DEPARTMENT OF NEUROLOGY SERIES OF REPORTS NO 58, 2001

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The Effects of Pharmacotherapy and Training on Functional Recovery after Global and Focal Cerebral Ischemia in Rats

Doctoral dissertation

To be presented with the assent of Faculty of Pharmacology of the University of Kuopio for public examination in the Auditorium L1 of Canthia Building of the University of Kuopio, on Saturday 8th December 2001, at 12 noon.

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ISBN 951-781-750-9
ISSN 0357-6043

Puurunen, Kirsi. The effects of pharmacotherapy and training on functional recovery after global and focal cerebral ischemia in rats. Series of Reports, No 58, Department of Neuroscience and Neurology, University of Kuopio 2001, 135 p. (*This page numbering applies to the print version of the thesis*)

ISBN 951-781-750-9
ISSN 0357-6043

ABSTRACT

The brain is an organ which is constantly modulated by our experiences. Moreover, the brain is capable of reorganization following injuries, such as stroke. There is evidence that adaptive reorganization can be supported by rehabilitative training and pharmacotherapy. We investigated the recovery promoting significance of two catecholaminergic agents: an α_2-adrenoceptor antagonist, atipamezole, and a monoamine oxidase type-B (MAO-B) inhibitor, selegiline, in experimental models of focal and global cerebral ischemia. We also studied the efficacy of spontaneous training in an enriched environment in these two stroke models. The outcome of these treatments was assessed by different behavioural tests. The effect of housing in an enriched environment on hippocampal function was studied by Fos-staining following a learning experience in a water-maze test. We found that atipamezole treatment facilitated recovery following focal cerebral ischemia in sensorimotor tests. However, it is not clear whether atipamezole treatment actually enhanced the recovery or only hastened it, since ischemic animals eventually reached the same performance level as the drug treated rats. Selegiline treatment was found to attenuate the cognitive deficit following focal cerebral ischemia, but this effect was dependent upon concomitant rehabilitative training. The rehabilitative training with an enriched-environment housing facilitated the recovery from a spatial learning deficit and abolished the hyperactivity following global cerebral ischemia. When assessed after a learning situation the enriched-environment housing increased the activation of hippocampus in the dentate gyrus revealed by activation of Fos-positive neurons in sham-operated rats and in their counterparts subjected to global ischemia. In conclusion, recent evidence has shown that atipamezole and selegiline can both be considered as good candidates for recovery promoting agents following stroke. Furthermore, these data show that rehabilitative training also plays an important role in the recovery and may even be necessary if one is to achieve the recovery promoting pharmacologic effects.

National Library of Medicine Classification: WL 355, QY 58

Medical Subject Headings: brain ischemia; ischemic attack, transient; brain/drug effects; environment; housing, animal; motor activity/drug effects; maze learning; adrenergic alpha-antagonists; receptors, adrenergic, alpha-2; selegiline; rats; animal

ACKNOWLEDGEMENTS

This work was carried out in the Department of Neuroscience and Neurology and A.I. Virtanen Institute, University of Kuopio, during the years 1995-1998.

I wish to express my deepest gratitude to my principal supervisor Professor Juhani Sivenius, M.D., for introducing me to the field of neuroscience and the fascinating subject of stroke and recovery, and for believing in me.

I am most grateful to my supervisor Docent Jouni Sirviö, Ph.D., for his contribution to this study and guidance, support and constructive criticism with manuscripts. I also wish to express my warm thanks to my other supervisor Professor Jari Koistinaho, M.D., for his guidance related to the molecular biology of stroke.

I am greatly indebted to Docent Jari Honkaniemi, M.D., University of Tampere, Tampere, and Docent Perttu Lindsberg, M.D., University of Helsinki, Helsinki, the official referees of this thesis for their valuable advice in ways to improve this manuscript.

I want to thank my colleague Docent Jukka Jolkkonen, Ph.D., for his crucial collaboration, advice, and fruitful debates during these years. I am also very grateful to Riitta Miettinen, Ph.D. for collaboration and advice with histology.

I owe my special thanks to Ms. Nanna Huuskonen for her excellent technical assistance, knowledge in many laboratory techniques and practical problem solving capability during these years. I also wish to thank the other members of our group Tiina Virtanen M.Sc, Heli Karhunen, M.Sc., Sanna Rantakömi, M.Sc. and. Katja Aho, M.Sc. and also Jaana Jääskö, M.Sc., Katariina Sivenius, M.Sc. and Ms. Hannele Ylitie for their skilful technical assistance. I am deeply grateful to Ms. Anna-Liisa Gidlund for introducing me to laboratory practices and her knowledge of histological matters. I also wish to thank Hannele Lahtinen, Ph.D. for her positive impact at the beginning of my career.

I am greatly indebted to Esa Koivisto, M.Sc. for being helpful and a continuous source of advice through his expert knowledge in technology, especially related to computers and computer programs, and rescuing me numerous times in technical matters.

It is my pleasure to thank Vice President of Research at Orion Ltd, Antti Haapalinna, for providing me with the drugs, atipamezole and selegiline, used in the present work and for fruitful collaboration during these studies.

I owe my thanks to my colleagues Alexandra Barbelevien, Ph.D., Maya Holmberg, M.D., Tiina Koskinen, Ph.D., Tiina Kotti, Ph.D., Minna Niittykoski Ph.D., Sirja Ruotsalainen, D.Pharm., for sharing these years.

I want to thank Ewen MacDonald, D.Pharm., for revising the language of this thesis.

I wish to thank Ms. Sari Palviainen, Ms. Hanna Turkki, Ms. Nilla Karjalainen and Ms. Tuija Parsons for their great help and kind collaboration.

I am deeply grateful to professor Timo Nevalainen Ph.D. for the advice in laboratory animal practices and for providing me with the surgical facilities, and to the personnel of the National Laboratory Animal Center of Kuopio University for their great help and collaboration during this study. My warm thanks belong also to the Technical Centre, the Computing Centre, the Photographic Laboratory, the Library and the Printing Office of the University of Kuopio for their collaboration.

I owe my warmest thanks to my mother, who has provided me with possibilities for education and always encouraged and believed in me.

Finally, I owe my sincere thanks to my husband, who has shared the feelings during these years on the bumpy road of science.

Study was supported by EVO grant 5510, University of Kuopio

Kuopio, October 2001

Kirsi Puurunen

ABBREVIATIONS

AMPA	α-amino-3-hydroxy-5-methyl-4-isoxasole propionic acid
ANOVA	analysis of variance
BDNF	brain derived neurotrophic factor
BFGF	basic fibroblast growth factor
CA1-3	subfields of hippocampus
DNA	deoxyribonucleic acid
DNMS/P	non matching to sample/position
GABA	γ-aminobutyric acid
GAP-43	growth associated protein-43
GDNF	glial derived neurotrophic factor
5-HT	5-hydroxytryptamine, (serotonin)
IU	international units
LTD	long-term depression
LTP	long-term potentiation
MAO-B	monoamine oxidase B
MCA	middle cerebral artery
mRNA	messenger ribonucleic acid
NBT	nitro blue tetrazolium
NGF	nerve growth factor
NE	norepinephrine
NMDA	N-methyl-D-aspartate
NO	nitric oxide
OP-1	osteogenic protein-1
PET	positron emission tomography
s.c.	subcutaneous
SOD	superoxide dismutase
TGF-β	transforming growth factor-β
TPA	tissue plasminogen activator

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following publications that are referred to in the text by the Roman numerals (**I-IV**):

I **Puurunen K**, Sirviö J, Koistinaho J, Miettinen R, Haapalinna A, Riekkinen P Sr and Sivenius J: Studies on the influence of enriched-environment housing combined with systemic administration of an α_2-adrenergic antagonist on spatial learning and hyperactivity after global ischemia in rats. Stroke 28: 623-631, 1997.

II **Puurunen K**, Koistinaho J, Sirviö J, Jolkkonen J and Sivenius J: Enriched-environment housing increases neuronal Fos-staining in the dentate gyrus after a water maze spatial learning task. Neuropharmacology 40: 440-447, 2001.

III **Puurunen K**, Jolkkonen J, Sirviö J, Haapalinna A and Sivenius J: An α_2-adrenergic antagonist, atipamezole, facilitates behavioral recovery after focal cerebral ischemia in rats. Neuropharmacology 40: 597-606, 2001.

IV **Puurunen K**, Jolkkonen J, Sirviö J, Haapalinna A and Sivenius J: Selegiline combined with enriched–environment housing attenuates spatial learning deficits following focal cerebral ischemia in rats. Exp Neurol 167: 348-355, 2001.

CONTENTS

I INTRODUCTION

Stroke is a major cause of death in the developed countries (Diener 1996; Nagahiro et al. 1998; Wolf et al. 1998). Stroke causes immense human suffering leaving the patient usually grossly disabled. Therefore, stroke is viewed as a leading cause for the loss of quality-adjusted life-years (Broderick et al. 1998). Stroke patients need intensive care and require major investments from society as well as from their relatives.

Stroke has been considered as untreatable and even today there is no effective drug therapy to help stroke patients. The search for the cure of stroke requires valid experimental models. Human ischemic stroke is very heterogenous in its manifestations, causes and anatomic sites and a wide variety of animal models for stroke have been developed in order to find different approaches to study ischemic brain injury (Ginsberg and Busto 1989; Hossmann 1998). Rats have a close resemblance of the cerebrovascular anatomy and physiology to the higher species and are widely used in cerebral ischemia studies (Ginsberg and Busto 1989). However, the rat is not a good model in terms of its collateral blood supply (Hunter et al. 1995). The validity of these cerebral ischemia models is highly dependent on the strict control of physiological variables, such as body temperature, blood pressure, blood gases and glucose levels, which may fluctuate and lead to variability in the results (Ginsberg and Busto 1998; Hossmann 1998). The functional deficits that are seen in rodents following focal and global cerebral ischemia have similarities with those typically seen in stroke patients (Squire 1992; Van der Staay 2000).

The ischemia models have enabled the effective study of pathological mechanisms of stroke and within the last decades the complex molecular mechanisms leading to cell death following cerebral ischemia have been partly elucidated (for review, see Dietrich 1998; Dirnagl et al. 1999; Lipton 1999; Snider et al. 1999). This has lead to attempts to find ways to interfere with these mechanisms. The attepts to help stroke patients have predominantly been concentrated on prevention of acute cell death. Indeed, more than one hundred agents have been proved to be neuroprotective in experimental models (Hachinski 1996). Unfortunately, despite these promising prospects in the prevention of cell death, the drugs that have been evaluated clinically have failed, usually because of an unsuitable time-window, lack of efficacy or the presenced unwanted side effects (Grotta 1994; The European Ad Hoc Consensus 1998). Although there have been some hopes concerning thrombolytic therapy (Alexandrov et al. 2000), it seems that thrombolytic compounds such as tPA cannot be used for the majority of patients, at least in the near future (Clark et al. 2000).

Another approach to help stroke patients, which has received less attention, is to enhance their recovery. It has been proved that the adult brain is not a stable structure but has a capacity for adaptation. Plastic changes occur after training and learning experiences (Jenkins et al. 1990; Karni et al. 1995; Xerri et al. 1996) and various lesions can cause reorganization in the brain (Nudo 1997; Steward 1989; Xerri et al. 1998). The framework of behavioral plasticity in the brain was provided by Dr. Hebb, who proposed that synaptic change underlies behavioral and cognitive plasticity (Hebb

1949). He also stated that strengthening of synaptic connections occurs when pre- and postsynaptic neurons are coactive. Later this theory was extended to recovery from brain injury, and it has been assumed that good recovery correlates with enhanced connectivity (Kolb 1999). Accordingly, poor recovery would correlate with an absence of reorganized connectivity.

It is possible to enhance the functional recovery after stroke by rehabilitation (Kwakkel et al. 1997; Ottenbacher and Jannell 1993a; Sivenius et al. 1985). However, at present it is not clear what practices are optimal for stroke rehabilitation (Hoenig et al. 1999). One possibility to study in an experimental setting the rehabilitative effects of training is to house animals in an enriched environment, which has been shown to facilitate recovery after various brain lesions (Rose 1988; Will and Kelche 1992), including cerebral ischemia (Ohlsson and Johansson 1995). Moreover, there is increasing evidence that the functional recovery after cerebral lesions and ischemia may be influenced by pharmacotherapy (Feeney and Sutton 1987; Goldstein 1990, 1993). Amphetamine is perhaps the best documented agent to be able to enhance recovery (Feeney et al. 1982; Feeney and Hovda 1983, 1985; Hovda and Fenney 1984; Hurwitz et al. 1991; Schmanke et al. 1996). There is also recent evidence, that certain trophic factors may enhance recovery after brain lesion (Kawamata et al. 1996; Kawamata et al. 1998; Kolb et al. 1996).

The optimal way to help stroke patients would be to minimize the extent of the damage during acute stroke phase and then to promote the plastic changes which would aid the recovery in order to minimize the permanent functional deficits. It is possible that even the drugs that have been proved to be ineffective as neuroprotective agents in clinical trials may be beneficial, when we learn to target the clinical trials optimally to better simulate the experimental conditions under which these drugs have been found to be effective (Grotta and Hickenbottom 1999). It may be possible that a combination of drugs should be directed at different sites in the ischemic cascade (Hickenbottom and Grotta 1998). Even though the recovery promoting action of amphetamine was discovered several decades ago (Mailing and Acheson 1946), it is still not used in the rehabilitation of stroke patients. It seems that also the other candidate drugs for recovery promotion still need further development before they will be accepted to clinical practice. For example, basic fibroblast growth factor (bFGF) did enter clinical trials (The FIBLAST Safety Study Group 1998), but these were terminated. It is possible that new stem cell therapies will be used in the future (Abe 2000; Fukunaga et al. 1999; Nishino and Borlongan 2000). To summarize, there are currently several different cellular, molecular and pharmacological approaches that may eventually enter development in clinical trials of stroke recovery.

II REVIEW OF THE LITERATURE

1 GLOBAL CEREBRAL ISCHEMIA

1.1 Animal models of global ischemia

In cerebral ischemia, blood flow within the brain is severely reduced or totally blocked (Hossmann 1999; Paschen et al. 1992). In global cerebral ischemia, the reduction of blood flow involves the whole brain or forebrain. Experimental models of global ischemia simulate hypoxic/ischemic state such as that occurring during cardiac arrest, severe hypotension or drowning.

Several global ischemia animal models have been designed, (see Ginsberg and Busto 1989; Hunter et al. 1995; McBean and Kelley 1998). The advantages and disadvantages of the most widely used models are listed in Table 1.

Table 1. Global ischemia models

Model	Advantages (+)/disadvantages (-)
Gerbil-model	+ simple operation: only short occlusion of common carotid arteries is needed - less possibilities for behavioral testing - variable outcome due to variations in cerebral circulation
Rats: two-vessel occlusion	+ one stage surgery: only common carotid arteries are occluded + occlusion is reversible + possibility to control respiration by ventilation - needs induction of hypotension - needs anaesthesia during occlusion, this may complicate the interpretation of outcome
Rats: four-vessel occlusion	+ no anaesthesia needed during ischemic occlusion + well-documented, the most used global ischemia model + solidly validated - two-stage operation, highly invasive operation for the closure of vertebral arteries - procedure only partly reversible: vertebral arteries are permanently closed - variable outcome within one strain and variation in susceptibility in different strains

1.2 Ischemic neuronal death

There are only a few reports on the pathological changes occurring in human hippocampus after global ischemia and much of the pathophysiology after global ischemia is based on experimental studies. However, hippocampal CA1 loss (Zola-Morgan et al. 1986) and atrophy (Fujioka et al. 2000) have been detected following global ischemia in humans.

There are differences between brain areas in their vulnerability to global ischemia. For example, some hippocampal neurons are sensitive to ischemic insults. The pyramidal neurons in area CA1 and some neurons in the hilus are most vulnerable, whereas most of the CA3 pyramidal neurons survive and the granular cells in dentate gyrus are resistant to ischemic damage (Ito et al. 1975; Pulsinelli et al. 1982). According to studies by Ito and colleagues, CA3 pyramidal cells undergo reactive changes, which can be reversible, in response to ischemia (Ito et al. 1975). However, the neuronal damage in CA3 inside the hilus (CA3c) commonly occurs and is thought to result from unspecific events such as edema formation within the hilus (Schmidt-Kastner and Hossmann 1988). There is also differential vulnerability along the temporoseptal axis within the hippocampus. The ventral CA1 is more resistant to the global ischemia than the dorsal one, and only minor cell loss is seen in this region (Ashton et al. 1989; Olsen et al. 1994a). Moreover, there is variability in ischemic resistance even within the dorsal hippocampus and more cell death occurs in the septal area than in the mid-dorsal level (Schmidt-Kastner and Hossmann 1988). When global ischemia is prolonged, cell death occurs also in extrahippocampal structures, such as striatum, certain thalamic nuclei and cortex (Pulsinelli et al. 1982). There are also major differences in the time course of cell death. The ischemic cell death becomes manifested first in the hilar neurons (Johansen 1993), but the cell death in CA1 occurs with a delay of 1 to 2 days (Crain et al. 1988; Kirino and Sano 1984; Pulsinelli et al. 1982) and has been called delayed cell death (Pulsinelli et al. 1982). Both necrotic and apoptotic cell death mechanisms are involved in cell death after global ischemia (Colbourne et al. 1999; Heron et al. 1993; Nitanori et al. 1995; Petito et al. 1997). The death of hilar and CA3 neurons can be mediated by toxic zinc translocation into these neurons (Koh et al. 1996; Tonder et al. 1990).

1.3 Behavioral deficit and recovery following global cerebral ischemia

The hippocampus is the brain structure most severely affected by global ischemia. It is also the region known to play an important role in certain learning and memory processes (Jarrard 1993; Zola-Morgan and Squire 1986). Deficits in learning and memory in rats have also been detected after global ischemia (for reviews, see Block 1999; Corbett and Nurse 1998; Nunn and Hodges 1994) and patients having hypoxic/ischemic hippocampal damage are amnesic (Squire and Zola 1996; Wilson 1996). Ischemic rats are impaired in tasks requiring working and spatial memory, such as T-maze, radial arm maze, water-maze, circular platform task and delayed non-matching to sample/position (DNMS/P) tasks (Milani et al. 1998; Nunn and Hodges 1994). Global ischemia also leads to locomotor hyperactivity in an open-field test (Green et al. 1995).

The studies examining the correlation between the extent of the cell death and behavioral outcome have had variability in results. Linkage between behavioral impairment and ischemic cell death in CA1 has been found in radial arm maze or T-maze tests in rats (Nunn and Hodges 1994). There are also studies that show the global ischemia can impair acquisition of the water-maze task (Jaspers et al. 1990; Olsen et al. 1994b), but not all results are consistent (Block 1999; Corbett and Nurse 1998; Nunn and Hodges 1994). The severity of global ischemia (Block and Schwarz 1997; Corbett and Nurse 1998; Kiyota et al. 1991; Olsen et al. 1994a) and difficulty of the task, e.g. learning-set paradigm, seem to be critical factors, whether the ischemic lesion causes impairment in spatial memory (Auer et al. 1989; Green et al. 1992; Rod et al. 1990; Whishaw et al. 1994). One possible factor is also the delay between the induction of ischemia and behavioral testing (Corbett et al. 1992). There are studies that have found a correlation between a functional deficit in water maze and the extent of CA1 pyramidal cell loss (Block and Schwarz 1997; Olsen et al. 1994b). The hyperactivity following global cerebral ischemia is pronounced in gerbils and has been used as a predictor of CA1 damage in that model (Mileson and Schwartz 1991). The reduction of hyperactivity has been used as an outcome measure of neuroprotection in CA1 in pharmacotherapy studies (Green et al. 1995; Judge et al. 1991).

2 FOCAL CEREBRAL ISCHEMIA

2.1 Animal models of focal ischemia

In focal cerebral ischemia, the disturbance in blood flow occurs in a localized area. There are several experimental models available for focal cerebral ischemia studies, (see Ginsberg and Busto 1989; Hunter et al. 1995; McAuley 1995). The favourable and unfavourable features of some of these models are compiled in Table 2.

Table 2. Rat focal ischemia models

Model	Advantages/disadvantages
MCA occlusion: permanent	+ selection of the occlusion site allows to some extent the choice of the affected brain area - penetration of drugs to the infarcted area is limited - no recanalization involved - variation in infarct size - some models need craniectomy
transient	+ probably the most widely used experimental stroke model + possible to assess reperfusion damage following recanalization + possible to have drug penetration to the occluded area - variation in infarct size
Photochemically induced focal cerebral thrombosis	+ possibility for precise location and size of infarcted cortical area + invasive procedures minimal + possibility to study antiplatelet and thrombolytic therapy - end-arterial occlusion, which is resistant to therapies based on enhancement of collateral perfusion - differs in some respects from human stroke, e.g. no penumbra
Miscellaneous models of cerebral embolism and thrombosis	- random and unpredictable location and size of the lesion

For references, (see (Ginsberg and Busto 1989; Hunter et al. 1995; McAuley 1995; Watson 1998).

In the commonly used focal cerebral ischemia animal models, the middle cerebral artery (MCA) is occluded either transiently or permanently (Longa et al. 1989; Tamura et al. 1981), which simulates the stroke type of thrombotic occlusion of MCA commonly seen in stroke patients (Derouesne et al. 1993). The size of the infarct after MCA occlusion varies depending on collateral blood supply (Menzies et al. 1992; Rubino and Young 1988). Generally when blood flow falls below 20 ml/100 g/min neurons lose their synaptic function and electrical communication between neurons ceases. A further fall in blood flow below 10 ml/100 g/min disrupts ionic homeostasis in neurons (Branston et al. 1976; Hakim 1987).

2.2 Ischemic neuronal death

The modern imaging techniques have shown that stroke volume expands for several days in humans (Karonen et al. 1999). The brain lesion induced by experimental MCA occlusion also matures and grows as a function of time. Acute changes are seen in an ischemic core after 30 min, and it has been reported that by 72 h these abnormalities are seen in all at risk areas supplied by the MCA (Garcia et al. 1993). The area surrounding the ischemic core is called the penumbra. The penumbra is characterized as an area of reduced cerebral blood flow that leads to electrical quiescence, but a site where ionic gradients are not irreversibly disturbed (Astrup et al. 1981; Strong et al. 1983; Symon 1980). Thus the penumbra offers an extended time window for drug therapy. The ischemic penumbra can be detected with different imaging techniques and its recovery or conversion to infarction has been documented in experimental models (Heiss 2000). Animal studies indicate that if the blood supply is not restored and the tissue protected metabolically within 6 hours, the penumbral area deteriorates and contributes to the centrifugal enlargement of the ischemic core (Ginsberg 1997). It is therefore thought that acute drug therapy also to stroke patients should be given within 6 hours (Muir and Grosset 1999). The cell death following focal cerebral cerebral ischemia involves necrotic and apoptotic mechanisms (Choi 1996; Li et al. 1998; Snider et al. 1999; Sorlano et al. 1996). The pathological process in focal cerebral ischemia is complex (for review, see Dirnagl et al. 1999; Dorman et al. 1996; Grotta and Hickenbottom 1999). A nonadequate blood supply results in impairment of energy dependent ionic pumps, increased release of glutamate and calcium, and potassium leakage. Excessive calcium overload exceeds the capacity of its tight regulation systems in cell and is followed by mitochondrial damage and activation of numerous secondary events such as activation of proteases, leading to digestion of cell proteins, activation of lipases, leading to digestion of cell membranes, activation of endonucleases, leading to DNA degradation, and formation of free radicals and NO, all of which induce cell death (Sattler and Tymianski 2000).

Focal ischemia by MCA occlusion in rats leads to cell death in striatum and different cortical areas (Longa et al. 1989; Tamura et al. 1981). This is followed, however, by secondary neuronal damage in remote brain regions, such as substantia nigra (Tamura et al. 1991) and thalamus (Fujie et al. 1990). The neuronal degeneration in thalamus progresses approximately from 6 days after MCA occlusion (Nordborg and Johansson 1996; Rupalla et al. 1998).

2.3 Behavioral deficit and recovery following of focal cerebral ischemia

In humans, large MCA territory infarcts causes neurological deficits, such as contralateral severe complete hemiplegia affecting the face, arm and the leg and are associated with complete loss of superficial and deep sensations. Also major higher function disturbances, including global aphasia with left-sided lesions, visuospatial impairment, and hemineglect with a right-sided lesions are involved (Neau and Bogousslavsky 1998).

Rats having focal cerebral ischemia following MCA occlusion have been shown to have deficits in several tests assessing sensorimotor and cognitive functions (Aronowski et al. 1996; Corbett and Nurse 1998; Markgraf et al. 1992), which are collated in Table 3. The ischemic rats are impaired in a test which assesses coordination and integration of motor movement, such as the beam-walking test (Ohlsson and Johansson 1995; Okada et al. 1995). Another test assessing accuracy of limb placement (Markgraf et al. 1992; Stroemer et al. 1995) is the foot-fault test, in which rats have to walk on a grid (Hernandez and Schallert 1988). Other similar tests are rota-rod (Rogers et al. 1997; Yamamoto et al. 1987) and running wheel (Aronowski et al. 1996). The behavioral deficit is also evident in Montoya's staircase test (Grabowski et al. 1993) which assesses skilled coordination of the forelimb in the ability to reach and grasp food pellets (Montoya et al. 1991). The ischemic rats are impaired also in tests assessing responses to various sensory stimuli. Focal cerebral ischemia leads to deficits in a limb-placing test (Markgraf et al. 1992; Ohlsson and Johansson 1995) developed by De Ryck et al. (De Ryck et al. 1989), which assesses the sensorimotor integration of forelimb and hindlimb responses to tactile and proprioceptive stimulation. The functional deficit is apparent also in the tape test (Aronowski et al. 1996; Markgraf et al. 1992), described by Schallert et al. (Schallert et al. 1986) assessing asymmetry to remove stickey tapes from the forepaws, and in a sensory inattention test (Grabowski et al. 1988) described by Marshal and Teitelbaum (Marshall and Teitelbaum 1974) measuring asymmetry in sensitivity to sensory stimulation. Muscular strength of the affected body side is decreased following focal ischemia. Thus tests measuring muscle strength, such as the prehensile traction test (DeGraba et al. 1994; Ohlsson and Johansson 1995) and the vertical screen test (Johansson and Olsson 1996; Tominaga and Ohnishi 1989) have been used following focal cerebral ischemia. The deficits of the ischemia affected body side may lead to its non-use. Ischemic rats have been demonstrated to have decreased motor activity on the side of the impaired forelimb (Kawamata et al. 1997a) in a test assessing asymmetry in the use of the forelimbs in vertical and horizontal movements of the body (Jones and Schallert 1992, 1994). Moreover, cognitive deficits following focal ischemia have been found in tests measuring associative memory such as passive avoidance (Hirakawa et al. 1994; Wahl et al. 1992; Yamamoto et al. 1987) or spatial memory such as water maze (Yonemori et al. 1996; Yonemori et al. 1999) and radial arm maze (Okada et al. 1995; Sakai et al. 1996).

Table 3.

Behavioral test	Behavioral task	Behavioral assessment
Beam-walking test	Walking on a beam	Ability to maintain balance and hindlimb slips during walking
Foot-fault test	Walking on a grid	Limb misplacement asymmetry while moving around a grid
Rota-rod test	Walking on a rotating rod	Time of staying on a rod
Running wheel test	Running in a wheel	Forelimb slips during running
Montoya's staircase test	Reaching and grasping of food pellets	Fine movements of forepaws
Limb-placing test	Forelimb and hindlimb placement	Response to proprioceptive and tactile stimuli
Tape test	Removing of sticky tapes from forepaws	Extinction, preference for removing of adhesive stimuli
Sensory inattention test	Orientation to sensory (visual, olfactory or tactile) stimuli	Tendency to orientate and investigate impinging stimuli
Prehensile traction test	Prehensile traction	Time of traction
Vertical screen test	Ability to stay on a vertical screen	Forelimb and hindlimb muscular strength
Cylinder test	Forelimb usage in vertical movements	Asymmetry in forelimb usage
Passive avoidance test	Avoidance of aversive stimulus	Associative memory
Water-maze test	Memorizing the location of a hidden platform	Spatial learning

It seems that rats have a great capacity for recovery and in many behavioral tests the impairment in performance after focal cerebral ischemia is transient, (for review, see Corbett and Nurse 1998). For example, in the study of Markgraf et al., (Markgraf et al. 1992) where multiple tests were used the reflex and sensorimotor function deficits recovered to pre-operative levels by day 30 post-ischemia. However, return of skilled paw movement take longer time, since rats are still impaired for at least 3 months in Montoya's staircase test, a test that assesses forepaw dexterity (Grabowski et al. 1993). Moreover, the cognitive deficits seem to be prominent in later phases after focal cerebral ischemia. Deficits in water-maze learning are still seen 5 weeks (Markgraf et al. 1992) and in passive avoidance retention 16 weeks after ischemia induction (Yamamoto et al. 1987).

It may be difficult to find tests that are sensitive at distinguishing the different grades of ischemic brain insults. For example, the foot-fault and tape tests seem to be sensitive in this respect (Aronowski et al. 1996), but abnormalities within the tape test were reversible within one week. Montoya's staircase test is sensitive to injury of the forelimb region of cortex as well as the dorsolateral striatum (Whishaw et al. 1986). Moreover, a correlation between water-maze deficit and ischemic lesion size has been observed (Smith et al. 1997; Yonemori et al. 1999). The change in search pattern was found to correlate with shrinkage of the caudate-putamen, whereas the cognitive deficit correlated with shrinkage of the cortex, especially the parietal cortex (Yonemori et al. 1999).

3. PLASTICITY IN THE NORMAL BRAIN

3.1 Mechanisms of plasticity

It is thought that brain plasticity inherently involves changes at the synaptic level (Donoghue 1995; Kolb 1995b). Synaptic plasticity may involve changes in the number of synapses or synaptic strength. (for review, see Greenough et al. 1994) A change in synaptic strength may involve alteration in synapse size, postsynaptic increases in the diameter of the spine head and a decrease in the length of the spine neck with or without any alteration in overall spine length or in neck width, number or distribution of vesicles, or in LTP/LTD. Recently much interest has been directed toward so called silent synapses, synapses that are inactive. The inactivity may be of presynaptic origin when little or no glutamate is released (Kimura et al. 1997), or postsynaptic, when no AMPA receptors are involved or they are nonfunctional (Rumpel et al. 1998). Silent synapses are thought to be recruited in activity dependent plasticity (Nicoll and Malenka 1999). It must be noted, however, that changes in synapses may involve axonal and dendritic growth, and plastic changes are seen also in glial cells and the brain vasculature (Kolb 1995b).

3.2 Plasticity following brain activation or inactivation

It is well known that training induces plastic changes in the brain (Jenkins et al. 1990; Karni et al. 1995; Nudo et al. 1996a; Xerri et al. 1996). Training has been shown to alter

representation areas in the brain. In humans, training which involved a rapid sequence of finger movements for 4 weeks resulted in the enlargement of the extent of activated cortical area (Karni et al. 1995). These changes persisted for several months. A brief daily exercise of a skill (picking up food pellets from wells) for several weeks remodelled the cortical representation area in the primary somatosensory cortex in monkeys (Xerri et al. 1999). In this remodelling, skin surfaces, which were important for the behavior were represented in enlarged areas and in a much finer representational grain. It may be that the specificity of a respective field is dependent on the nature of the stimulation. When learning-dependent enlargement of cortical representation was examined after the stimulation of a row of vibrissae, which was paired with a tail shock, the overlap between neighbouring rows became larger (Kossut and Siucinska 1998). The importance of this type of modulating activity has been shown also in the study of Kilgard and Merzenich (Kilgard and Merzenich 1998). Receptive field sizes could be narrowed, broadened or left unaltered, depending on the specific parameters of the acoustic stimulus paired with nucleus basalis activation.

Sensory deprivation has been used in studies of plastic changes in the brain. Changes in excitability and expansion of representation areas adjacent to the deprived area are seen within minutes (Byrne and Calford 1991; Calford and Tweedale 1991; Doetsch et al. 1996). Following deactivation due to subcutaneous injection of lidocaine, immediate and simultaneous sensory reorganization was detected at the cortical as well as subcortical areas (ventral posterior medial nucleus of the thalamus and trigeminal brainstem complex) (Faggin et al. 1997). There is evidence that representation maps in sensorimotor cortex overlap (Godde et al. 1995), which may enable the immediate change in the representation area. A recent study showed that the plastic changes after sensory deprivation can also be two directional and depend on the animal's interaction with its environment during the sensory deprivation period. Large-scale expansion of a single whisker's functional representation was observed following innocuous removal of all neighbouring whiskers (Polley et al. 1999). However, this manipulation induced a large-scale contraction of the representation if the animal was removed from its home cage and given a brief opportunity to use its whiskers to actively explore a different environment.

When morphological changes after training have been examined, it has been shown that motor learning generates new synapses and glial hypertrophy, but a simple motor exercise leading to increased synaptic activity, induced angiogenesis in cerebellar cortex (Anderson et al. 1994; Black et al. 1990). Moreover, it has been proposed that an increase in synaptic efficacy in the existing synaptic neural circuits via LTP could be involved in the early stages of motor learning, whereas the retention of motor skills involves the formation of new synapses (Asanuma and Keller 1991; Asanuma and Pavlides 1997). An increased number of synapses have been shown in primary motor cortex after motor learning for five days (Kleim et al. 1996).

3.2.1 Effects of enriched-environment housing

Housing animals in an enriched environment induces morphologic and functional changes in the brain. Enriched-environment housing is reported to increase the brain size, cortical thickness, and the magnitudes of astrocytic material and blood capillaries. An increased number of neurons and neuronal cell bodies, dendritic field and synaptic contact size as well as increased dendritic branching, spine density, number of synapses per neuron, protein synthesis and expression of mRNA have been also detected (for review, see Kolb and Whishaw 1998; Rosenzweig and Bennett 1996). It has also been reported that enriched-environment housing increases the levels of neurotrophic factors, such as glial derived neurotrophic factor (GDNF), brain derived neurotrophic factor (BDNF), and nerve growth factor (NGF) (Pham et al. 1999; Young et al. 1999). Furthermore, the enriched-environment housing is able to extend the cortical forepaw representation area (Coq and Xerri 1998). The glaborous receptive fields of enriched-environment housed rats were smaller and more clustered on the digit tips and palmar pads compared to those of standard cage housed rats.(Coq and Xerri 1998). Cutaneous maps of enriched-environment housed rats contained distinct representations of digit phalangeal glaborous skin and were more sensitive to light tactile stimulation.

Increased survival of newly born neurons is an interesting new finding and might explain the consequences of enriched-environment housing. Neurogenesis persists in the dentate gyrus and olfactory system throughout life in adult rodents, but in a broader context, neuronal stem cells appear to be found throughout the brain (Kaplan and Hinds 1977; Kee et al. 2001; Kempermann et al. 2000). A recent finding showed that housing in an enriched environment increases the number of proliferating cells, possibly by promoting the survival of dividing neuronal stem and progenitor cells (Kempermann and Gage 1999), and promotes the survival of newly formed neurons in dentate gyrus, thus leading to a larger granule cell layer and 15% more granule cell neurons (Kempermann et al. 1997). It has been suggested that this is based on increased voluntary running exercise (van Praag et al. 1999), but also hippocampus-dependent learning has been shown to double the number of adult-generated neurons in dentate gyrus (Gould et al. 1999). Another study, however, which examined the effect of learning experience on the formation of new neurons and their survival at a later time point, did not find any enhancement in neurogenesis (van Praag et al. 1999). Furthermore, it has also been proposed that it is the novelty of the complex stimuli, rather than continued exposure to complex stimuli, which elicits the effects of enriched-environment housing on neurogenesis (Kempermann and Gage 1999). It has been proposed that at least in the hippocampus, physical activity stimulates neurogenesis by promoting the proliferation of neuronal stem cells, but more specific functions such as learning may be able to recruit new neurons from the pool of cells with neurogenic potential (Kempermann et al. 2000).

4 PLASTICITY AFTER BRAIN INJURY

Patients recover spontaneously after stroke or brain injury, at least partially. Usually the functional recovery is most prominent during the first 3 months following stroke, but

may continue thereafter (Fig. 1) (Kotila et al. 1984; Nakayama et al. 1994). Furthermore, chronic motor deficits can be improved by rehabilitation (Taub et al. 1993). When the affected hand after recovery from hemiparesis following stroke is moved, the activation in brain has been shown to be shifted into new areas, such as ipsilateral sensorimotor and premotor cortical areas, and to the opposite sensorimotor cortex and contralateral cerebellum (Chollet et al. 1991).

The reinstitution of functions in brain can occur even after very drastic changes. For example, following hemispherectomy (removal or disconnection of an entire hemisphere), a procedure that has been used for treatment in epilepsy (Rasmussen 1983), residual somatosensory function in the hand opposite to the ablated hemisphere is mediated by the second somatosensory area and other cortical regions in the intact hemisphere instead of by the primary somatosensory area (Bittar et al. 2000). However, it is believed that there must be at least a survival of 5-10% residual in the affected brain structure if a functional recovery is to occur (for review, see Sabel 1997).

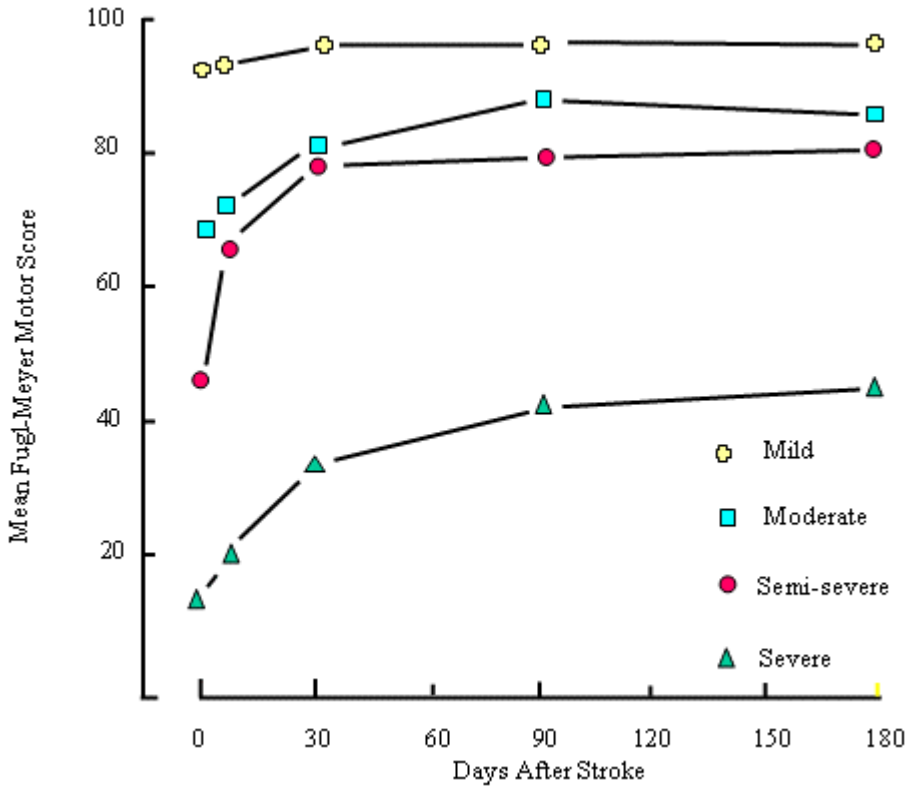

Fig. 1. Motor recovery after human stroke measured with the Fugl-Meyer Assessment. Modified from Duncan et al. 1992.

It must be noted that all plastic changes in brain are not beneficial. Experimental brain lesions may induce plastic changes that are detrimental to the behavioral outcome (Harrell and Parsons 1988; Kolb et al. 1994). Furthermore, prevention of cell death in a target-deprived area may also interfere with behavioral outcome. Prevention of the ipsilateral substantia nigra neuron degeneration following striatal damage exaggerated

the functional impairment of the forelimb contralateral to the lesion (Schallert and Lindner 1990). An example of a maladaptive plastic change in humans is phantom pain after sensory deprivation following amputation (Birbaumer et al. 1997; Harris 1999). It is also usual that following recovery of the paretic limb, its movement induces simultaneous mirror movements in the healthy side (Nelles et al. 1998). In 5 to 15 percent of patients, stroke leads to epileptic seizures (Bladin and Norris 1998; Kotila and Waltimo 1992) which probably are a consequence of hyperexcitability of neurons, a phenomenon detected after experimental stroke (Buchkremer-Ratzmann et al. 1996; Hagemann et al. 1998).

4.1 Theories of recovery following brain injury

4.1.1 Resolution of edema

Brain injuries, such as stroke, are commonly followed by severe brain edema and further deterioration by brain swelling is common in MCA occlusion patients (Wijdicks and Diringer 1998). The pathologic cascade in cells following cerebral ischemia includes the loss of function of ionic pumps and cell swelling as well as vasogenic edema caused by increased leakage of brain vessels (Rosenberg 1999). Cerebral edema is likely to produce local functional perturbation in the area immediately surrounding the primary injury (Goldstein 1993). The rapid improvement in performance after stroke could be due partially to the resolution of the cerebral edema (Dombovy and Bach-y-Rita 1988). After MCA occlusion in the rat, the infarcted hemisphere remains swollen for 7 days (Persson et al. 1988).

4.1.2 Amelioration of diaschisis

The term diaschisis was introduced by von Monakow already at the beginning of the last century (von Monakow 1914). It means the depressed function of brain areas that are remote, but anatomically connected with the damaged areas. Diaschisis can be observed by metabolic and functional depression as well as hypoperfusion and it is probably caused by loss of excitatory inputs (Serteser et al. 2001). Several animal (Buchkremer-Ratzmann et al. 1996; Ginsberg et al. 1989; Kataoka et al. 1989) and human studies (Bowler et al. 1995; Dobkin et al. 1989; Infeld et al. 1995) have demonstrated this phenomenon. Differential forms of diaschisis exist: cortico-cerebellar diaschisis, cerebello-cortical diaschisis, transhemispheric diaschisis, cortico-thalamic diaschisis, thalamo-cortical diaschisis and basal ganglia-cortical diaschisis (Nguyen and Botez 1998). It seems that diaschisis is relatively persistent, since it has been demonstrated up to 5 years after stroke onset (Miura et al. 1994). Furthermore, it has been shown that hypoperfusion in the cerebellum (crossed cerebellar diaschisis) correlated with the infarct volume as well as with the degree of neurological deficits but persisted despite neurological recovery (Infeld et al. 1995). However, in a recent study by Seitz et al. (Seitz et al. 1999) the networks that were affected by the lesion and the ones related to recovery overlapped topographically in the contralesional thalamus and extrastriate occipital cortex.

4.1.3 Hyperexcitability

There is also experimental electrophysiological data, suggesting that an ischemic brain lesion might lead to cerebral hyperreactivity. It has been demonstrated in animal studies following a photothrombotic ischemic lesion that LTP induction is facilitated in the vicinity of the cortical infarction (Hagemann et al. 1998). This change in excitability was detectable one day after ischemia formation and still existed 30 days later (Buchkremer-Ratzmann et al. 1996). Also the contralateral hemisphere showed hyperexcitability, which was not restricted to the homotopic brain areas to the lesion. There is an imbalance between excitatory and inhibitory systems, NMDA receptors are up-regulated whereas the $GABA_A$ receptors are down-regulated in the ipsi- and contralateral neocortex after photothrombotic focal cerebral ischemia (Qu et al. 1998). Moreover, double or multiple epileptiform discharges occurred in more than 30 % of the recordings (Buchkremer-Ratzmann et al. 1996). However, there are no reports of epileptic seizures *in vivo* following experimental stroke in rats. However, it is known, that in 5 to 15 percent of stroke patients a brain infarct can provoke epileptic seizures (Bladin and Norris 1998; Kotila and Waltimo 1992).

4.1.4 Multiple representation areas and unmasking

It has been proposed that there is redundancy in the functional organization of brain. In humans, integrative cerebral functions, such as language, cognition, perception, memory, sensory information processing and fine motor movements involve brain areas that are widely distributed throughout the cortex, diencephalon and brainstem, see (Lee and van Donkelaar 1995; Stein 1998). Unmasking is a hypothesis proposed by Lashley (Lashley 1929) and Luria (Luria 1963, 1966) suggesting that redundant neural networks can perform these functions lost following brain lesion.

Findings from brain imaging techniques, such as PET, show that new brain areas are able to take over the lost function after brain damage (Chollet et al. 1991; Thulborn et al. 1999; Weiller et al. 1992). It is known that in humans 10-15% of fibers in the corticospinal tract descend uncrossed (Davidoff 1990). It seems, that this uncrossed proportion in the pyramidal tract is responsible for the activation of the ipsilateral primary sensorimotor cortex during paretic hand movements (Cao et al. 1998). However, the significance of this activation to the recovery of function is not clear (Netz et al. 1997).

4.1.5 Synaptogenesis

Morphological alterations after damage in the sensorimotor cortex forelimb representation area by increased dendritic arborization of layer V pyramidal neurons in the respective contralateral cortex have been detected in rats. Arbor size was maximal 18 days after the lesion, and was followed by partial elimination or pruning of dendritic processes (Jones and Schallert 1994). This overgrowth of dendrites was related with the disuse of the affected forelimb and over-reliance of the non-affected forelimb. Furthermore, it seems that motor training enhances lesion induced plastic changes.

When complex acrobatic training was given to rats with damage in the forelimb sensorimotor cortex enhanced synaptogenesis was shown in layer V of the motor cortex opposite to the lesion relative to sham-operated acrobatic trained rats or rats having sensorimotor cortex lesion and receiving simple repetitive exercise (Jones et al. 1999).

4.1.6 Compensation of function

It must be noted that the recovery of function may be based on compensation rather than reinstitution of function. Dr. Kolb has given an example of this from his own life after losing vision in 1/4 of the foveal representation in the left eye (Kolb 1995a). He learned to fixate his vision in a way that the blind point did not disturb recognition. Some experimental studies have examined the role of compensation in recovery of function. When rats with unilateral dopamine depletion produced by injection of 6-hydroxydopamine into the nigrostriatal bundle were studied after training, their locomotion was still impaired, but the affected limbs were able to support their weight (Muir and Grosset 1999). The impaired hindlimb provided significant propulsive force and a relatively large laterally directed force, and was used partly as a spring. The most prominent abnormalities were seen during the diagonal couplet of the impaired forelimb and the unimpaired hindlimb, which revealed the important compensatory role of the unimpaired hindlimb. Moreover, dopamine depleted rats have been shown to be impaired in both their mouth and paw movements (Whishaw et al. 1997). They did not use the affected side of their mouth to chew, but relied upon the non-affected side of their mouth. Rats were seen to improve over a 30-day recovery period, much of the recovery being attributable to compensatory adjustments. As already noted, morphologic plastic changes in the opposite hemisphere related to the disuse of the affected forelimb and over-reliance of the nonaffected forelimb have been shown to occur (Jones and Schallert 1994). It has been suggested, that motor disability after certain types of injury involves a learned suppression of movement, so called learned nonuse (Taub et al. 1994).

4.2 Effects of rehabilitation and training

There is a wide variety of tests assessing recovery of stroke patients (Duncan et al. 2000b). For example, different aspects of disabilities of stroke patients can be assessed by tests measuring motor (e.g. Fugl-Meyer Assessment of Motor Recovery), cognitive (e.g. Stroke Unit Mental Status Examination, Mini Mental State) or functional abilities (e.g. Barthel Index of Activities of Daily Living) or neurological state (e.g. National Institute of Health Stroke Scale) (Duncan et al. 2000b; Hajek et al. 1997). This lack of consistency may complicate interpretation of stroke recovery studies (Duncan et al. 2000a). It is known that it is possible to enhance the functional recovery after stroke with rehabilitation (Kwakkel et al. 1997; Ottenbacher and Jannell 1993b; Ronning and Guldvog 1998; Sivenius et al. 1985). However, it is still not known which is the optimal rehabilitation procedure (de Pedro-Cuesta et al. 1992; Ernst 1990). The early initiation (Ottenbacher and Jannell 1993b) and intensity (Kwakkel et al. 1997) of rehabilitation correlate positively to better recovery of stroke patients. When one attempts to predict the extent of recovery in stroke patients, the severity of stroke is an important factor

(Jorgensen et al. 1999). Furthermore, independent of the effects of physical disability, the presence of cognitive impairment and depression have functional importance for stroke patients (Pohjasvaara et al. 1998; Robinson-Smith et al. 2000).

4.2.1 Training by enriched-environment housing

Housing animals in an enriched-environment offers one way to rehabilitate animals after brain lesion. It seems that the beneficial effect of enriched-environment housing is composed of social and physical components. When the relative importance of these two factors was studied, rats housed together in a large cage with no activity–stimulating facilities improved more than rats housed in individual cages with access to a running wheel, but both groups were inferior to rats housed in an enriched environment (Johansson and Olsson 1996). Enriched-environment housing can facilitate the recovery after different brain lesions (Rose 1988; Will and Kelche 1992), including focal ischemia (Johansson 1996; Ohlsson and Johansson 1995). Most commonly enriched-environment housing has been found to have a beneficial effect on recovery from lesions in the cerebral cortex, but also facilitates recovery from lesions in other brain areas, such as hippocampus, septum, amygdala, hypothalamus and thalamus (Rose 1988).

4.2.2 Role of training and rehabilitation in recovery process

There are some recent experimental studies concerning training induced plastic changes of the brain after ischemia (Biernaskie and Corbett 2001; Borlongan 2000; Briones et al. 2000; Nudo and Milliken 1996; Ohlsson and Johansson 1995; Xerri et al. 1998). The importance of training is emphasized by the studies of Nudo et al. (Nudo and Milliken 1996; Nudo et al. 1996b). In the absence of rehabilitative training following a small subtotal infarct in the hand representation area, the surrounding brain tissue underwent a further functional loss. This was prevented by rehabilitation, which may help the surrounding brain tissue to take over the lost function. In some cases, the representation area expanded into the area formerly occupied by elbow and shoulder. Conversely an excessively intensive training early after a brain lesion may even be harmful. Forced overuse of an impaired limb by immobilization of the intact forelimb after sensorimotor cortex lesion results in an exaggeration of the neuronal injury and severe and chronic behavioral deficits in rats (Kozlowski et al. 1996). A recent study showed also that the exclusive use of the affected forelimb immediately after moderate focal ischemia has detrimental effects on sensorimotor function (Bland et al. 2000b). Furthermore, early training in an enriched environment without immobilization exacerbated brain damage after focal cerebral ischemia, but still seemed to be beneficial at the behavioral level (Risedal et al. 1999). The exacerbation is probably based on increased release of glutamate or on hyperexcitability in neighbouring areas adjacent to the lesion. However, new findings suggest that the effects of intense early overuse may depend on the location and extent of the primary injury (Bland et al. 2000a). A recent experimental study showed, that intensive training combined with enriched-environment housing starting 15 days following focal cerebral ischemia improved the performance of fine digit and forelimb function by approximately 30 % in the staircase task where the deficit

is considered quite resistant (Biernaskie and Corbett 2001) The examination of the arborization of layer V pyramidal cells suggested that this task-specific rehabilitative therapy is capable of augmenting intrinsic neuronal plasticity within noninjured, functionally connected brain regions.

A recent study investigated the relationship between morphological and behavioral plasticity following exposure to an enriched environment after a brief global cerebral ischemia (Briones et al. 2000). After housing the rats for 4 days in enriched environment, the animals were tested in the water maze. The ischemic rats housed in an enriched environment had shorter escape latencies and minor heading errors towards the platform. Furthermore, sham-operated and ischemic rats that were housed in an enriched environment had increased dendritic length as well as an increased number of dendritic segments in the apical hippocampal region.

It is not clear how the modulation of brain activation during the recovery following stroke proceeds nor how it influences the rehabilitation. A recent study suggests that the ratio of contralateral to ipsilateral sensorimotor cortex activity during movement of the paretic hand increases as the paretic hand regains function when compared with the nonparetic hand (Marshall et al. 2000). The enlargement of the representation area in the hemisphere affected by stroke has been documented as corresponding to the improved motor performance of the paretic hand due to the rehabilitative training (Liepert et al. 2000).

4.3 Effects of pharmacotherapy

There is increasing experimental evidence, that recovery from differential brain injury can be modulated by pharmacotherapy. Most of the studies have used simple ablation lesions of the motor cortex. Three general principles concerning recovery promoting pharmacotherapy seem to be evident (Goldstein 1998b): 1) the drug effects are dose-dependent, 2) some drugs may have opposite effects depending on whether they are given during an acute phase or later following brain injury and 3) the effects of certain drugs are dependent of concomitant behavioral experience. Some of the data concerning beneficial or detrimental effects of the pharmacotherapy following differential brain lesions are summarized in Table 4.

Table 4. Selected experimental studies of drug effects on functional recovery

Transmitter/Drug	Action	Effect
Norepinephrine		+
Amphetamine	Sympathomimetic	+
Phentermine	Sympathomimetic	+
Phenylpropanolamine	Sympathomimetic	+
Methylphenidate	Sympathomimetic	+
Yohimbine	α_2-adrenoceptor antagonist	+
Idazoxan	α_2-adrenoceptor antagonist	+
Desipramine	NE reuptake blocker	+
Clonidine	α_2-adrenoceptor agonist	-
Haloperidol	α_1-adrenoceptor antagonist	-
Prazosin	α_1-adrenoceptor antagonist	-
Propranolol	β-adrenoceptor antagonist	+/-
GABA		-
Diazepam	GABA agonist	-
Muscimol	GABA agonist	-
Phenobarbital		-
Phenytoin		-
NMDA		
MK-801	NMDA-antagonist	- or +/-
Serotonin		
Trazodone	5-HT-uptake blocker	-
Fluoxetine	5-HT-uptake blocker	+/-
Amitriptyline	5-HT- & NE- uptake blocker	- or +/-
Dopamine		
Haloperidol	Butyrophenone, dopamine antagonist	-
Fluanisone	Butyrophenone	-
Droperidol	Butyrophenone	-
Spiroperidol	Dopamine antagonist	-
Apomorphine	Dopamine agonist	+
Acetylcholine		+
YM796	Muscarinic agonist	+
Scopolamine	Antagonist	-
Others		
bFGF	neurotrophic factor	+
OP-1	trophic factor	+
ORG2766	ACTH analog	+
BIM-22015	ACTH analog	+/-
JTP-2942	TRH analog	+/- or +
YM-14673	TRH analog	+

+ indicates a beneficial effect on recovery; - indicates a detrimental effect; and +/- indicates neutral effects on recovery.

Modified from (Goldstein 1998a). (See also Attella et al. 1992; Goldstein 1993; Kawamata et al. 1998; Kawamata et al. 1997b; van Rijzingen et al. 1996; Yamaguchi et al. 1995; Yamamoto et al. 1989; Yonemori et al. 2000).

4.3.1 Sympathomimetics

Amphetamine is a psychostimulant that has dopaminergic, noradrenergic and serotonergic effects (West et al. 1995). Amphetamine has been reported to enhance recovery after various experimental brain lesions (Feeney et al. 1982; Feeney and Hovda 1985; Hovda and Feeney 1984; Hurwitz et al. 1991). The beneficial effect of amphetamine pharmacotherapy has been found also in small clinical trials after stroke or traumatic brain injury (Crisostomo et al. 1988; Hornstein et al. 1996; Walker-Batson et al. 1990; Walker-Batson et al. 1995), even when initiated 15-30 days following stroke (Walker-Batson et al. 1995). Amphetamine administration induces an immediate and enduring acceleration of recovery even after a single dose (Feeney et al. 1982). Furthermore, it is possible that amphetamine increases the degree of recovery (Feeney and Hovda 1985; Hurwitz et al. 1991), not only hastening it.

Usually the amphetamine treatment has been found to be effective, but there may also be some limitations. The effect of amphetamine on recovery after damage may depend on the location of the lesion as well as the behavioral requirements of the task. In a study in rats conducted by Mintz and Tober (Mintz and Toner 1986), amphetamine administration interfered with recovery from rotational asymmetry behavior after a substantia nigra lesion. Furthermore, amphetamine produced a facilitation of recovery on the beam-walking test (Feeney et al. 1982; Goldstein and Davis 1990c) but not in the foot-fault test (Schmanke et al. 1996), where the rat has to place its limbs on the rungs of the elevated grid during locomotion, suggesting that amphetamine may facilitate recovery when the requirements of the task produce a deficit in the initiation of locomotion but not when the animal is required to use somatosensory and proprioceptive cues to guide performance in the task. Furthermore, amphetamine was unable to hasten the recovery of spatial mapping ability of gerbils after global ischemia (Colbourne and Corbett 1992) or to proffer additional benefit in rats housed in an enriched-environment (Johansson et al. 1997). The inability to improve the effects of enriched-environment housing was suggested to result in a common action mechanism, such as arousal.

Previous studies with amphetamine have shown that it is important to combine the drug administration with appropriate training (Feeney et al. 1982; Feeney and Hovda 1985). However, it seems that after multiple amphetamine injections, task-specific practice is not necessary (Hovda and Feeney 1984). When multiple amphetamine doses were administered, the recovery was no longer dependent on a concomitant beam-walking experience (Goldstein and Davis 1990b; Hovda and Feeney 1984). Also amphetamine doses administered 24 hours prior to each test day promoted recovery from sensorimotor integration deficit in a T-maze test after infarction in vibrissae cortical barrel-field within the primary somatosensory cortex (Hurwitz et al. 1991). Rats receiving task-specific practice after amphetamine-injection did not differ from rats receiving amphetamine without practice following lesion in sensorimotor forelimb representation area in vibrissae-evoked forelimb-placing task (Schmanke and Barth 1997). However, it was noted that rats receiving amphetamine show the classical signs of amphetamine-induced behavior, such as increased locomotion, circling, rearing, and

grooming responses. This generalized locomotor experience may be sufficient to evoke amphetamine-accelerated recovery (Hovda and Feeney 1984; Schmanke and Barth 1997).

There are several possible ways by which amphetamine can induce its effects on recovery. It has been proposed that amphetamine could enhance recovery by alleviating neuronal metabolic depression after brain trauma in rats (Queen et al. 1997). On the other hand, amphetamine treatment after focal ischemia has been found to enlarge the cortical receptive field, and it has been suggested that amphetamine allows normally depressed circuits to respond to sensory stimulation in normal rats and in rats having a brain infarct (Dietrich et al. 1990). Amphetamine has also been found to facilitate the development of LTP in a dose dependent manner (Gold et al. 1984). When reorganization of functional pathways was assessed by the distribution of a growth associated protein (GAP-43), a molecular marker of axonal growth, and synaptophysin, a protein found in synapses following amphetamine treatment after MCA occlusion, a significant increase in both proteins was found compared to vehicle treated ischemic control rats (Stroemer et al. 1998). The GAP-43 level was elevated in forelimb, hindlimb and parietal cortical regions ipsilateral to the lesion for two weeks after ischemia. Elevation of synaptophysin was detected in these regions as well as in the contralateral parietal cortex after two weeks and still 60 days after infarct induction. In conclusion, amphetamine seems to be a very efficient agent to help the injured brain to regain its function. It possibly augments the effects of sensorimotoric stimulation by different mechanisms involved in plasticity.

Methylphenidate is another psychostimulant, that inhibits the uptake of norepinephrine and dopamine (Kuczenski and Segal 1997) and is used for the treatment of attention deficit/hyperactivity disorder and narcolepsy (Challman and Lipsky 2000). There is also some data from small scale clinical trials, which suggest that methylphenidate could expedite the recovery following stroke (Plenger et al. 1996) and traumatic brain injury (Grade et al. 1998).

4.3.2 Noradrenergic modulation

The stimulative effect on noradrenergic system is thought to play a key role as a basis of the beneficial amphetamine action following brain trauma. The intraventricular administration of norepinephrine, but not dopamine (Boyeson and Feeney 1990) or serotonin (Boyeson et al. 1994) has been found to facilitate recovery after injury to the sensorimotor cortex in the beam-walking task. The importance of the noradrenergic system is further emphasized by pharmacological studies modulating its function. The α_2-adrenergic autoreceptors regulate the firing of noradrenergic neurons and the release of norepinephrine; thus, α_2-adrenergic receptor antagonists (e.g., yohimbine and idazoxan), which increase the release of norepinephrine, have been shown to facilitate recovery after specific cortical lesions (Goldstein 1989; Sutton and Feeney 1992). In contrast, decreased noradrenergic activity in the brain by α_1-adrenergic receptor antagonists (e.g. prazosin) or α_2-adrenergic receptor agonists (e.g. clonidine) is detrimental to recovery (Feeney and Westerberg 1990; Goldstein and Davis 1990a).

These drugs are able to retard the recovery and transiently reinstate the symptoms after recovery has occurred (Feeney and Westerberg 1990; Sutton and Feeney 1992).

Norepinephrine plays an important role in plasticity. Norepinephrine is involved in LTP (Brocher et al. 1992; Harley 1991) and its depletion has been found to block the ocular dominance shift in the kitten visual cortex (Kasamatsu and Pettigrew 1976) and experience dependent changes of enriched-environment housing (Benloucif et al. 1995; Brenner et al. 1983; Mohammed et al. 1986). The locus coeruleus, a major source of noradrenergic neurons in the brain, has a widespread efferent innervation to functionally diverse regions of the brain (Ungerstedt 1971) and is involved in "alertness and vigilance" (Aston-Jones et al. 1991; Berridge and Foote 1996). It has been suggested that the potential for remodelling the cortical representation may depend on the attentional and behavioral states (Chapin and Lin 1990; Recanzone et al. 1992). Furthermore, the activation of the locus coeruleus efferent system has been shown to enhance the efficacy of signal transmission through the sensory network (Waterhouse et al. 1998).

The mechanism underlying the facilitative effect of noradrenergic stimulation to the recovery of function after brain damage is not clear. There are only few somatosensory cortex ablation studies, which have tried to solve the mechanism. It has been proposed that norepinephrine alleviates diaschisis after brain injury (Boyeson and Feeney 1990). On the other hand, norepinephrine has been found to modulate plastic enlargement of the activated barrel following vibrissectomy in the partially deafferentiated sensorimotor cortex (Levin et al. 1988).

4.3.3 Dopaminergic drugs

Dopaminergic antagonists are detrimental to recovery (Goldstein 1998b). For example, haloperidol is considered to be deleterious on motor recovery following stroke (Goldstein 1993) and experimental studies show that haloperidol can disrupt recovery (Feeney et al. 1982; Hovda and Feeney 1985). The haloperidol-induced deficit can be partially restored by apomorphine (Feeney and Hovda 1983). Haloperidol inhibits also the noradrenergic system (Fang and Yu 1995), this may be an important facet of its effects, since intraventricular dopamine injection had no major importance on recovery (Boyeson and Feeney 1990).

Selegiline is an irreversible MAO-B inhibitor, which impairs the degradation of dopamine and inhibits the uptake of dopamine and norepinephrine, but also has various other pharmacologic modes of action (for review, see Magyar et al. 1996). There is evidence that selegiline might improve cognitive function following brain injury (Zhu et al. 2000).

4.3.4 Neurotrophic factors

Recent studies examining the effects neurotrophic factors on functional recovery have been promising. Furthermore, it seems that neuroprotection against secondary cell death

can be achieved even if they are administered relatively late after focal cerebral ischemia. For example, bFGF has been found to prevent thalamic atrophy when administered even 24 hours after cortical infarction (Yamada et al. 1991). Furthermore, bFGF seems to enhance behavioral recovery following focal cerebral infarction administered at later time points after ischemia without affecting infarct size (Kawamata et al. 1996) possibly by stimulation of axonal sprouting, since increased GAP-43 reactivity has been noted in the intact contralateral sensorimotor cortex after bFGF-treatment (Kawamata et al. 1997b). One trophic factor that has shown to be beneficial to functional recovery is osteogenic protein-1 (OP-1), a member of the bone morphogenic protein subfamily of the transforming growth factor-β (TGF-β) superfamily. OP-1 appears to enhance recovery of sensorimotor function dose dependently without affecting infarct size by enhancing dendritic sprouting (Kawamata et al. 1998). Both of the former agents seem to increase the rate of recovery, at least when assessed one month following brain infarction. Also nerve growth factor (NGF) has been suggested to promote behavioral recovery after cortical injury by preventing atrophy of dendritic fields and spine density (Kolb et al. 1996).

4.3.5 Cholinergic drugs

It seems that administration of acetylcholine may enhance recovery of function (Feeney and Sutton 1987) and anticholinergic drugs, such as scopolamine, may interfere with recovery (De Ryck et al. 1990). These results are to be expected, since the cholinergic system is involved in brain excitability and has an important role in attention and arousal (Brown and Marsden 1998; Wenk 1997). Furthermore, the cholinergic nucleus basalis has been shown to have a role in representational plasticity allowing the cortex to selectively improve neural representations from behaviorally important stimuli while ignoring irrelevant stimuli (Kilgard and Merzenich 1998).

4.3.6 GABAergic drugs

Drugs that increase inhibition in brain via the GABAergic system seem to have a detrimental influence on recovery after brain lesion. For example, benzodiazepines, such as diazepam and barbiturates, e.g. phenobarbital can disrupt or interfere with recovery (Hernandez and Holling 1994; Schallert et al. 1986). The results of GABAergic modulation on recovery after brain damage are not surprising because of its major inhibitory role in brain (MacDonald and Olsen 1994). Inhibition of the GABAergic system seems to be crucial in modification of plastic changes (Skangiel-Kramska et al. 1994; Ziemann et al. 1998). GABAergic intracortical connections are a candidate pathway for mediating the cortical reorganization and unmasking of existing lateral excitatory connections (Jacobs and Donoghue 1991).

5 CONCLUSIONS

There is clear evidence that recovery after brain injury can be modulated by pharmacotherapy (Feeney and Sutton 1987; Goldstein 1993). Generally, it seems that drugs that support plastic changes are beneficial for recovery, but activation of

inhibitory circuits is harmful. However, there has not been much enthusiasm to develop clinical regimes involving this kind of pharmacotherapy. There have been no large clinical studies that would evaluate whether amphetamine actually benefits stroke survivors, even though there is relatively abundant supportive experimental data. The unwanted side effects would not prohibit the therapeutic usage of amphetamine (Unwin and Walker-Batson 2000). However, there is a study in progress using amphetamine in stroke patients being conducted by Dr. Delaina Walker-Batson (Walker-Batson 2000). Furthermore, the lack of uniform practice in stroke patient rehabilitation and its evaluation makes the comparison of drug studies difficult (Duncan et al. 2000a). This would necessitate large-scale multicenter trials to determine long-term efficacy.

III AIMS OF THE STUDY

The purpose of the present thesis was to investigate the effects of pharmacotherapy and training on functional recovery after focal and global cerebral ischemia in rats. The specific aims were:

1. To investigate the effect of enriched-environment housing on functional recovery after global (**I, II**) and focal (**III**, **IV**) cerebral ischemia.

2. To investigate the effect of enriched-environment housing on hippocampal function on cellular level using an immediate early gene, Fos as a marker of cellular activity in sham-operated rats and in rats following global ischemia hippocampus lesion (**II**).

3. To investigate catecholaminergic stimulation using atipamezole (**I, III**), an α_2-receptor antagonist, and selegiline (**IV**), an irreversible MAO-B inhibitor, on functional recovery after global (**I**) and focal (**III, IV**) cerebral ischemia. The interaction between drug therapy and training in enriched environment was investigated (**I**, **III**, **IV**).

IV MATERIALS AND METHODS

1 ANIMALS

Male Wistar rats (HsdBrl:WH [Hannover origin]; National Laboratory Animal Centre, Kuopio, 260-400 g) were used in the experiments. The rats were housed in standard cages or in enriched-environment cages in a temperature (22 ºC), humidity (40-60%), and light period (07.00-19.00 h) controlled environment. The rats had free access to food and water, except those rats that were fasted overnight before focal cerebral ischemia lesion (**III**) and the ones which were food deprived 36 h before Montoya's staircase test (**IV**) and were given restricted amount of food (15 g) during test period. The studies were approved by the Ethics Committee of the University of Kuopio and by the Provincial Goverment of Kuopio.

2 SURGICAL AND PHARMACOLOGICAL PROTOCOLS

2.1 GLOBAL ISCHEMIA MODEL (I-II)

The rats used for global ischemia studies weighed 260-400 g (N=84). Global ischemia was induced using a slightly modified version of the four-vessel occlusion model by Pulsinelli and Brierley (Pulsinelli and Brierley 1979). The rats were anesthetized with a mixture of sodium pentobarbital (9.7 mg/ml) and chloral hydrate (10 mg/ml) administered intraperitoneally (2 ml/kg). The vertebral arteries were exposed under an operating microscope and closed permanently by cauterization. The next day, anesthesia was induced with 3% halothane in 30% O_2/70% N_2O and then 0.5% halothane was used to maintain a surgical depth of anesthesia. The common carotid arteries were exposed and anesthesia was stopped before the arteries were closed with clips for 20 min. The rectal temperature was monitored and maintained at 37°C using a heating pad. After both surgeries, the animals were placed in an incubator (30°C) until they recovered. Sham-operated control rats underwent the same surgery procedure without artery occlusion. The surgical procedure did not involve monitoring of blood gases, blood pressure or cerebral blood flow during ischemia. The loss of blood flow during ischemia period was verified by checking the loss of the righting reflex.

2.2 FOCAL ISCHEMIA MODEL (III-IV)

Focal cerebral ischemia was used to attain a more clinically relevant model of stroke, since the major human strokes are focal. The rats weighing 260-320 g were used for focal ischemia studies (N=95). Focal cerebral ischemia was induced using a slightly modified version of the intraluminal filament technique described by Longa et al. (Longa et al. 1989). Anesthesia was induced with 3% halothane in 30% O_2/70% N_2O and then decreased to 0.5 to 0.6% halothane to maintain a surgical depth of anesthesia. The right common carotid artery was exposed with a midline cervical incision. Heparin (90 IU (**III**) or 20 IU (**IV**); 100 IU/ml in 0.9% saline) was administered intraperitoneally before the heparinized intraluminal filament (Ø 0.28 mm, rounded tip) was introduced *via* the external carotid artery 1.9 to 2.1 cm into the internal carotid artery to occlude

blood flow to the MCA. Head temperature was monitored with a probe inserted into the temporalis muscle and rectal temperature was monitored and maintained at 37ºC using a heating pad. After 120 min of MCA occlusion, the filament was gently removed and the external carotid artery was permanently closed by cauterization. In sham-operated rats, carotid arteries were only exposed. The surgical procedure did not involve monitoring of blood gases, blood pressure or cerebral blood flow during ischemia.

2.3 TRAINING

2.3.1 Enriched-environment housing

The enriched environment consisted of two cages (61 x 46 x 46 cm) that were connected by a short tunnel and ladders (Fig. 2). One of the walls was constructed of bars, and the cages contained tunnels, shelves, a running wheel, and different kinds of manipulatable objects (*e.g.*, glass balls, jars, wooden objects), which were changed every week (**I**) or every other day (**III**). The rats were housed in the enriched environment in groups of 6-10 rats. The purpose was to offer the animals an opportunity to have complex sensorimotor stimulus and motor training. The enriched-environment housed rats had also more social activation, since the other rats were housed in standard cages (53 x 32.5 x 20 cm) individually (**I, II**) or in groups of two or three animals (**III, IV**).

Fig. 2. Enriched-environment cage.

2.3.2 Labyrinth

The enriched-environment housing was combined with 30-min exercise in a labyrinth (127x127 cm with 37-cm walls) providing the animals with the possibility to explore a complex environment and to practise spatial memory (**I, IV**). The spatial exercise was combined with sensorimotor exercise when the labyrinth was filled with traversable

objects (**IV**). The labyrinth exposure occurred 30 min after the atipamezole/saline (**I**) and selegiline/saline (**IV**) administration.

2.4 DRUG ADMINISTRATION

2.4.1 Atipamezole (I, III)

Atipamezole (4-(2-ethyl-2,3-dihydro-1H-inden-2-yl)-1H-imidazole; Orion Corporation, Orion Pharma, Turku, Finland) is a relatively novel, highly selective and specific α_2-adrenoceptor antagonist (Scheinin et al. 1988; Virtanen et al. 1989).

In experiment I atipamezole was dissolved in 0.9% saline, and was administered (0.5 mg/kg subcutaneously, s.c.) once a day beginning on the third day after global cerebral ischemia. Administration continued in three periods of 7 days with 2-day breaks between each drug treatment period. Since atipamezole may interfere with water-maze behavior (Sirviö et al. 1992), there was a wash-out period after each drug-treatment period, before behavioral testing. Control groups were injected with 0.9% saline (2 ml/kg, s.c.). The atipamezole/saline was administered either combined with the training in the enriched environment and spatial exercise in a labyrinth or without training.

In experiment III atipamezole was dissolved in sterile water and administered (1 mg/kg, s.c.) once a day 30 minutes before behavioral testing, which served also as a training, beginning on the second day after induction of focal cerebral ischemia and continuing for 10 days. Atipamezole is well tolerated at this dose level and blocks central α_2-adrenoceptors increasing central norepinephrine release for hours (Haapalinna et al. 1997). The distribution half-life of atipamezole is about 10 min and the half-life of elimination is 1.3 hours (unpublished observations, Orion Pharma). The behavioural testing was performed at the times when there is increased norepinephrine levels in brain (Laitinen et al. 1995). The other groups were injected with 0.9% saline (2 ml/kg, s.c.).

2.4.2 Selegiline (IV)

Selegiline HCl (Orion Corporation, Orion Pharma, Turku, Finland, 0.5 mg/kg, s.c.), an irreversible MAO-B inhibitor (Magyar et al. 1996), or an equivalent volume of 0.9% NaCl (2 ml/kg, s.c.) was administered once a day, beginning on the second day after focal cerebral ischemia induction, continuing for 30 days. The selegiline dose used was selected on the basis of previous behavioural studies (Bickford et al. 1997; Gelowitz et al. 1994) and was expected to induce almost complete MAO-B inhibition (Jolkkonen et al. 2000). Selegiline and saline were administered either combined with the training in enriched-environment or without training.

3 BEHAVIORAL TESTS

3.1 Water-maze test (I-IV)

To assess spatial learning, a modified version of the Morris water-maze task was used. The water-maze pool (Ø 150 cm, depth 74 cm, filled to a height of 52 cm with clear water at temperature 20±2 ºC) was a circular fiber-glass tank, painted black. The pool was divided into four quadrants of equal surface area. The starting locations were called north, south, east, and west, and were located arbitrarily at equal distances on the pool rim. The platform (10 cm diameter, composed of black rubber) was located with its top surface 2.5 cm below the water line in the middle of the quadrant 25 cm from the pool rim. However, in the north-west quadrant (**I**) the center of the platform was 22 cm from the north-south axis and 20 cm from the pool rim. The swim paths were monitored by a video camera connected to a computer through an image analyzer. If the rat failed to find the hidden platform within 70 s, it was placed on the platform. The animal was allowed to remain on the platform for 10 s and to rest for either 30 s or 1 min. The first, third, and fourth trials of the day were started from one of the points located farthest from the platform. The start point was changed after each trial. Escape latency (time to reach the platform) and path length the animal swam to find the platform were used to assess the acquisition of the water-maze task. Swimming speed (path length/escape latency) was used to assess the motor activity of rats in this task. The shorter the latency to find the platform, the better the memory for its location was considered to be. At the end of the testing period, a probe trial of 70 seconds without the platform was used to assess how well the animals remembered the location of the platform (i.e. by number of passes over the previous platform location) (**III, IV**). Different searching strategies were also analyzed (percentage of time spent in three equal zones of the pool). A visible platform in a new location was used to determine whether the groups had differences in recognition ability or escape motivation (**III**).

In experiment I testing was performed for 2 days after each of the three atipamezole/saline treatment periods (on days 11, 12, 20, 21, 29 and 30 after global ischemia induction). The rats had 6 trials on each test day. The platform was changed to a different quadrant each day. After the last two water-maze test days the animals were tested in another room to evaluate their performance in a new environment (days 31 and 32).

In experiment II rats were exposed to a water-maze task of eight trials 12 days after global ischemia induction. On the previous day, the animals had been allowed a habituation swim of 90 s without the platform.

In experiment III the spatial learning ability of rats was assessed in the water maze on days 22-24 after focal cerebral ischemia. Rats were given five trials from the first through the third test day. There was also an additional probe trial without the platform given on the third day to test how well the animals remembered the location of the hidden platform. On the fourth day (postoperative day 25), the rats were given four trials to find a visible platform placed in the south-east quadrant.

In experiment IV the water-maze paradigm was the same as the previous one, except that no visible platform was used.

3.2 Open-arena test (I)

The open-arena test was performed in the same room as the first series of water-maze tests in experiment **I**. The open-arena test was used for assessing of the exploratory activity on postoperative days 2, 10, 19 and 28. The apparatus was placed on the rim of the water-maze. Each rat was placed in the middle of a black painted square (110 x 110 cm, walls 30 cm), and was monitored for 15 min by a video-camera connected to a computer through an image analyzer (in 3-minute sessions that were interrupted by a 25-second break during which the computer loaded the next program). The computer system registered the distance traveled, and the number of rearings (rearing up on hind legs), number of fecal boli, and time spent grooming were observed by the experimenter.

3.3 Limb-placing test (III, IV)

This test was a modified version of a test described by De Ryck et al. (De Ryck et al. 1989). The rats were habituated to handling before the induction of ischemia. The limb-placing test was used for assigning ischemic animals to behaviorally equal groups the day after induction of ischemia and the same test was used to assess recovery of rats on postoperative days 2 through 11, 16, and 21 (**III**) and on days 3, 5, 7, 9, 11, 16, 21 and 32 (**IV**). This test had seven limb-placing tasks to assess the integration of forelimb and hindlimb responses to tactile and proprioceptive stimulation. The tasks were scored as follows: 2 points, the rat performed normally; 1 point, the rat performed with a delay (2 s) and/or incompletely; and 0 points, the rat did not perform normally. Both sides of the body were tested. In the first task, the rat was suspended 10 cm over a table. Rats normally stretch both of their forelimbs towards the table. In the second task, the rat was positioned towards the table and its forelimbs were placed on the table. Each forelimb was gently pulled down and retrieval and placement were checked. Rats normally replace the limb on the table. The third task was the same as the second except that, by keeping the rat's head upward in a 45º angle, the rat was prohibited from seeing the table or contacting it with its vibrissae. Next, the rats were placed along the table edge to check for lateral placement of the each forelimb (fourth task) and hindlimb (fifth task). In the sixth task, the rat was again positioned towards the table, the hindlimbs just over the table edge. Each hindlimb was pulled down and gently stimulated by pushing it towards the side of the table. In the seventh task, the forelimbs were placed on the edge of the table and the rat was gently pushed from behind toward the edge. Rats normally rats resist the pushing, but injured rats cannot keep their grip and the injured limb slips off the edge.

3.4 Beam-walking test (III)

The beam-walking test was used to assess deficits in coordination and integration of motor movement, especially in the hindlimb. The rats were trained to traverse the beam for 3 days before the induction of ischemia and by the end of the training period all rats had learned the task. The animals were tested from days 2 to 7 after ischemia. A beam-walking apparatus consisted of a square beam (2.5 cm wide, 122 cm long, at 42 cm high) connected to a black box (20.5 x 25 cm, 25 cm). A bright light was placed above the start point to motivate the rats to traverse the beam. The performance of the rats was rated as follows: the rat was not able to stay on the beam, 0 points; the rat did not move, but was able to stay on the beam, 1 point; the rat tried to traverse the beam, but fell, 2 points; the rat traversed the beam with more than 50% footslips of the affected hindlimb, 3 points; the rat traversed the beam with more than one footslip, but less than 50%, 4 points; the rat had only one slip of the hindlimb, 5 points; the rat traversed the beam without any slips of the hindlimb, 6 points.

3.5 Foot-slip test (III, IV)

The rats were trained to run in a wheel for 4 days before the induction of ischemia. To assess motor coordination and proprioception, the accuracy of forelimb placement of rats was quantified using running in a wheel in a foot-slip test (on days 2 to 11, 16, and 21 (**III**) and 2, 3, 5, 7, 9, 11, 16, 21 and 32 (**IV**) after induction of focal cerebral ischemia). The running wheel (Ø 29 cm, with transparent plastic walls, rungs 2 cm apart) had an adjustable motor and rotated 6 times per minute. Performance was recorded *via* a camera connected to a video recorder and a monitor. The performance of the rats was assessed by calculating the slip ratio of the affected forelimb (number of slips/number of steps taken) over 2 min.

3.6 Staircase test (IV)

A modified version of the staircase test by Montoya et al. (Montoya et al. 1991) was used for evaluation of the forelimb food pellet reaching and grasping abilities of the rats from different levels of a staircase on days 27 to 32 after induction of ischemia. The test was preceded by 36 h of food deprivation. The Plexiglas™ test apparatus has an elevated central platform with a staircase on both sides. The staircases have six steps, of which the five upper steps were each baited with a chow pellet (45 mg, Campden Instruments Ltd, UK). The rat was placed on the platform and was allowed to collect the pellets during four trials each of five min duration. During each trial, the number of pellets reached but dropped as well as successfully retrieved pellets from both sides were calculated. After each test, the rats were given approximately 15 g of standard food pellets.

4 HISTOLOGICAL METHODS

4.1 Tissue preparations

In global ischemia studies, the rats were deeply anesthetized and perfused transcardially initially with saline (2 min) and then with 4% paraformaldehyde (400 ml). The brains were removed and postfixed for 3 (**II**) or 6 (**I**) hours. The hippocampal area was cut into 50-μm sections using a vibratome.

In focal ischemia studies, the animals were decapitated and the brain were rapidly removed from the skull and frozen on dry ice. Coronal sections of 30 μm or (**IV**) 40 μm (**III**) at 1.24 mm (**III**) or 1.0 mm (**IV**) intervals were cut throughout the brain on a cryostat and sections were collected on SuperFrost® slides (Menzel-Gläser).

4.2 Cresyl fast violet staining (I, II, IV)

Cresyl fast violet staining was used for the assessment of the cell loss in hippocampus (**I, II**).

4.3 Nitro blue tetrazolium staining (III, IV)

Sections were stained for 20 minutes with a solution containing 1.2 mmol/l nitro blue tetrazolium (NBT) and 0.1 mol/l sodium succinate in 0.1 mol/l sodium phosphate buffer, pH 7.6, at 37^{o}C (Nachlas et al. 1957). Sections were then rinsed in water, dehydrated in an ascending series of alcohol, cleared in xylenes, and coverslipped with Depex.

4.4 Fos-immunostaining (II)

Three hours after the swim task, the rats were anesthetized and perfused**.** Sections from four different levels (approximately -3.0; -3.9; -5.1, and -6.0 relative to bregma (Swanson 1992) were analyzed for Fos-immunostaining. The sections were washed in PBS containing 0.3% Triton X-100 and 1% bovine serum albumin, and were then incubated in the Fos primary antibody (4 cat.# sc-0.52, rabbit polyclonal, Santa Cruz Biotechnology, Santa Cruz, CA) diluted 1:2000 in the same buffer at 4°C for 48 to 72 h. After washing, the sections were incubated in biotinylated goat anti-rabbit serum and ABC complex (Vectastain Elite Kit, Vector Laboratories, Burlingame, CA) for 2 h. The ABC complex was visualized with 0.05% diaminobenzidine and 0.02% H2O2.

4.5 Estimation of cell death after global ischemia (I, II)

The semiquantitative analysis of neuronal damage in CA subfields of each hemisphere was carried out by determining the grade of pyramidal cell loss. The neuronal damage was scored in the same manner as in the study by Block and Pulsinelli (Block and Pulsinelli 1987): score 1 = 0-10%, score 2 = 10-50%, and score 3 = 50-100% loss of

pyramidal cells. The assessment was done in a blind manner on coded slides from different levels of the dorsal hippocampus.

4.6 Assessment of infarcted volume after focal ischemia (III, IV)

The infarct volumes were determined from NBT-stained sections. Estimations of the infarct areas in the cortex, striatum (**III, IV**) and thalamus (**IV**) were performed using an image analysis system (MCID). The area of infarction was determined according to the indirect method of Swanson et al. (Swanson et al. 1990) by an observer blind to the experimental conditions. The image of each section was stored as a 1280 x 1024 matrix of calibrated pixel units. The digitized image was then displayed on a video screen and areas of interest were outlined separately for each hemisphere and automatically recognized according to optical densities above threshold levels. The difference between the size of an intact area in the contralateral hemisphere and its respective residual area in the ipsilateral hemisphere was defined as the infarcted area (infarct area = area of the contralateral hemisphere-noninfarcted area of the ipsilateral hemisphere). Total infarct volume was calculated by multiplying the infarct area by the distance between the sections and summing together the volumes for each brain.

4.7 Quantification of the Fos-immunostained nuclei (II)

The Fos staining was analyzed with a Nikon Optiphot-2 microscope and the distribution of stained nuclei in the different hippocampal fields was plotted with a computer-aided digitizing system (Minnesota Datametrics, St. Paul, MN). The number of Fos-stained nuclei of CA1, CA3, granular, and subicular cells was counted in each section from rats having water-maze exposure and those without exposure.

5 STATISTICS

Parametric statistical tests were chosen when continuous variables were being analysed and non-parametric when class variables were analyzed. The water-maze data (**I-IV**), (escape latency, path length, and swimming speed) were analyzed using ANOVA for repeated measures. In experiment **I** also four ischemic groups of rats were regrouped according to whether or not they were treated with atipamezole, and whether or not they were housed in the enriched environment. These new groups were used as grouping factors in an ANOVA for repeated measures and an interaction between those factors was analyzed (over 10 trials). The water-maze probe trial data (**III, IV**)(removed platform), however, were analyzed using one-way ANOVA with Duncan's post hoc test. The open-arena data (**I**), path length, number of rearings and fecal boli, and time spent grooming, of the first test day were analyzed by one-way ANOVA with Scheffe's post hoc test to determine if there were differences between groups in the baseline levels, and the data of the next three tests were analyzed by ANOVA for repeated measures. Beam walking (**III**) and limb-placing test (**III**, **IV**) data were analyzed using Kruskal-Wallis nonparametric analysis of variance to determine the overall group effect on each test day. Comparisons between the ISCH group and other experimental groups were performed using the Mann-Whitney test. Foot-slip data (**III, IV**) for the overall

group effect were analyzed using ANOVA for repeated measures. Comparisons between groups were made using one-way ANOVA with Duncan's post hoc test. The staircase data (**IV**)(number of reached-and-eaten and reached-but-dropped pellets) were analyzed using ANOVA for repeated measures.

To test whether ischemia and/or enriched-environment housing accounted for the variability in Fos staining (**II**), the four groups of rats were regrouped according to whether or not they were ischemic, and whether or not they were housed in the enriched environment. These new groups were used as grouping factors in an ANOVA for repeated measures and an interaction between those factors was analyzed (over four sections) in each hippocampal field to determine whether the influence of the enriched environment on Fos staining was different between ischemic and sham-operated rats. The Mann-Whitney U-test (two-tailed) was used to analyze differences in the histological damage between the ischemic groups (**II**). The infarct volumes (**III, IV**)(cortical, striatal, and hemispheric) were analyzed using one-way ANOVA with Duncan's post hoc test. Spearman's rank correlation between total and cortical infarct volumes and behavioral deficit in limb-placing tests used for group assignment was investigated (**III**).

V RESULTS

1 GLOBAL ISCHEMIA MODEL

1.1 Histological findings (I, II)

1.1.1 Histological cell death

Cell loss in the dorsal hippocampal CA1 field was usually nearly complete (Fig. 3). Only very few rats had more 50 % of pyramidal cells left in CA1 area, and this was unilateral except in one case where it was bilateral. Also some unilateral hippocampal damages in CA1 area were noticed and these rats were excluded. Some animals had cell death in the most distal and proximal parts of CA3, i.e. in the CA3 next to CA2, and in CA3c inside the hilus. There were also rats, which had damage in all parts of CA3 hippocampal areas. Loss of the hilar neurons in the dentate gyrus also was commonly observed.

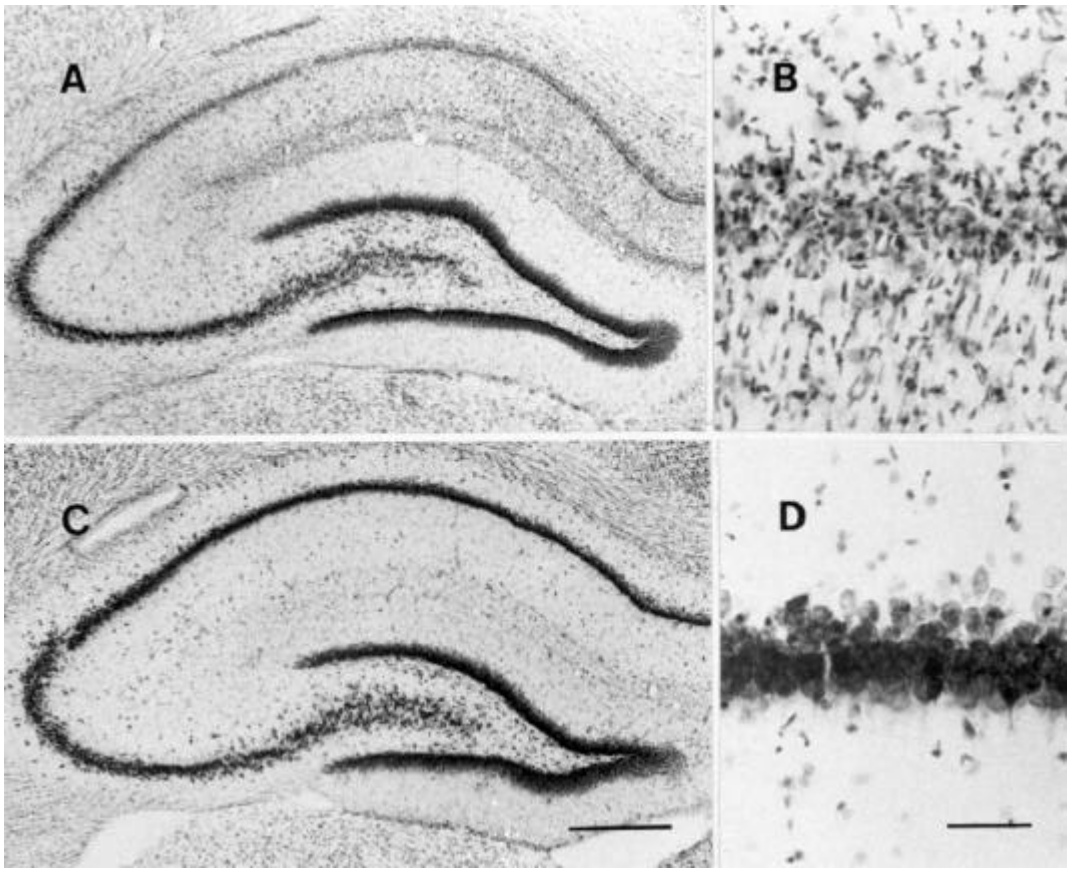

Fig 3. Typical example of a global ischemia lesion in hippocampus. Photographs represent coronal sections of the hippocampus stained with cresyl fast violet from a rat operated 12 days after the 20-min ischemia (A, B), with sections from a corresponding sham-operated animal (C, D). The CA1 pyramidal cell layer is shown at higher magnification on the right side. Scale bar in B = 500 μm, in D = 50 μm.

1.1.2 Effects of enriched-environment housing on the number of Fos-immunopositive nuclei and cell death (II)

Enriched-environment housing increased the number of Fos-positive granular cells 3 hours after a learning experience in the water maze. This was seen more clearly in sham-operated animals, but to some extent also in rats with global cerebral ischemia following enriched-environment housing. The enriched-environment housing had no effect on the number of Fos-positive neurons following water-maze test in the CA3 or in the CA1 field pyramidal cell layer or in subiculum. When basal Fos-expression following differential housing without the water-maze task was quantified, neither the enriched-environment housing nor the global ischemia had any significant effect on the number of Fos-positive cells in the dentate gyrus. No differences were found in any other hippocampal area. These results imply that enriched-environment housing possibly increases the number of dentate granule cells that fire when receiving information in a water-maze learning situation.

1.2 Behavioral outcome (I, II)

1.2.1 Effects of global ischemia

When water-maze acquisition was assessed at the first time 11 and 12 days after global cerebral ischemia induction, rats with global ischemia had longer escape latencies than sham-operated rats (**I**). In this water-maze paradigm, the rats had 5 trials on both days and the location of the platform was changed. On the following test days, differences in escape latencies between ischemic and sham-operated rats were no longer found, even when testing occurred in a novel environment. Global ischemia did not affect the swimming speed of rats. However, when a water-maze paradigm that had a habituation swim without the platform and single testing of 8 trials on the following day (day 12 after ischemia induction) was used, rats with global ischemia showed no deficit in water-maze learning (**II**).

In the open-arena test, rats with global cerebral ischemia showed an increase in the distance traveled (path length) and in the number of rearings compared to sham-operated rats. Global ischemia was not found to affect the time spent on grooming.

1.2.2 Effects of enriched-environment housing

The enriched-environment housing improved the water-maze performance of ischemic animals during the first two days of testing (**I**). In the later phase of the testing, the ischemic control rats were no longer inferior to sham-operated ones. However, the effect of enriched-environment housing was more profound in the open-arena test. When the open-arena test was used as a measure of the start level, 2 days after global ischemia before differential housing had begun, no differences in the path length, number of rearings, or number of fecal boli were found between the groups. The ischemic rats that had experienced training in an enriched environment and labyrinth were not hyperactive. Enriched-environment housed ischemic rats had a shorter path

length than the ischemic rats that were housed in standard cages. Furthermore, training decreased the number of rearings whereas these rats spent more time grooming than the ischemic rats housed in standard cages.

1.2.3 Effects of atipamezole administration (I)

The atipamezole administration was not beneficial either in water-maze or open-arena tests after global ischemia. However, in the final water-maze tests days (7 and 8) in a novel environment, rats that has been treated with atipamezole and which did not receive training were inferior to saline treated ischemic rats which were housed in standard cages.

2 FOCAL ISCHEMIA MODEL

2.1 Histological findings (III, IV)

The infarct volumes were large: in experiment III 144.1 ± 17.4 mm^3 for total hemisphere, 66.7 ± 11.6 mm^3 for the cortex, and 28.8 ± 1.4 mm^3 for the striatum and in experiment IV 179.5 ± 18.3 for total hemisphere, 93.5 ± 10.8 mm^3 for the cortex and 32.8 ± 3.1 mm^3 for the striatum. One animal with no detectable lesion was excluded. When histologic data were analyzed, there was no difference in infarct volumes between ischemic groups.

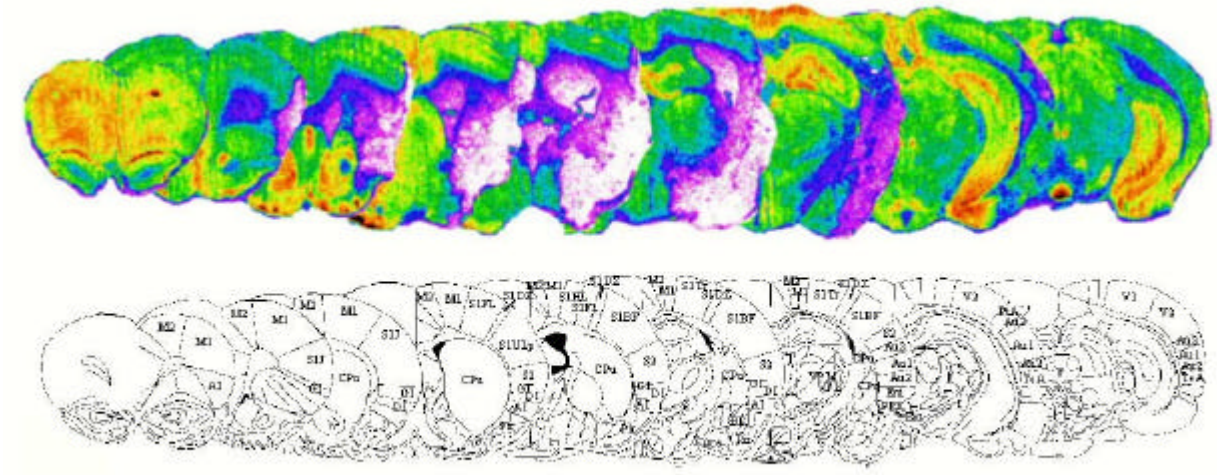

Fig. 4. A typical example of a brain having focal cerebral ischemia lesion. The infarct volumes are 176 mm^3 for total hemisphere, 73 mm^3 for the cortex, and 35 mm^3 for the striatum. The respective functional anatomy of the brain areas is presented below (Paxinos and Watson 1996). Aco anterior cortical nucleus; AI agranular insular cortex; Au1 primary auditory cortex; Au2 secondary auditory cortex; BL basolateral amygdaloid nucleus; CPu caudate putamen (striatum); DI dysgranular insular cortex; Ent entorhinal cortex; GI granular insular cortex; M1 primary motor cortex; M2 secondary motor cortex; Pir piriform cortex; PRh perirhinal cortex; PtA parietal association cortex; S1BF primary somatosensory cortex, barrel field; S1DZ primary somatosensory cortex, dysgranular region; S1FL primary somatosensory cortex, forelimb region; S1HL primary somatosensory cortex, hindlimb region; S1J primary somatosensory cortex, jaw region; S1Tr primary somatosensory cortex, trunk region; S1ULp primary somatosensory cortex, upper lip region; S2 secondary somatosensory cortex; TeA temporal association cortex; V1 primary visual cortex; V2 secondary visual cortex; VPL ventral posterolateral thalamic nucleus; VPM ventral posteromedial thalamic nucleus.

2.2 Behavioral outcome

2.2.1 Effects of focal ischemia

The ischemic rats were found to be inferior to sham-operated rats in different sensorimotor tests. The scoring of the limb-placing test one day after induction of ischemia was used to assign rats to comparable ischemic groups (**III, IV**). After the experiment, when relations between limb-placing scores and infarct volumes were made, a significant Spearman's rank correlation between cortical and total infarct volumes and limb-placing scores on the first day following focal ischemia induction was found (Fig. 5) (**III).** The rats with focal cerebral ischemia were impaired in the limb-placing performance from the second day on (except on day 10, **III**) throughout the testing period until day 32 (**IV**) following ischemia induction compared to sham-operated rats. Rats having focal ischemia were also impaired in successful retrievals of food pellets and made fewer attempts to reach pellets with their affected left forepaw than the sham-operated rats during days 27-31 following focal ischemia induction. The rats with focal ischemia lesion also made fewer attempts to reach the food pellets with the nonaffected forepaw than the sham-operated rats with their respective right forepaw. Moreover, rats with focal cerebral ischemia made more slips than sham-operated control rats. However, the deficit in foot-slip test was transient and ischemic rats reached the level of performance of the sham-operated rats on postoperative day 16. The rats having focal ischemia lesion showed the most rapid improvement in the beam-walking task, but ischemic rats were found to be inferior to sham-operated rats during the first 2 days of testing on days 2 and 3 after ischemia induction

Water-maze testing occurred in the later phase of recovery on postoperative days 22-24. The rats with focal cerebral ischemia were still impaired in a spatial learning task. The sham-operated rats had shorter escape latencies and path lengths than the rats having focal ischemia lesion (**III, IV**). Ischemic rats preferred to swim close to the pool rim and spent more time in the outermost zone of the pool (**IV**). Ischemic rats showed no decrease in motor activity, since there was no difference in swimming speeds between these animals. Furthermore, ischemic rats had no difficulties in swimming to a visible platform on day 25 after ischemia lesioning and there were no differences in escape latency and path length between sham-operated and ischemic rats (**III**).

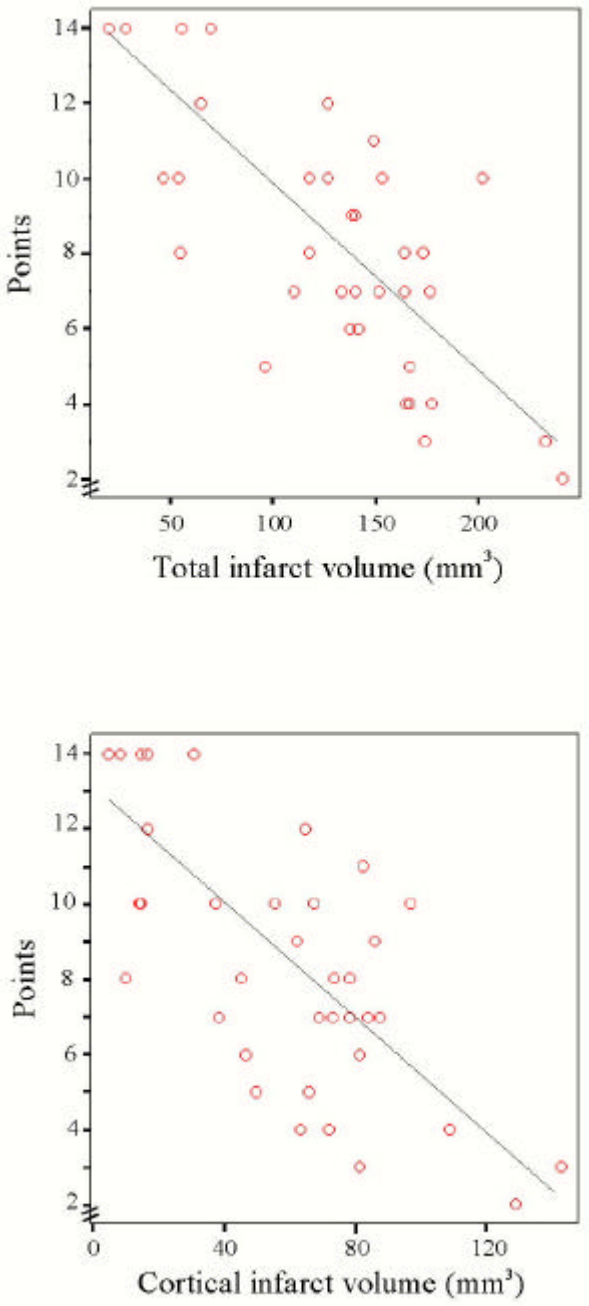

Fig. 5. Spearman rank correlation between total and cortical infarct volumes and limb-placing deficit ($r = –0.7$, $P < 0.001$ and $r = –0.6$, $P<0.001$, respectively) on the day following ischemia induction.

2.2.2 Effects of enriched-environment housing

The enriched-environment housing improved the performance of rats in a limb-placing test that was seen in a later phase of testing (**III, IV**). There was a significant difference between the enriched-environment and standard-cage housed ischemic rats 16 days after ischemia (**III**). However, no difference between differentially housed rats was seen in experiment **IV**. This may be due to the difference in severity of focal cerebral ischemia lesion. In experiment **III,** all animals which had had proper occlusion and showed a detectable lesion were accepted, but in experiment **IV** only rats having less than 10 points in the limb-placing task were included to the final experiment. Therefore, also a few rats with minor deficit in behavior were tested. It is possible that the enriched-environment housing benefits to the greater extent those rats that still possess more potential for recovery. The results of the foot-slip test were also mixed. The beneficial effect of enriched-environment housing became manifested on days 8 to 10 after induction of ischemia (**III**). However, in experiment **IV,** the rats housed in the enriched environment after focal ischemia slipped more than the standard cage housed control rats on days 11 and 32 after induction of ischemia. In the staircase test (**IV**), the enriched-environment housed rats made more attempts to reach the pellets with their nonaffected forepaw than rats housed in standard cages. Enriched-environment housing had no effect on the number of attempts made with the affected forepaw or in the number of successfully retrieved food pellets with either forepaw. Enriched-environment housing did not affect the performance in the beam-walking test.

Perhaps the most prominent effect of the enriched-environment housing was found in the water-maze task (**III, IV**). In experiment **III,** the rats which were housed in the enriched environment after focal cerebral ischemia had shorter escape latencies, but not path lengths than the standard cage housed rats. The enriched-environment housed ischemic rats also had significantly faster swimming speeds than the standard cage housed rats. When data from the probe trial was analyzed, the enriched-environment housed rats had significantly longer path lengths and thus faster swimming speeds during the 70-second trial when compared to standard cage housed rats. There was no difference between groups in passes over the removed platform. There was a difference, however, in swim-path profiles of the enriched-environment housed ischemic rats and other rats. The enriched-environment housed rats swam more in the central and less in the outer parts of the pool than all the other groups in the experiment. In the later experiment (**IV**) the enriched-environment housing was found to significantly reduce the tendency of ischemic animals to swim in the outermost annulus of the pool during all trials. However, no difference in escape latency was found.

2.2.3 Effects of atipamezole administration (III)

Atipamezole administration improved the performance in a limb-placing test immediately following the first administration (except for day 4 after induction of ischemia) lasting to day 8 after induction of ischemia when compared to ischemic control group. Rats that received atipamezole made also fewer slips than those that were

treated with saline on day 2 after the induction of ischemia. There was no significant effect of atipamezole treatment in the beam-walking test or on the water-maze learning after focal ischemia. However, in the probe trial, the saline treated ischemic rats swam significantly slower than the atipamezole treated rats. Atipamezole treatment did not affect the infarct size.

2.2.4 Effects of selegiline administration (IV)

The beneficial effect of selegiline administration after focal cerebral ischemia was noted in the water-maze test measuring spatial learning ability. Rats that received selegiline combined with the enriched-environment housing and labyrinth training had shorter latencies to find the hidden platform in the water-maze test when compared to saline treated standard cage housed rats. In the probe trial, these selegiline treated rats spent significantly more time than the saline treated rats in the quadrant in which the platform had been located. The rats which received selegiline treatment and training made more attempts to reach pellets with the affected and the nonaffected forelimbs than the ischemic rats which were housed in standard cages, but did not retrieve more pellets with their affected forepaw in the staircase test. These effects of selegiline administration were linked to the training and no enhancement of recovery in any test was observed with drug administration without the training. The selegiline administration combined with training was not beneficial in limb-placing or foot-slip tests. Selegiline treatment had no effect on the infarct size.

VI DISCUSSION

1 METHODOLOGICAL ASPECTS

1.1 Global ischemia

1.1.1 Global ischemia model

So called four-vessel occlusion was used to achieve global ischemia (Pulsinelli and Brierley 1979) (**I, II**). A four-vessel occlusion model is a widely used but highly invasive global ischemia model. Furthermore, it involves some variability in the severity of the lesion, since some rats have collateral blood flow. The sources of continued cerebral blood flow in rats subjected to successful occlusion of the common carotid and vertebral arteries are the anterior spinal and the collateral arteries in the cervical and paravertebral muscles (Pulsinelli and Buchan 1988). Usually this compensatory blood flow is revealed by the return of the righting reflex in these animals (Ordy et al. 1993). Furthermore, a total absence of blood flow is found to involve also complete binocular pupillary dilation and non-responsiveness to somatosensory stimulation. Even though the fulfilment of these criteria was checked, a few exceptional animals with unilateral brain damage were accepted to the final experiment, but were excluded when data was analyzed.

1.1.2 Behavioral tests

It has been recommended that at least two behavioral tests should be used for the behavioral outcome measures in drug studies (STAIR 1999). The recovery of animals after global ischemia was assessed by two behavioral tests (**I**), which fulfils this criterion. The open-arena test was used for the evaluation of the initial level of animals and thus for the verification of equal group assignment. The experimental ischemic groups were found to be equal in this respect. The differential drug treatment and housing began two days after global ischemia induction, beyond the timetable of cell death after global cerebral ischemia (Pulsinelli et al. 1982). Later, the open-arena was used for assessment of behavioral outcome together with the water-maze test. Since atipamezole may interfere with water-maze behavior (Sirviö et al. 1992), there was a wash-out period after each drug treatment period, before behavioral testing. In the water-maze paradigm, a so called learning-set task was used. This is a more difficult version, since the rats have to learn the new location of the platform instead of remembering the previously learned location. Furthermore, the spatial learning ability of rats was assessed also in a new environment at the end of the experiment.

When hippocampal function after housing in the enriched-environment was studied in rats exposed to a water-maze learning situation, a different water-maze paradigm had to be used (**II**). The delay after ischemia induction was the same as in the first test in the experiment (**I**). In order to optimize Fos-induction related to the learning experience, the rats had a habituation swim to inhibit Fos-expression due to novel stressful situation and the test paradigm had 8 trials on the following single day. Repetition of the previously

learned task would have lowered the expression of immediate early genes (Anokhin and Rose 1990). Therefore it was not possible to perform the previous water-maze paradigm. The number of Fos-immunopositive nuclei was quantified from all hippocampal areas to evaluate possible plastic changes such as a compensatory shift of function inside the hippocampus formation as a consequence of the profound ischemic lesion.

1.2 Focal ischemia

1.2.1 Focal ischemia model

Focal cerebral ischemia was used to attain better clinically relevant model of stroke, since major human strokes are focal. Focal ischemia was induced using the so called intraluminal tread model (Longa et al. 1989) (**III, IV**). Broadly the focal cerebral ischemia models can be categorized into permanent and transient models (Hunter et al. 1995). This model simulates the situation where reperfusion in the ischemic area is achieved which enables the drug action in ischemic area. However, in this study, the drug administration took place so late after ischemia induction, that this factor most probably was of no importance. Spontaneous reperfusion may be involved also in humans (Hakim et al. 1987). Our occlusion period of 120 min produces a large cerebral infarct. It is thought that approximately 180 min duration of ischemia is sufficient to attain the maximal infarction observed after permanent ischemia in the rat MCA occlusion model (Kawamura et al. 1994). The cell death in the tissue at risk is seen by about 72 h following ischemia induction (Garcia et al. 1993). In order not to interfere with the cell death process, the drug administration was initiated only after two days (**III, IV**). Secondary cell death e.g. in thalamus and substantia nigra, is more delayed and might still be influenced by drug treatment (Rupalla et al. 1998; Tamura et al. 1990).

Several variables, such as brain temperature (Busto et al. 1987), blood glucose level (Wass and Lanier 1996), oxygen saturation (Simon et al. 1993) and blood pressure (McCulloch 1996) affect the size of infarction. Furthermore, the individual pattern of blood circulation in the MCA area by collateral arteries is a factor which can affect the infarct size (Rubino and Young 1988). Blood gases, glucose, and blood pressure were not monitored in the present studies because closing of the femoral artery after blood sampling might interfere with the performance of rats in the behavioral tests (Aronowski et al. 1996; Mattson et al. 1997). The lack of monitoring of these parameters may have increased the variability in lesion size. Therefore, an appropriate way for assignment of animals to equal groups had to be devised. In humans, the size of the lesion does not always correlate well with the functional impairment (Pantano et al. 1996). Some previous experimental studies have used simple neurological scoring to estimate the severity of the lesions (Bederson et al. 1986; Menzies et al. 1992). However, in the present studies the limb-placing test devised by De Ryck et al. (De Ryck et al. 1989) proved to be sensitive for evaluation of severity of ischemia lesion

1.2.2 Behavioral tests

The recovery after focal cerebral ischemia was assessed by behavioral outcome in different tests measuring sensorimotor (limb-placing, beam-walking, foot-slip and staircase tests) and cognitive (water-maze test) functions. It was found that rats with focal cerebral ischemia possessed a remarkable ability to recover after ischemia lesion. In many tests, the difference between sham-operated and ischemic rats was eventually no longer apparent. This makes it difficult to predict whether the drug treatment was able to enhance recovery or simply to hasten it.

1.2.3 Drug treatment

The drug doses used were chosen on the basis of previous experiments e.g. (Haapalinna et al. 1998; Haapalinna et al. 1997; Scheinin et al. 1988) and no recovery dose-response curves were made. It is possible, that those doses were not optimal. It is also possible that a different timing of drug administration, earlier or later, would have given different results. For example, an increase in norepinephrine release early after traumatic brain injury is protective possibly by reduced edema formation (Dunn-Meynell et al. 1998). Furthermore, bFGF has been found to prevent thalamic atrophy administered 24 hours after cortical infarction (Yamada et al. 1991). On the other hand, initiation of enriched-environment housing as early as 24 hours following focal cerebral ischemia may exacerbate the brain damage (Risedal et al. 1999).

1.3 Clinical relevance of used ischemia models

The clinical relevance of the ischemia models used remains open (Wiebers et al. 1989). It has been claimed, that animal studies provide an important tool to study ischemia, and indeed are relevant to human disease (Zivin and Grotta 1990). It is clear, however, that no animal model can exactly mimic stroke in humans (STAIR 1999). Much of the functional recovery studies have used ablation lesions of the cortex, but that kind of lesion lacks the complex pathophysiology of ischemic stroke. It can be claimed that the pathophysiology after MCA occlusion resembles sufficiently well the human stroke. It has been stated that focal cerebral ischemia is of greater relevance of human condition of stroke, since global ischemia lesion is more rare and thus has less clinical value (Macrae 1992). However, global ischemia serves well as a model to study the plastic recovery mechanisms of memory functions in brain. There have been major discrepancies between acute stoke treatment studies in humans and animals, since none of the potential neuroprotective drugs has succeeded in clinical trials. This can probably be attributed to the failures in the design of animal studies (Grotta 1994). Often drug treatment is given at an earlier time point than would be possible in a real clinical situation. However, as already stated, in the present experiments no acute treatment was given.

Furthermore, it must be noted that young healthy rats were used. People, who suffer stroke are usually older and have additional diseases and risk factors (Kernan et al. 2000). Old brains evidently have less plastic potential because of cell death during

aging. The remarkable ability of rats to undergo a rapid functional recovery differs from humans. In humans, recovery following stroke takes place in a much longer time frame (Kotila et al. 1984).

2 FACILITATION OF RECOVERY BY ENRICHED-ENVIRONMENT HOUSING

2.1 The facilitation of recovery after global ischemia

2.1.1 Abolishment of hyperactivity

In the open-arena test, ischemia resulted in hyperactivity as indicated by the increased locomotor activity and number of rearings. This is consistent with previous studies, since hippocampal lesions have been found to increase locomotor activity in the open-field (McDaniel et al. 1994; Nadel 1968). Hyperactivity after global cerebral ischemia is more pronounced in gerbils and the activity level has been used as a behavioral test in ischemic drug studies (Judge et al. 1991) and as a predictor of CA1 damage in the gerbil global ischemia model (Mileson and Schwartz 1991). Hyperactivity can result from deficits in adaptive responses to novelty vs. familiarity, and behavioral inhibition (Kelley et al. 1989). It has also been suggested that hyperactivity after global ischemia may represent an impaired ability to form spatial maps (Wang and Corbett 1990). In the present series, hyperactivity lasted for up to 4 weeks. Ischemia did not significantly affect the number of fecal boli, which is a crude index of emotional reactivity (Kelley 1993).

We made a novel finding that training diminished hyperactivity as indicated by the reduced locomotor activity and the reduced number of rearings of ischemic rats in the open-arena test. Consequently, the time spent grooming became increased. The results of some studies suggest that social isolation induces hyperactivity in rats in a novel environment (Wilkinson et al. 1994). The present result may not simply be a consequence of the isolation of the animals, because sham-operated animals did not exhibit any marked change in behavior, and in this experiment rats were housed individually in standard cages and were handled daily when given saline/drug injections. In addition, the rats underwent an intensive testing procedure.

2.1.2 Effects on the water-maze deficit

There is extensive evidence indicating that a deficit in spatial learning and memory, especially in working memory, can be induced by hippocampal damage (e.g. Eichenbaum et al. 1990; Moser et al. 1993). Global ischemia is known to impair acquisition of the water-maze task (Jaspers et al. 1990; Olsen et al. 1994a), although not all results are consistent (see Block 1999; Corbett and Nurse 1998; Nunn and Hodges 1994), probably due to the different kinds of experimental procedures used in global ischemia models and in water-maze paradigms.

The results of the present study, where the platform location was changed each day, are consistent with previous findings that global ischemia impairs performance of a learning set version of the water-maze (Auer et al. 1989; Green et al. 1992; Rod et al. 1990; Whishaw et al. 1994), although in the present study the impairment in the water-maze task was short-lasting. Behavior in the water-maze learning set task appears to be more sensitive to global ischemia damage, and a learning impairment in this kind of task is evident when only half of the CA1 area has been damaged (Auer et al. 1989; Whishaw et al. 1994). The novel finding was that enriched-environment housing facilitated the rate of spatial learning in rats with global ischemia. In experiment **I,** the rats with global ischemia were not inferior to sham-operated rats when tested in another room at the end of the experiment. In a previous study, rats with 15-min four vessel occlusion were impaired also in a novel pool (Netto et al. 1993). On the other hand, the ischemic rats showed recovered spatial learning abilities after training in a radial arm maze and learned the spatial memory task equally as well as sham-operated rats when the task was performed in a new room (Mizumori et al. 1995).

In these experiments (**I, II**), the ischemic rats learned to find the platform, even though anatomical examination of brain tissue confirmed that there was extensive damage in the hippocampus, especially in the dorsal CA1 field. The possible structural or functional substrates in the hippocampus for the novel finding on the facilitative influence of training in global ischemia were studied in experiment **II**. In this study, no differences between ischemic and sham-operated rats were found in escape latency. However, the spatial learning deficit after global ischemia is not a consistent finding. One factor that has an influence is the difficulty of the paradigm used (for review, see Nunn and Hodges 1994). The lack of a water-maze deficit might be attributed to the training paradigm (eight trials on one day, 30-s trials intertrial interval), which might not be very sensitive to hippocampal damage. It is possible that the rats used extrahippocampal brain structures to solve the problem of the hidden platform. This has been suggested to contribute to the preserved spatial learning ability after a fimbria-fornix lesion (Whishaw et al. 1995) and near total cell death in dorsal CA1 after global ischemia (Olsen et al. 1994b). Rats with hippocampal lesions may use ‘nonmapping’ strategies (DiMattia and Kesner 1988). When the swim paths were analyzed, it seemed that some rats with global ischemia could have been using taxon strategies, (see experiment **II)**. These rats preferentially approached the platform from a certain direction or swam at a fixed distance from the pool rim. Furthermore, even though the latencies to find the platform became shorter in all groups, the localization of the platform was not perfect even in sham-operated rat groups. It is interesting to note that analysis of swim paths of experiment **I**, however, does not support the use of a taxon strategy by ischemic rats, since the rats learned to swim rather directly toward the correct location, (see experiment **I)**. In addition, on the next day, the rats initially mapped the position of the platform relevant to its previous location, which also suggests that they did not see the platform in our clear-water, black-pool water-maze apparatus.

2.1.3 Effects on hippocampal function

Fos-immunostaining showed that the rats that were housed in the enriched environment had increased number of Fos-positive nuclei in dentate gyrus following exposure to a learning task in a water maze. This increase was more profound in sham-operated rats, but it was seen also to some extent in rats with global ischemia. It is interesting to note that no increase of Fos-positive nuclei in pyramidal neurons was detected. The plastic changes seen in dentate gyrus are consistent with the previous findings stating that neuronal transmission is enhanced in the perforant path synapses on granule cells as a consequence of enriched-environment housing (Foster et al. 1996; Green and Greenough 1986; Sharp et al. 1985). However, we do not know the extent to which the water-maze task -induced stress has contributed to the Fos expression observed. When the effect of plain enriched-environment housing without the water-maze learning task was studied, no significant differences in the number of Fos-positive neurons in any hippocampal area were detected between the groups.

2.2 Facilitation of recovery after focal ischemia

Ischemic control rats housed in standard cages improved their performance to the level of sham-operated rats within 2 to 3 weeks in the limb-placing task, which assesses the function of both fore- and hindlimbs, and foot-slip tests, which assess the function of the forelimb. However, in the beam-walking task, which measures mainly the hindlimb function, the ischemic rats recovered to the level of sham-operated rats within a few days. The cortical areas representing the hindlimb were, however, partially spared following transient MCA occlusion and the rapid spontaneous improvement of ischemic rats in the beam-walking task could be due to the resolution of cerebral edema and the improvement of the local circulation, which is suggested to account for the rapid recovery occurring in stroke patients (Dombovy and Bach-y-Rita 1988). Ischemic control rats had significantly longer escape latencies and path lengths in the water-maze test compared to sham-operated rats (**III, IV**), consistent with previous studies (Markgraf et al. 1992; Yonemori et al. 1999). However, there was no difference between the groups in their escape latency or the path length on the last day of water-maze testing with the visible platform, suggesting that there was no gross decline in their motivational or visual abilities (*e.g.*, neglect) of the ischemic rats.

The enriched-environment housing facilitated improvement in performance to some extent in sensorimotor tasks. The previous studies on recovery after focal ischemia have found that enriched-environment housing enhances recovery in brain-infarcted rats (Ohlsson and Johansson 1995), even when started 15 days after the induction of ischemia (Johansson 1996). In experiment **III,** the effect of enriched-environment housing was beneficial on days 8 to 10 in the foot-slip test. Furthermore, the enriched-environment housing decreased the latency to find the hidden platform in the water-maze test. The enriched-environment housed rats found the hidden platform faster, but they also swam faster and their escape path lengths were not significantly shorter than those of ischemic standard cage housed rats. It is possible that the enriched-environment housed rats used a different strategy to find the hidden platform. In a recent study by

Yonemori et al. (Yonemori et al. 1999), some ischemic rats were found to use a circular search strategy, *i.e.* rats had less turning behavior and swam more in the outermost annulus of the pool. The change in search pattern correlated with the shrinkage of the caudate-putamen, whereas the cognitive deficit correlated with the shrinkage of the cortex, especially the parietal cortex. In experiment **III,** the sham-operated rats housed in the enriched-environment swam more in the central part of the pool than the other groups when the platform was removed on the probe trial of the third water-maze test day.

In experiment **IV**, the enriched-environment housed rats did not differ from ischemic control rats in any of the sensorimotor tests or in the water-maze test. However, when swimming paths of all trials were analyzed, the enriched-environment-housed rats were found to swim more in the central parts of the pool, suggesting they were using a different kind of strategy to find the hidden platform. The reason for this discrepancy between the two experiments is not evident. However, these two experiments differed in severity of focal cerebral ischemia lesion. In experiment **III,** all animals which had had proper occlusion and showed detectable lesion were accepted, but in experiment **IV** only rats having less than 10 points in the limb-placing task were included to the final experiment. Therefore, also a few rats with minor deficits in behavior were tested. It is possible that the enriched-environment housing benefits to the greatest extent those rats that still possess more potential for recovery. It is also possible that the great variability in the severity of ischemic damage may have prevented us from detecting fine outcome differences due to enriched environment housing that otherwise would have been detectable.

3 FACILITATION OF RECOVERY BY ATIPAMEZOLE

3.1 Pharmacology of atipamezole

Atipamezole is highly selective and specific α_2-adrenoceptor antagonist (Haapalinna et al. 1997; Scheinin et al. 1988). In receptor binding studies, atipamezole is reported to have approximately 100 times greater affinity for α_2-adrenoceptors and an α_2/α_1-selectivity ratio over 100 times greater than that of either idazoxan or yohimbine. In studies with isolated organs, atipamezole is a more potent α_2-adrenoceptor antagonist and has a relative α_2/α_1-blocking ratio approximately 200 times greater than idazoxan (Virtanen et al. 1989). Atipamezole has an almost equal affinity for the different α_2-adrenoceptor subtypes (Renouard et al. 1994). Atipamezole penetrates rapidly into the brain (Biegon et al. 1992), and causes a dose dependent increase in the release of central norepinephrine (Scheinin et al. 1988). There are α_2-heteroreceptors, which are not located in noradrenergic nerves, that participate in the regulation of the release of other neurotransmitters, such as serotonin (Raiteri et al. 1990), dopamine (Trendelenburg et al. 1994) and histamine (Gulat Marney et al. 1989). Thus, one cannot completely exclude the possibility that atipamezole also increases the excitability of the brain through other neurotransmitter mechanisms.

3.2 Facilitation of recovery after global ischemia (I)

The atipamezole treatment was not beneficial in the paradigm used after global ischemia. It has been previously reported that atipamezole improves learning and enhances consolidation processes in adult rats when administered prior to or immediately after behavioral testing (Haapalinna et al. 1998). However, atipamezole treatment did not significantly improve the rate of spatial learning in ischemic rats when combined with training. Instead, atipamezole treatment impaired water-maze performance in the ischemic rats, which were housed in standard cages when assessed in a novel pool at the end of the water-maze paradigm. It is possible that the wash-out period between atipamezole treatment and water-maze testing was too short, especially since the drug was administered subchronically. It is known that pre-training administration of atipamezole (at doses of 300 µg/kg or higher) interferes with the acquisition of the water-maze task (Sirviö et al. 1992). The reasons for the impairment in a novel pool may be related to the fact that the water-maze task is rather stressful and it is known that atipamezole potentiates reaction to novelty (Haapalinna et al. 1999). Acute administration of atipamezole impaired performance in active avoidance learning tests, causing a learned helplessness-like behavior. However, after subchronic treatment, there was an improvement in the learning of a mildly stressful active avoidance test.

3.3 Facilitation of recovery after focal ischemia (III)

The behavioral improvement in limb-placing and foot-slip tests was further facilitated by the α_2-adrenergic antagonist, atipamezole, and the beneficial effect was evident immediately after the drug was first administered. Compared to ischemic controls, atipamezole-treated rats had better performance from the beginning of treatment to day 8 after ischemia in the limb-placing test. In particular, atipamezole treatment improved the placing of the affected forelimb (data not shown). In the foot-slip test, the atipamezole-treated rats performed better on days 2 and 4 after ischemia. Since atipamezole was administered shortly before testing, it might transiently improve the performance of rats in the behavioral tests rather than truly enhancing their functional recovery. However, there was no clear decline in performance after termination of drug treatment.

In contrast to the limb-placing and foot-slip tests, the atipamezole treatment did not improve the performance in the beam-walking test. The rapid recovery of rats subjected to focal ischemia lesion is perhaps one reason why a beneficial effect of atipamezole administration could not be observed. Another possibility for the lack of a drug effect in the beam-walking test is that noradrenergic stimulation worsens the symptoms of striatal damage in this model, which in turn could counteract the overall beneficial effect (Mintz and Toner 1986).

In addition, the performance of the ischemic rats following atipamezole treatment was not improved in the water-maze test. The water-maze testing, however, was done quite a long time after drug treatment had ceased. As already noted, it has been previously reported that atipamezole improves learning and enhances consolidation processes in

adult rats when administered prior to or immediately after behavioral testing (Haapalinna et al. 1998). Therefore, it remains to be determined whether atipamezole also improves cognitive recovery under different experimental conditions, such as combined drug treatment with enriched-environment housing or administration prior to the cognitive task. However, it is interesting to note, that amphetamine treatment has not been beneficial in different cognitive tests, and it could be possible that noradrenergic modulation after brain injury is effective only in the restoration of sensorimotoric functions. Amphetamine did not improve memory after global ischemia (Colbourne and Corbett 1992) and metamphetamine was also found to be ineffective in Hebb-Williams maze after bilateral cortical lesions (Will et al. 1977). However, in humans, amphetamine treatment may be beneficial in the treatment of aphasic stroke patients (Walker-Batson 1998).

4. FACILITATION OF RECOVERY BY SELEGILINE

4.1 Pharmacology of selegiline

Selegiline (l-deprenyl) is a relatively selective irreversible monoamine oxidase B (MAO-B) inhibitor, which is used in the treatment of Parkinson's disease (Oerthel and Quinn 1997). Selegiline is believed to have neuroprotective and neuronal rescuing properties (Magyar et al. 1998; Tatton 1993). For example, selegiline protects neurons in different experimental models of cerebral ischemia (Knollema et al. 1995; Lahtinen et al. 1997; Matsui and Kumagae 1991; Semkova et al. 1996).

Selegiline cannot be considered simply as a MAO-B inhibitor because it has different pharmacologic modes of action (for review, see Magyar et al. 1996)). Selegiline impairs the degradation of dopamine and inhibits the uptake of dopamine and norepinephrine. Furthermore, selegiline forms amphetamine-like metabolites. Selegiline treatment also seems to increase superoxide dismutase (SOD) activity. It has been shown that selegiline has antiapoptotic actions (Paterson and Tatton 1998) possibly by preventing altered protein synthesis in the mitochondrial and nuclear fractions (Tatton et al. 1994). Moreover, selegiline induces reactive astrocytes which in turn increased their secretion of trophic factors that promote neuronal survival and growth (Biagini et al. 1994). Neurotrophic factors that selegiline might increase include bFGF (Riva et al. 1997), NGF (Semkova et al. 1996), and GDNF (Tang et al. 1998).

4.2 Facilitation of recovery after focal ischemia

Selegiline treatment combined with the enriched-environment housing diminished spatial learning deficits induced by focal cerebral ischemia. Furthermore, rats housed in an enriched-environment that received selegiline treatment were more likely to make attempts to reach food pellets with the affected (contralateral to the lesion) and non-affected (ipsilateral to the lesion) forelimbs. There was no significant improvement in sensorimotor tasks (limb-placing or foot-slip tests) when selegiline treatment was combined with the enriched-environment housing. There was no benefit of selegiline treatment alone (without the enriched-environment housing) in any of the behavioral

tests. Subsequent histologic examination revealed that there was no difference in the infarct volumes between the experimental groups. The neurobiologic basis of the cognitive improvement is unknown and remains to be further explored. However, in many respects, selegiline actions are consistent with those of neurotrophic factors.

The attenuating effect of the selegiline treatment combined to enriched-environment housing to the water-maze deficit was drastic. The escape latencies of the selegiline treated enriched-environment housed group were identical to those of the sham-operated group. Focal ischemia by MCA occlusion leads to cell death in the striatum and different cortical areas, as well as secondary neuronal damage in remote brain regions, such as the thalamus. All of these areas are involved in learning and memory (Devan et al. 1999; Eichenbaum 1997; Savage et al. 1997; Zis et al. 1984). It has been suggested, however, that the cognitive deficit induced by MCA occlusion is a result of a disturbance in the neuronal network rather than a specific brain region (Okada et al. 1995) and some studies have found a correlation between the water-maze deficit and the ischemia-induced infarct volume (Smith et al. 1997; Yonemori et al. 1996). In particular, the infarct volume in the parietal cortex correlates with the memory deficit (Yonemori et al. 1999). Furthermore, ischemic rats appeared to use a circular-shaped search strategy, *i.e.* a strategy with less turning behavior and using more of the outermost annulus of the pool. The use of this search pattern correlates with shrinkage of the striatum.

The selegiline treated group which received training made more attempts to obtain food pellets with both affected and non-affected forelimbs compared to the ischemic control group, but there was no improvement in the number of successfully retrieved pellets. It is surprising that there was no attenuation of the sensorimotoric deficit following the selegiline treatment, but this finding is consistent with the result of a previous study in which chronic selegiline treatment was examined in aged rats (Bickford et al. 1997). Selegiline improved the performance in spatial learning of the water-maze task, but not in sensorimotor tasks.

Although the therapeutic window for neuroprotection after focal cerebral ischemia could be quite long, as there are still viable cells within the ischemic tissue 46 h after 2-h of MCA occlusion (Li et al. 1998), the beginning of selegiline administration 2 days after the induction of ischemia was beyond the time window for rescue of the cells in the ischemic area. This is consistent with the finding that basic fibroblast growth factor (bFGF) administered at later time points after the induction of ischemia enhances behavioral recovery following focal cerebral infarction without affecting the infarct size (Kawamata et al. 1996), possibly by stimulating neuronal sprouting in the intact brain (Kawamata et al. 1997b). It is possible that plastic changes occurred in the surviving brain areas, which is supported by the fact there were no differences in infarct volumes between experimental groups. For example, selegiline administration increases dendritic arborization in the CA3 neurons of the hippocampus (Lakshmana et al. 1998) and in pyramidal neurons of layer III in the prefrontal cortex (Shankaranarayana Rao et al. 1999).

Selegiline treatment without the enriched-environment housing did not attenuate the water-maze deficit or increase the attempts in the staircase test. This indicates that the results of the present study cannot be explained by symptomatic drug actions. Furthermore, in experiment **III**, ischemic rats swam equally well as sham-operated rats to a visible platform, suggesting that lack of motivation or impaired visual acuity were not responsible for their impaired performance in this task. Instead it seems likely that the beneficial effect of the drug is dependent on simultaneous rehabilitative training. The original findings of the effects of amphetamine administration on recovery also point to the necessity of simultaneous rehabilitative training (Feeney et al. 1982; Feeney and Hovda 1985). On the other hand, neurotrophins increase synaptic efficacy and are involved in synaptic plasticity (Lessmann 1998; Schuman 1999). This increase in synaptic efficacy might be activity dependent because a brief presynaptic depolarization in the presence of BDNF markedly potentiated both evoked and spontaneous synaptic transmission, whereas exposure to either BDNF or depolarization alone had no effect (Boulanger and Poo 1999). Further evidence of a generalized mechanism in which Trk activation and afferent discharge interact to modulate neuronal plasticity is suggested by the finding that the effects of NGF during the development of the visual cortex require afferent electrical activity (Caleo et al. 1999).

VII CONCLUSIONS

1. Rehabilitative training achieved by housing animals in an enriched environment facilitated recovery after global ischemia by reducing hyperactivity. The facilitation of recovery after spatial learning deficit was seen in a more difficult version of the water-maze task, e.g. the learning set task. The easier paradigm used was not sensitive to global ischemia damage. The beneficial effect of enriched environment housing following focal cerebral ischemia was subtle, and may be overwhelmed by large infarctions.

2. Enriched-environment housing alters the hippocampal function. The enriched-environment housing increased the number of Fos-immunopositive granular neurons when the rats were exposed to a water-maze learning situation. The other hippocampal structures analyzed did not show this kind of plastic effect. The effect was seen in sham-operated rats, but also in rats with global ischemia lesion.

3. The blockade of α_2-adrenergic receptors by atipamezole facilitated behavioral recovery in motor and sensorimotor tasks in rats after transient focal cerebral ischemia, but not in the cognitive task. The drug treatment did not affect the infarct size. Furthermore, selegiline treatment was effective in attenuating cognitive deficits assessed in water-maze task of rats subjected to transient MCA occlusion. Furthermore this treatment increased the number of attempts made in Montoya's staircase test. The drug effect was dependent on the concomitant training, since no beneficial effect was seen when drug treatment was given alone. The drug treatment did not affect the infarct size.

Taken together, these results show that an α_2-adrenergic antagonist, atipamezole, facilitated sensorimotoric recovery and a MAO-B inhibitor, selegiline, attenuated cognitive decline following focal cerebral ischemia. This indicates that pharmacotherapic agents can have differential beneficial characteristics as recovery promoting agents. In addition, rehabilitative training facilitates recovery following cerebral ischemia and may be crucial in mediating the benefits of drug therapy.

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