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Damaged neuronal energy metabolism and behavior are improved by Ginkgo biloba extract (EGb 761)

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Summary. The standardized extract EGb 761 from the dried green leaves of Ginkgo biloba is a complex mixture of ingredients with an uniquely broad spectrum of pharmacological activities on the central nervous system e.g. in memory enhancing properties and in the regulation of cerebral glucose/energy metabolism. To test these effects on both behavioral and metabolic brain parameters, the animal model of intracerebroventricular (icv) streptozotocin (STZ) treatment was used. To quantify the experimental data more precisely, animals that were good performers were separated from poor performers by means of the holeboard test before icv administration of STZ. Good performers only were considered for the study. After a 1-week training period on the holeboard improvement was seen in all animals in learning, memory and cognition, and the improvement was maintained over the investigation period of 12 weeks in the control group. In this group, the energy pool in the cerebral parietotemporal cortex was found to be large and the energy turnover high. After triplicate icv STZ injection, working memory (WM), reference memory (RM), and passive avoidance (PA) behavior fell off and continued to deteriorate throughout the investigation period. Otherwise there were no significant differences in locomotor activity, excluding the possibility that activity per se might have contributed to the behavioral abnormalities. These were accompanied by a permanent deficit in cerebral energy metabolism. The ongoing deterioration in behavior and the maintained deficit in cerebral energy metabolism occurring after a triplicate icv STZ injection were significantly slowed down by EGb761. The deficits in learning, memory and cognition were partially compensated, and the disturbances in cerebral energy metabolism returned to almost completely normal values. These findings underscore the beneficial effect of EGb761 that had been reported in dementia disorders.

Keywords: Brain, behavior, energy pool, streptozotocin, Ginkgo biloba, dementia.

Introduction

Availability of reliable animal models of progressive decline in cognitive functions resembling dementia e.g. sporadic dementia of Alzheimer type (SDAT) in human beings is not only a basic requirement for better understanding of the cellular and molecular mechanisms underlying these deficits, but also for improved changes of success in the search for the much needed therapeutic agents potentially useful for the management of such a devastating neurodegenerative disorder. Since both, brain glucose utilization and metabolism are basic to the functions of learning, memory and cognition (Benton and Owens, 1993; Benton et al., 1994; Gold, 1995; Wenk, 1989), and since the disturbance in both, the control and metabolism of glucose in the brain has apparently been found to be an early and major abnormality in SDAT (Hoyer et al., 1991; Craft et al., 1998; Frölich et al., 1998; Hoyer, 1998; for review see Hoyer, 1996), animals in which cerebral glucose utilization/metabolism is experimentally compromised can be expected to serve as models for such purposes.

Attempts to develop such an animal model led us to damage brain glucose metabolism by means of the intracerebroventricular (icv) administration of streptozotocin (STZ). It has long been known that systemic administration of this diabetogenic agent selectively destroys the insulin producing pancreatic β-cells (Meyerovitch et al., 1989). Furthermore, it decreases both insulin receptor autophosphorylation and intrinsic tyrosine kinase activity (Ar'Rajab and Ahrén, 1993; Burant et al., 1986; Kadowaki et al., 1984) and elevates the activity of phosphotyrosine phosphatase (Begum et al., 1991; Meyerovitch et al., 1989). In the mammalian brain, insulin is formed in a subpopulation of pyramidal cells and insulin receptors are widely distributed in different densities. Both are assumed to have a neuromodulatory role and to control the neuronal glucose metabolism (for review see Henneberg and Hoyer, 1995). In a series of previous studies it was demonstrated that disturbances in learning, memory and cognition and abnormalities in global and local cerebral glucose utilization/metabolism accompanied by an energy deficit and a cholinergic deficit and cholinergic denervation became apparent after a single icv STZ injection (Mayer et al., 1990; Nitsch and Hoyer, 1991; Hellweg et al., 1992; Plaschke and Hoyer, 1993; Blokland and Jolles, 1993, 1994; Duelli et al., 1994; Prickaerts et al., 1995) whereas no structural changes were found (unpublished data). These metabolic disturbances were assigned to the effect of STZ on neuronal glucose metabolism and its control by the insulin/insulin receptor signal transduction cascade (Hoyer et al., 1994).

In all studies reported so far, a single icv STZ injection was used to study the short-term effect of this regimen on behavior and brain metabolism. To induce a long-term and progressive deterioration in behavior and in cerebral metabolism such as is characteristic for SDAT, pilot studies were performed to investigate the duration of the effect of a single icv STZ injection on the above parameters. It became obvious that either a single or duplicate icv STZ challenge deteriorated behavior over the period of three months. In contrast, the changes found in energy metabolism were not invariable in that slight but

inconsistent improvements developed in some of the energy rich phosphates (data not shown). Otherwise, a triplicate icv STZ injection did progressively deteriorate both behavior and energy rich compounds over the experimental period chosen, and induced more pronounced abnormalities as were seen 3 weeks after a single icv STZ (Nitsch and Hoyer, 1991; Lannert and Hoyer, 1998). We, therefore, tentatively assume that the present approach which leads to progressive disturbances in behavior and in glucose/energy metabolism can be considered as an appropriate animal model for sporadic Alzheimer type dementia (Lannert and Hoyer, 1998).

The standardized extract EGb761 from the dried green leaves of Ginkgo biloba is a complex mixture of ingredients with an uniquely broad spectrum of pharmalogical activities on the central nervous system. The major till now identified active constituents of EGb761 are the flavone glycosides possessing antioxidant and radical scavenging potentials and the Ginkgo biloba specific terpene lactones i.e. ginkgolides and bilobalides with neuroprotective and diverse other therapeutically pharmacological properties such as e.g. protection against hypoxia or membrane degeneration (DeFeudis, 1998; Klein et al., 1998). Numerous controlled clinical trials demonstrate its therapeutic usefulness and currently it is widely used for the treatment of dementia and other cerebral insufficiencies. Although the ginkgo flavonoids possessing radical scavenging properties, the PAF-antagonists ginkgolides and bilobalides with neuroprotective and cerebral edema inhibiting activities are considered to be the main active ingredients of the extract, its active components responsible for several known pharmalogical activities have not yet been defined (for review see DeFeudis, 1998).

Earlier reports suggested the putative usefulness of EGb761 in the treatment of SDAT (Kleijnen and Knipschild, 1992a,b; Hofferberth, 1994; Kanowski et al., 1996) but also on the current understanding of its mode of action. The survival time after hypobaric hypoxia was increased nearly 3-fold when rats were pretreated with EGb 761. The same experimental condition caused falls in creatine phosphate and ATP and an increase in lactate in cerebral cortex. After EGb 761 these changes were less pronounced after acute hypoxia but they were not clearly influenced by long-term therapy (Karcher et al., 1984). Similar results were obtained in in vitro studies (Janssens et al., 1995). The elevated water and electrolyte contents, and the size of vacuoles were noticeably reduced by EGb 761 in triethylin-induced cytotoxic rat brain edema (Otani et al., 1986). EGb 761 was shown to prevent apoptosis of cerebellar neurons and to protect these cells after oxidative stress (Ni et al., 1996; Oyama et al., 1996). The latter finding may also explain, at least in part, the beneficial effect of EGb 761 on the composition of membrane lipid metabolism after electroconvulsive shock and ischemia in rat brain (Rodriguez de Turco et al., 1993; Rabin et al., 1996), and also on the hypoxia-induced membrane breakdown (Klein et al., 1998).

A recent finding clearly demonstrated the beneficial effects of EGb761 on locomotor behavior and cortical histopathology after brain contusion in rats. The loss of cholinergic neurons was reduced in the basal forebrain nucleus basalis magnocellularis of Meynert (Hoffmann and Stein, 1997).

Our choice of this drug for the present study was based not only on its broad effect on metabolic abnormalities occurring at least in part in SDAT, too, but also on potential anti-dementive effect in clinical terms. Here we report that EGb 761 counterbalanced the long-term and progressive deterioration in learning, memory and cognition, and improved the cerebral energy pool, after repetitive icv STZ-induced damage to the neuronal glucose metabolism.

Material and methods

Animals

One-year-old (adult) male Wistar rats weighing between 420 and 540 g (breeder: R. Janvier, France) were used in the present study. They were housed in individual cages in a temperature-controlled animal room with a reversed 12 : 12 h light dark cycle (lights on at 19.00 h). Experiments were conducted during the dark portion of the cycle. Four groups of animals were formed randomly (n = 15/group in the final experiment).

– 1. Control (icv CSF*)
– 2. icv STZ
– 3. icv CSF + EGb 761
– 4. icv STZ + EGb 761

While all animals had free access to drinking water and food (Altromin standard pellets no 1320, Lage, Germany) intake was limited to 25 g/animal/day. In groups 3 and 4, EGb 761 (50 mg/d) was offered in the same amount of pellets which was eaten completely by the animals. EGb 761-treatment started after the first injection of icv STZ or CSF.

Psychometric testing

Psychometric testing comprised three different phases: 1. habituation, 2. training, and 3. retest. The time course of the psychometric procedure is demonstrated in Fig. 1. Three different behavioral tests were applied to test learning, memory and cognition, and locomotor activity.

1. The holeboard test

– Apparatus
A holeboard of the type devised by Oades and Isaacson (1978) was slightly modified as was described in detail in Lannert and Hoyer (1998; Lannert et al., 1998).

– Time course
• Habituation phase (4 days). The rats were at first habituated to the holeboard apparatus on 4 consecutive days. They were deprived of food during this period, which caused a reduction in their body weight to 85% of that at the start of this phase. During habituation each hole was baited with a 50 mg food pellet. The animals were placed singly in the start box, the guillotine door was opened, and a trial started when the guillotine door was closed and the animal had entered the testing area. The trial ended after 10 min or after all food pellets had been consumed. The number of hole visits was recorded by a microcomputer. On the 4th day the rat had to find at least 15 of 16 pellets; rats with a poorer result were excluded from further experiments (poor performers).

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*CSF means artificial cerebral spinal fluid; for composition see below

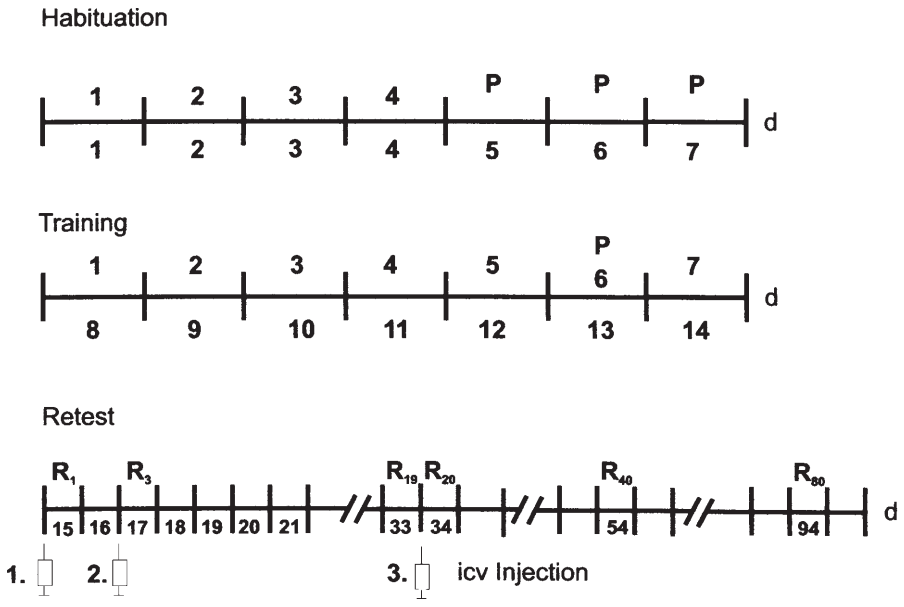

Fig. 1. Time course in days (d) of the experimental design for psychometric testing. Upper files of numbers demonstrate the duration of the repective test periods; *P* pause. Lower file of number show the duration of the whole psychometric procedure

• Training phase (7 days). For the remaining rats a training phase starting 3 days after habituation followed for the next 7 days, with a pause, P, on the 6th day (see experimental design). They were trained to collect pellets from a set of four holes (A1, B3, C2, and D4; one trial) within 5 min. Four trials were executed, each starting after placement of a rat in the start box. The actual trial was initiated by raising the guillotine door between the start box and the holeboard, and it was terminated after the rat had found four food pellets or after 5 min had elapsed, whichever occurred first. The number of hole visits was recorded by a microcomputer, which identified nose pokes by light beam crossings. At the end of a trial, the rat was replaced in the start box and food pellets were placed in appropriate holes of the area before the next trial. After each trial, visible traces of urine and feces were removed with a dry cloth. The interval between trials was approximately 1 min. Training sessions of four consecutive trials were given each morning. At the end of the training phase, the animals were separated in good and poor performers. The study started with 80 rats as a whole. 15 animals out of them were defined as poor performers (Stecher et al., 1997; Lannert and Hoyer, 1998). However, every experimental group consisted of 15 animals defined as good performers. So, 60 well performing rats were included in the present investigation.
• Retest phase (79 days). After icv injections (see below) the rats had to collect pellets in four trials from a fixed set of four holes (A4, B2, C3, D1). On days R19, R40 and R80 different holes were baited than in the training phase. The rats were deprived of food (5 g/d) for 2 days before each test. No arterial hypoglycemia was caused by this procedure, what had been proven in numerous former investigations (data not shown).

The experimental set-up of the holeboard permits a definition of two distinct memory functions, i.e., working memory (WM) and reference memory (RM). Performance of both components can be expressed as ratios of different types of hole visits (see below) (van der Staay et al., 1990).

1. Working memory ratio was defined as (number of food rewarded visits)/(number of visits and revisits to the baited set of holes). Thus, this measure represents the percent-

age of all visits to the baited set of holes that had been reinforced with food (van der Staay et al., 1986, 1988, 1990). It refers to short-term memory.

2. Reference memory ratio was defined as (number of visits and revisits to the baited set of holes)/(number of visits und revisits to all holes). This measure expresses the number of visits to the baited set of holes as a percentage of the total number of visits to all holes. In the present study WM ratio and RM ratio were calculated (Beldhuis et al., 1992; van der Staay et al., 1990) using block means of four trials (Lannert and Hoyer, 1998; Lannert et al., 1998). It refers to long-term memory, and in concern with the retest procedure, to cognition, too.

2. Single-trial passive avoidance learning

Step-through passive avoidance behavior was evaluated by using the light-dark avoidance box test (Lannert and Hoyer, 1998). The inhibitory apparatus (70 × 45 × 40 cm wooden box) consisted of a light (30 × 45 × 40 cm) and a dark (40 × 45 × 40 cm) compartment. The light compartment was illuminated by a 60-W lamp fixed 40 cm above its floor in the center. The interior of the dark chamber was painted black and had a ceiling.

The floor consisted of a metal grid connected to a shock scrambler. The two compartments were separated by a guillotine door (10 × 10 cm) that could be raised by 10 cm. On day R17, rats were placed in the light compartment and the latent period (initial) before each animal entered the dark compartment and had all four paws inside it was measured. Twenty-four hours later (day R18) the rats were placed directly in the dark compartment; after 20 s the guillotine door was opened and a single scrambled footshock (AC, 1 mA, 1 s) was delivered. After the rats had left the dark compartment, they stayed in the light compartment for a maximum of 300 s. A further step-through latency from the light to dark compartment was measured 24 h after the shock (day R19). The rat was placed in the illuminated starting box again and the test was concluded when the animal had all four paws in the dark compartment or after 5 min if it failed to go right into the dark compartment. The test was repeated on days R40 and R80.

To improve the reliability and validity of the footshock avoidance test, the grid was moistened with water before each footshock, significantly reducing the wide interindividual variability in paw skin resistance of the rats. The first icv injection of STZ or artificial CSF was given 17 days before the test at day R19.

3. Closed field activity test

Spontaneous locomotor activity was assessed on days R19, R40 and R80 after the first STZ administration. Each animal was observed over a period of 300 s in a square closed-field arena (80 × 80 × 40 cm) equipped with a row of 6 infrared light-sensitive photocells placed 5 cm above the wooden floor of the pen. The 3 photocells on each wall of the square were spaced 20 cm apart, and the last photocell in each row was 20 cm from the next wall. Interruption of photocell beams were recorded by means of a microcomputer allowing a record of all horizontal activity as measured by the total number of interruption of the 6 photocell beams. The closed-field apparatus was sited in a darkened, light- and sound-attenuated and ventilated testing room together with the other behavioral testing apparatus. During behavioral testing, only one animal and the tester were in the testing room at any time.

The sequence of the behavioral tests in the retest phase on days R19, R40 and R80 was locomotor activity, followed by memory performance in the holeboard and, finally, passive avoidance behavior.

Surgery

After habituation and training, and at the beginning of the retest phase (day R1; Fig. 1), the animals were divided randomly into two groups of 30 animals each. Animals were

anesthetized with chloral hydrate (240 mg/kg body weight i.p.). The head was positioned in a stereotactic frame (Uhl, Asslar, Germany), the skin over the skull was incised sagittally, and bur holes were drilled into the skull 2 mm lateral (right and left) and 0.7 mm caudal to bregma as described earlier (Mayer et al., 1990; Nitsch and Hoyer, 1991). Injection cannulas were lowered into the cerebral ventricles under stereotactic guidance (4 mm ventral to the brain surface). On days R1, R3, and R20 in the first group each rat received a bilateral icv. injection of STZ (Sigma, Munich, Germany) in a subdiabetogenic dose (0.25 mg STZ dissolved in 2 ml artificial CSF/injection site). The injections were repeated on day R3 and day R20 (see above). In the second group, which served as the control group, artificial CSF containing 120 mM NaCl, 3 mM KCL, 1.15 mM $CaCl_2$, 0.8 mM $MgCl_2$, 27 mM $NaHCO_3$, and 0.33 mM NaH_2PO_4, pH adjusted to 7.2 by $C0_2$ insufflation (all chemicals from Merck, Darmstadt, Germany) was injected, the time-course being the same as in STZ treated animals. The cannulas were left in place for a further 2 min before slow removal. The bur holes were closed with bone wax, and the skin incision was sutured.

These two groups were each subdivided in two groups to give the four groups for the final experiment mentioned above (n = 15/group).

In situ freezing of the brain

After completion of psychometric testing, the animals were sacrificed in the final experiment. To obtain tissue levels of energy-rich phosphates that reflect in vivo conditions, brains were frozen in situ under intubation anesthesia and during a steady state of physiological blood pressure, blood gases and acid-base parameters. Animals were anesthetized with 3 vol% halothane (2 min), tracheotomized, intubated with a Teflon tube and ventilated with a small animal respirator. Anesthesia was continued with 1.5 vol% halothane in nitrous oxide/oxygen 70:30 (vol/vol). One femoral artery was cannulated to monitor arterial blood pressure and to sample blood for the determination of blood gases ($PaC0_2$, $Pa0_2$), pH, hemoglobin, hematocrit, glucose and lactate. One femoral vein was cannulated to inject pancuronium bromide (2 mg/kg body weight) and to balance acid-base metabolism if necessary. The skin above the skull was sagittally incised to form a skin funnel for later in situ freezing. A warm (37°C) sponge moistened with phosphate buffered saline was placed in the funnel to preserve normal brain temperature. Anesthesia was continued with 0.5 vol% halothane in 70:30 (vol/vol) nitrous oxide/oxygen until steady state conditions of arterial normotension (mean arterial blood pressure 100–120 mmHg), normocapnia (36–41 mmHg $PaC0_2$), normoxia (100–140 $Pa0_2$), and normal pH 7.4 were established. This physiological steady state was maintained for 20 min before brains were frozen in situ by liquid nitrogen poured into the skin funnel. The frozen brains were chiselled out of the skull under liquid nitrogen. The parietotemporal cerebral cortex was dissected at −20°C in a cryostat chamber. The tissue was stored at −80°C until biochemical analyses.

Determination of energy-rich phosphates

The frozen tissue samples were weighed and homogenized at −20°C in 20 vol $HClO_4$ with an ultraturrax homogenizer (Janke & Kunkel, Staufen, Germany), then centrifuged at 10,000 × g for 10 min at 4°C. The pellets were discarded and the supernatant fluids were neutralized to pH 7.2 with 0.4 M imidazole base, 1.5 N KOH and 0.3 M KCl. ATP, ADP, GTP and CrP were determined by HPLC analysis after disruption of cell membranes with an ultraturrax in a chloroform/acetic acid mixture (1:2) at −20°C. Thereafter, samples (0.1 ml) for determination of the energy rich phosphates were automatically injected into a Partisil SAX column (4.6 ID, 25 cm, 10 æm pore size). The linear gradient started with 100% 0.01 M H_3PO_4, and the 0.75 M KH_2PO_4 buffer was increased from zero to 100% after 20 min, the flow rate being 2.0 ml/min. Absorbance of the column eluate was continuously monitored on a Shimadzu IV-Detector SPD 6A at 210 nm.

The following functional parameters were calculated:

1) ~P, representing the total load of available energy (0.18 × ATP + 0.15 × CrP) and
2) energy turnover, calculated from the ATP/ADP ratio.

Statistics

Data are displayed as mean values + standard deviation (SD). The statistical comparison of the steady state parameters, the energy rich phosphates, and the behavioral data among the groups were performed by means of the Mann-Whitney U nonparametric statistic test using the program "statgraphics" (version 6.0). An alpha level of 0.05 was the criterion of statistical significance. Statistical advice from the Institute of Medical Biostatistics, University of Heidelberg is gratefully acknowledged.

Ethics

The experimental protocol was approved by the review committee for animal experimentation of the Medical Faculty of the University of Heidelberg and by the responsible government agency.

Results

Steady state parameters

No differences were found between the physiological steady state parameters of the four groups studied except a slight fall in Hc in group 4 (Table 1). However, this lower value was still in the physiological range and is assumed to be due to the known effect of EGb 761 on hemodilution. Nor were any differences found between the groups in body weight. Thus, the groups can be

Table 1. Mean values and standard deviations of the steady state parameters of the four experimental groups. No statistically significant differences were found except a fall in Hc in group 4. However, the latter is in the physiological range

		Physiological steady state parameters			
		1 CSF	2 STZ	3 CSF + EGb 761	4 STZ + EGb 761
Hb	g/100ml	14.0 ± 1.1	14.4 ± 0.6	14.3 ± 1.0	13.4 ± 0.4
Hc	%	42.3 ± 2.8	43.6 ± 2.5	41.6 ± 3.3	38.9 ± 1.3*
MABP	mmHg	108 ± 6	112 ± 8	112 ± 6	113 ± 8
$paC0_2$	mmHg	36.5 ± 2.3	38.4 ± 2.6	38.5 ± 2.3	38.8 ± 2.4
$pa0_2$	mmHg	132 ± 19	124 ± 20	119 ± 13	120 ± 15
pH		7.46 ± 0.03	7.43 ± 0.04	7.45 ± 0.04	7.46 ± 0.04
t	°C	37.1 ± 0.1	37.2 ± 0.1	37.2 ± 0.1	37.1 ± 0.1
art glucose	mM	11.7 ± 1.5	11.7 ± 3.1	11.4 ± 1.8	12.2 ± 2.9
art lactate	mM	1.6 ± 0.5	2.2 ± 0.3	1.8 ± 0.6	2.1 ± 0.5
b.w.	g	578 ± 56	544 ± 51	573 ± 53	528 ± 66

* p 0.05 vs CSF. *Hb* hemaglobin, *Hc* hematocrit, *MABP* mean arterial blood pressure, $pa0_2$ partition pressure arterial oxygen, $paC0_2$ partition pressure arterial $C0_2$, *b.w.* body weight, *t* body temperature

compared, and the changes found can be attributed to either the STZ-induced damage (group 2) or its treatment with EGb 761 (group 4).

Holeboard test (working and reference memories)

Mean values for WM and RM during the training and retesting periods before and after STZ, and during EGb 761 treatment are summarized in Table 2. With training, the memory parameters of all animals increased and by day T7 were almost double the day T1 values indicating a robust training effect on WM and RM in these animals. Since no statistically significant differences were observed on day T7 among the different experimental groups, these data were pooled and used for comparison with the retest data of the different groups. On day R19, i.e. 16 days after the two initial icv injections of either STZ or CSF, the mean values of groups 1 and 3 were comparable to the mean value for all animals on day T7. In contrast, WM capacity dropped by 59%, and RM capacity by 28% after icv STZ on day R19 compared with day T7. However, this fall in WM and RM was completely abolished when STZ-damaged animals were treated with EGb 761. Even 20 days after (day R40) or 60 days after (day R80) the third icv CSF injection to the animals in groups 1 and 3, their memory functions remained high and unchanged in the holeboard paradigm. In contrast, icv STZ (group 2) induced a gradual loss of the capacities of WM by 71% and of RM by 65% by day R40, and of both capacities by 82% by day R80 compared with T7. Both WM and RM were found to be even worse on days R40 and R80 than on day T1. These observations demonstrated that the icv STZ regimen chosen induced a progressive alteration in the

Table 2. Mean values (±SD) of working and reference memory during the training and retest periods in the experimental groups studied

	Experimental day	All animals	Experimental groups			
			1 CSF	2 STZ	3 CSF + EGb 761	4 STZ + EGb 761
Working memory	T1	0.376 ± 0.17				
	T7	0.657 ± 0.10*				
	R19		0.586 ± 0.10	0.271 ± 0.17[a,b]	0.606 ± 0.17	0.586 ± 0.14
	R40		0.575 ± 0.07	0.191 ± 0.12[a,b]	0.574 ± 0.17	0.530 ± 0.17
	R80		0.583 ± 0.04	0.119 ± 0.09[a,b,c]	0.550 ± 0.17	0.445 ± 0.17[c]
Reference memory	T1	0.339 ± 0.13				
	T7	0.547 ± 0.09*				
	R19		0.524 ± 0.11	0.393 ± 0.14[a,b]	0.546 ± 0.13	0.517 ± 0.11
	R40		0.522 ± 0.10	0.192 ± 0.14[a,b]	0.450 ± 0.11	0.457 ± 0.15
	R80		0.547 ± 0.09	0.096 ± 0.09[a,b,c]	0.502 ± 0.14	0.427 ± 0.21[c]

* $p \leq 0.05$ between T1 and T7, [a] $p \leq 0.05$ between CSF and STZ, [b] $p \leq 0.05$ between STZ and STZ + EGb 761, [c] $p \leq 0.05$ between R19 und R80

memory capacities in the holeboard paradigm. EGb 761 treatment after STZ damage (group 4) prevented the sharp fall of both WM and RM at day R19, but could not block the decrease completely. At day R80, both these mental capacities were slightly but significantly reduced compared with day R19. Otherwise on day R80, WM was 3.7-fold and RM was 4.4-fold the corresponding levels after STZ damage alone.

Passive avoidance

The data are summarized in Figure 2. On day R17, i.e. 14 days after the second icv injection of either STZ or CSF, the animals were exposed to the test box for the first time. As becomes obvious from Fig. 2, the mean step through latencies of all groups were short (between 11.7 s and 19.4 s) and indicated the habitual dark preference of the animals independently of the treatments given. In the control group, latency was at its maximum capacity (279 s and 300 s) what held true also when EGb 761 was applied to undamaged animal (group 3; 233 s, 281 s and 285 s). Icv STZ (group 2) caused a statistically significant decline at day R19 as compared to all other groups (107 s), and an ongoing deterioration at days R40 and R80 (38 s and 39 s). The mean latency

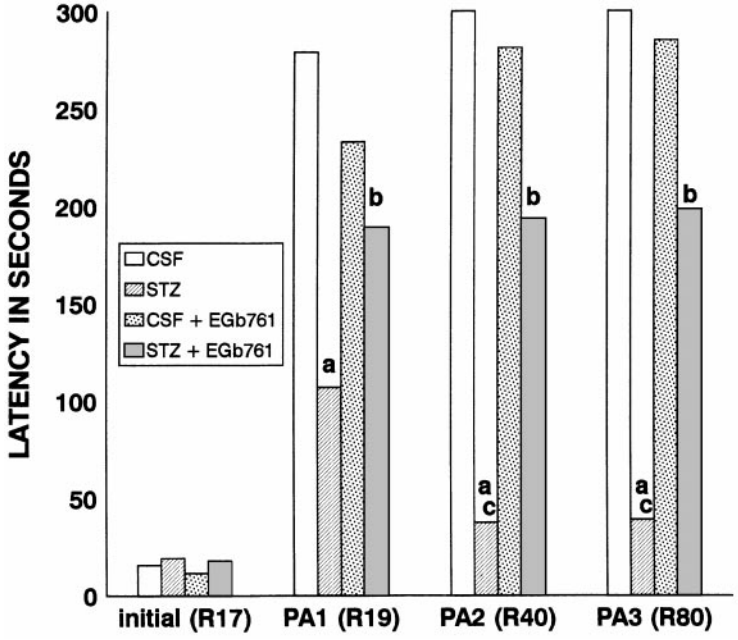

Fig. 2. Passive avoidance behavior after icv STZ application and under treatment with EGb 761. Initially, no differences were found between the experimental groups. Icv STZ reduced latency significantly at R19, which decreased further during the experiment. EGb 761 improved latency significantly after icv STZ damage as compared to icv STZ. *PA1*: passive avoidance test at day R19; *PA2*: passive avoidance test at day R40; *PA3*: passive avoidance test at day R80. [a] $p \leq 0.05$ between CSF and STZ; [b] $p \leq 0.05$ between STZ and STZ + EGb 761; [c] $p \leq 0.05$ between R19 and R40/R80

in group 4 (STZ damage and EGb 761 treatment; 189 s, 193 s, 198 s) was found to be statistically significantly higher than that following STZ damage only (group 2). While days R40 and R80, the latencies in the STZ-damaged animals had fallen further from day R19, the STZ-damaged and EGb 761-treated animals maintained latencies of around 200 s, as at day R19. These data clearly indicate that acquisition or retrieval of memory of the aversive shock had become severely deficient after icv STZ, and that this effect could be reversed only partially by EGb 761 treatment, irrespectively of its duration.

Locomotor activity

The spontaneous locomotor activity in the open field did not differ significantly between the experimental groups studied. In the control group, 83 ± 37.41 counts/5 min were found on day R19, 92.7 ± 37.4 counts/5 min on day R40, and 66.1 ± 36.0 counts/5 min on day R80. The mean values in the STZ group were 100.0 ± 38.6 counts/5 min on day R19, 122.1 ± 48.4 counts/5 min on day R40, and 89.5 ± 28.0 counts/5 min on day R80. In group 3 (icv CSF and EGb761-treatment) 100.13 ± 39 counts/5 min were found on day R19, 74.26 ± 33.72 counts/5 min on day R40, and 57 ± 25.85 counts/5 min on day R80. A tendency of slightly enhanced locomotor activity could be observed in group 4 (icv STZ and Egb 761-treatment) 119.58 ± 60.43 counts/5 min were found on day R19, 113 ± 73.57 counts/5 min on day R40, and 95.53 ± 59.88 counts/5 min on day R80.

Cortical energy metabolism

The mean levels of energy rich phosphates in parietotemporal cerebral cortex assayed on day R80 are summarized in Table 3. After triplicate icv STZ application, ATP, CrP, the total load of available energy ~P, and GTP decreased significantly compared with the levels in the control group to 77% (ATP), 84% (CrP), 82% (~P) and 76% (GTP) of normal. In contrast, ADP increased by 83% and the ATP turnover rate (as judged by the ATP/ADP ratio) increased 2.25-fold compared with the control group.

After EGb 761 treatment, icv STZ damaged animals showed a significant improvement of the high energy phosphates ATP and CrP, and of the pool of available energy ~P. Whereas CrP and ~P increased to 93% and to 91% of

Table 3. Mean values and standard deviations of energy rich phosphates in parietotemporal cerebral cortex of rats after icv STZ and treatment with EGb 761

	ATP	ADP	ATP/ADP	CrP	GTP	~P
CSF	2.48 ± 0.33	0.42 ± 0.07	6.05 ± 1.17	5.76 ± 0.99	0.62 ± 0.08	1.31 ± 0.20
STZ	1.90 ± 0.29[a]	0.77 ± 0.21[a]	2.69 ± 0.89[a]	4.85 ± 0.62[a]	0.47 ± 0.06[a]	1.07 ± 0.14[a]
CSF + EGb 761	2.20 ± 0.26[a]	0.49 ± 0.16	4.88 ± 1.47[a]	5.17 ± 0.85	0.56 ± 0.04[a]	1.17 ± 0.16
STZ + EGb 761	2.13 ± 0.39[a,b]	0.68 ± 0.24[a]	3.52 ± 1.48[a]	5.36 ± 0.94[b]	0.51 ± 0.09[a]	1.19 ± 0.20[b]

[a] $p \leq 0.05$ vs CSF, [b] $p \leq 0.05$ between STZ and STZ + EGb 761

normal, ATP rose to only 86% and GTP to only 82% of normal. ADP was found to be significantly elevated, and the ATP turnover was increased 1.7-fold compared with controls.

With EGb 761 treatment only, ATP concentration fell significantly by 11% and GTP by 10% whereas ATP turnover increased 1.2-fold compared with the control group. These latter data clearly indicate that EGb 761 exerts a minor effect on the neuronal energy metabolism, what may, however, be without any functional meaning as becomes obvious from the behavioral data. Otherwise these changes are easy to distinguish from those that follow after icv STZ damage without or with treatment with EGb 761.

Discussion

Although a variety of procedures and devices are now available to study behavioral functions in experimental animals (Heise, 1984; Sarter et al., 1992a,b; Porsolt et al., 1995), suitable models for the evaluation of both the rate and progression of behavioral decline are rare. Such models should mimic dementia in human beings the major form of which is sporadic Alzheimer disease. A recently supported working hypothesis is that a dysfunction of the neuronal insulin receptor signal transduction cascade is the pivotal abnormality in the early pathogenesis of this neurodegenerative disorder (Craft et al., 1998; Hoyer, 1998). It was, therefore, proposed that a damage of the neuronal insulin receptor by a single icv STZ-challenge could be an appropriate animal model for this neurodegenerative disorder (Mayer et al., 1990; Blokland and Jolles, 1993; Hoyer et al., 1994). However, as mentioned above, this procedure caused a progressive deterioration in behavior but did not initiate an invariable progressive deterioration in cerebral energy metabolism. In contrast, a triplicate icv STZ challenge did lead to progressively advancing abnormalities of both, behavior and energy metabolism of the brain (see below).

A clear result of this present study was that mental training performed over 6 days enhances and maintains learning, memory and cognition capacities longer in well performing animals (Stecher et al., 1997). Only the latter animals were used in this study to allow better discrimination between controls and STZ-damaged animals, and EGb761-treated animals. Subsequent studies in which the cerebral energy pool was directly compared between mental activity and mental rest over a longer period of time confirmed that mental activation enhanced the size of the energy pool in cerebral parietotemporal cortex (Hoyer and Haag, 1999; Nitsch and Hoyer, 1991).

In contrast to the results of the training phase and the retest phase in control animals, an STZ-induced disturbance in the neuronal insulin signal transduction cascade (Ar'Rajab and Ahrén, 1993) by triplicate icv application caused long-term abnormalities in learning, memory and cognition abilities. Both WM and RM deteriorated progressively in a stepwise manner during the same period of time, and no improvement could be observed. STZ-damaged animals revealed significantly reduced latencies in passive avoidance on day R19 with a further significant fall by day R40. Passive avoidance was also found to not improve at all during the course of the whole investigation period

in rats damaged with icv STZ. The ongoing deterioration from day R19 to day R80 after STZ-damage may be due to the fact that these animals had made the "good" experience not to receive a further foot-shock on day R19. In these terms, PA2 and PA3 are not independent from PA1. These findings are consistent with deficits found in WM and RM.

It can therefore be concluded that the data arising from different behavioral tests indicate that icv STZ-induced damage of the neuronal insulin receptor caused progressive deteriorations in the mental capacities of learning, memory and cognition and that these deteriorations were maintained at a low level over a long period of time as is equivalent to sporadic Alzheimer disease. The effect of EGb 761 on WM was liken to all degrees of STZ-induced damage. The capacity of WM increased by around 50% at days R19, R40, and R80 each. Because of the most severe STZ damage at day R80, the progression of WM-deterioration could be slowed by EGb 761 but not be stopped by EGb 761. The same effect of EGb 761 became also obvious in RM. However, at day R80, the improvement was more pronounced as compared to WM. It should be clarified in studies on human beings suffering from dementia whether or not EGb 761 elicits different beneficial effect on short-term and long-term memory. Indeed, a beneficial drug effect on memory function in general, and on social performance, too, has recently be demonstrated in a placebo-controlled double-blind and randomized trial in Alzheimer dementia patients over a period of 52 weeks (Le Bars et al., 1997). The beneficial effects of EGb761 have recently summarized on the basis of a metaanalysis of clinical data (Oken et al., 1998).

When compared with WM and RM, the icv STZ-induced deficits in acquisition and retrieval of memory after the shock were found to be more pronounced quantatively, particularly at day R40. (R19 38%; R40 13%; R80 13% compared to control). Although the rate of improvement due to EGb761-treatment was found to be of approximately the same degree as was in WM and RM at days R40 and R80, acquisition and retrieval memory were far from being normal. Thus, it may be assumed that icv STZ exerts a dual effect on learning, memory and cognition capacities: a rapid on acquisition and retrieval of (short-term) memory, and also long-lasting on long-term memory and cognition.

The experimental manipulations of mental training performed are suggested to account for an enhancement of the cerebral energy pool. Icv STZ counteracted this effect resulting in an impairment of brain energy metabolism characterized by significant decreases in the cortical tissue concentrations of ATP, GTP and CrP and, thus, in available energy, ~P. The disturbances found in this study were of the same quality, albeit slightly more pronounced in quantity, as abnormalities seen 3 week after a single icv STZ application (Nitsch and Hoyer, 1991). This indicates that brain energy metabolism can be kept at a lower level than normal over a longer period of time after triplicate icv STZ. The decreases in CrP and ATP, together with the increased ADP concentration, and the resulting reduction in the ATP/ADP ratio, may be indicative of an imbalance between energy production and energy utilization. This imbalance of energy metabolism may reflect a state of metabolic neu-

ronal stress, as has been shown to occur after cerebral ischemia (Hoyer and Betz, 1988).

The question now arises whether or not the STZ-induced abnormalities in behavior and metabolism are related to any pathological cerebral condition, and if so, what its pathophysiological significance might be.

Indeed, in the pathologic condition of sporadic dementia of Alzheimer type, the primary abnormality in cerebral glucose utilization (Hoyer et al., 1991) was found to be accompanied by a remarkable fall in acetylcholine synthesis (Sims et al., 1981), changes in glutamate metabolism (Hoyer and Nitsch, 1989), and a reduction in brain ATP concentration (Hoyer, 1992). Reduced availability of both acetylcholine and ATP can be assumed to set a cascade of cellular and molecular events in motion (for review see Lannert and Hoyer, 1998) and may also have drastic impacts on the abnormal processing of the amyloid precursor protein and on hyperphosphorylation of the tau protein (Roder and Ingram, 1991; Nitsch et al., 1992; Hoyer, 1993; Gabuzda et al., 1994). Impairments in cerebral glucose metabolism, including acetylcholine and glutamate, have been found to be closely related to the intellectual decline in Alzheimer's disease and related conditions (Collerton, 1986; Ohta et al., 1996; Deary and Caryl, 1997). We assume, then, that the icv STZ model tested in this study is appropriate to mimic changes in both the behavior and the oxidative metabolism that are characteristic for sporadic Alzheimer's dementia.

EGb761 has been clearly demonstrated to act effectively and long-lastingly on memory disturbances in sporadic Alzheimer disease (Le Bars et al., 1997; Oken et al., 1998). However, although multifold positive effects of this drug on different metabolic parameters had been shown in in vitro and acute in vivo models (see above), long-term effects on cerebral metabolism were lacking in animal models and Alzheimer patients.

The beneficial effect of EGb761 treatment on energy metabolism after icv STZ-induced damage becomes obvious in a 9%-increase of the energy-rich compounds ATP and CrP, and of energy load ~P. Since several ATP-dependent processes are abolished in a hierarchical fashion when ATP drops (Buttgereit and Brand, 1995), the reestablishment of such processes may be assumed when ATP raises. Thus, this nearly 10% improvement in energy availability may be considered as one of several reasons for the improvement in behavioral functions via the maintenance of synaptic transmission (Kadokaro et al., 1985; Huganir and Greengard, 1990). In this context, one major effect of EGb 761 may be seen in the improving fluidity, stability and permeability of membranes (Rodriguez de Turco et al., 1993; Rabin et al., 1996; Stoll et al., 1996; Klein et al., 1998) and in the improvement of receptor function (Huguet et al., 1994) reduced as a consequence of an energy deficit (Wu et al., 1996). Another effect of EGb 761 found in STZ-induced diabetes mellitus was the increase in glucose uptake in liver and muscle (Rapin et al., 1997). Future studies on the neuronal insulin receptor tyrosine kinase, and the glucose transport proteins 1 and 3 after icv STZ will be necessary for a more comprehensive explanation for the efficacy of EGb 761 in relation to sporadic Alzheimer disease. In this pathologic condition, neuronal insulin receptor

function has been found to be perturbed (Frölich et al., 1997) and the concentrations of the glucose transport proteins 1 and 3 to be diminished (Harr et al., 1995; Stoll et al., 1996).

In conclusion, 12-mth-old male Wistar rats that were good performers developed progressively deteriorating and ultimately severe disturbances in learning, memory and cognition function and a severe deficit in the cerebral energy pool after triplicate icv STZ injection. So far, these abnormalities resemble the psychological and metabolic deficits found in sporadic Alzheimer's disease. Treatment of the animals with the antidementive drug EGb 761 considerably slowed down the progression of the deteriorating memory functions and partially compensated the learning deficit. EGb 761 also improved the icv STZ-induced disturbance in the cerebral energy pool. However, further studies are necessary to elucidate the pharmacological effect of the multiple EGb compounds on cellular and molecular processes.

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