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Cisplatin-induced neuropathy in mature rats: effects of the melanocortin-derived peptide ORG 2766

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Abstract. A major side effect of cisplatin treatment is peripheral neuropathy. In the past few years we have provided evidence that the $ACTH_{4-9}$ analogue ORG 2766 provides protection against this neuropathy in rats and man. In this study we investigated the development of a cisplatin-induced neuropathy and the protective and therapeutic actions of ORG 2766 in mature rats. We also studied the effects of the peptide and of growth impairment caused by food restriction on nerve conduction velocities in healthy young adult rats (not subjected to any other treatment). In the neuropathy experiment, cisplatin induced a significant decrease in sensory nerve conduction velocity (SNCV), which could be prevented by concomitant administration of ORG 2766. The SNCV of the neuropathic animals recovered to control values within 10 weeks of discontinuation of cisplatin treatment. ORG 2766 did not enhance the rate of recovery. In the young adult rats neither ORG 2766 administration nor restricted weight gain significantly influenced either the motor or the sensory nerve conduction velocity. These results validate the animal model of cisplatin-induced neuropathy.

Introduction

Cisplatin has greatly improved the therapeutic efficacy of combination regimens for the treatment of various malignancies, such as ovarian and testicular cancer. Its use, however, is restricted by its serious side effects. The most pronounced acute side effects are nausea and vomiting; late side effects include nephrotoxicity, ototoxicity and neurotoxicity. Since nephrotoxicity can be avoided by forced diuresis, neurotoxicity and ototoxicity are the dose-limiting side effects of cisplatin [10]. The neuropathy is of a purely sensory nature, with a preference for the thickest myelinated fibres. The first symptoms are those of a peripheral neuropathy, with paraesthesias and tingling sensations, followed by progressively worsening sensory ataxia. Pain and temperature sensitivity are relatively spared [5, 15, 20]. Even if treatment with cisplatin is discontinued as soon as the first neuropathic signs and symptoms become apparent, this neuropathy may continue to develop for several months and spontaneous regression is both slow and incomplete [10, 11].

In the past decade, several drugs have been shown to enhance post-damage recovery of the peripheral nervous system. One of these drugs is the neuropeptide ORG 2766, an analogue of $ACTH_{4-9}$ [1]. In 1987 De Koning [4] described an animal model of cisplatin-induced neuropathy. With this model it was shown that ORG 2766 can prevent cisplatin-induced neuropathy without interfering with cisplatin's anti-tumour efficacy [4, 6, 9]. These observations have been confirmed in humans [7].

The animal model of cisplatin-induced neuropathy is based on young adult rats. The nerves of these rats may have a better natural potency to recover than the nerves of mature rats. Moreover, in young rats parameters such as body weight, and motor and sensory nerve conduction velocities increase substantially during the course of experiments. It may be that the beneficial effect of peptide treatment found in previous experiments with young rats originated from maturation per se. Moreover, cisplatin-treated animals initially display a lower weight gain than age-matched controls and eventually lose weight. Does the poor nutritional status itself influence the maturation of the peripheral nervous system?

Follow-up of several patients co-treated with ORG 2766 in the clinical study mentioned above showed that some did develop neuropathic signs and symptoms after discontinuation of both cisplatin and peptide treatment [11], albeit fewer than patients previously co-treated with placebo. Although the number of patients in this exploratory study was too small to draw firm conclusions, it would be interesting to see whether such a retarded neuropathy develops in rats previously co-treated with cisplatin and ORG 2766.

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We performed two experiments to answer the above questions. In the first, cisplatin neuropathy was induced in mature rats. During the first part of this study, a decrease in the sensory nerve conduction velocity was observed in the cisplatin-treated animals, and this decrease was prevented by co-treatment with ORG 2766. During the second part of this study, recovery from this neuropathy was studied in the neuropathic group.

In the second experiment, one group of young adult rats was treated with ORG 2766, one group was treated with placebo, and one group was subjected to food restriction to limit their weight gain to 25% of that of controls. Neither ORG 2766 injections nor food restriction influenced motor and sensory nerve conduction velocities.

Materials and methods

Animals and animal care. Wistar rats of an inbred strain (originally obtained from TNO, Zeist, The Netherlands) were used in both experiments. Animals were housed on sawdust in macrolon cages (two to four animals/cage, depending on body weight) and maintained on a 12 hours light/12 hours darkness cycle (lights on at 7.30 a. m.) with free access to food and water, unless stated otherwise in one of the following sections.

Evaluation of the neuropathy. The development of a cisplatin-induced neuropathy was evaluated by electrophysiological measurement of the H-related sensory nerve conduction velocity (SNCV). Treatment with cisplatin decreases this parameter.

Electrophysiology. Measurements were performed with the animals under general anaesthesia (Hypnorm, Janssen Pharmaceutica BV, Tilburg, The Netherlands, containing 10 mg/ml fluanisone and 0.315 mg/ml fentanyl citrate, dose 0.4 ml/kg body weight, administered subcutaneously).

The method is described in detail elsewhere [4]. In short: The sciatic and tibial nerves were stimulated at the sciatic notch and the ankle, respectively, by means of monopolar needle electrodes. The anode was placed 5 mm proximal to the cathode. Upon stimulation of these peripheral nerves, two responses can be elicited from the small muscles of the sole of the foot by means of surface electrodes: the short latency M-response, due to stimulation of α-motor fibres, and the long latency H-response, due to stimulation of the afferent I^A-fibres which monosynaptically excite α-motor neurons in the spinal cord. The H-reflex-related SNCV can be calculated from the latencies of the H-response at both stimulation points and the distance between the two stimulation points (as measured between sciatic notch and ankle in the gently stretched paw). The same applies for the M-responses and the motor nerve conduction velocity (MNCV).

Drugs. Cisplatin (Bristol Myers BV, Weesp, The Netherlands) was dissolved in saline to a final concentration of 0.05 mg/ml and administered intraperitoneally two times a week in a dose of 1 mg/kg body weight (20 ml/kg between 9.00 and 11.00 a.m.). Saline served as placebo.

In order to minimize kidney damage caused by cisplatin administration, all animals were given furosemide 12.5 mg/kg s.c. 20 min before the cisplatin injection. In combination with the large volume of saline in which cisplatin is administered this treatment minimizes nephrotoxic damage without influencing neurotoxicity.

ORG 2766 (a gift of Organon BV, Oss, The Netherlands) was dissolved in saline. A dose of 75 μg/kg in 0.5 ml saline was administered every 48 h subcutaneously. Saline served as placebo.

Experiments. In the first experiment, 60 full-grown rats (age 7 months, weight ca. 460 g) were divided into three groups. One group ($n = 15$) served as age-matched controls. The other two groups were treated for 10 consecutive weeks with cisplatin as outlined above (cumulative cisplatin dose, 20 mg/kg). One of these groups ($n = 15$) was co-treated with ORG 2766; the other group ($n = 30$) and the age-matched controls were co-treated with saline. SNCV was measured immediately before the start of the experiment at week 0 and after completion of cisplatin treatment at week 10. After treatment with cisplatin and saline stopped, the 30 animals previously treated with cisplatin and saline were randomly divided into two subgroups, one treated with ORG 2766 and the other treated with saline for another 10 weeks. All animals previously treated with ORG 2766 now received saline. The SNCV was measured every 2 weeks for the next 10 weeks (weeks 12, 14, 16, 18 and 20). The experimenter who evaluated the neuropathy was unaware of the treatment the animals had received.

In the second experiment, 30 young adult rats (age 2.5 months, weight ca. 200 g) were divided into three groups of 10 animals each. Two of these groups had unlimited access to food and water and the other group was given food once a day in an amount that would restrict animals' weight gain to about 25% of that of the other groups. We started with about 60% of the normal requirement, i.e. about 8 g per animal. This amount was eventually adjusted on the basis of actual weight gain. Body weight was measured every day at 11 a.m. Wood for gnawing was provided. Rats with approximately the same body weight were housed in groups of three or four. Animals from one of the freely feeding groups were injected with ORG 2766 every 48 h, and the other group and the food-restricted animals received saline injections at the same intervals. The MNCV and the SNCV were measured weekly for 8 weeks. As in the first experiment, the experimenter who evaluated the neuropathy was unaware of the treatment the animals had received.

Data analysis. In both experiments the conduction velocities (SNCV in the first experiment and both MNCV and SNCV in the second experiment) were analysed with an analysis of variance for repeated measurements (ANOVAR), followed by supplementary *t*-tests at the end of the treatment periods. Data from animals that died during the experiment were excluded from statistical analysis.

Results

Experiment 1

During the 20 weeks of this experiment 8 of 60 animals died. Two died during the first period of anaesthesia at week 0, 3 died during the period of cisplatin treatment, 2 died during anaesthesia at week 10 and 1 died during the electrophysiological measurement in week 18. Significant differences in body weight developed between the cisplatin-treated and age-matched control groups (ANOVAR week 0 to 20, all three groups: $F_{3,48} = 13.72$, $P < 0.001$). Body weight in the animals that received cisplatin decreased by about 10% during the first 10 weeks (Fig. 1 A). There were no statistically significant differences between the cisplatin-treated groups, neither during the first 10 weeks nor during the second 10 weeks of treatment (ANOVAR week 0 to 20, cisplatin-treated groups: $F_{2,36} = 0.55$, $P < 0.58$). The body weight of age-matched controls increased by about 15% over the experimental period.

The SNCV was initially 63 m/s. The SNCV remained in the range of 60–63 m/s in the age-matched controls during the experimental period, whereas the SNCV decreased significantly in the cisplatin/saline-treated animals (to 54 m/s) during cisplatin treatment. The conduction velocity at the end of the experiment was significantly different from the value at the start of the experiment (paired *t*-test week 0 vs week 10: $t = 6.23$, $df = 22$, $P < 0.001$) and was significantly

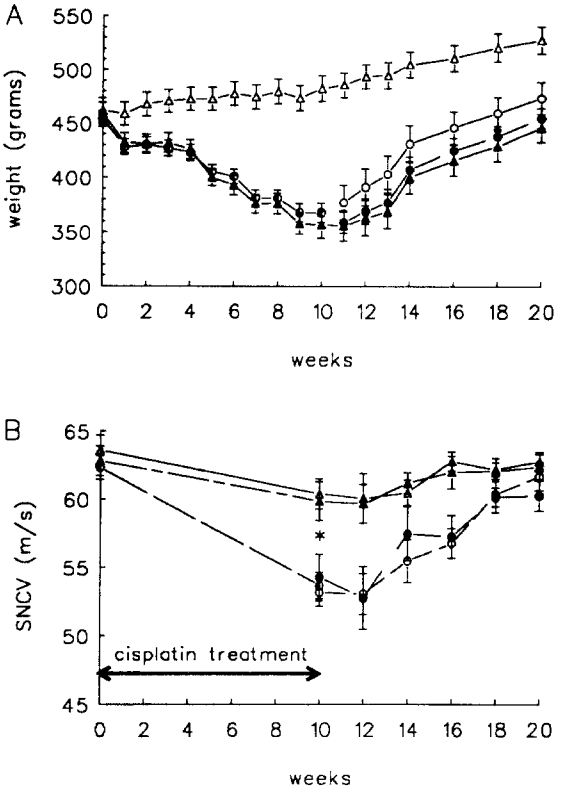

Fig. 1 A, B. Experiment 1: neurotoxicity experiment. Animals were treated with cisplatin 1 mg/kg two times a week for the first 10 weeks. Values are given as means ± SEM. Groups are coded as follows: △ age-matched controls (n = 13); ◐ cisplatin + saline (first period) (n = 24); ▲ cisplatin + ORG 2766 (first period), saline (last period) (n = 15); ● neuropathy + ORG 2766 (second period) (n = 12); ○ neuropathy + saline (second period) (n = 12). **A** Body weight; **B** sensory nerve conduction velocity (t-test after induction of neuropathy: cisplatin/saline treated group vs age-matched controls.* P <0.001)

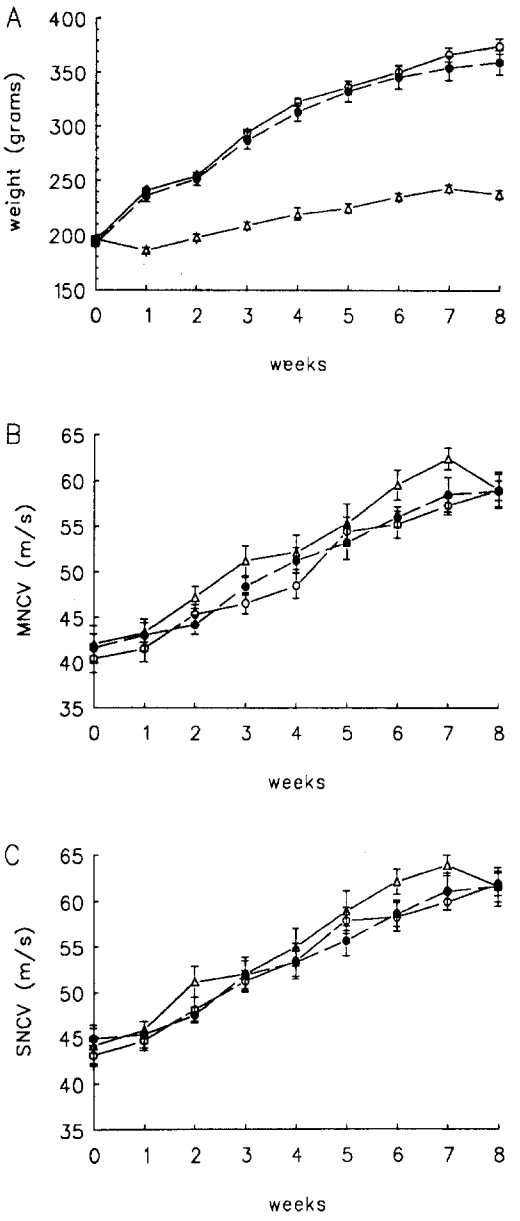

Fig. 2 A – C. Experiment 2: development and food restriction experiment. Values are given as means ± SEM. Groups are coded as follows: ○ age-matched controls (n = 9); ● ORG 2766 administration (n = 9); △ food restriction (n = 10). **A** Body weight; **B** motor nerve conduction velocity; **C** sensory nerve conduction velocity

different from that of the age-matched controls at this time point (ANOVA, all groups at week 10: $F_{3,47} = 12.49$, P <0.001; t-test cisplatin/saline vs age-matched controls at week 10: $t = 3.59$, $df = 34$, P <0.001). Peptide treatment completely inhibited the fall in conduction velocity observed in the placebo-treated animals, as evidenced by similar SNCVs in the age-matched control and cisplatin/peptide-treated groups (Fig. 1B). During the subsequent weeks no significant changes were observed in the SNCV of the animals formerly treated with cisplatin/peptide as compared to age-matched controls (ANOVAR week 10 to 20, cisplatin/peptide and age-matched controls: $F_{1,26} = 0.32$, ns).

The neuropathic animals gradually recovered and SNCV increased gradually to control values during the second experimental period. The SNCV of both the peptide- and the saline-treated groups increased at the same rate (ANOVAR week 10 to 20, neuropathy/saline and neuropathy/peptide: $F_{1,21} = 0.22$, ns).

Experiment 2

After a weight loss of about 10% in the first few days, the animals on a restricted food intake gained weight at a rate 25% of that of the animals in the freely feeding groups. After 8 weeks the freely feeding animals weighed about 360 g (160 g more than at week 0), whereas food-restricted animals weighed about 240 grams (40 g more than at week 0; Fig. 2A).

In this experiment one animal in the peptide-treated group and one in the saline-treated group died after the electrophysiological measurements in week 3; all other animals survived until the end of the experiment.

ANOVAR demonstrated a slight but significant difference between the MNCVs of the three groups ($F_{2,25} = 5.64$, P <0.01). Inspection of the graphs (Fig. 2B) suggests that this could be due to a somewhat faster increase in conduction velocity in the group with a restricted food intake.

Testing at the end of the treatment period, however, failed to detect a significant difference between this group and the saline-treated freely feeding group (t-test: $t = 0.05$, $df = 17$, ns). No differences were observed between the saline-treated and the ORG 2766-treated freely feeding groups. Neither peptide administration nor restricted food intake had an effect on the SNCV (ANOVAR: $F_{2,25} = 2.86$, $P < 0.089$; Fig. 2C).

Discussion

Previously we demonstrated that in young adult rats the $ACTH_{4-9}$ analogue ORG 2766 can prevent the neurotoxicity of cisplatin without affecting the anti-tumour activity of this drug [6]. Histochemical and morphological studies suggest that this amelioration is not directly related to displacement of cisplatin from its DNA complexes in rat dorsal root ganglion neurons and satellite cells [14] or to a counteracting of cisplatin-induced changes at the ultrastructural level in these cells [12]. In fact it has been hypothesized that the peptide aids the regenerative repertoire of inflicted nerves irrespective of the nature of the threatening condition (mechanical, intoxication, metabolic disturbance etc.) [8].

In the first experiment we demonstrated that the neurotrophic peptide ORG 2766 prevented the development of cisplatin-induced neuropathy in mature rats but did not enhance recovery from a existing cisplatin neuropathy. These data support and extend previous observations. The efficacy of ORG 2766 in preventing cisplatin-induced neuropathy has been established in young rats [4, 6, 9] and humans [7]. It has been suggested that this animal model is not valid as a paradigm for the neuropathy observed in humans. Young adult rats probably have a greater regenerative capacity than mature rats [16], and the differences in conduction velocities between neuropathic and control groups might be explained by a block in myelin maturation rather than by primary disease of the sensory neuron. The present results indicate that cisplatin treatment decreases the SNCV in mature (and myelinated) nerves and that ORG 2766 prevents this decrease in conduction velocity. Furthermore, no deterioration of the SNCV was observed after discontinuation of both cisplatin and peptide treatment during the second part of the experiment. This is in contrast to humans, in whom cisplatin-induced neuropathy continues to develop up to 4 months after the last cycle of cisplatin and then starts to regress. The most important reason for performing this experiment was to determine whether ORG 2766 enhances the rate of recovery from an existing cisplatin-induced neuropathy. We failed to collect evidence for accelerated recovery in the peptide-treated group. It could be that the regenerative machinery of rats is already maximally stimulated after 10 weeks of neurotoxic treatment. This could explain both the observation that the SNCV in previously cisplatin/peptide-treated animals did not deteriorate and the observation that peptide treatment of an existing neuropathy did not increase the recovery rate further. The failure of ORG 2766 to enhance recovery could otherwise be explained by the fact that melanocortins are known to stimulate the initial reaction of peripheral nerves to trauma, both on functional [3] and histological [18] parameters, but that treatment after a critical period (about 8 days after nerve crush) does not further enhance regeneration [3]. Moreover, the fact that ORG 2766 stimulates the initial sprouting response after a crush lesion [19] indicates yet again the beneficial effects of the peptide in an early phase of nerve damage.

The results of the second experiment indicate that ORG 2766 does not influence the age-related increment in nerve conduction velocity in young adult animals. ACTH-like peptides are known to influence prenatal and early postnatal development of the neuromuscular system [13], and although any influence of ORG 2766 on nerve conduction velocity was unlikely, it was not possible to exclude such an influence completely. A reduced rate of weight gain, induced by food restriction, did not by itself restrict the age-related increase in nerve conduction velocity. Others have shown that severe food restriction apparently resulting in weight gain only 25% of that in age-matched controls leads to some differences in morphometric parameters without signs of degeneration or demyelination. However, also under these severe food restriction conditions no changes in the peripheral nerve conduction velocity were observed [2].

This observation provides additional support for the neuropathy models used in our laboratory (cisplatin as well as diabetic neuropathy and experimental allergic neuritis [1], as in these models weight gain is often impaired and weight loss is not exceptional. The weight gain in the food-restricted group was comparable to that of cisplatin-treated animals during the period in which they developed a neuropathy. The most important conclusions from this study are that ORG 2766 prevents cisplatin-induced neuropathy, not only in young adult but also in adult rats, and that it does not accelarate spontaneous recovery from an existing cisplatin-induced neuropathy in our model. In humans, recovery mechanisms in cisplatin-induced neuropathy are probably insufficient, as deterioration continues for about 4 months followed by incomplete and protracted recovery. These differences between rats and humans may suggest that in humans it is advisable to continue ORG 2766 administration up to 4 months after the last cycle of cisplatin treatment [17], although this needs further investigation in clinical studies.

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