Chapter 9

Summary and conclusion

Summary

Neuropathic pain is a disabling disease with a mechanism consisting of several pathways 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. Pharmacological treatment is often inadequate and coincides with intolerable side effects. New treatments are arising that may be able to target neuropathic pain more efficiently, 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 particular 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 preparation, 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 following spared nerve injury. We showed that a 10 day regimen in which 5 administrations 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 observed 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 suppresses 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 accompanied 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 Traxoprodil 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 neuropathic 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 contributors 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 $\beta cR^{-/-}$ 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