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A single injection of a biodegradable microsphere formulation of the ACTH-(4-9) analogue ORG 2766 accelerates functional recovery after brain damage

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Abstract—The functional recovery from impaired motor activity induced by 6-hydroxydopamine lesions in rat nucleus accumbens was accelerated by subcutaneous treatment with the ACTH-(4-9) analogue Met/O_2/-Glu-His-Phe-D-Lys-Phe (ORG 2766). Treatment was effective after daily injections of ORG 2766 dissolved in saline during the first 6 days following the lesion (ED50: 28·5 ng kg^{-1} day^{-1}) or after a single injection of the peptide in a biodegradable microsphere formulation administered after the lesion (ED50: 8·9 ng kg^{-1} day^{-1}). This study shows that a single injection of a microsphere preparation can replace multiple injections with ORG 2766 in order to facilitate functional recovery after brain damage.

Nerve damage can stimulate processes resulting in functional recovery from injury. A number of recovery mechanisms have been proposed such as reinnervation of lesioned tissue (Onn et al 1986), increased transmitter turnover (Zigmond et al 1984) and development of denervation supersensitivity (Trendelenburg 1966). The mechanisms mediating nerve tissue recovery have been subject to investigation. A number of factors with neurotrophic activity have been described (Verhaagen & Gispen 1990), amongst these are peptides derived from ACTH and the structurally related melanocyte-stimulating hormone (MSH). These and closely related peptides have beneficial effects on the morphological and functional recovery from peripheral nerve tissue damage (Verhaagen & Gispen 1990). These peptides also accelerate functional recovery from lesions in the central nervous system. The impaired reversal learning of rats with bilateral lesions of the parafascicular area is restored by chronic treatment with the ACTH-(4-9) analog Met/O_2/-Glu-His-Phe-D-Lys-Phe (ORG 2766) (Nyakas et al 1985). ORG 2766 also facilitates recovery of hyperemotionality of rats with lesions in the septal area (Isaacson & Poplawsky 1983). Destruction of the dopamine system in the nucleus accumbens induced by bilateral injection of the neurotoxin 6-hydroxydopamine (6-OHDA) into this brain structure causes a temporary reduction in motor activity which is followed by spontaneous functional recovery in approximately 3–4 weeks (Winn & Robbins 1985; Wolterink et al 1990a). When rats are treated with ORG 2766 during the first 6 days after the lesion, functional recovery is present one week later (Wolterink et al 1990b). This effect of the peptide has been observed following intra-accumbal, subcutaneous (s.c.) and oral treatment (Wolterink & Van Ree 1990), but was effective when ORG 2766 was given for more than 3 days during the period just after the lesion.

In the present study we have investigated the effect of a single injection of ORG 2766 in the form of a microsphere depot preparation and compared this effect to that of chronic s.c. treatment with the peptide. We found that the microsphere preparation of ORG 2766 accelerated the functional recovery from a 6-OHDA lesion and was as effective at comparable doses as the daily injections with ORG 2766 dissolved in saline.

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Materials and methods

Male Wistar rats (130–150 g), from our own stock were housed in a dimly lit room (lights on 07.00–19.00 h) in groups of 5–6 animals per cage (40 × 26 × 20 cm). The animals had free access to food and tap water.

Induction of the lesion. One hour before the operation, the rats received an i.p. injection of desipramine (DMI, 25 mg kg^{-1}), to prevent destruction of noradrenergic nerve cells. The rats were anaesthetized with Hypnorm (0·08 mL/100 g, i.m.) and secured in a stereotaxic apparatus. Stainless steel guide-cannulae were bilaterally implanted into the brain, their tips ending in the nucleus accumbens (coordinates; 2·6 mm anterior to bregma, 2·7 mm lateral of midline, 6·2 mm below the surface of the skull at the point of penetration, inserted at an angle of 12°, incissor bar at horizontal zero level). The cannulae served to guide the needle of a Hamilton syringe through which 6-hydroxydopamine (6-OHDA) (8 μg/2 μL dissolved in 0·9% NaCl (saline) containing 0·1% ascorbic acid) or vehicle was injected over a 2 min period. After the injection the guide cannula, together with the syringe, was removed and the skin stitched.

Test conditions. Seven and 14 days after the operation behavioural activity of the rats was assessed in a small open field in a sound attenuated room. The animals were brought to the test room at least one h before testing. The small open field consisted of a transparant Plexiglass Tube (diam. 19·5, h 30 cm) placed on a plastic board which was divided into four equal sections. During a 3 min observation period motor activity (number of sections explored) was measured.

Microspheres preparation. Microspheres containing approximately 0·5% (w/w) of ORG 2766 were prepared from 50:50 (mol/mol) poly (DL-lactide-co-glycolide), a biocompatible and biodegradable polymer (Visscher et al 1985). This polymer elicits minimal inflammatory tissue response and is hydrolytically biodegraded to lactic acid and glycolic acid in about one month. The microsphere product was a free flowing powder consisting of spherical particles and was not sieved before use. The in-vitro release rate of the microspheres was determined in 250 mL of demineralized water at 37°C. Typically 50 mg of microspheres showed the following matrix-type of release:

Day	1	2	3	4	5	7	9	11	14	22
ORG 2766 released (μg)	50·2	29·0	32·1	30·8	12·3	12·3	13·6	12·0	7·0	7·4

Microsphere dosage forms with a 0·1, 0·01, 0·001, 0·0001 and 0·00001-fold lower release rate were prepared by diluting the loaded microspheres with placebo microspheres, in such a way that doses of microspheres 50 mg kg^{-1} could be administered. Just before administration, the microspheres were suspended in an injection vehicle (0·3 mL per rat) containing 5% mannitol and 0·005% benzalkonium chloride in water. Assuming that the in-

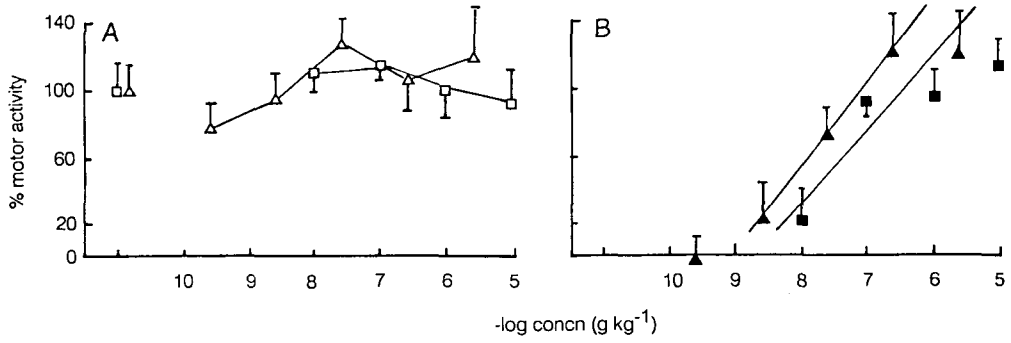

FIG. 1. Effect of subcutaneous treatment with ORG 2766 dissolved in saline (□,■) or in a microsphere form (△,▲) on motor activity of rats with bilateral sham (A) or 6-OHDA (B) lesions in the nucleus accumbens. ORG 2766 dissolved in saline was administered daily from day 1 to day 6 after the lesion. ORG 2766 in the microsphere preparation was injected once on day 1 following the lesion. Motor activity was measured in a small open field 7 days after the lesion. The motor activity of each individual animal was related to that of sham and 6-OHDA-lesioned placebo-treated animals. For each experiment the mean motor activity of sham-lesioned placebo-treated animals was taken as 100%; and the mean motor activity of 6-OHDA lesioned placebo-treated rats as 0%. The mean (± s.e.m.) motor activity of sham-lesioned rats treated daily with placebo was 16·3 (± 0·9) and that of 6-OHDA-lesioned rats treated daily with placebo 10·2 (± 1·1). The number of animals per group was 5. The mean (± s.e.m.) motor activity of sham-lesioned animals treated with microsphere vehicle on day 7 was 16·5 (±0·8) and that of 6-OHDA-lesioned rats treated with microsphere vehicle on day 7 was 10·9 (±0·7). The number of animals per group varied from 7 to 25. Presented are the mean calculated % ± s.e.m. (vertical bars) versus the dose of the peptide administered.

vivo release of ORG 2766 from the microspheres is the same as in-vitro, a mean in-vivo release rate of approximately 25 μg kg^{-1} day^{-1} was calculated for undiluted microspheres during the first week and approximately 10 μg kg^{-1} day^{-1} during the second week.

Experimental procedure. Groups of 6-OHDA lesioned or sham operated rats (n = 5) were s.c. treated with 0·9% NaCl (saline) or graded doses of ORG 2766 dissolved in saline once daily for 6 days, starting 1 day after the operation. The doses of ORG 2766 were 0·01, 0·1, 1·0 and 10·0 μg kg^{-1} day^{-1}. Motor activity of these rats was measured on day 7 after the lesion. Other groups of 6-OHDA lesioned or sham operated rats (n = 7–25) received a single injection of the microcapsule preparation loaded with graded doses of ORG 2766 or vehicle 1 day after the operation. A diluted microsphere preparation was used such that 0·00025, 0·0025, 0·025, 0·25 and 2·5 μg kg^{-1} day^{-1} was administered. Motor activity of these rats was assessed 7 and 14 days after the lesion.

Statistical analysis. The data were analysed using analysis of variance (ANOVA) and Student-Newman-Keuls parametric tests. The dose-related effects were analysed by calculating dose-response lines using the method of least squares. Three dose levels of the peptide were considered in the analysis. Subsequently, the calculated lines were compared using ANCOVA analysis, which permits the calculation of ED50 values and 95% confidence limits.

Drugs. ORG 2766 (Met/O_2/-Glu-His-Phe-D-Lys-Phe) in either dry powder or microsphere form was from Organon International BV, Oss, The Netherlands. 6-OHDA.HBr was obtained from Sigma Chemical Co. Desipramine was a gift from Ciba-Geigy. All solutions were freshly prepared on the day of use.

Results

In the sham lesioned animals, s.c. injected ORG 2766 dissolved in saline or incorporated in microspheres did not influence motor activity of rats (Fig. 1A). 6-OHDA, injected bilaterally into the nucleus accumbens, induced a decrease in motor activity in placebo-treated animals when tested on day 7 after the lesion (Fig. 1B). Subcutaneous treatment with graded doses of ORG 2766, either dissolved in saline or incorporated in microspheres, dose-dependently reversed the 6-OHDA-induced reduction of motor activity when tested on day 7. The ED50 values (±95% confidence limits) for ORG 2766 treatment in the form of saline solution or as microspheres were 28·5 (0·068–5537) and 8·9 (0·187–335·9) ng kg^{-1} day^{-1}, respectively. Similar effects were found in the microsphere treated rats when tested 14 days after the 6-OHDA lesion (ED50 (±95% confidence limits) 7·0 (0·096–247·5) ng kg^{-1} day^{-1}) (Fig. 2). Analysis of covariance revealed no significant difference between the dose-response curves of the treatment with ORG 2766 dissolved in saline and the microsphere treatment. No significant difference was observed between the dose-response curves of the microsphere adminis-

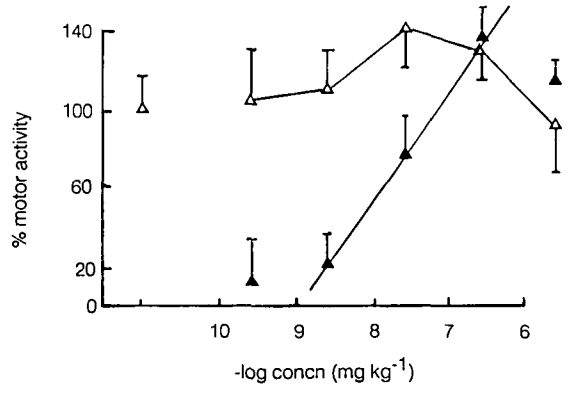

FIG. 2. Effect of subcutaneous treatment with ORG 2766 in a microsphere form on motor activity of rats with bilateral sham (open symbols) or 6-OHDA lesions (closed symbols) in the nucleus accumbens as measured 14 days after the lesion. ORG 2766 in the microsphere preparation was injected once on day 1 following the lesion. The motor activity of each individual animal was related to that of sham-and 6-OHDA-lesioned placebo-treated animals. The mean motor activity of sham-lesioned placebo-treated rats was taken as 100%; and the mean motor activity of 6-OHDA-lesioned placebo-treated rats as 0%. The mean (s.e.m.) motor activity of sham-lesioned animals treated with microsphere vehicle on day 14 was 13·2 (±0·8) and that of 6-OHDA-lesioned rats treated with microsphere vehicle on day 14 was 8·7 (±0·9). The number of animals per group varied from 7–25. Presented are the mean calculated % ± s.e.m. (vertical bars) versus the dose of the peptide administered.

tration 7 and 14 days after the lesion. There were also no differences in the calculated slopes of the three curves (ANCOVA: $F = 0{\cdot}206$ (2,49)).

Discussion

6-OHDA, when injected bilaterally into the nucleus accumbens of rats, induces motor hypoactivity 7 days later. Spontaneous functional recovery of this impaired motor activity occurs in 3–4 weeks. Subcutaneous treatment with ORG 2766, either dissolved in saline or incorporated in microspheres, shortens this recovery period to one week. Other studies have shown that ORG 2766 is also effective after intra-accumbal or oral administration (Wolterink & Van Ree 1990). Detailed analysis has shown that administration of ORG 2766 dissolved in saline is only effective when treatment is started the day after the lesion and if it is continued for at least 4 days, which indicates that the peptide must be present during the first day following the lesion in order to exert its beneficial effect (Wolterink & Van Ree 1990). The present study shows that the effect of daily administration of ORG 2766 can be mimicked by a single injection of the peptide in the biodegradable microsphere formulation.

In the 6-OHDA-lesioned animals treated with ORG 2766 microspheres, the 6-OHDA-induced motor hypoactivity was dose-dependently reversed by the peptide treatment both 7 and 14 days after the lesion. Although ORG 2766 probably was also released during the second week after the lesion, no significant shift in the dose-response curve was found between day 7 and day 14 after the lesion. This indicates that the effectiveness of the treatment does not increase with time. Treatment during the first week is crucial for the beneficial effect of ORG 2766 on functional recovery (Wolterink & Van Ree 1990). Once the effect of ORG 2766 is established it appears to be long-lasting. In rats which had been treated with ORG 2766 from day 1 to day 6 after the lesion, motor activity was completely recovered 14 days after the lesion i.e. 8 days after the treatment with the peptide was stopped (Wolterink & Van Ree 1990). Furthermore, treatment with ORG 2766 during the second week following the lesion was not effective. Thus, once the impaired behaviour has recovered, treatment with the peptide is no longer necessary.

The treatment with the microspheres appeared to be effective at comparable doses as the daily injections with ORG 2766 dissolved in saline (calculated ED50 values of 8·9 and 28·5 ng kg^{-1}, respectively). Apparently, as with the daily s.c. administration of ORG 2766 in a saline solution, the continuous release of ORG 2766 from the microspheres injected at day 1 maintains a peptide concentration at a level needed to stimulate the recovery processes. Dekker et al (1987) showed that microsphere treatment also accelerates functional recovery from sciatic nerve tissue damage. The effectiveness of the microsphere preparation in this system is not yet clear since only one dose (40 $\mu g\ kg^{-1}\ day^{-1}$) was tested.

In conclusion, ORG 2766 accelerates functional recovery of a behavioural impairment induced by bilateral 6-OHDA lesions in the rat nucleus accumbens. The peptide in a microsphere form is effective after a single administration one day after the lesion while daily s.c. administration of a saline solution of the peptide is necessary to induce the same effect. The microsphere form of the ORG 2766 appears to be as effective at comparable doses as the saline solution of the peptide. These properties of ORG 2766 in the form of a microsphere make it an interesting drug preparation for clinical use.

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