasts to nerve injury models such as the spinal nerve ligation (Kim and Chung, 1992), chronic constriction injury (Bennett and Xie, 1988) and spared nerve injury models (DeCosterd and Woolf, 2000) that produce more persistent behavioral effects. This difference in the pattern of behavioral changes in the neuritis model compared to these other neuropathic pain models probably reflects the minor nature of the neuritis injury and the lack of nerve degeneration. The recovery of the behavioral changes is consistent with electrophysiological findings of ongoing activity and axonal mechanical sensitivity that also peak within the first week of neuritis induction (Bove et al., 2003; Dilley and Bove, 2008; Bove and Dilley, 2010).

The therapeutic effects of ARA290 were clearly evident on day 4 post neuritis, when ARA290 treatment produced effective relief of mechanical allodynia. These findings were comparable to a previous study that has shown 30 μg/kg ARA290 to be efficacious in limiting mechanical allodynia in the spared nerve injury model (Swartjes et al., 2011). The current ARA290 injection regime was broadly comparable to Swartjes and colleagues (2011) who initially administered five intraperitoneal injections of ARA290 at two-day intervals from day 1 post-surgery. In the present study, a higher 120 μg/kg dose of ARA290 was also tested, although there was no evidence that this dose significantly improved the degree or rate of mechanical allodynia recovery. Interestingly, it has been suggested that in the central nervous system, EPO receptors on hippocampal neurons respond within a limited range and that high concentrations of EPO induce a down-regulation of the receptor (Sakanaka et al., 1998). It is therefore possible that within the peripheral nervous system, ARA290 also functions within a limited range. Despite the lack of studies on ARA290, there have been several investigations that have examined the effects of EPO on pain behavior in nerve injury models. Similar to the therapeutic effects of ARA290, the systemic administration of EPO reduced mechanical allodynia in both the L5 spinal nerve injury (Campana and Myers, 2003; Jia et al., 2009a,b) and chronic constriction injury models (Campana et al., 2006).

ARA290 administration did not appear to significantly reverse the development of heat hyperalgesia in the neuritis model. Animals treated with ARA290 continued to develop signs of heat hyperalgesia up to day 2, after which there were indications of a transient, although insignificant, partial recovery. These results contrast from previous studies that have shown a clear reversal of nerve injury-induced heat hyperalgesia following the systemic administration of recombinant human EPO (Campana et al., 2006; Jia et al., 2009b). The lack of significant effect of ARA290 on neuritis-induced heat hyperalgesia may reflect differences in underlying hyperalgesic mechanisms between models. It may also indicate functional differences between ARA290 and EPO. On the contralateral side, there was some variability in withdrawal latency in all groups. Responses on the contralateral side are likely to be determined by the level of arousal due to prior exposure to the stimuli on the opposite (ipsilateral) foot. This is consistent with the tendency for faster responses from the second (contralateral) foot to be tested during baseline testing. Differences in the level of arousal between days (which may also be influenced by the neuritis as well as drug treatment) could account for the observed variability, as well as explain the significant decrease in contralateral withdrawal latency on day 3 in the 120 μg/kg ARA290 group. Alternatively, the neuritis animals in the higher dose group may have developed bilateral heat hyperalgesia. Bilateral pain behaviors following the induction of neuritis have been previously reported (Chacur et al., 2001).

It is generally accepted that ongoing activity from the periphery is necessary to drive central mechanisms that lead to allodynia and hyperalgesia (LaMotte et al., 1991; Gracely et al., 1992; Campbell and Meyer, 2006; Woolf, 2011). In the neuritis model, ongoing activity is generated from apparent uninjured neurons (Bove et al., 2003; Dilley et al., 2005; Bove, 2009), whereas in nerve injury models, a high proportion of ongoing activity
arises from axotomized A-fiber neurons (Kajander and Bennett, 1992; Tal and Eliav, 1996). These pathophysiological differences are of particular interest when the therapeutic actions of either EPO or ARA290 on pain behavior are examined. Since EPO protects neurons against apoptosis and Wallarian degeneration (Sakanaka et al., 1998; Bernaudin et al., 1999; Campana and Myers, 2003; Keswani et al., 2004; Campana et al., 2006), in pain models where nerve injury is a significant component, EPO may help to reduce the number of damaged neurons (Toth et al., 2008). The result will be less aberrant firing into the spinal cord, and therefore reduced pain behavior. In the neuritis model, where axonal degeneration is not a major feature, the therapeutic effects of ARA290 on pain behavior are likely to be via an alternative mechanism.

Interactions between the immune system and nervous system are important for the development of neuritis-induced pain behaviors (Bennett, 2000). For example, TNF-α as well as CCL2 can cause ongoing activity in primary sensory neurons (Sorkin et al., 1997; Leem and Bove, 2002; Schafer et al., 2003; Richards et al., 2011). Furthermore, in the chronic constriction injury model, which has both immune and structural components, pain behaviors can be partially reversed by administering thalidomide or antibodies that neutralize TNF-α (Sommer et al., 1998, 2001). Since EPO can decrease TNF-α expression in Schwann cells in the chronic constriction injury model (Campana et al., 2006) as well as other pro-inflammatory cytokines in the spinal nerve ligation model (Jia et al., 2009a,b), we have hypothesized that ARA290 reverses neuritis-induced allodynia by inhibiting the production of TNF-α or CCL2.

In this study we measured the levels of TNF-α and CCL2 mRNA in nerve as well as the Gelfoam, since in the neuritis model immune cells are predominantly found extraneurally (Bove et al., 2009). However, contrary to our hypothesis, TNF-α mRNA levels peaked on day 1 post neuritis within the Gelfoam, which was before the administration of ARA290. At day 4, when withdrawal thresholds were lowest, the levels of TNF-α mRNA in the nerve and Gelfoam were comparable between the drug and vehicle groups. Furthermore, at day 11 when behavioral changes are largely resolved (see Eliav et al., 1999; Bove et al., 2003), the levels of TNF-α mRNA remained unchanged in both groups compared to day 4. Similarly, there were no differences between the levels of CCL2 mRNA in the ARA290 and vehicle-treated groups on days 4 and 11 post-surgery. Despite a lack of effect at these time points, it is possible that if the drug was administered from the time of surgery (i.e. before mRNA levels peak), it may reduce TNF-α and CCL2 mRNA levels. However, delivery of ARA290 at surgery would be counterintuitive, since the aim of this study was to determine whether ARA290 could be used to treat neuritis-induced pain behaviors rather than prevent the development of neuritis.

Importantly, the results from this study suggest that the reversal of neuritis-induced pain behaviors was due to ARA290 acting on targets other than TNF-α and CCL2 production. These targets may include the production of different inflammatory mediators. For example, EPO is reported to inhibit the production of interleukin (IL)-1β as well as IL-6 following spinal nerve ligation (Jia et al., 2009a). Since IL-1β and IL-6 can increase ongoing activity in A- and C-fiber neurons (Hoheisel et al., 2005; Ozaktay et al., 2006; Eliav et al., 2009), their inhibition may reverse central mechanisms that are reputed to lead to pain behaviors. It is also possible that ARA290 exerts an anti-inflammatory effect by inhibiting the action of inflammatory cytokines rather than their production. For instance, EPO was reported to prevent some effects induced by TNF-α directly (Pregi et al., 2009; Contaldo et al., 2011). EPO is also reported to have antioxidant as well as anti-nitric oxide effects (Calapai et al., 2000; Kilic et al., 2005; Oztürk et al., 2008). Since nitric oxide signaling plays an important role in the development and maintenance of pain hypersensitivity (Holthusen, 1997; Aley et al., 1998; De Alba et al., 2006; Chen et al., 2010; Kim et al., 2011), ARA290 may act by inhibiting this pathway. Alternatively, activation of EPO receptors on primary sensory neurons (see Campana and Myers, 2001) might lead to the direct modulation of neuronal excitability. For example, within the central nervous system, EPO has been shown to activate calcium channels (Koshimura et al., 1999), although a similar activation on primary sensory neurons would presumably increase excitability.

The mechanisms of action of ARA290 are intriguing, since it has a short plasma half-life of only 2 min (Brines et al., 2008) yet its effects on behavior occur over hours to days. Although ARA290 is able to cross the blood–brain barrier (Brines et al., 2008), its short half-life suggests a peripheral mechanism that is likely to involve the activation of multiple signaling pathways (Brines and Cerami, 2008), which lead to more persistent biological effects.

CONCLUSION

ARA290 may be beneficial in the treatment of neuritis without the potential side effects of EPO. In particular, it may be useful in the treatment of chronic pain conditions where on routine clinical examination signs of nerve injury are absent. Such conditions include non-specific arm pain, complex regional pain syndrome type 1 and back pain. In these conditions, patients are likely to have a persistent inflammatory nerve lesion similar to the neuritis model (Dilley and Bove, 2008; Dilley, 2011), which may be partly responsible for the development of pain hypersensitivity, radiating and spontaneous pain. Treatment with ARA290 may therefore help alleviate the pain hypersensitivity and possibly some of these other symptoms.

Acknowledgments—The authors would like to thank Dr. Michael Brines for his helpful comments on the manuscript. This study was supported by US Army Medical Research Acquisition Activity Grant W81XWH-11-2-0024 to AraiM Pharmaceuticals, Inc.
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(Accepted 11 December 2012)
(Available online 20 December 2012)