Erythropoietin Improves Place Learning in an 8-Arm Radial Maze in Fimbria-Fornix Transected Rats

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ABSTRACT

Systemically administered human recombinant erythropoietin (EPO) may have the potential to reduce the cognitive and behavioral symptoms of mechanical brain injury. In a series of studies, we address this possibility. We previously found that EPO given to fimbria-fornix transected rats at the moment of injury could substantially improve the posttraumatic acquisition of an allocentric place learning task when such a task is administered in a water maze. Due to the clinical importance of such results, it is important to scrutinize whether the therapeutic effect of EPO is specific to the experimental setup of our original experiments or generalizes across test situations. Consequently, here we studied the effects of similarly administered EPO in fimbria-fornix transected and control operated rats, respectively, evaluating the posttraumatic behavioral/cognitive abilities in an allocentric place learning task administered in an 8-arm radial maze. The administration of EPO to the hippocampally injured rats was associated with a virtually complete elimination of the otherwise severe behavioral impairment caused by fimbria-fornix transection. In contrast, EPO had no detectable effect on the task acquisition of non-lesioned animals. The results of the present study confirm our previous demonstration of EPO's ability to reduce or eliminate the behavioral/cognitive consequences of mechanical injury to the hippocampus, while adding the important observation that such a therapeutic effect is not restricted to the specific experimental setup previously studied.

KEYWORDS

hippocampus, place learning, functional recovery, brain damage, neuronal plasticity, neuroprotection, neurotrophic effects

INTRODUCTION

Specific receptors for erythropoietin (EPO), a hormone that also stimulates the body to produce red blood cells, 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 manner (Bernaudin et al., 2000; Chin et al., 2000;

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Digicaylioglu et al., 1995; Marti et al., 1996; Masuda et al., 1994; Tan et al., 1992). Such results have in recent years led to a dramatic increase in the interest in the potential neuroprotective roles of EPO (for reviews see e.g., Buemi et al., 2002; Dame et al., 2001; Olsen, 2003). It has even been suggested that EPO might be able to act therapeutically in patients suffering certain neurodegenerative conditions (e.g., Ehrenreich et al, 2004). A number of studies (for references, see below) have demonstrated that EPO may be able to act therapeutically in case of various types of brain injury. Peripherally administered human recombinant EPO penetrates the blood-brain barrier and can exert neuroprotective effects in case of both brain ischemia and experimental subarachnoid hemorrhage (e.g., Alafaci et al., 2000; Brines et al., 2000; Buemi et al., 2000; Calapai et al., 2000; Grasso 2001; Siren et al., 2001; Springborg et al., 2002). The neuroprotective potentials of EPO have been demonstrated mainly in animal models of various types of vascular incidents (e.g., Alafaci et al., 2000; Brines et al., 2000; Buemi et al., 2000; Calapai et al., 2000; Grasso 2001; Siren et al., 2001; Springborg et al., 2002). It is clear, however, that EPO even has the potential of exerting such effects in case of nonvascular types of neural trauma. For instance, EPO increases the survival of septal cholinergic neurons in rats subjected to transection of the fimbria-fornix (Konishi et al., 1993) and protects against the neurotoxicity of MPTP (Genc et al., 2001). Additionally, EPO modifies the consequences of spinal cord injury (e.g., 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).

Most studies addressing the potentially therapeutic effects of EPO after brain injury evaluated such effects by utilizing histological and/or neurochemical methods exclusively. Only a few studies have addressed the question whether the administration of EPO also improves the posttraumatic behavioral and cognitive outcome after a given lesion. Until recently, the rare studies dealing with this issue (e.g., Catania et al., 2002; Kumral et al., 2004; Sadamoto et al., 1998) have exclusively used animal models of various vascular types of brain injury. Recently, however, we addressed the issue of whether EPO can improve the posttraumatic cognitive/behavioral performance of mechanically brain injured rats. We did so by studying the posttraumatic acquisition of a water maze-based allocentric place learning task of the mapping type after transection of the fimbria-fornix. Such a task is known to be highly sensitive to hippocampal dysfunction (e.g., Cassel et al., 1998; DiMattia & Kesner, 1988; Hannesson & Skelton, 1998; Mogensen et al., 2004a; Morris et al., 1982; Morris et al., 1986; Packard & McGaugh, 1992; Sutherland & Rodriguez, 1989; Sutherland et al., 1982; Sutherland et al., 1983; Whishaw & Jarrard, 1995; Whishaw et al., 1995). We have by now repeatedly (Mogensen et al., 2004b; Mogensen et al., submitted) demonstrated that systemically administered EPO can improve a water maze-based place learning task in fimbria-fornix transected subjects, thereby showing an ability to support posttraumatic behavioral/ cognitive recovery.

The ability of systemically administered EPO to reduce the cognitive/behavioral consequences of hippocampal injury and/or significantly support the posttraumatic functional recovery after such lesions is of substantial clinical importance. It should, however, be noticed that to evaluate the therapeutic potentials of EPO, it is necessary to further scrutinize the generality of such EPO-associated effects. As emphasized elsewhere (e.g., Mogensen, 2003) one frequently encounters a surprisingly high degree of method dependency when evaluating the consequences of brain injury and/or pharmacological interventions. For instance, the interhemispheric transfer of information relevant to the performance of a visual discrimination task in cats subjected to transections of callosal connections

appear to be possible when tested in a Lashley-type jumping stand but absent when tested in a classic two-choice discrimination box (Lepore et al., 1985). Additionally, rats subjected to lesions within the prefrontal system are severely impaired in a spatial delayed alternation task when tested in a T-maze but perform at a normal proficiency when encountering the same task in an operant chamber (Mogensen et al., 1987). As a final example, until we realized the differentiation between allocentric place learning tasks of the mapping and non-mapping types (e.g., Mogensen et al., 1995b; 2002; 2004a; Wörtwein et al., 1995) differential setups of water mazes might result in contradictive results of brain injury or pharmacological manipulation. Compare for instance the consequences of hippocampal lesions in Mogensen et al. (2004a) and Wörtwein et al. (1995). Consequently, the ability of EPO to counteract the posttraumatic impairment of allocentric place learning demonstrated so far in water mazes (Mogensen et al, 2004b; Mogensen et al., submitted) should also be evaluated in a different experimental setup. We therefore decided to test whether systemically administered EPO can reduce the behavioral/cognitive consequences of fimbria-fornix transections, even when the post-traumatic acquisition of an allocentric place learning task is tested in an 8-arm radial maze. Lesions of the hippocampus, including transections of the fimbria-fornix, have been shown to be associated with impairments of allocentric place learning when tested in 8-arm radial mazes (Cassel et al., 1998).

For the present study, we chose to repeat our original design, in which two groups of rats were subjected to transections of the fimbria-fornix. One group was administered EPO at the moment of injury, whereas the other group received control injections of saline (vehicle); two groups were subjected to sham surgery and received pharmacological treatments similar to those of the two fimbria-fornix transected groups.

EXPERIMENTAL

Subjects

Thirty-six experimentally naive, male Wistar albino rats with an initial body weight of approximately 300 g each served as subjects. The animals were housed two per cage with water always available in a colony room with a 12 h light cycle (on at 6.00 h). The animals were fed commercial rat chow once daily after training and were maintained at approximately 85% of their ad libitum body weights. The rats were randomly divided into four experimental groups:

1. sham surgery accompanied by a saline (vehicle) control injection (Sham/Sal) (n=7),
2. sham surgery accompanied by injection of EPO (Sham/EPO) (n=10),
3. bilateral transection of the fimbria-fornix accompanied by saline (vehicle) control injection (FF/Sal) (n=9), and
4. bilateral transection of the fimbria-fornix accompanied by injection of EPO (FF/EPO) (n=10).

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

Apparatus

All training and testing was performed in an open, black, one-unit 8-arm radial maze with 2.7 cm high walls and 9.2 cm wide corridors. The 8 arms radiated equidistantly from a circular central area with a diameter of 50.0 cm. Each arm was 60.0 cm long, and at the end of the arm a circular food well (diameter: 4.8 cm, depth: 2.3 cm) contained the reinforcements in the form

of 45 mg food pellets (Precision Food Pellets of Campden Instruments, England). The maze was placed in the centre of a well lit room, in which no other animals were present during training and testing, and in which a multitude of two as well as three dimensionally arranged distal cues were available.

Behavioral procedures

Preoperatively all animals were habituated to the maze and shaped. The habituation lasted for two sessions. Each session allowed the rats 25 min of undisturbed exploration of the maze. During the first habituation session, 45-mg reinforcement pellets were scattered all over the maze, whereas in the second habituation session, the reinforcement pellets were present only in and around the food wells. In the third session, the shaping procedure was initiated. During shaping, 15 trials (runs) were given per (daily) session, and the start arm of each trial was randomly selected. The reinforcement pellets were present in the food wells of all arms but the start arm, and the animal was released from the end of the start arm. After reaching the end of any of the response arms, the animal was allowed to eat freely four reinforcement pellets. Subsequently, the animal was picked up and the next trial initiated. The shaping procedures continued until all animals promptly (within less than 10 s) entered one of the response arms when released. The postoperative behavioral procedures were initiated 6 or 7 days after surgery. During the first three postoperative sessions, the animals were briefly reshaped. In the fourth postoperative session, training and testing of the place learning task was initiated. All animals were given one daily place learning session on 20 consecutive days. At each acquisition session, 15 trials (runs) were given and 4 reinforcement pellets were present in all arms but the start arm. One arm (defined according to its spatial location within the experimental room) was defined as the goal arm for all trials throughout the task acquisition period. The remaining seven arms served as (for each trial randomly selected) start arms. To avoid the possibility of successful task solution being based on local (intramaze) cues, we rotated the maze between sessions (in such a way that only the spatial position but not the intramaze identity of the goal arm remained constant between sessions). When entering the goal arm, the rat was allowed to reach the food well and consume the four reinforcement pellets. If, however, an incorrect arm was entered, the animal was picked up before reaching the food well and returned to a holding cage where it remained until the next trial. After a correct trial, the animal was likewise transferred to a holding cage until the next trial. The inter-trial periods were of approximately 1 min duration. From each trial, two parameters were recorded: the total number of errors and the number of "distal" errors (defined as all errors but those to the two arms adjacent to the goal arm). Although all animals continued behavioral training and testing for all 20 task acquisition sessions, when an animal reached a behavioral criterion defined as two consecutive days with no errors, this was recorded.

Statistical analysis

Non-parametric statistics were chosen because the normal distribution of Behavioral data could not be expected, and the sample sizes were too small for the proper testing of the underlying distributions (Pett, 1997). The Kruskal-Wallis non-parametric analysis of variance was initially performed (Siegel, 1956). If the analysis of variance revealed significant group differences, then Mann-Whitney U-tests were applied (Siegel, 1956). The latter were two-tailed, except for the analysis comparing the quality of task performance in the two vehicle-injected groups, for which a

predicted impairment in the lesioned group allowed the use of a one-tailed analysis. The two parameters from all sessions, as well as the number of sessions required to reach the Behavioral criterion, were analyzed in this manner (in case an animal failed to reach the Behavioral criterion within the 20 sessions, the "number of sessions to criterion" was indicated as 21).

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 non-sterile conditions. The animals were anesthetized by intraperitoneal injection of Equithesin (3.3 ml/kg body weight) and 1% Atropin sulphate (0.9 mg/kg body weight). Bilateral transections of the fimbria-fornix were performed stereotaxically using a wire-knife. Detailed descriptions of the surgical procedures have previously been published (e.g., Mogensen et al., 2004a; 2004b; 2005).

Simultaneously with the performance of surgery, all animals received one intraperitoneal injection in a volume of 1.0 mL, either a vehicle (saline) injection or an administration of EPO (Eprex™, 10,000 IU/ml, Janssen-Cilag, Denmark) in the dosage of 5,000 IU/kg body weight.

Histology

After the completion of the Behavioral testing, all animals were deeply anesthetized (Equithesin injection) and transcardially perfused with saline, followed by 10% formalin in saline solution. After perfusion, the brains were removed and allowed to sink at 4°C in 10% formalin in saline solution containing 20% sucrose. The brains were cut horizontally at 50 μm on a vibratome. The Nissl-stained sections were examined with the help of a microfiche reader, and the locus and lesion size were verified.

RESULTS

Anatomy

Histological examination of the fimbria-fornix transected brains established that all lesioned animals had transections of the major portion of this fiber bundle, although a minor portion of the fibers of the fimbria-fornix remained intact. Only minor variations between the extents of lesion in individual animals were apparent, and the lesions of the fimbria-fornix-transected groups were of similar extent.

Behavior

The Behavioral results are illustrated in Figs. 1 and 2. As shown in Fig. 1, the fimbria-fornix transected group receiving saline injections (FF/Sal) required significantly more sessions to reach the Behavioral criterion when compared with the Sham/Sal, as well as with the FF/EPO groups. The fimbria-fornix-transected EPO-treated group, on the other hand, fulfilled the Behavioral criterion as quickly as the two control-operated groups did. Administration of EPO to sham-operated control animals did not significantly influence the number of sessions required to fulfill the criterion. For this parameter, the analysis of variance was significant at the $p < 0.01$ level. The levels of significance for the individual group comparisons are given in Fig. 1 (note that the indicated values are medians and ranges). Although the distance between minimum and maximum values differ somewhat between groups, the general variability does not differ to any major extent.

As reflected in the learning curves illustrated in Fig. 2, the fimbria-fornix transected saline injected (FF/Sal) group demonstrated a certain level of Behavioral recovery in the form of gradual task acquisition, but remained significantly impaired when compared to both the saline injected sham operated group (Sham/Sal) and the

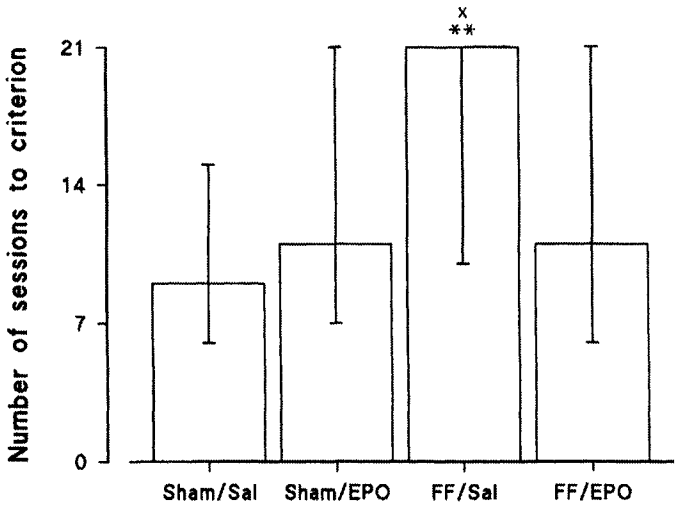

Fig. 1: Number of sessions required by the four experimental groups to reach the task acquisition criterion (see Experimental section for details). Values are given as medians with ranges. **: Significantly ($p < 0.01$) different from the Sham/Sal group. x: Significantly ($p < 0.05$) different from the FF/EPO group.

EPO-treated fimbria-fornix transected group (FF/EPO) throughout most of the 20 d of the task acquisition period. In contrast, neither the FF/EPO group nor the Sham/EPO group differed significantly from the normal animals of the Sham/Sal group throughout the task acquisition period. For the total number of errors, the analysis of variance indicated the presence of significant group differences on the following sessions:

Total errors			
session 3	($p < 0.05$)	session 13	($p < 0.05$)
session 4	($p < 0.01$)	session 14	($p < 0.05$)
session 5	($p < 0.01$)	session 15	($p < 0.001$)
session 6	($p < 0.05$)	session 16	($p < 0.05$)
session 10	($p < 0.05$)	session 18	($p < 0.001$)
session 11	$p < 0.001$)	session 19	($p < 0.01$)

For the number of "distal" errors, the analysis of variance indicated the presence of significant group differences on the following sessions:

"Distal" errors			
session 6	($p<0.05$)	session 13	($p<0.01$)
session 10	($p<0.01$)	session 15	($p<0.01$)
session 11	($p<0.001$)	session 16	($p<0.05$)
session 12	($p<0.05$)		

For both parameters, the levels of significance for the individual group comparisons are given in Fig. 2.

DISCUSSION

The results of the present study (Figs. 1 and 2) clearly demonstrate that systemically administered human recombinant EPO can dramatically improve the cognitive/behavioral performance of rats after mechanical brain injury in the form of bilateral transections of the fimbria-fornix. Although we previously demonstrated such an ability in the form of complete or near-complete

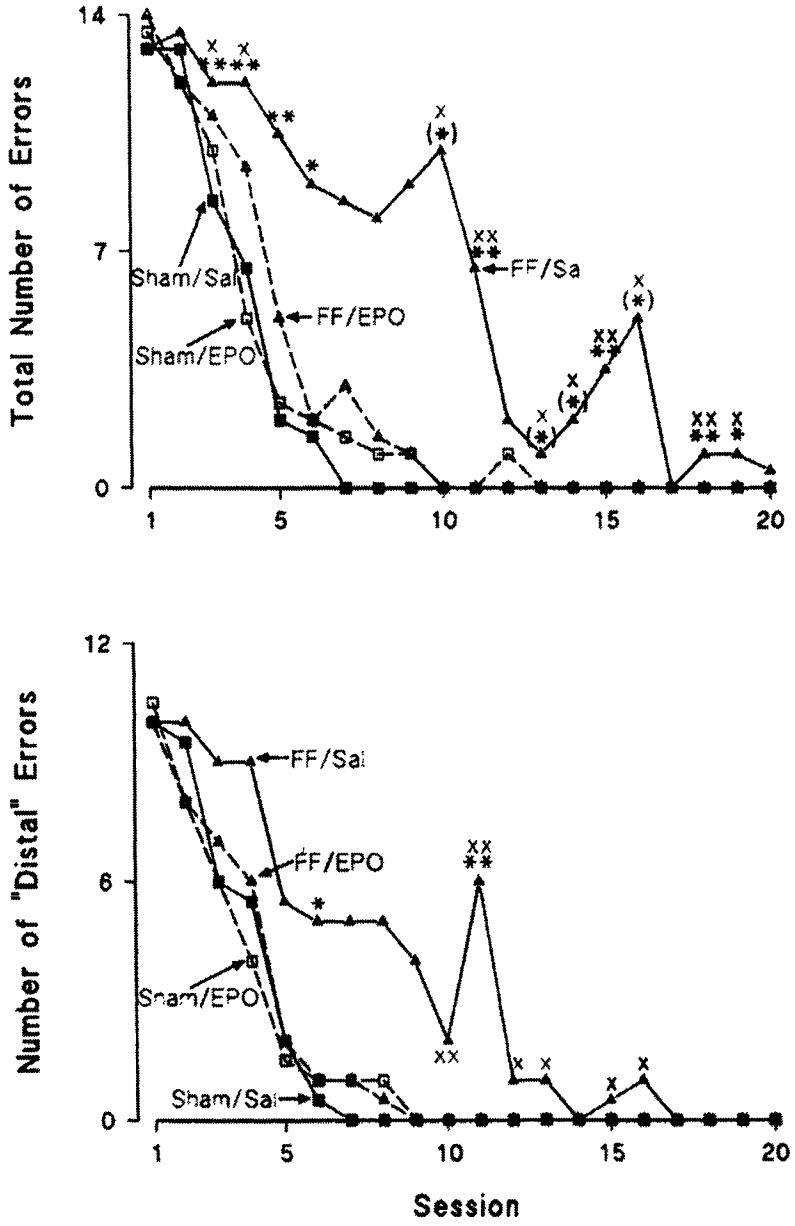

Fig. 2: Performance of the four experimental groups (black square and solid line: Sham/Sal; black triangle and solid line: FF/Sal; open square and broken line: Sham/EPO; open triangle and broken line: FF/EPO) on the 20 sessions of the place learning acquisition period. Values are given as medians. *: Significantly ($p<0.05$) different from the Sham/Sal group. (*): Significantly ($p<0.05$, one-tailed) different from the Sham/Sal group. **: Significantly ($p<0.01$) different from the Sham/Sal group. x: Significantly ($p<0.05$) different from the FF/EPO group. xx: Significantly ($p<0.01$) different from the FF/EPO group.

normalization of posttraumatic acquisition of a water maze-based allocentric place learning task (Mogensen et al., 2004b; Mogensen et al., submitted), in the present study we obtained a similar effect in an allocentric place learning task performed in a completely different experimental setup—an 8-arm radial maze.

As expected (Cassel et al., 1998), the saline injected fimbria-fornix-transected group had a significantly impaired acquisition of the 8-arm radial maze-based allocentric place learning task. Even this group, however, eventually managed to acquire the task to a relatively high proficiency of task performance, thereby demonstrating a functional recovery (Fig. 2). In contrast, the similarly lesioned group treated with EPO at the moment of brain injury did not differ significantly from the saline-injected, sham-operated control group. Consequently, it has to be concluded that EPO can either completely eliminate the normally occurring negative consequences of the fimbria-fornix transections or promote compensational processes, which were so immediate that even initial behavioral symptoms could not be observed. (See for instance Mogensen et al., 2002, for an example of immediate and complete compensation for the consequences of a neural disturbance preventing even initial behavioral symptoms.)

The two sham-operated groups receiving saline control injections and systemic injections of EPO, respectively, did not differ significantly on any behavioral measure (Figs. 1-2). Consequently, it has to be concluded that when administered in one high dose 6 or 7 days before the initiation of training on the presently studied type of place learning, EPO does not influence the performance of the task either negatively or positively. This result seems to be in agreement with the results of Hengemihle et al. (1996), who found an improved quality of place learning in a water maze in non-lesioned EPO treated animals after 19 wk of EPO administration every other day, but absence of such an effect after 8 wk of a similar treatment regime. On the background of such results, one would not expect the task performance of intact animals to be influenced by the presently studied administration of EPO. In two previous studies, however, we found EPO, administered as in the present study, to influence place learning of intact rats in a water maze. In our initial study (Mogensen et al., 2004b) animals treated in this way displayed a modified search pattern but no change in the quality of task performance, whereas subsequently (Mogensen et al., submitted) we found a minor but significant impairment of the task performance of EPO-treated, non-lesioned rats. On the other hand, EPO has been suggested to act as a "cognitive enhancer" in normal animals (see for instance Ehrenreich et al., 2004, for a suggestion of such a role for this hormone). Our three studies of allocentric place learning in EPO-treated sham operated animals (Mogensen et al., 2004b; Mogensen et al., submitted; and present results) do not lend support to such a notion however.

As we have previously emphasized (Mogensen et al., 2004b; Mogensen et al., submitted), EPO-associated improvements of posttraumatic Behavioral/cognitive performance in lesioned individuals can be, in principle, obtained via one or both of two categories of mechanisms:

1. The magnitude of injury inflicted on the neural substrate of the normally employed task solution can be reduced by EPO administration. In the case of tasks like that currently studied here, which normally rely heavily upon hippo-campal contributions to task mediation, it may be of relevance that Konishi et al. (1993) found an increased survival of septal cholinergic neurons after transections of the fimbria-fornix.
2. Erythropoietin can facilitate a process in which the task is mediated by an alternative neural substrate (e.g., Mogensen & Holm, 1994; Mogensen et al., 1995a-d; 1996; 2002; 2004a; 2005). Such an EPO-associated facilitation of the neural reorganizations that are necessary

for the application of an alternative solution strategy can, for instance, be obtained by facilitating synaptic plasticity—e.g., 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). Erythropoietin might also protect significant parts of the neural substrate of alternative behavioral strategies by diminishing or eliminating the secondary or tertiary consequences of the primary neural trauma. This task might be accomplished, for instance, 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 acting as an indirect free radical scavenger.

By addressing some cognitive mechanisms of EPO-assisted posttraumatic recovery after fimbria-fornix transections, we found indications that with at least some administration schedules and certain tasks, EPO facilitates the application of an alternative solution strategy rather than preserving one identical to that applied by normal animals (Mogensen et al., submitted). In the same study, however, we also saw indications that this might not be the only mechanism via which EPO improves the posttraumatic functional recovery of a place learning task.

The results of the present study do not point directly to one or the other of these therapeutic mechanisms of EPO. We have now clearly established, however, that the EPO-associated dramatic improvement of allocentric place learning in fimbria-fornix transected rats is not specific to such tasks administrated in a water maze. Presently, a cognitively rather similar task was administered in a setup requiring not only motoric responses clearly different from those of a rat swimming in a water maze but also presumably solution strategies somewhat dissimilar to those applicable in the previously studied, experimental setups. Yet, the administration of EPO to fimbria-fornix-transected rats led to a virtual elimination of the lesion-associated task impairment. Whether obtained via one or the other of the two above mentioned classes of hormonal action, the results clearly testify that administration of human recombinant EPO is likely to have cognitive/behavioral therapeutic effects, which even in case of mechanical brain injury, can be effective across the situations in which a particular neural/cognitive mechanism is required.

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