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Erythropoietin improves place learning in fimbria–fornix-transected rats and modifies the search pattern of normal rats

Jesper Mogensen[a,*], Kamilla Miskowiak[a], Thomas Alrik Sørensen[a], Christopher Trier Lind[a], Niels Vidiendal Olsen[b,c], Jacob Bertram Springborg[c], Hana Malá[a]

[a]Department of Psychology, University of Copenhagen, Amager, Njalsgade 88, DK-2300 Copenhagen S, Denmark
[b]Department of Pharmacology, University of Copenhagen, Copenhagen, Denmark
[c]Department of Neuroanaesthesia, The Neuroscience Centre, Copenhagen University Hospital, Copenhagen, Denmark

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Abstract

The acquisition of a water-maze-based allocentric place learning task was studied in four groups of rats: two groups subjected to bilateral transections of the fimbria–fornix and two groups undergoing a sham control operation. At the moment of surgery all animals were given one systemic (intraperitoneal) injection of either human recombinant erythropoietin (EPO) (at a dosage of 5000 IU/kg body weight), given to one of the fimbria–fornix-transected groups and one of the sham-operated groups, or vehicle (saline), given to the two remaining groups. The 25-day task acquisition period (one session/day) began 6 or 7 days after the day of surgery. The fimbria–fornix-transected and saline-injected group exhibited a pronounced and long-lasting impairment of task acquisition. In contrast, the fimbria–fornix-transected and EPO-treated group demonstrated a less pronounced and more transient lesion-associated impairment. The two sham-operated groups did not differ with respect to the proficiency of task acquisition. But administration of EPO to intact animals caused a significant modification of swim patterns—apparently reflecting a somewhat modified strategy of task solution. It is concluded that systemic administration of EPO significantly improves the posttraumatic functional recovery of the presently studied place learning task after transections of the fimbria–fornix. Additionally, administration of EPO influences the strategy, although not quality, of task solution in normal (sham-operated) rats.

Keywords: Erythropoietin; Fimbria–fornix; Hippocampus; Place learning; Functional recovery; Brain damage; Plasticity; Neuroprotection; Neurotrophic effects; Water maze; Rat

1. Introduction

The interest in and understanding of the neuroprotective effects of erythropoietin (EPO) have risen drastically in recent years (for reviews, see, for instance, Buemi et al., 2002; Dame et al., 2001). Specific EPO receptors have been found on neurons, glial cells, and brain capillary endothelial cells (Bernaudin et al., 1999; Brines et al., 2000; Juul et al., 1998; Masuda et al., 1993; Morishita et al., 1997; Yamaji et al., 1996). Neurons as well as astrocytes produce EPO in an oxygen-dependent fashion (Bernaudin et al., 2000; Chin et al., 2000; Digicaylioglu et al., 1995; Marti et al., 1996; Masuda et al., 1994; Tan et al., 1992). It was originally assumed that systemically administered EPO would not cross the blood–brain barrier and reach central neurons. It has, however, now been demonstrated that systemic administration of recombinant EPO exerts neuroprotective effects in cases of experimental brain ischaemia (e.g., Brines et al., 2000; Calapai et al., 2000; Siren et al., 2001) and experimental subarachnoid haemorrhage (e.g., Alafaci et al., 2000; Buemi et al., 2000; Grasso, 2001; Springborg et al., 2002). Such results emphasize the therapeutic potentials of systemic administration of EPO. The original demonstrations of neuroprotective effects of intracerebroventricular administration of EPO in animal models of ischemic damage (e.g., Bernaudin et al., 1999; Calapai et al., 2000; Catania et al., 2002; Sadamoto et al., 1998; Sakanaka et al., 1998) were interesting but therapeutically less promising.

* Corresponding author. Tel.: +45-353-28793; fax: +45-353-28745.
E-mail address: jesper.mogensen@psy.ku.dk (J. Mogensen).

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Most studies addressing the potential neuroprotective and neurotrophic effects of EPO in animal models have focused on various types of vascular incidents (references above). Neuroprotective effects of EPO are, however, not restricted to ischemic or in other ways vascular types of brain damage. This is emphasized by a limited number of studies. For instance, EPO protects against the neurotoxicity of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) in mice (Genc et al., 2001). It modifies the consequences of spinal cord injury (Celik et al., 2002; Gorio et al., 2002; Iwasaki et al., 2002), kainate-induced seizures (Brines et al., 2000), and blunt trauma (Brines et al., 2000). EPO also increases the survival of septal cholinergic neurons in rats subjected to transections of the fimbria–fornix (Konishi et al., 1993).

It therefore seems relatively safe to conclude that the administration (and apparently even systemic administration) of recombinant EPO is able to exert neuroprotective and/or neurotrophic effects in cases of vascular as well as at least certain types of nonvascular damage to the brain. The demonstrations of such effects have, however, rarely included an examination of whether or not behavioural and cognitive symptoms were diminished. The few studies addressing such issues (e.g., Catania et al., 2002; Sadamoto et al., 1998) have found that EPO is able to reduce or eliminate behavioural symptoms but have exclusively dealt with vascular types of brain damage. Furthermore, potential behavioural and cognitive effects of the administration of EPO in nonlesioned individuals have only rarely been studied. One of the few exceptions is the demonstration by Hengemihle et al. (1996) that subcutaneous administration of EPO every other day for 19 weeks (but not 8 weeks) improved the acquisition of a water-maze-based place learning task in mice.

In the present study we decided to examine whether the systemic administration of one high dosage of human recombinant EPO would influence the postoperative acquisition of a water-maze-based place learning task of the allocentric mapping type after bilateral transections of the fimbria–fornix. Transections of the fimbria–fornix deprive the hippocampus of its major cholinergic input and, furthermore, disrupt substantial parts of the output from the hippocampal formation. Normal hippocampal function cannot be expected after such transections. Hippocampal lesions, often in the form of damage to the fimbria–fornix, in the rat have repeatedly been demonstrated to be associated with impaired acquisition of water-maze-based place learning of the allocentric mapping type (e.g., Cassel et al., 1998; DiMattia and Kesner, 1988; Hannesson and Skelton, 1998; Morris et al., 1982, 1986; Packard and McGaugh, 1992; Sutherland and Rodriguez, 1989; Sutherland et al., 1982, 1983; Whishaw and Jarrard, 1995; Whishaw et al., 1995; Mogensen et al., submitted).

The primary purpose of the present study was to investigate whether the administration of EPO influences the lesion-associated impairment of place learning. We, however, also addressed whether systemic administration of a single high dosage of EPO to normal rats modifies acquisition of the presently studied task.

2. Methods

2.1. Subjects

Forty experimentally naive, male Wistar albino rats with an initial body weight of approximately 300 g served as subjects. The animals were housed two per cage with commercial rat chow and water always available. The animals' living quarters were maintained on a 12-h light/dark cycle (lights on 0600 h). The rats were randomly divided into four experimental groups: sham surgery accompanied by a saline (vehicle) control injection (Sham/Sal) ($n = 11$), sham surgery accompanied by injection of EPO (Sham/EPO) ($n = 10$), bilateral transection of the fimbria–fornix accompanied by a saline (vehicle) control injection (FF/Sal) ($n = 10$), and bilateral transection of the fimbria–fornix accompanied by injection of EPO (FF/EPO) ($n = 9$).

The experimental protocol was approved by the Danish National Review Committee for the use of Animal Subjects (''Dyreforsøgstilsynet'') and all procedures were in compliance with the European Communities Council Directive of 24 November 1986 (86/609/EEC).

2.2. Apparatus

The water maze, a circular water tank measuring 1.85 m in diameter, was constructed according to a basic design similar to that of Morris (1984) and has been described in detail elsewhere (e.g., Mogensen et al., 1995a,d). Four points along the circumference of the water tank were arbitrarily designated North (N), South (S), East (E), and West (W), thus dividing the maze into four ''quadrants.'' Throughout all parts of the experiment one circular, submerged platform (diameter, 12.5 cm) remained in a fixed position in the middle of the SE quadrant. All parameters involving time were measured in seconds and all distances were measured in arbitrary units (''pixels'').

2.3. Behavioural procedures

The behavioural procedures were similar to those described by Mogensen et al. (1995a,d). In short, each animal was given five trials (swims) per session. Each trial had as its start position one of the locations N, S, E, or W. Within a session a given start position was not allowed to be selected on more than two trials and the start positions were otherwise randomly selected. The following parameters were considered: the total swim distance, the

total duration of a swim, the average speed of a swim, the "mean distance to platform," the "heading angle error," and the percentage of the swim duration during which the animal was found in the outer maze centered annulus. All animals were given one daily session on 25 consecutive days. The first session was given after a postoperative pause lasting 6 or 7 days.

2.4. Statistical analysis

Nonparametric statistics were chosen since normal distribution of the behavioural data could not be expected and the sample sizes were too small for proper testing of the underlying distributions (Pett, 1997). The Kruskal–Wallis nonparametric analysis of variance was initially performed (Siegel, 1956). If the analysis of variance revealed significant group differences Mann–Whitney *U* tests were applied (Siegel, 1956). These tests were performed two-tailed, except for the analysis comparing the quality of task performance in the two vehicle-injected groups where a predicted impairment in the lesioned group allowed the use of a one-tailed analysis. All parameters from all sessions were analysed in this manner and, additionally, three parameters—the total swim distance, the total duration of

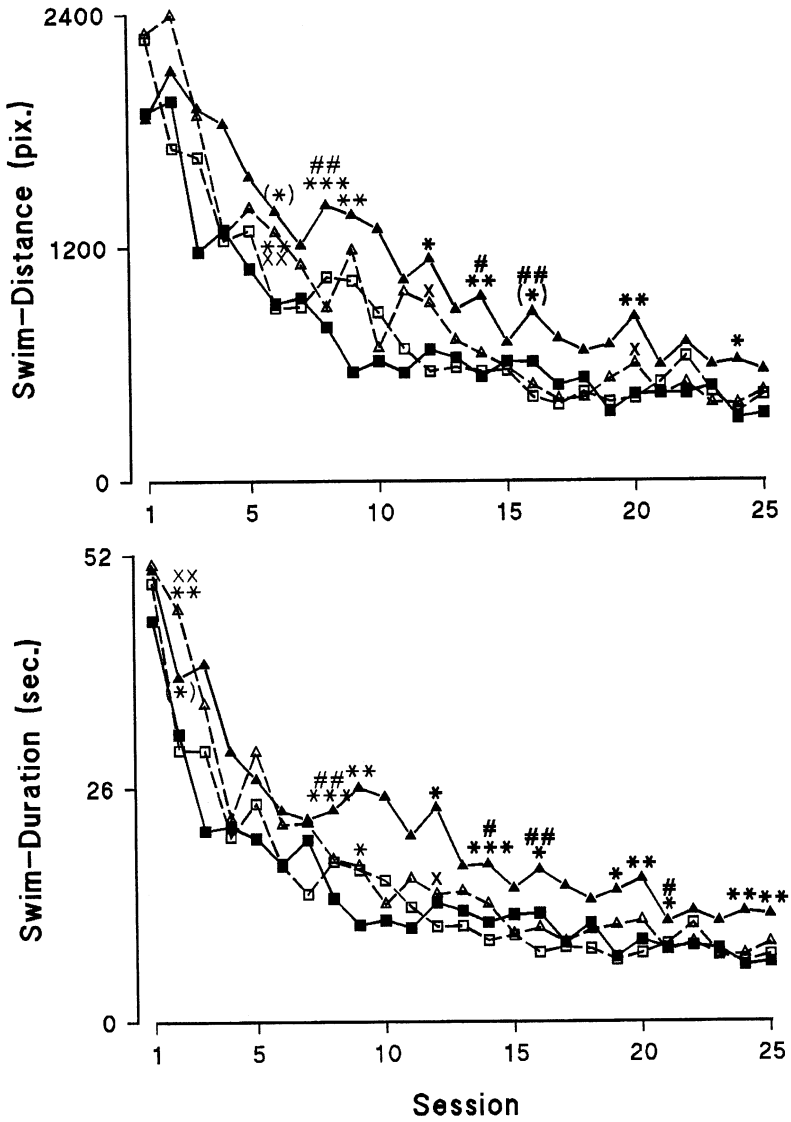

Fig. 1. Performance of the four experimental groups (black square and solid line: Sham/Sal; black triangle and solid line: FF/Sal; open square and broken line: Sham/EPO; open triangle and broken line: FF/EPO) on the 25 sessions of the place learning acquisition period. Values are given as medians. *: $P<.05$, significantly different from the Sham/Sal group. (*): $P<.05$, one-tailed, significantly different from the Sham/Sal group. **: $P<.01$, significantly different from the Sham/Sal group. ***: $P<.001$, significantly different from the Sham/Sal group. ×: $P<.05$, significantly different from the Sham/EPO group. ××: $P<.01$, significantly different from the Sham/EPO group. #: $P<.05$, significantly different from the FF/EPO group. ##: $P<.01$, significantly different from the FF/EPO group.

Table 1
Swim distance

	FF/Sal	FF/EPO	Sham/EPO
Sham/Sal	Session 6 ($p<0.05$, one-tailed) Session 8 ($p<0.001$) Session 9 ($p<0.01$) Session 12 ($p<0.05$) Session 14 ($p<0.01$) Session 16 ($p<0.05$, one-tailed) Session 20 ($p<0.01$) Session 24 ($p<0.05$)	Session 6 ($p<0.01$)	
Sham/EPO		Session 6 ($p<0.01$) Session 12 ($p<0.05$) Session 20 ($p<0.05$)	
FF/Sal		Session 8 ($p<0.01$) Session 14 ($p<0.05$) Session 16 ($p<0.01$)	

Sessions on which significant group differences were found on the parameter swim distance. Shaded boxes indicate comparisons that were not performed.

swims, and the percentage of the swim duration during which the animal was found in the outer maze centered annulus—were selected for a further analysis: for each parameter the values were summed within each of five 5-session blocks (Sessions 1–5, 6–10, 11–15, 16–20, and 21–25). For every 5-session block of each of the three parameters, statistical analysis was performed according to the methods described above.

2.5. Surgery and administration of EPO

Surgery (which lasted approximately 30 min per animal) was performed with the aid of a surgical microscope under clean but nonsterile conditions. Animals were anaesthetised by intraperitoneal injection of Equithesin (3.3 ml/kg body weight) and 1% atropine sulphate (0.9 mg/kg body weight). Transections of the fimbria–fornix were performed stereotaxically using a wire knife. Holes were drilled in the skull bilaterally at a point 1.1 mm posterior to bregma and 1.2 mm lateral to the sagittal suture. The guiding cannula of the wire knife was lowered to a position 3.2 mm ventral to the dura, and the knife was extended laterally to a length of 1.6 mm. After extension of the knife the wire knife was lowered to a position 5.0 mm ventral to the dura and left in this position for 1 min. Then the wire knife was raised again to a position 3.2 mm ventral to the dura, the knife was drawn into the guiding cannula, and the instrument was rotated 180°. The knife was then reextended to a length of 1.6 mm (now medially) and lowered to a position 5.0 mm ventral to the dura, where it was left for 1 min. The knife was then again raised to a position 3.2 mm ventral to the dura, and drawn

Table 2
Swim duration

	FF/Sal	FF/EPO	Sham/EPO
Sham/Sal	Session 2 ($p<0.05$, one-tailed) Session 8 ($p<0.001$) Session 9 ($p<0.01$) Session 12 ($p<0.05$) Session 14 ($p<0.001$) Session 16 ($p<0.05$) Session 19 ($p<0.05$) Session 20 ($p<0.01$) Session 21 ($p<0.05$) Session 24 ($p<0.01$) Session 25 ($p<0.01$)	Session 2 ($p<0.01$) Session 9 ($p<0.05$)	
Sham/EPO		Session 2 ($p<0.01$) Session 12 ($p<0.05$)	
FF/Sal		Session 8 ($p<0.01$) Session 14 ($p<0.05$) Session 16 ($p<0.01$) Session 21 ($p<0.05$)	

Sessions on which significant group differences were found on the parameter swim duration. Shaded boxes indicate comparisons that were not performed.

into the guiding cannula, which was immediately withdrawn from the brain. Identical procedures were performed in both hemispheres.

Simultaneously with the performance of surgery all animals received one intraperitoneal injection in the volume of 1.0 ml. The injection was either a vehicle (saline) injection or an administration of EPO (Eprex, 10,000 IU/ml, Janssen-Cilag, Denmark) at a dosage of 5000 IU/kg body weight.

2.6. Histology

After completion of behavioural testing all animals were deeply anaesthetised by injection of Equithesin and transcardially perfused with saline followed by a 10% formalin in saline solution. After perfusion the brains were removed and allowed to sink at 4 °C in a 10% formalin in saline solution containing 20% sucrose. The brains were cut horizontally at 50 μm. All cutting was performed on a vibratome. The Nissl-stained sections were examined with the help of a microfiche reader and the locus as well as size of lesions was verified.

3. Results

3.1. Anatomy

The histological examination of the fimbria–fornix-transected brains established that all lesioned animals had transections of the major portion of this fibre bundle, although a minor portion of the fibres of the fimbria–fornix remained intact. Only minor variations between the extents of lesion in individual animals were apparent and

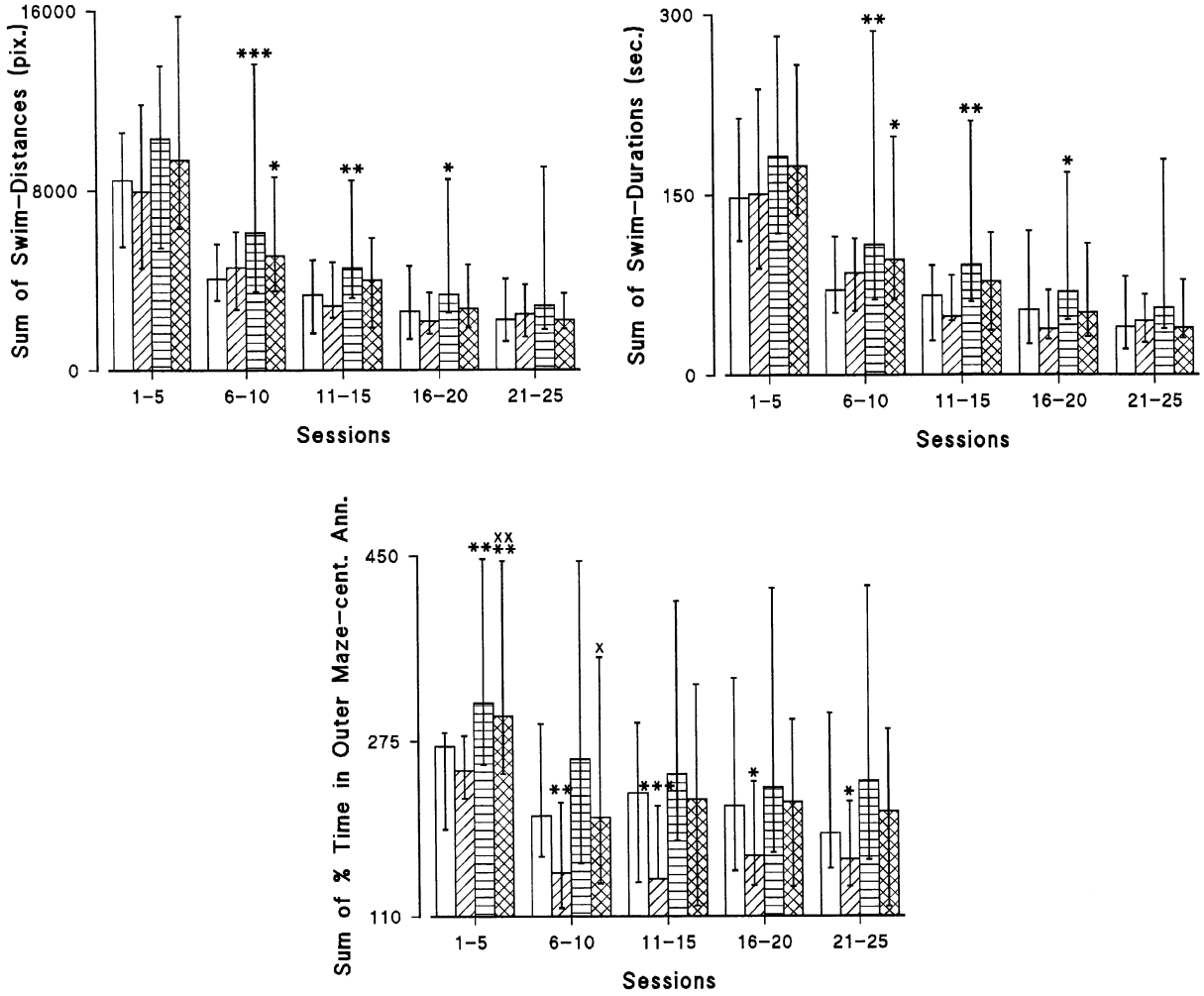

Fig. 2. Performance of the four experimental groups (open bars: the Sham/Sal group; single-hatched bars: the Sham/EPO group; horizontally lined bars: the FF/Sal group; and doubled-hatched bars: the FF/EPO group) during the five blocks of trials (see the Methods section) of the place learning acquisition period. Values are given as medians with ranges. *: $P<.05$, significantly different from the Sham/Sal group. **: $P<.01$, significantly different from the Sham/Sal group. ***: $P<.001$, significantly different from the Sham/Sal group. ×: $P<.05$, significantly different from the Sham/EPO group. ××: $P<.01$, significantly different from the Sham/EPO group. When analysed in this way, the two fimbria–fornix transected groups did not differ significantly from each other.

the lesions of the two fimbria–fornix-transected groups were of similar extent.

3.2. Behaviour

Aspects of the behavioural results are illustrated in Figs. 1–3 and Tables 1–3.

Significant group differences on the 25 acquisition sessions are illustrated in Figs. 1–3 and Tables 1–3. Additionally, significant group differences were found on the heading angle errors on Session 2 (the heading angle errors of the FF/EPO group were significantly ($P<.01$) larger than those of both of the two sham-operated groups) and the mean distances to platform on Session 4 (the mean distances to platform of the FF/Sal group were significantly ($P<.05$) larger than those of the Sham/Sal group).

As can be seen from both the detailed learning curves of Fig. 1 (plus Tables 1 and 2) and the general trends emphasized in Fig. 2, the FF/Sal group demonstrated that transections of the fimbria–fornix impaired the place learning task. Although this group underwent a substantial degree of functional recovery during the period of the 25 training sessions, both the parameters swim distance and swim duration demonstrated significant lesion-associated impairments even on the last sessions of the experiment. In contrast, the FF/EPO group had a much more transient and limited degree of impairment—both when compared to the Sham/Sal and the Sham/EPO group. Neither of the two "quality parameters," swim distance and swim duration, showed any clear differences between the two sham-operated groups.

As demonstrated in Fig. 3 significant differences between the swim speeds of the various experimental groups were only found on two sessions—and both occurred in the later phases of the experiment.

The "strategy parameter" percentage swim time spent in the outer maze centered annulus (Figs. 2 and 3; Table 3) demonstrated rather independent effects of the lesion and EPO, respectively. Transection of the fimbria–fornix caused the rats to remain significantly closer to the edges of the maze (especially in the early phases of the experiment). Administration of EPO, especially in the Sham/EPO group,

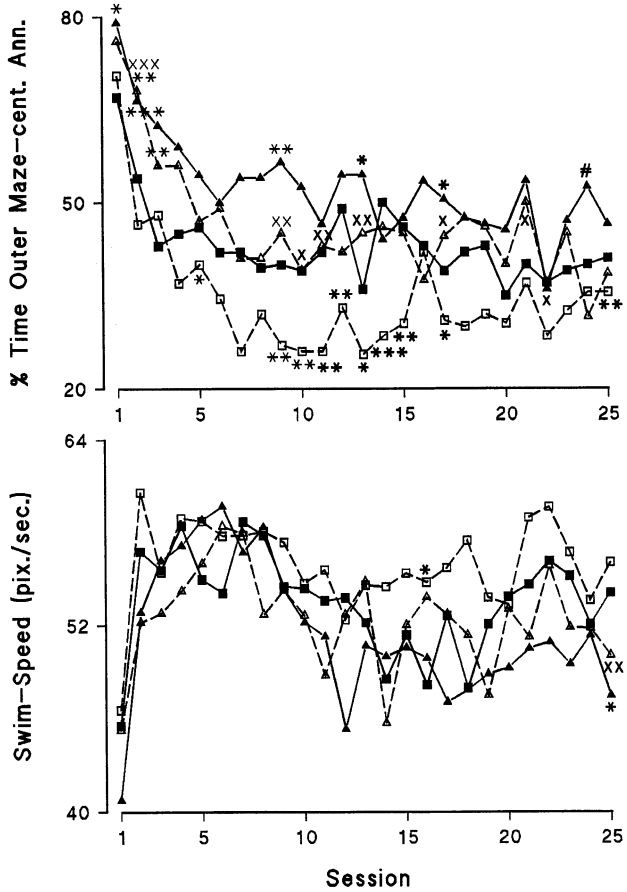

Fig. 3. Performance of the four experimental groups (black square and solid line: Sham/Sal; black triangle and solid line: FF/Sal; open square and broken line: Sham/EPO; open triangle and broken line: FF/EPO) on the 25 sessions of the place learning acquisition period. Values are given as medians. *: $P<.05$, significantly different from the Sham/Sal group. **: $P<.01$, significantly different from the Sham/Sal group. ***: $P<.001$, significantly different from the Sham/Sal group. ×: $P<.05$, significantly different from the Sham/EPO group. ××: $P<.01$, significantly different from the Sham/EPO group. ×××: $P<.001$, significantly different from the Sham/EPO group. #: $P<.05$, significantly different from the FF/EPO group.

Table 3
Percentage swim time in the outer maze centered annulus

	FF/Sal	FF/EPO	Sham/EPO
Sham/Sal	Session 1 ($p<0.05$) Session 2 ($p<0.01$) Session 3 ($p<0.05$) Session 9 ($p<0.01$) Session 13 ($p<0.05$) Session 17 ($p<0.05$)	Session 2 ($p<0.01$) Session 3 ($p<0.01$)	Session 5 ($p<0.05$) Session 9 ($p<0.01$) Session 10 ($p<0.01$) Session 11 ($p<0.01$) Session 12 ($p<0.01$) Session 13 ($p<0.05$) Session 14 ($p<0.001$) Session 15 ($p<0.01$) Session 17 ($p<0.05$) Session 25 ($p<0.01$)
Sham/EPO		Session 2 ($p<0.001$) Session 9 ($p<0.01$) Session 10 ($p<0.05$) Session 11 ($p<0.01$) Session 13 ($p<0.01$) Session 17 ($p<0.05$) Session 21 ($p<0.05$) Session 22 ($p<0.05$)	
FF/Sal		Session 24 ($p<0.05$)	

Sessions on which significant group differences were found on the parameter percentage swim time in the outer maze centered annulus. Shaded boxes indicate comparisons that were not performed.

significantly decreased the swim time in the outer maze centered annulus.

4. Discussion

In the present study, human recombinant EPO administered systemically at the moment when the lesion was inflicted significantly reduced the magnitude of behavioural symptoms associated with bilateral transections of the fimbria–fornix in the rat. The "quality parameters" swim distances and swim durations are indicators of the proficiency of task performance. As illustrated in Figs. 1 and 2 (plus Tables 1 and 2) these variables demonstrated that the vehicle (saline)-injected and fimbria–fornix-transected group had a substantial and long-lasting impairment of task acquisition. The group subjected to similar lesions, but given one intraperitoneal injection of human recombinant EPO, however, only demonstrated a milder and more transient behavioural impairment.

When comparing the two sham-operated groups (given EPO and saline, respectively) on the parameters that reveal the proficiency of task performance (Figs. 1 and 2) no clear difference was found. In contrast, administration of EPO had a highly significant and long-lasting effect on the search patterns selected by the animals, as revealed by the percentage swim time spent in the outer maze centered annulus. This parameter is considered one of the strategy parameters that primarily reflect the employed solution strategies rather than the proficiency of task performance. Relative to the saline-injected normal animals, the nonlesioned and EPO-treated group spent significantly less time close to the edge of the water maze.

Rats subjected to lesions of the hippocampus tend to spend a higher percentage of their swim time close to the edges of the maze, thereby demonstrating "wall-hugging" or "thigmotaxis" (e.g., O'Keefe and Nadel, 1978). In the present study, hippocampal lesions increased the percentage swim time in the outer maze centered annulus, both within the saline-injected groups and within the EPO-treated groups. On this parameter the effects of EPO was to reduce thigmotaxis to more or less similar extents in both sham operated and fimbria–fornix-transected animals, thereby modifying the swim patterns of all EPO-injected rats rather than eliminating the effects of fimbria–fornix transection.

The present results emphasize the ability of systemically administered human recombinant EPO to reach and influence the function of the brain (both in intact and mechanically lesioned rats). However, the mechanisms via which EPO exerts such influences remain obscure. With respect to the reduced lesion-associated behavioural symptoms and/or improved functional recovery potential mechanisms can be divided into two principally different classes.

1. EPO may reduce the magnitude of damage inflicted on the neural substrate of the normally employed task solution. As indicated by the impairment of task acquisition seen in hippocampally lesioned rats, the mediation of the presently studied task will normally receive major contributions from the hippocampus. Consequently, the demonstration of Konishi et al. (1993) of increased survival of septal cholinergic neurons

after transections of the fimbria–fornix indicates one mechanism by which the EPO might exert such an influence.

2. EPO might facilitate a process in which the task would be mediated by an alternative neural substrate (e.g., Mogensen and Holm, 1994; Mogensen et al., 1995a,b,c,d, 1996, 2002). EPO might be able to facilitate the neural reorganizations necessary for the application of an alternative solution strategy and thereby an alternative neural substrate, e.g., by facilitating synaptic plasticity—for instance, via an effect on Ca^{2+} channels and the NO system (Assandri et al., 1999; Koshimura et al., 1999; Miller et al., 1999) or synaptic transmission (Weber et al., 2002). EPO might also protect significant parts of the neural substrate of alternative behavioural strategies by diminishing or eliminating secondary or tertiary consequences of the primary trauma. This might for instance be accomplished via the ability of EPO to increase the activity of antioxidant enzymes (Chattopadhyay et al., 2000; Sakanaka et al., 1998; Sela et al., 2001), thereby being able to act as an indirect free radical scavenger.

Planned and ongoing research will address whether one or both of these mechanisms is involved in the presently studied effects of EPO.

As can be seen from Figs. 1 and 2, EPO clearly reduces the behavioural symptoms associated with the lesion during most of the 25-day task acquisition period. For approximately the first week of this period, however, both the fimbria–fornix-transected groups performed at approximately the same (impaired) proficiency of task solution. This implies that administration of EPO supports and enhances the training-induced functional recovery rather than a priori diminishing the level of lesion-associated impairment. Since the present study only included groups that started the behavioural procedures 6–7 days postoperatively, an alternative interpretation could be that the symptom-reducing effects of EPO required approximately 2 weeks to manifest themselves (whether or not behavioural training occur during the second of these weeks).

Utilizing pharmacological, surgical, and/or behavioural "challenges" (see Mogensen et al., 1995d) we have studied the neural and cognitive mechanisms of functional recovery after various types of brain damage. In the present context it is of special relevance that the functional recovery of allocentric place learning of the mapping type after transections of the fimbria–fornix receives significant contributions to its mediation from the anteromedial prefrontal cortex (Mogensen et al., submitted). Additionally, the dopaminergic systems as well as the prefrontal cortex contribute significantly to the mediation of the functional recovery of an allocentric place learning task of the "nonmapping" type after fimbria–fornix transections (Wörtwein et al., 1995). Also after transections of the fimbria–fornix the posttraumatic functional recovery of a water-maze-based task requiring egocentric navigation was found to be mediated by the prefrontal cortex (Mogensen et al., submitted). It may also be of relevance to note that L-nitro-arginine induced inhibition of nitric oxide synthase (NOS) reduced or eliminated contributions from the hippocampal formation (Mogensen et al., 1995c) and the cholinergic systems (Mogensen et al., 1995d) to mediation of the presently studied task. Under such circumstances a relative increase of the importance of dopaminergic task mediation occurred (Mogensen et al., 1995d). Note, however, that in the case of L-nitro-arginine-induced inhibition of NOS, the shift away from hippocampal task mediation seems not to occur primarily in the direction of an increased importance of prefrontal cortical contributions to the task mediation (Mogensen et al., 1996). These studies demonstrate that within a variety of spatial navigational tasks the functional recovery after fimbria–fornix transections is at least partly mediated by dopaminergic and/or prefrontal cortical mechanisms. On this background it is noteworthy that EPO seems to stimulate the release of dopamine (Koshimura et al., 1999; Yamamoto et al., 2000; see, however, Kawakami et al., 2000). It should be mentioned that the prefrontal cortex is the cortical area that receives the highest density of dopaminergic innervation (e.g., Björklund and Lindvall, 1984; Divac et al., 1978) and that the prefrontal system depends on normal levels of dopaminergic activity for at least certain aspects of its activity (e.g., Brozoski et al., 1979; Simon et al., 1980).

In contrast to the results of Hengemihle et al. (1996) we did not see an improved quality of place learning in the nonlesioned and EPO-treated animals. This is, however, not surprising since the demonstration of such an effect by Hengemihle et al. was performed after 19 weeks of EPO administration every other day (and not found after 8 weeks of such a treatment regime). In the present study, only one (high) dosage of EPO was administered. It may even be considered somewhat surprising that (as illustrated in Figs. 2 and 3) significant EPO-associated effects were seen on the swim patterns of the sham-operated rats throughout a period of around 3 weeks starting approximately 2 weeks after the one administration of the hormone.

In the present study we focused on the effects of one relatively high dosage of EPO administered at the moment of brain damage. The "therapeutic" effect of this treatment can hardly be questioned, but it is obviously important that future studies expand this research in two directions:

1. A dosage–response relationship needs to be established by studying effects of a variety of (lower) dosages of EPO.
2. It must be addressed whether administration of EPO can have similar effects to those seen in the present study if the substance is administered after the moment of brain damage.

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