The study of behavioral dysfunctions

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Behavioral effects of stroke induced by occlusion of the middle cerebral artery (MCA) in rodents

The third leading cause of death in the major industrialized countries is stroke (Hunter, Green & Cross, 1995). Stroke also causes long-lasting functional impairments in the afflicted. About 50% of patients who survive suffer from persistent neurological impairments (Gorelick, 1995). Stroke might be defined as *"rapidly developed clinical signs of focal (or global) disturbance of cerebral function, lasting more than 24 hours or leading to death, with no apparent cause other than of vascular origin"* (Aho et al., 1980, p. 114). The prevalence of stroke increases dramatically with advancing age (Zippel, 1994; Gorelick, 1995; Reuter, 1997; see also Chapter 1).

Red infarct or hemorrhage versus white infarct or ischemic stroke

Two main groups of cerebrovascular accidents or strokes can be distinguished: rupture of cerebral vessels, which causes hemorrhage, and occlusion of large or small blood vessels in the brain by an embolus or thrombus, which induces an ischemic infarct. The very complex neurological symptomatology, often characterized by sudden headache, seizures, dizziness and vertigo, visual disturbances, aphasia, movement impairments, or, more severe, hemiplegia, provides a first hint about the precise type and location of the stroke (Wiebers, Feigin & Brown, 1997; Adams, Victor & Ropper, 1997).

Cerebral hemorrhage

Hemorrhagic cerebrovascular disorders cause approximately 20% of all strokes. About 9% of strokes appear to be caused by intracerebral hemorrhage (Jørgensen et al., 1995). Intracerebral hemorrhages are caused by damage to vessels deep in the cerebrum or cerebellum, although damage to more superficial vessels also occurs (van Dellen & Becker, 1988, p. 56). Hemorrhagic stroke can be subdivided into epidural, subdural, subarachnoid, intracerebral, and intraventricular hemorrhage, depending on the location of the primary bleeding (Wiebers, Feigin & Brown, 1997). In general, cerebral hemorrhage induces more severe neurological dysfunctions and higher mortality than ischemic stroke, most probably because the lesions are more extensive (Jørgensen et al., 1995).

Ischemic cerebrovascular diseases

Hypertension, i.e. systolic blood pressure of 160 mmHg or higher, or a diastolic blood pressure of 95 mmHg or higher, is the main cause of atherosclerotic cerebrovascular changes in small vessels. Cardioembolic infarction causes 20 to 25% of all ischemic stroke and mostly involves the cortex. The embolus is often found in the cortical branches of the middle cerebral artery (MCA).

Atherosclerosis is the most common underlying cause of cerebral ischemia: it accounts for 15 to 30% of all ischemic strokes through either hemodynamic or thromboembolic mechanisms, or a combination of both. In large vessels, the lumen of the atherosclerotic vessel narrows progressively - a

hemodynamic process that develops over a long period of time, e.g. years. When 75% of the luminal area is compromised, the blood flow across the stenotic area becomes severely impaired. This process eventually results in a complete occlusion of the affected vessel. Insufficient collateral circulation distal to the occlusion produces the ischemic symptoms. The anterior, middle and posterior arteries are most frequently affected by this process.

The second mechanism mentioned above is thromboembolism. *"Atherosclerotic deposits in the process of evolution tend to ulcerate and form necrotic areas capable of attracting blood products, and clot formation results. This atherothrombotic material may either stenose or occlude the vessel lumen, or it may break off to embolize distally in the arterial tree."* (Wiebers, Feigin & Brown, 1997, p. 192).

Cerebral infarction, induced by occlusion of the MCA

Cerebral ischemia is the most frequently observed type of stroke in humans (Tamura, Kawai & Takagi, 1997), and the most commonly encountered cause is the occlusion of the MCA or its branches (Adams, Victor & Ropper, 1997). The clinical symptomatology of patients with complete occlusion of the MCA consists of *"(...) contralateral hemiplegia (face, arm, and leg), hemianesthesia, and homonymous hemianopia, with deviation of the head and eyes toward the side of the lesion; in addition, there is a global aphasia with left hemispheric lesions and anosognosia and amorphosynthesis with right-sided ones (...). In the beginning the patient is dull or stuporous because of an ill-defined effect of widespread paralysis of function. Once fully established, the motor, sensory, and language deficits remain static or improve very little as months and years pass. If globally aphasic, the patient seldom ever again communicates effectively. Occlusion of branches of the middle cerebral artery give rise to only part of the symptom complex."* (Adams, Victor & Ropper, 1997, p. 790). Occlusion of the MCA in humans thus has severe consequences for sensorimotor and cognitive functions. The deficits are often long-lasting and the prognosis for recovery is poor.

Therapeutic approaches

Compared with only a few years ago, when treatment of stroke was characterized by a type of 'therapeutic nihilism', the prognosis of patients suffering from stroke has improved dramatically. This has been possible due to a number of factors. First, a major cause of cerebrovascular accidents or strokes, hypertension, can effectively be treated by, for example, calcium channel blockers of the dihydropyridine type, α- and β-adrenergic blockers, or angiotensin-converting enzyme (ACE) inhibitors. Second, patients receive medical care earlier, and treatment is increasingly being taken over by specialized stroke-units (Bath, 1997; Sulter & De Keyser, 1999). Third, new treatments have become available, and even more are currently under clinical development.

If treatment starts early after ischemic stroke, i.e. within 3 to 6 hours, then a thrombolytic therapy using, for example, tissue plasminogen activator (t-PA), streptokinase, or urokinase might help to resolve the embolus or thrombus (Caplan, 1995). A broad range of other putative therapeutic approaches to induce arterial reperfusion are under investigation (for a review see: McAuley, 1995; del Zoppo, Wagner & Tagaya, 1997; Read et al., 1999). Early reperfusion can significantly reduce both the extent of brain damage after occlusion and the severity of neurological dysfunctions (Caplan, 1995). An absolute prerequisite for this treatment, however, is that there are no signs of cerebral hemorrhage as detected by CT scan, magnetic resonance angiography, or other examinations such as ultrasound.

A second therapeutic option which is within reach of clinical application concerns drugs which are able to reduce the stroke-induced damage to cerebral tissue. After a stroke a cascade of biochemical processes in the penumbra increases the size of the infarct. The penumbra is the region that

surrounds the core of the infarct. Whereas the core is irretrievably lost, the damage in the penumbra might be reversible (Stevens et al., 1997). As a result of the severely impaired or interrupted blood supply distal to the embolus or thrombus, the concentrations of noxious metabolites, such as lactic acid, or excitatory neurotransmitters, such as glutamate, increase over time in necrotic cells and invade the adjacent areas. The released glutamate opens calcium channels through which extracellular calcium rushes into the cells (Garcia, 1997; Luiten et al., 1997). Excessive calcium threatens the cell, and eventually leads to cell death (Siesjö, 1994; Luiten et al., 1997). Free radicals are released from endothelial cells in response to the changed perfusion pressure, and when endothelial cells interact with circulating polymorphonuclear leukocytes. Free radicals act as neurotoxins and further damage the cells. These processes might act sequentially, or in parallel, in the region that is directly supplied by the occluded vessel and surrounding tissue (Garcia, 1997).

Animal models of cerebrovascular diseases

Animal models of ischemic stroke or hemorrhage are an important tool to identify and characterize new therapeutics. In recent years, several animal models of cerebrovascular diseases have been developed. These models are characterized by a great variety of techniques used to induce cerebral infarcts. Table 1 provides an overview of currently available models (see also Hunter, Green & Cross, 1995; Tamura, Kawai & Takagi, 1997). The main aim of using these models is:

- to study the pathophysiology of stroke in order to identify the processes which cause the damage; and
- to test the efficacy of putative neuroprotective or recovery promoting agents, which might be useful for the treatment of stroke patients, either prophylactically or therapeutically.

Most models of ischemic stroke fall into one of two categories: global or focal models (Hunter, Green & Cross, 1995). Global ischemia is induced by surgical occlusion of the major blood vessels, which leads to (nearly) complete interruption of the cerebral circulation. By contrast, focal ischemia is induced by occlusion of a single trunk artery (e.g. Tamura, Kawai & Takagi, 1997), such as the middle cerebral artery.

Permanent versus transient occlusions

Most strokes are caused by a transient or permanent thrombotic occlusion of blood vessels. Permanent occlusion produces a region that develops intense ischemic damage, the so-called core (Memezawa, 1993; Hunter, Green & Cross, 1995), from which pathophysiological processes spread to the surrounding region, the penumbra. The penumbra is characterized by *"I) the reduction of blood flow and II) the fundamental reversibility of the ischemic injury."* (Zhao, 1995, p. 8). Ginsberg and Pulsinelli (1994) defined the ischemic penumbra as *"brain tissue in which the CBF* (cerebral blood flow) *has decreased to a point of causing electrophysiological silence (i.e., an isoelectric electroencephalogram) and transient but recurrent losses of membrane ion gradients and energy metabolites."* The core of the infarct is extremely vulnerable, because it is permanently detached from the blood supply.

However, in most patients the thrombus that causes stroke disappears because of thrombus disintegration or thrombolysis, eventually leading to reperfusion of the infarcted area, i.e. the vessel is occluded transiently. Various animal models of focal transient occlusion have been developed (e.g. Borlongan, Cahill & Sanberg, 1995; Gartshore et al., 1995; Marston et al., 1995).

Table 1. *Animal models of cerebral stroke and head trauma. A great variety of techniques to produce cerebral infarction have been developed. As new techniques are continuously developed, and existing techniques are refined and modified, this list is not complete. In particular, models of traumatic brain injury and closed head injury are developing very rapidly. Where appropriate, it has been indicated whether the model is based on a technique which allows reperfusion of the occluded vessel(s). In addition, selected publications are listed.*
[1]: Combination of different occlusion techniques
Abbreviations used: CCA: common carotid artery; MCA: middle cerebral artery

Model		Reperfusion		Selected publications
		yes	no	
Ischemic stroke				
global	complete ischemia induced by controlled cardiac arrest in rats	●		Wauquier, Melis & Janssen, 1989
	bilateral CCA occlusion in gerbil	●		Kirino, 1982; Mayevsky, 1990
	bilateral CCA occlusion in mouse	●		Himori et al., 1990
	bilateral, photochemically induced CCA occlusion in rats		●	Alexis et al., 1995
	two vessel occlusion: CCA occlusion with hypotension in rats	●		Eklof & Siesjö, 1972; McBean et al., 1995
	triple vessel occlusion in rat[1]	●	●	Cockroft et al., 1996
	four vessel occlusion: transient occlusion of CCA and permanent occlusion of vertebral arteries[1]	●	●	Pulsinelli & Brierley, 1979; Merlo Pich et al., 1993
focal	permanent MCA occlusion by ligation or electrocoagulation		●	Robinson, 1979; Tamura et al., 1981; Bederson et al., 1986
	photothrombotic permanent MCA occlusion in rat		●	Markgraf et al., 1994
	transient MCA occlusion in rat, mouse	●		Borlongan, Cahill & Sanberg, 1995
	transient MCA occlusion by endothelin-1 infusion	●		Marston et al., 1995; Gartshore et al., 1995
Embolic stroke				
	photochemically induced thromboembolytic lesion	●		Watson et al., 1985; De Ryck et al., 1989; Wood et al., 1996
	injection of autologous, or human clotted blood	●		Yang et al., 1994; Zhang et al., 1997
	injection of microspheres			Lyden et al., 1997
Traumatic brain injury				
	closed head injury induced by weight drop in rat, mouse			Chen et al., 1996; Xiong et al., 1997
	cortical impact injury to exposed rat, mouse brain			Dixon et al., 1991; Hamm et al., 1992; Fox et al., 1998
Cerebral hematoma and hemorrhage				
experimentally induced	subdural hematoma* in rats			Miller et al., 1990; Klapdor et al., 1997a
	subarachnoid hemorrhage			Sobey, Heistad & Faraci, 1997
	intracerebral hematoma			Rosenberg et al., 1993; Lyden, Jackson-Friedman & Lonzo-Dokter, 1997
naturally occurring	spontaneous hypertensive, stroke-prone rat			Okamoto, Yamori & Nagaoka, 1974; Yamori et al., 1991

* *but see Haines, Harkey and Al-Mefty (1993), who argue that the so-called subdural hematoma is a 'dural border' hematoma.*

Because the basic nature of the ischemic penumbra appears to be its bioenergetic instability due to a reduced cerebral blood flow, the early restoration of the blood flow might be sufficient to rescue the ischemic tissue (Ginsberg & Pulsinelli, 1994). Delayed reperfusion by itself has been identified as a process that at best has no beneficial effect, but which can cause additional damage (White, Grossman & Krause, 1993; Caplan, 1995, p. 4, and p. 52, Table 1; Garcia, 1995; Margaill et al., 1996). The duration of the occlusion appears to be critical in this context.

In rats, reperfusion shortly after occlusion reduces the resulting infarct and neurological deficits are less severe (Zhao, 1995). Beneficial effects of reperfusion are no longer seen when reperfusion is delayed beyond about 1.5 hours. The time window in which reperfusion is able to ameliorate the consequences of occlusion seems to be species-dependent (Zhao, 1995). In humans, the time window in which thrombolysis might be successful lies between 3 to 6 hours after the onset of symptoms of stroke (Wiebers, Feigin & Brown, 1997).

Animal models of permanent occlusion

The MCA occlusion (MCA-O) at present is the most frequently used animal model for permanent focal ischemia (Hunter, Green & Cross, 1995). The infarcts induced by transient MCA-O resemble those seen in patients with embolic stroke (Naritomi, 1991). Permanent MCA-O is usually induced by cauterization, or clips or threads which are left in place. These techniques require craniotomy (Memezawa, 1993; Rogers et al., 1997). Proximal MCA-O (i.e. close to origin of the artery) and distal MCA-O (above the lenticulostriate branch) might affect infarct volume (Shigeno et al., 1985; Niiro et al., 1996) and the behavioral impairments induced to a different extent (Shigeno et al., 1985). Proximal MCA-O has been found to produce reproducible infarcts in certain rat strains (e.g. Niiro et al., 1996), whereas between-strain comparisons have revealed a considerable variability in the susceptibility of the brains to develop infarcts (Hunter, Green & Cross, 1995).

Animal models of cerebral hemorrhage

Animal models of hemorrhage-induced damage in the brain are still scarce compared with those of ischemic stroke. Hematomas are usually induced by injection of saline or (own) blood under the dura, producing subdural hematoma (e.g. Miller et al., 1990; but see Haines, Harkey & Al-Mefty, 1993), or by injection of saline or blood into the cerebrospinal fluid, producing subarachnoid hemorrhage (e.g. Sobey et al., 1997). Intracerebral hematoma can experimentally be induced by stereotaxically guided injection of compounds, such as bacterial collagenase (Rosenberg et al., 1993), which disrupt cerebral blood vessels (e.g. Lyden, Jackson-Friedman & Lonzo-Dokter, 1997), subsequently causing hemorrhage.

Description of the experiments performed

Because of its prominent role in the investigation of occlusion-induced brain infarcts and its neuropathological and behavioral consequences, and because of the high incidence of this type of stroke in humans, we used the MCA-O model in rats and mice to assess infarct-induced *behavioral* deficits in batteries of sensorimotor tests and in two versions of the Morris water escape task.

- Various rat strains have been used in studies of the effects of MCA-O, among them the Wistar Kyoto (WKY) strain (e.g. Duverger, Lecoffre & MacKenzie, 1985; Barone et al., 1992; Nordborg & Johansson, 1980; Sauter & Rudin, 1995), which is considered as the normotensive control strain for the spontaneous hypertensive rat strain. The first aim of the experiment reported in Chapter 4.1 was to identify those behavioral and neurological tests which are sensitive enough to detect deficits

caused by MCA-O in WKY rats. The second aim was to investigate whether recovery of sensorimotor function occurs in the MCA-occluded WKY rat.

- There is growing evidence that the volume of the infarcted area (Duverger & MacKenzie, 1988) and the extent of neurological dysfunctions after MCA-O are strain dependent (e.g. Wahl et al., 1992). In Chapter 4.2, we studied several aspects of strain-dependent effects of MCA-O in three experiments. In the first experiment, we assessed the effects of unilateral MCA-O on sensorimotor functions in eight different rat strains [male Brown Norway (BN), Fischer 344 (F344), Long Evans (LE), Lewis (LEW), Sprague Dawley (SD), Spontaneous Hypertensive Stroke-Prone (SHR-SP), Wistar (WISW), and WKY] by comparing pre-occlusion behavioral scores with those 2 days after surgery. In the second experiment we compared the effects of proximal and distal MCA-O in the LE, LEW and SHR-SP rat strains. We expected that the proximal occlusion would produce larger infarcts that would eventually affect not only cortical, but also subcortical (striatal) areas (Shigeno et al., 1985). Finally, in the third experiment, we determined whether there is a relation between behavioral deficits and volume of the cortical infarct, a question that is currently highly controversial.
- In Chapter 4.3, we assessed the effects of MCA-O on spatial learning and memory in two experiments with mice in the standard Morris task. The question we addressed in the first experiment was whether CFW1 mice are able to learn to escape onto an invisible platform in the place version of the water-escape task (Morris, 1984), and whether unilateral occlusion of the MCA affects the retention of the water-escape response acquired before surgery. In addition, we assessed the acquisition of a new position of the escape platform (reversal learning) after MCA-O in these animals. In the second experiment, we studied the effects of MCA occlusion on the acquisition of the water-escape task in naive mice.
- Rats and mice appear to find the working memory (i.e. repeated acquisition) version of the Morris water escape task more difficult than the standard water escape task (Petrie, 1995). We therefore decided to assess the effects of occlusion of the MCA in C57BL mice using this task. This experiment is described in Chapter 4.4. We used three versions to experimentally manipulate the degree of difficulty of this task: a massed trials version, consisting of four trial pairs per daily session; a spaced version, in which only one trial pair was given per session; and a spaced delay version, in which only one trial pair was given per session but there was a 90-minute interval between the first and the second trials of the trial pair. The MCA was occluded after the mice had acquired the repeated acquisition task and then we assessed the effects of the occlusion on their working memory performance.

4.1

Sensorimotor impairments in Wistar Kyoto rats with cerebral infarction, induced by unilateral occlusion of the middle cerebral artery: recovery of function*

Abstract

Wistar Kyoto (WKY) rats with cerebral infarction induced by permanent unilateral occlusion of the middle cerebral artery (MCA) and sham-operated rats were tested in a series of simple behavioral tests 2, 16 and 37 days after surgery. In addition, the motility of the animals was measured over a period of 62 hours, after the third test series. A subset of the tests appeared to be suitable to assess the effects of cerebral infarction, namely, grasping reflex of contralateral hindpaw, circling behavior, forelimb flexion, hindlimb flexion, and latency to falling off a square bridge. Except for the impaired grasping reflex of the contralateral hindpaw, there was spontaneous complete recovery of function by the third test session, 37 days after surgery. Some of the other tests might not have been sensitive enough to detect the effects of the unilateral MCA-occlusion (MCA-O) on behavior. Moreover, the WKY rats were very inactive in some of the tests, so that reliable scoring of the effects was not always possible. A rat strain other than the WKY strain might be more suitable for studying the behavioral consequences of MCA-O.

Introduction

Patients with cerebral infarcts suffer from functional deficits such as sensorimotor impairments, coordination deficits, hemiparesis, and cognitive and speech disturbances (Adams, Victor & Ropper, 1996), depending on the site and size of the infarcted area. A wide range of techniques to induce ischemic stroke have been developed (Tamura, Kawai & Takagi, 1997), among them being the occlusion of the middle cerebral artery (MCA; Hunter, Green & Cross, 1995). The MCA can be occluded permanently or transiently (Garcia et al., 1995). The permanent MCA occlusion (MCA-O) probably is the most widely used technique for inducing a focal ischemic cortical infarct in rodents. Permanent MCA-O in rats and mice is believed to provide a valid animal model for investigating questions related to the pathophysiology of focal cerebral ischemia (Tamura et al., 1981; Welsh et al., 1987), and to the behavioral impairments associated with an infarct. Moreover, this model has been used to assess the validity of putative neuroprotective therapeutic principles. Based on approaches deduced from these principles, MCA-occluded rodents have been used to screen for and characterize

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substances which are believed to ameliorate or counteract the aversive consequences of a stroke with respect to the infarct size (e.g. Obana, Pitts & Nishimura, 1988; Gotti et al., 1990; Hara et al., 1991; Wahl et al., 1993; Park & Hall, 1994; Katsuta et al., 1995), the neurological (e.g. Tamura et al., 1985; Bederson et al., 1986; Yamamoto et al., 1988; Wahl et al., 1993; Park & Hall, 1994) and/or cognitive deficits (e.g. Tamura et al., 1985; Yamamoto et al., 1988, 1991; Markgraf et al., 1992; Wahl et al., 1993; Okada et al., 1995a,b).

The most frequently used rat strains in studies on the effects of MCA-O are the Sprague Dawley (e.g. Robinson, 1979; Duverger, Lecoffre & MacKenzie, 1985; Obana, Pitts & Nishimura, 1988; Shiraishi & Simon, 1989; Sauter & Rudin, 1995), spontaneously hypertensive (SHR, e.g. Pearlson, Kubos & Robinson, 1984; Duverger, Lecoffre & MacKenzie, 1985; Sauter & Rudin, 1995; Tamura et al., 1985), Wistar (e.g. Garcia et al., 1995; Sauter & Rudin, 1995), and, to a lesser extent, the normotensive Wistar Kyoto (WKY; e.g. Duverger, Lecoffre & MacKenzie, 1985; Barone et al., 1992; Nordborg & Johansson, 1980; Sauter & Rudin, 1995) rat strains. Using inbred WKY rats, we assessed the effects of unilateral MCA-O on sensorimotor function using a battery of simple sensorimotor tests. This strain was chosen, because we had previously investigated the effects of MCA-O in SHR rats (unpublished data) and wanted to characterize the genetic control of this strain under our experimental conditions.

The first aim of our study was to identify those behavioral and neurological tests which are sensitive enough to detect behavioral deficits caused by MCA-O in WKY rats. The functional state of different brain regions can be assessed by using a series of different tests. Additionally, because the location and size of the infarcted area shows considerable variation between and within strains, the use of different tests makes it more likely that ischemia-induced deficits will be detected (Markgraf et al., 1992).

Persson and colleagues (1989) found that many rats with a unilateral occlusion of the MCA showed neurological improvement within weeks of surgery. Markgraf and co-workers (1992) observed complete recovery of the postural reflexes and of sensorimotor function in rats within 30 days of MCA-O. Similarly, neurological abnormalities disappeared within the first four weeks after MCA-O in a study by Yamamoto and colleagues (1988).

The second aim of our study was to investigate whether recovery of sensorimotor function occurs in the MCA-occluded WKY rat. To this end behavior was assessed 2, 16, and 37 days after surgery. We knew from previous work at our laboratory that the minimum recovery period from MCA-O is about 2 days. We decided to perform the tests at intervals of approximately two to three weeks in order to avoid too frequent testing, as some of the tests involved were expected to be influenced by learning processes. Knowledge about the rate and degree of recovery of function may be of value for the development of animal models with which to study the pharmacological facilitation of post-ischemic recovery (Goldstein, 1989).

Material and Methods

Subjects

Eighteen inbred male WKY rats were supplied by Møllegaard ApS (LI. Skensved, Denmark) at the age of approximately 11 weeks. Three to four animals were group-housed in standard Makrolon type IV cages. The rats were kept under an artificial light/dark regimen (lights on from 7:00 to 19:00) in a

temperature (ca. 21.5°C) and humidity (50%) controlled vivarium. From the operation onwards the rats were housed individually in standard Makrolon type III cages. Food and water were always available ad libitum.

One week after arrival at our laboratory, the animals were randomly assigned to one of two conditions. Ten rats of a first group received a unilateral occlusion of the MCA. Eight rats of a second group received a sham operation which was identical to that of the MCA-O condition, except that the muscle and skin were closed immediately after exposure of the MCA. The average weight of the rats was (mean ± SEM) 268 ± 2.7 grams.

Middle cerebral artery occlusion

Under general anesthesia (chloral hydrate, Fluka Chemie AG, Buchs, Switzerland; 400 mg/kg i.p.) the MCA was occluded unilaterally according to the standard surgical procedure described by Bederson and colleagues (1986) with minor modifications. Briefly, the left temporal-parietal region of the head was shaved, and the skin was disinfected and opened between the orbit and the external ear canal. A midline incision was made, and the temporal muscle was divided and pulled aside with hooks to expose the lateral aspect of the skull. The facial nerve, major facial arteries and veins, the lateral eye muscle, the intra- and extra orbital lacrimal glands and the zygomatic bone were left intact. Under an operation microscope a small burr hole was drilled directly under the zygomatic arc, 1 to 2 millimeters rostral to its caudal origin.

After the dura was carefully opened, the exposed MCA and its branches were permanently occluded between the olfactory tract and the inferior cerebral vein by electro-coagulation (Bipolator 50, Fischer MET GmbH, Freiburg, Germany). To avoid recanalization, the occluded vessels were removed. The operation area was covered with a small piece of sterile absorbable gelatine sponge (Marbagelan, Behringwerke AG, Marburg, Germany). Muscle and skin wounds were closed with tissue glue (Histoacryl, B. Braun Melsungen AG, Melsungen, Germany). As it has been reported that hypothermia might act neuroprotectively in animal models of ischemia (Green et al., 1992; Barone, Feuerstein & White, 1997; Corbett, Nurse & Colbourne, 1997) and cortical impact injury (Dixon et al., 1998), the body temperature was monitored during the surgery and maintained between 36.5 and 37.5℃ by using a heating pad. The animals recovered from anesthesia, lying on a heating pad and covered with some layers of tissue. In earlier, unpublished experiments, we had monitored both rectal temperature and subdural temperature and found that brain hypothermia did not occur when the body temperature was maintained in this range. After recovery from anesthesia, the rats were returned to their home cage.

Histological verification: the rats were decapitated between 41 and 49 days after the operation. The brains were rapidly removed and frozen in n-methyl butane at -40℃. Coronal sections (20-µm thick) were cut throughout the entire infarcted area with a standard distance of 500 µm, using a cryostat microtome (Reichert-Jung, Leica Vertrieb GmbH, Cologne, Germany). Slide-mounted tissue sections were stained with cresyl fast violet.

Functional examination

The effects of the MCA-O were assessed by rating the severity of deficits with a series of simple neurological tests. The observer was not informed about the lesion condition of the individual rats (blind procedure).

On days 2, 16, and 37 after the operation, and at least one hour before neurological testing started, the rats were transported from the animal vivarium to the testing laboratory. The entire battery of tests, except the paw-test and the motility test, which were performed only once, was run three times in close

succession under normal light conditions between 9:00 and 12:00 a.m. The tests were run in triplicate, because aggregation of data reduces their variance and increases their reliability (Ossenkopp & Mazmanian, 1985). Within each testing series, the tests were performed in the order in which they are described below. The experimental protocol is summarized in Table 1.

Grasping reflex of the hindpaw: a rat was held in the left hand, with thumb and index around the chest, immediately under the rat's forelegs. Then, the experimenter gently touched the palm of the hindpaw contralateral to the operated side with the index finger of his right hand. Grasping was scored as zero (no neurological abnormality on this test). When a rat failed to grasp, the score one was given.

Walking initiation: this task was adapted from Whishaw, O'Connor, and Dunnett (1985). The rat was placed on a horizontal surface in the center of two concentric circles with diameters of 20 cm and 60 cm, respectively. When a rat moved the length of its own body (i.e. left the inner circle), or made a 180° turn within 60 seconds, this was registered as walking initiation and given a score of zero. Otherwise, the score one was given. In addition, the latency to leave the outer circle (position of the hindlegs) was monitored, as was *unilateral circling* (i.e. whether they rotated toward the lesioned side).

Table 1. *Experimental protocol of behavioral testing of male WKY rats which had undergone unilateral sham operation or unilateral occlusion of the left MCA.*

Day after surgery	Events
0	Operation: MCA-O (n = 10), sham operation (n = 8)
2	First neurological test
16	Second neurological test (same battery of tests)
37	Third neurological test (same battery of tests plus ‘paw test’)
39-47	Motility: 62 hours, starting 2 hours before the start of the night cycle
41-49	Preparation of brains for histological examination

Forelimb flexion: the rat was gently lifted by its tail and was held one meter above the table. Whether the animal showed forelimb flexion, or not, was observed (Bederson et al., 1986; note that this behavior was called *forelimb clasping* by Whishaw, O'Connor & Dunnett, 1985). The absence of forelimb flexion was scored as zero (i.e. the rat showed no neurological abnormalities on this test). The presence of forelimb flexion was scored as one.

Hindlimb flexion: at the same time, hindlimb flexion (called *hindlimb clasping* by Whishaw, O'Connor & Dunnett, 1985) was assessed. Scoring was as for forelimb flexion.

Visually triggered placing: the test was adapted from Marshall (1982). A rat was picked up by its tail and was slowly lowered toward the edge of a table until its nose was approximately 10 cm from the edge. Then, the rat was moved toward the edge. Care was taken that the vibrissae did not touch the edge. A score of zero was awarded when a rat extended its forepaws towards the edge (the rat showed visual placing). Otherwise, a score of one was given.

Contact placing: this task resembles *visually triggered placing*. The rat was lowered until the animal touched the edge of the table with its vibrissae (Whishaw, O’Connor & Dunnett, 1985). If a rat

extended the forelimbs toward the edge as soon as it had made tactile contact with the table with its vibrissae, a score of zero was given. Otherwise, a score of one was given.

Suspension from a horizontal wire: the task was adapted from Wallace, Krauter, and Campbell (1980b). A rat was held against a horizontal wire (diameter: 0.5 cm, length: 60 cm, elevated about 40 cm above the surface) until it grasped it with its forepaw(s). Then the rat was released and the time elapsed before it fell off was measured (maximum duration of a trial was 60 s). A pad of crêpe paper (about 10 cm thick) was placed beneath the wire to cushion the rat's fall. In addition, a five-point scale was used to score whether a rat pulled itself up and supported itself with one or two hindlegs and successfully climbed onto the wire (score zero), or supported itself with its hindleg(s) without succeeding to climb onto the wire (score one), or held itself with both forepaws (score two), with only one forepaw (score three), or whether it was unable to grasp the wire (score four).

Traversing a square bridge: a rat was placed on a square bridge (2 cm x 2 cm x 60 cm, elevated about 40 cm above the surface), equidistant from two escape platforms (20 cm x 20 cm) (Wallace, Krauter & Campbell, 1980b). The duration the rat stayed on the bridge was measured to a maximum of 120 s. When a rat escaped onto one of the platforms, the duration was ascribed as the maximum. A pad of crêpe paper (about 10 cm thick) was placed beneath the bridge to cushion the rat's fall.

The next two tests were performed using a grid (75 cm width x 100 cm height) made of a sheet of stainless steel in which a 13 x 17 matrix of 5 x 5 cm holes were punched with an interspace of 0.5 cm. One short side of the grid was attached to a holding device. The experimenter could manipulate the inclination of the grid by moving the opposite short side up or down.

Turning on the inclined grid was assessed by a modification of the procedure described by Marshall (1982). The grid was held in a horizontal position. A rat was placed on it approximately in the center. The nose of the rat pointed to the edge of the grid that was to be lowered. Then, the grid was lowered until it attained a negative inclination of 30° with respect to the horizontal plane. The latency to turn on the grid was measured to a maximum of 120 s. If a rat turned 90° or more within 2 minutes of lowering of the grid, a score of zero was given. If the rat failed to turn on the grid, a score of one was given and the latency was set to 120 s.

Climbing on the inclined grid was assessed by a modification of the procedures described by Marshall (1982) and by Whishaw, O'Connor, and Dunnett (1985). Observation started immediately after a rat had turned 180°. Rats which failed to turn were turned by the experimenter. A step was operationally defined as the movement of a paw from one side of a square to one of the three other sides of the same square or to one of the sides of the adjacent squares. The number of steps was counted. Steps were classified as correct whenever a paw was placed on the grid. A step was classified as incorrect (mis-step) whenever the rat put a paw through one of the holes, irrespective of whether or not the rat corrected this step.

Vocalization, urination, and defecation were assessed for each of the complete three series of behavioral tests. For each individual behavior, a score of zero was awarded when the behavior did not occur during handling. Otherwise, a score of one was given. Differences in vocalization, urination, or defecation might indicate differences in emotional reactivity.

The *paw test* (Ellenbroek & Cools, 1988; Ellenbroek et al., 1987; Vrijmoed-de Vries, Tönissen & Cools, 1987) was performed as part of the third series of tests, 37 days after surgery. The test apparatus consisted of a box made of polyvinyl chloride. Two holes for the forelimbs and two holes for the hindlimbs were drilled in the upper surface of the box. In addition, there was a V-shaped opening for

the rat's tail. A rat was held in one hand, with thumb and index around the chest, immediately under the forelegs. First, its hindlimbs were placed into the holes of the test apparatus and then the forepaws were put into the appropriate holes. As soon as its four legs were in the holes the rat was released, and the latency to retract the contralateral fore- and hindlegs from the holes was registered to a maximum of 60 s.

Motility was assessed by using three identical motility meters (MFU 2100, Rhema-Labortechnik, Hofheim, Germany), housed in sound insulated cubicles (inner dimensions: width: 59 cm, height: 42 cm, depth: 52 cm). Fresh air was provided continuously through a ventilation system that also produced masking noise. The light/dark rhythm within the cubicles was the same as that in the animal vivarium.

The test was performed once, between postoperative days 38 and 49. Each rat was semi-randomly assigned to one of three motility counters. Each motility apparatus was used for approximately the same number of sham-operated and MCA-occluded rats. The registration of rats' motility for an uninterrupted period of 62 hours started at 5:00 p.m., two hours before the beginning of the dark cycle. The motility counts per hour were used for statistical analysis. The difference score between the motility counts of the first and the second hours in the apparatus can be taken as a measure for the speed of adaptation to a novel environment (van der Staay, 1989), or as measure of the habituation rate (Kolb, 1974).

A cosine curve fitting procedure (Monk & Fort, 1983) estimated mesor, amplitude, and acrophase of the motility of each individual rat over the two and a half consecutive days of testing. The mesor reflects the mean motility level of a rat. The acrophase reflects the point of maximum motility, that is, the point where the sine curve reaches its maximum deviation from the mesor, as defined by the estimate of the amplitude.

Results

Body weight

Body weights were analyzed by a two-way analysis of variance (ANOVA) with the factor Treatment (sham-operated vs. MCA-occluded) and with the repeated measures factor Days after operation (days 0, 6, 13, 20, 27, and 34, where day 0 is the day of surgery). Because of the unequal intervals between measurements, days were used as level values of the repeated measures factor (SAS Institute, 1990, p. 956). Where appropriate, ANOVAs on weights on a particular day are reported.

One of the eight sham-operated rats and two of the ten MCA-occluded rats died before the behavioral testing was completed. The mean body weights (grams ± SEM) of the two groups were similar before operation (sham lesioned: 265.8 ± 5.3, MCA-occluded: 270.1 ± 3.5; $F_{1,13} < 1$, n.s.; see Fig. 1). The body weight of the MCA-occluded rats decreased more than that of the sham-operated rats after surgery (Treatment by Days interaction: $F_{6,78} = 3.6$, $p < 0.01$), and remained at a lower level during the entire period of behavioral testing (General mean, i.e., body weight averaged over days: $F_{1,13} = 5.6$, $p < 0.05$). Because of the strong decrease in body weight in the MCA-occluded group and of some animals of the sham-operated group, rats were daily given about five crushed pellets (standard rodent chow, Altromin, Germany) supplemented with sunflower seeds. This feeding regimen was continued until a rat regained at least 90% of its preoperation free-feeding body weight.

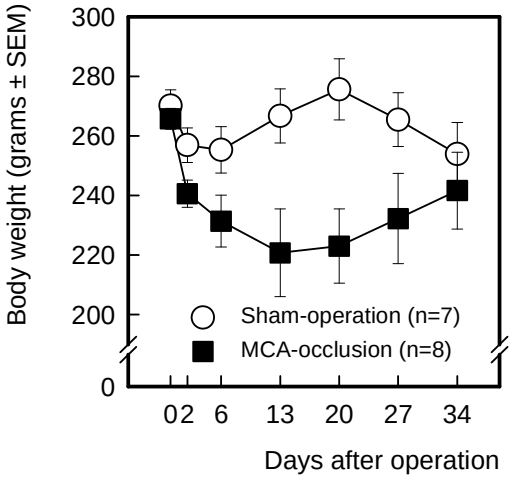

Figure 1. *Mean body weights (grams) and standard errors of the means (SEM) of male Wistar Kyoto rats which had either undergone unilateral proximal MCA-O or sham operation.*

Histological verification

Histological evaluation revealed that the occlusion of the MCA had been successful in all rats. Because of the strong shrinkage of the infarcted area, no reliable measurements of the infarct volume could be obtained after a survival period of more than 40 days. This also precluded the assessment of a correlation between the volume of the infarcted brain tissue and the degree of the sensorimotor disturbances. Data are, however, available from a strain comparison study in which the WKY strain was included, together with 7 other rat strains (see Chapter 4.2, third experiment). The infarct volumes were determined after a survival period of 7 days. The infarcts in the WKY rats were relatively small (mean volume ± SEM in cortex and striatum, 31.08 ± 11.44, and 24.84 ± 10.54, respectively), and an example of the extent and location of the infarcted cortical area in WKY rats is shown in Fig. 2.

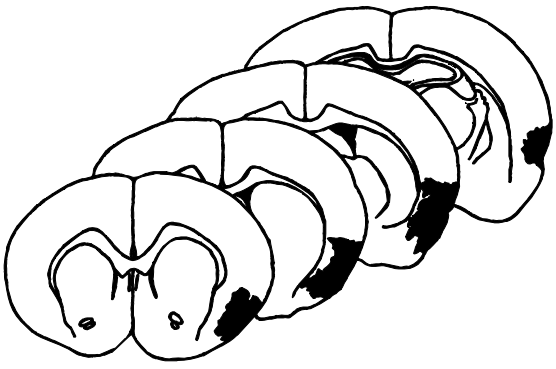

Figure 2. *Diagram of brain sections illustrating the typical location of the infarcted area in the cortex, 7 days after MCA-occlusion, in a Wistar Kyoto rat with a small infarct. In some rats, the infarcted area extended into the striatum (not shown). The coronal sections correspond approximately to the levels 1.2, 0.2, -1.3, and -2.56 mm from bregma in the stereotaxic atlas of Paxinos and Watson (1986).*

Neurological testing

For each individual test, the sum of scores over the three runs within a test session was determined and analyzed. The results of the functional tests are summarized in Table 2. Only results of tests that yielded differences between the two treatment groups will be discussed.

Table 2. *Summary of the results of the behavioral testing of seven sham-operated and eight MCA-occluded male WKY rats.*

Test	Short description of the effects
Grasping reflex of contralateral hindpaw	On average, the sham-operated animals showed a better grasping reflex than the MCA-occluded rats did.
Walking initiation: behavioral rating	No effects of MCA-O
Walking initiation: latency to leave outer circle	No effects of MCA-O
Circling behavior	MCA-occluded animals showed more unidirectional circling than the sham-operated rats did during the first test series. Repeated measures revealed a stronger decrease of circling behavior in MCA-occluded animals than in sham-operated rats.
Forelimb flexion	Flexion occurred in MCA-occluded animals only. The forelimb flexion decreased over sessions and finally vanished.
Hindlimb flexion	Flexion occurred in MCA-occluded animals only. The hindlimb flexion decreased over sessions and finally vanished.
Visual placing	MCA-occluded rats showed less visual placing than sham-operated rats during the first session. There was a tendency for functional recovery after MCA-O.
Contact placing	No effects of MCA-O
Wire suspension: latency to fall off	No effects of MCA-O
Wire suspension: behavioral rating	No effects of MCA-O
Square bridge: latency to fall off	There was a higher overall latency in sham-operated animals. Both groups showed a similar rate of improvement over sessions.
Turning on inclined grid: latency	No effects of MCA-O
Turning on inclined grid: behavioral rating	No effects of MCA-O
Number of steps on grid	No effects of MCA-O
Number of mis-steps on grid	MCA-occluded rats made more mis-steps than sham-operated rats during the third session.
Vocalization	No effects of MCA-O
Urination	No effects of MCA-O
Defecation	No effects of MCA-O
Paw test: retraction of contralateral hindleg	No effects of MCA-O
Motility first hour	No effects of MCA-O
Speed of adaptation (motility first hour minus motility second hour)	No effects of MCA-O
Mesor of motility over 60 hours	No effects of MCA-O
Amplitude of motility over 60 hours	No effects of MCA-O
Acrophase of motility over 60 hours	No effects of MCA-O

The scores from the functional tests were analyzed by a two-way analysis of variance (ANOVA) with the factor Treatment (sham-operated vs. MCA-occluded) and with the repeated measures factor Session. Because of the unequal intervals between measurements (tests were performed 2, 16, and 37 days after operation), number of days after operation were used as level values of the repeated measures factor (SAS Institute, 1990, p. 956). The ranks of measures, which represent *ratings of behavior*, were used for the statistical evaluations in addition to the raw data, because the raw data might violate the assumption of equal variances within groups. For all other variables, raw scores were analyzed. Where appropriate, scores of a particular session were analyzed by t-statistics or Z*-

statistics, depending on whether the raw scores or ranks were used. t-statistics were used to test the hypothesis that the scores of a particular treatment group in a particular session deviated from zero.

Grasping reflex of the contralateral hindpaw (Fig. 3, upper left panel): the repeated measures analysis revealed that, averaged over the three test sessions, MCA-occluded rats showed a severely disturbed grasping reflex, whereas the sham-operated animals showed only very mild disturbances or a normal reflex (General mean: $F_{1,13} = 5.71$, $p < 0.05$). There was no differential improvement over sessions (Treatment by Session interaction: $F_{2,26} = 2.56$, n.s.), indicating that the MCA-O induced persistent neurological deficits in this task.

Circling behavior (Fig. 3, upper center panel): circling contralateral to the lesioned side was observed in MCA-occluded rats in the first test session ($Z^* = -2.71$, $p < 0.01$). From the second test session onward, contralateral circling occurred to the same extent in both groups of rats (second session: $Z^* = -1.40$, n.s.; third session: $Z^* = 0.94$, n.s.). Repeated measures analysis revealed that there was a strong decrease in circling behavior in the MCA-occluded rats (Treatment by Session interaction: $F_{2,26} = 8.40$, $p < 0.01$); this behavior was no longer observed in the last session.

Forelimb flexion (Fig. 3, upper right panel): no forelimb flexion was observed in the sham-operated rats. The MCA-occluded rats showed very consistent forelimb flexion in the first session ($Z^* = -2.79$, $p < 0.01$), and a reduction of the severity of this behavioral abnormality over sessions (Treatment by Session interaction: $F_{2,26} = 5.60$, $p < 0.01$). In the last session, the behavior of the MCA-occluded rats returned to normal; the (raw) scores no longer deviated from zero ($t_7 = 1.0$, n.s.).

Hindlimb flexion (Fig. 3, lower left panel): whereas no hindlimb flexion was observed in the sham-operated rats, the MCA-occluded animals showed very consistent hindlimb flexion in the first session ($Z^* = -2.74$, $p < 0.01$), and a reduction in the severity of this behavioral abnormality over sessions (Treatment by Session interaction: $F_{2,26} = 4.47$, $p < 0.05$). In the last session, the behavior of the MCA-occluded rats had returned to normal; the (raw) scores no longer deviated from zero ($t_7 = 1.0$, n.s.).

Visual placing (data not shown): visual placing was unimpaired in the sham-operated rats. The MCA-occluded rats showed severe disturbances on this task in the first session ($Z^* = -2.05$, $p < 0.05$). From the second test session on, however, this disturbance was no longer observed, and the neurological (raw) scores for visual placing in the MCA-occluded group no longer deviated from zero (second session: $t_7 = 1.4$, n.s.; third session: $t_7 = 1.0$, n.s.). Repeated measures analysis revealed that there was a tendency for an improvement in the MCA-occluded rats over sessions (Treatment by Session interaction: $F_{2,26} = 2.67$, $0.1 > p > 0.05$).

Latency to fall off the square bridge (Fig. 3, lower center panel): in the first two sessions, the MCA-occluded rats had marginally shorter fall off latencies than the sham-operated rats (first session: $t_{13} = 1.98$, $0.10 > p > 0.05$; second session: $t_{13} = 1.87$, $0.1 > p > 0.05$). In the third session the rats from both treatment groups were able to stay on the bridge for the entire 120 s. Repeated measures analysis revealed that the MCA-occluded rats had, on average, shorter fall off latencies (General mean: $F_{2,26} = 515$, $p < 0.05$). Both treatment groups improved their performance over sessions ($F_{2,26} = 1479$, $p < 0.01$), but the rate of improvement appeared to be similar (Treatment by Session interaction: $F_{2,26} < 1.0$, n.s.).

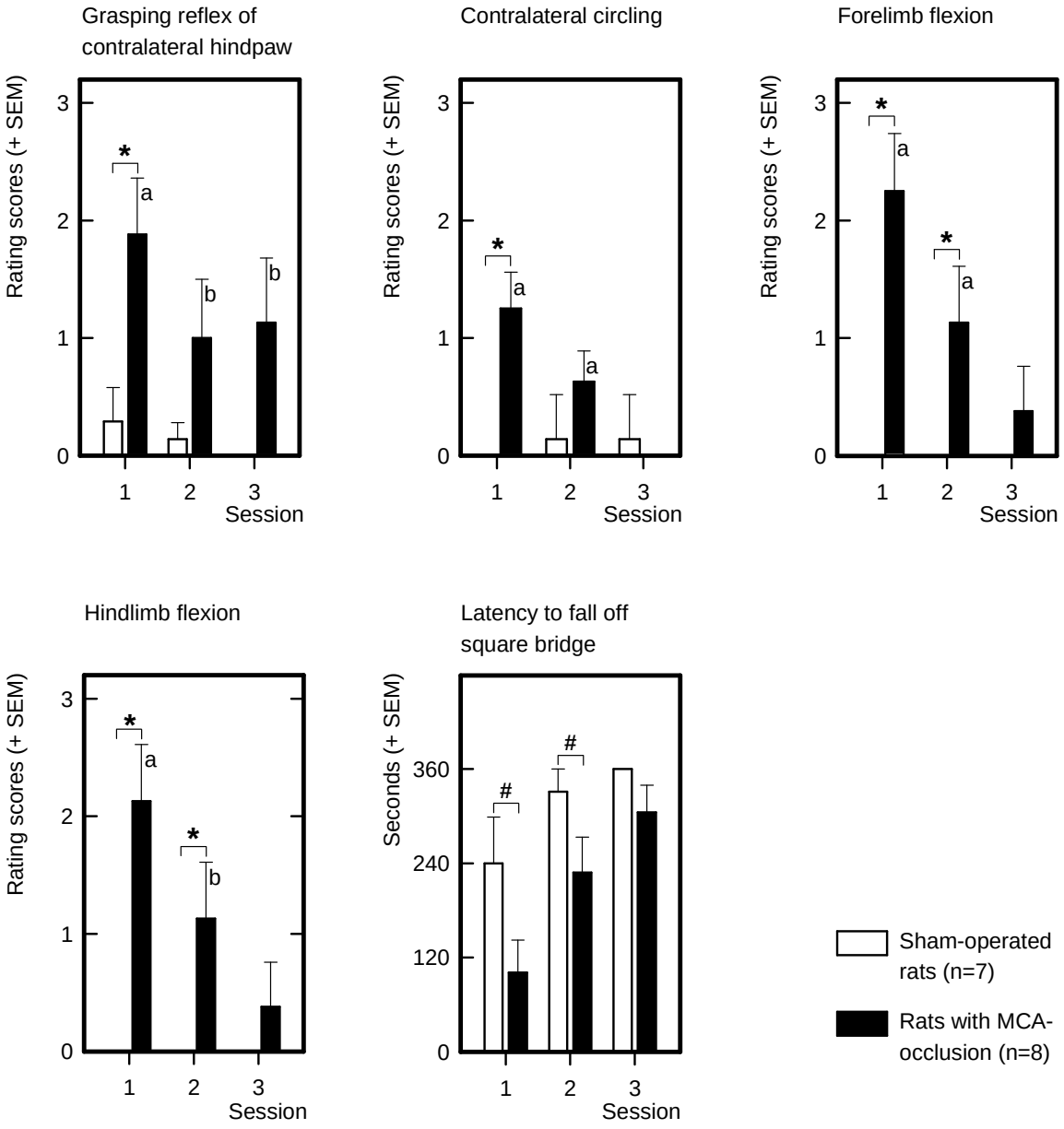

Figure 3. *Behavioral impairments in Wistar Kyoto rats which had either undergone permanent unilateral occlusion of the left proximal MCA or were sham-operated. The scores for grasping reflex of contralateral hindpaw (upper left panel), contralateral circling (upper center panel), flexion of the forelegs (upper right panel), and flexion of the hindlegs (lower left panel) are depicted as mean summed neurological ratings (+ SEM). The scores range from zero (no neurological abnormality) to three (severest impairment). In the lower center panel the latencies to fall off a square bridge are shown as summed means + SEM.*
**: MCA-occluded rats differ from sham-operated rats ($p < 0.05$)*
#: MCA-occluded rats differ marginally from sham-operated rats ($0.1 > p > 0.05$)
a: Score different from zero (t-statistics; $p < 0.05$)
b: Score marginally different from zero (t-statistics; $0.1 > p > 0.05$)

Number of mis-steps on grid (data not shown): analysis of the number of mis-steps was somewhat problematic because the WKY rats made only a few steps or were completely immobile within a trial. This precluded analysis of the ratio measure (number of mis-steps / total number of steps). Instead, an analysis of covariance of the number of mis-steps per session was performed, with the total number of

steps as covariate. Analysis revealed that there was no difference between the two groups for the number of mis-steps on the first two sessions (first and second sessions: $Z^* = -0.18$, and 0.95, respectively, $p > 0.10$). During the third session, the sham-operated rats made fewer mis-steps than the MCA-occluded rats ($Z^* = -2.59$, $p < 0.05$). These results were confirmed by the analysis of covariance on mis-steps, with total number of steps as covariate, revealing that the treatment groups differed for the number of mis-steps in the third session ($F_{1,12} = 13.73$, $p < 0.01$). The sham-operated rats appeared to improve walking performance on the grid over sessions (mirrored by a reduction in the number of mis-steps in relation to total number of steps), whereas the MCA-occluded rats did not.

Discussion

We found that a subset of behavioral tests in a test battery appeared to be suitable to assess the effects of cerebral infarction induced by unilateral MCA-O. These tests were grasping reflex of contralateral hindpaw (Wahl et al., 1992), circling behavior (Bederson et al., 1986), fore- and hindlimb flexion (Bederson et al., 1986), and latency to fall off a square bridge. These simple tests can easily be applied and scoring is easy and unambiguous. The tests can be performed within about 15 minutes, and in most of them learning effects are not likely to occur. Learning could eventually interfere with interpretation of the degree of sensorimotor recovery, due to cross-over effects from one series of tests to the other. There might have been a learning component in the bridge task and grid tasks: the rat must adopt a posture to keep its balance. The increase in time spent on the bridge and the reduction of the number of mis-steps over sessions shown by the sham-operated rats might be indicative of an underlying learning process.

Some of the tests used need further comment. Visually triggered placing (Marshall, 1982) could not be applied in a sufficiently reliable manner. The animals with ischemic damage showed strong flexion of the entire body when held head down and directed toward the edge of the table. It was not possible to hold a rat at a distance from the table that would induce visually triggered extension of the forelimbs while reliably preventing contact of the vibrissae with the edge of the table. Thus, some of the visually triggered extensions of the forelimb might in fact have been triggered through contact with the vibrissae. Wahl and co-workers (1992) observed similar abnormal postures when they tested visually triggered placing in rats. They suggest that *thorax twisting* might have been responsible for incorrect visual placing.

In the test *traversing a square bridge* only one WKY rat escaped onto one of the platforms at the end of the square bridge. Typically, as soon as the rats had attained postural equilibrium they froze in that particular posture and did not move for the remainder of the 120 s observation period.

The WKY rats showed little activity on the *inclined grid*. Although *turning on the inclined grid* was performed by most rats within the time limit of 120 s, qualitative differences were observed between the sham-operated and the MCA-occluded animals. It appeared that the ischemic rats turned predominantly ipsilaterally to the lesioned side around the ipsilateral hindleg (note that ischemic rats turn contralaterally on a horizontal surface). The grip of that leg on the grid was not released until maximal turning was reached. Often, turning stopped at that point, and the rat became immobile.

Most animals did not meet the minimum requirement that at least six steps should be made to evaluate the effects of the occlusion on *climbing on the inclined grid.* Typically, the animals with ischemic

damage rapidly [at the very first step(s)] put their contralateral fore- and hindleg through the grid and stayed in that position for the remainder of the 120 s observation period. Although these animals obviously tried to do so, they were unable to reposition their contralateral legs and to place their paws on the grid. Because of this observation, the paw test was added to the battery of tests used.

The *paw-test* (Ellenbroek & Cools, 1988) can be expected to be a valuable extension of the test battery to assess effects of MCA-O, if it is used early after occlusion. This test was applied only at the end of the third test session. Most of the neurological deficits produced by the MCA-O had already disappeared when the rats were tested for the third time. Therefore, the paw test should be used for acute neurological testing soon after the occlusion has been produced.

The same might be true for the *motility test.* No differences were found on this test, perhaps due to the fact that testing was performed too long after the operation. Neither the spontaneous activity measured during the first hour in the motility boxes, nor the speed of adaptation, nor the circadian activity pattern was affected by the effects of the MCA-O .

However, findings from cortical suction ablation experiments and from MCA-O experiments might provide an alternative explanation for the absence of effects on motility in the present study. Ablation of small cortical regions of the right hemisphere induced hyperactivity, whereas no effect was found after ablation of the corresponding regions of the left hemisphere (Pearlson, Kubos & Robinson, 1984). Ligation of the left MCA did not affect rats' spontaneous activity, whereas ligation of the right MCA increased locomotor activity in rats for about 3 weeks following surgery (Robinson, 1979; Robinson & Coyle, 1980). The data suggest a functional asymmetry after unilateral MCA-O. The latter finding, however, was not replicated by others. Andersen, Andersen, and Finger (1991), for example, found spontaneous activity to be in the normal range after occlusion of the right MCA. To clarify whether left MCA-O does affect spontaneous activity, motility should be assessed earlier after the operation, because Robinson and Coyle (1980) found that spontaneous activity returned to normal levels in the rats with right MCA-O within about three to four weeks of the operation.

Some of the tests might not have been sensitive enough to detect the disruptive effects of the unilateral occlusion of the MCA on behavioral parameters. However, the genotype used might have been part of this problem. The WKY rats appeared to be extremely inactive on the square bridge and on the grid, so that reliable scoring of effects was not possible. Previously, we had observed low activity levels of WKY rats in the open field and in a light-dark preference test, when compared to seven other rat strains (van der Staay, Kerbusch & Raaijmakers, 1990). Thus, the WKY strain appears to be less suited to study the behavioral consequences of MCA-O.

In our study, the unilateral MCA-O induced a stronger decrease in body weight than the sham operation did, corroborating data by Yamamoto and colleagues (1988). This result, however, contrasts with the findings of Barone and co-workers (1992), who reported that in, among others, the WKY strain MCA-O did not induce a greater weight loss than the sham operation did. The stronger decrease in body weight after MCA-O found in our study most likely is centrally mediated, as both the MCA-occluded and the sham-operated rats underwent identical surgery, except for occlusion and removal of the occluded vessels. Thus, differences between the results of different studies may be related to the infarction size or site, and to the consequences of the infarction on feeding behavior.

There was a complete recovery of function by the third test session, 37 days after surgery, except for the grasping reflex of the contralateral hindpaw, which was still slightly impaired. Markgraf and colleagues (1992), using a series of sensorimotor tests, also found complete recovery when they

compared the preoperative performance of MCA-occluded Sprague Dawley rats with their post-occlusion performance. Similar observations were also reported by Yamamoto and coworkers (1988) with respect to sensorimotor disturbances in MCA-occluded Wistar rats, whereas learning of a passive avoidance task was impaired, even 16 weeks after occlusion. Spontaneous and dramatic recovery has also been observed in patients after acute ischemic stroke (Biller et al., 1990). This supports the notion that MCA-O in rodents possesses face validity.

It would be of interest, using this ischemia model and these tests, but a different rat strain, to compare the speed of recovery of function in rats treated with substances that might reduce the infarction volume with that of rats which did not receive treatment after surgery.

4.2

Sensorimotor impairments in rats with cerebral infarction, induced by unilateral occlusion of the MCA: strain differences and effects of the occlusion site*

Abstract

Enormous differences exist between rat strains with respect to the infarct volume induced by unilateral middle cerebral artery (MCA) occlusion. We performed three experiments to address the following questions: first, whether the pattern of MCA-occlusion (MCA-O) induced sensorimotor impairments in rats are strain dependent; secondly, whether proximal (i.e. close to its origin) and distal occlusions (above the lenticulostriate branch) of the MCA affect infarct volume and the behavioral impairments to a different extent; and thirdly, whether there is a relationship between the infarct volume and behavioral deficits.

We found that the pattern of sensorimotor malfunctions induced by proximal unilateral MCA-O were highly strain dependent. Of the eight strains tested, Winkelmann Wistar rats, spontaneously hypertensive stroke-prone rats, and Wistar Kyoto rats were most severely affected. By contrast, Brown Norway rats showed only mild behavioral deficits after the MCA-O. The second experiment confirmed that proximal occlusions induced slightly more behavioral malfunctions than distal occlusions did. Histological evaluation of the brain damage caused by proximal and distal MCA-O, confirmed that distal MCA-O damaged nearly exclusively cortical areas, and spared the caudate/putamen. An exploratory analysis of the relationship between infarct volume and behavioral deficits did not indicate that the severity of sensorimotor malfunctions can be predicted from the size of the infarct.

Introduction

Occlusions of the middle cerebral artery (MCA) in rats or mice provide an animal model to investigate the pathophysiology of the permanent focal cerebral ischemia (Welsh et al., 1987) to screen potentially neuroprotective substances (e.g. Obana, Pitts & Nishimura, 1988; Gotti et al., 1990; Hara et al., 1991; Yamamoto, et al, 1991; Park & Hall, 1994; Hunter, Green & Cross, 1995; Sauter & Rudin, 1995), or to assess ischemia-induced behavioral and neurological disturbances (e.g. Tamura et al., 1985; Bederson et al., 1986; Yamamoto et al., 1988; Markgraf et al., 1992; van der Staay, Augstein & Horváth, 1996a). The majority of studies on the effects of MCA-occlusions (MCA-O) have been performed with rats. Enormous differences with respect to the infarct volume (e.g. Duverger, Lecoffre

* This chapter is based on the publication: van der Staay, F.J., Augstein, K.-H., & Horváth, E. (1996b). Sensorimotor impairments in rats with cerebral infarction, induced by unilateral occlusion of the left middle cerebral artery: strain differences and effects of the occlusion site. *Brain Research*, **735**, 271-284.

& MacKenzie, 1985) and to the behavioral deficits induced by MCA-O have been reported, depending, among other factors, upon the strains involved (e.g. Barone et al., 1992; Oliff et al., 1995a,b; Sauter & Rudin, 1995), the operation technique used (e.g. Shigeno et al., 1985; Shirashi & Simon, 1989), the anatomical location of the occlusion (Shigeno et al., 1985; Bederson et al., 1986), the survival time of the animals (e.g. Persson et al., 1989), and even the housing conditions of the animals (Ohlsson & Johansson, 1995).

In the present study, we performed three experiments to assess strain differences and the effects of the occlusion site on MCA-O induced behavioral deficits. To this end we used a battery of simple sensorimotor tests which included assessment of grasping reflex, walking initiation, circling behavior, C-shaped lateral bending of the body, forelimb and hindlimb flexion, latency to retract the fore- and hindpaws from a paw test apparatus, and corneal reflex. These simple tests were selected because we had previously found that they are sensitive to sensorimotor deficits induced by unilateral MCA-O in Wistar Kyoto (WKY) rats (van der Staay, Augstein & Horváth, 1996a; see Chapter 4.1).

In the first experiment, we studied the effects of unilateral MCA-O on sensorimotor functions in eight different rat strains [male Brown Norway (BN), Fischer 344 (F344), Long Evans (LE), Lewis (LEW), Sprague Dawley (SD), Spontaneous Hypertensive Stroke-Prone (SHR-SP), Wistar (WISW), and WKY], by comparing their pre-occlusion behavioral scores with those two days after surgery. The SD, F344, SHR and WKY rat strains are frequently used ones in studies on behavioral effects of MCA-O.

Rat strains differ with respect to the cerebrovascular anatomy: the degree of branching of the MCA (e.g. Shiino, 1989), the diameter and distribution of cerebral vessels (e.g. Nordborg & Johansson, 1980), and of the collateral supplier system. As a consequence, the extension and area of infarction would be expected to differ between strains. Duverger and MacKenzie (1988), Oliff and co-workers (1995a,b), and Sauter and Rudin (1995), for example, found that the infarct volume strongly differed between rat strains, and between different lines of the same strain from different suppliers (Oliff et al., 1995a,b). Niiro and co-workers (1996) even reported a considerable degree of variablitiy in the branching patterns of the MCA within a rat strain. Differences in braching patterns are expected to affect the size of the infarcted area after MCA-O. As chronic hypertension aggravates the histological damage induced by MCA-O, the infarcts of the SHR-SP rats are bigger than those of their normotensive controls, the Wistar Kyoto rats (Duverger & MacKenzie, 1988; Sauter & Rudin, 1995). We expected that the MCA-O would affect the behavior of strains differently.

In the second experiment, we compared the effects of proximal and distal MCA-O in the LE, LEW and SHR-SP rat strains. We expected that the distal occlusion would produce smaller infarcts that would eventually affect only cortical, but not subcortical (striatal) areas (Shigeno et al., 1985). Therefore, we expected that sensorimotor dysfunctions would be less severe in the rats that had undergone distal MCA-O (Bederson et al., 1986).

In the third experiment, we determined whether there is a relationship between behavioral deficits and volume of the cortical infarct, using the eight rat strains tested in the first experiment. Reports on this relationship are inconclusive. Bederson and co-workers (1986), Markgraf and co-workers (1992), and Rogers and colleagues (1997) for example, found that the infarct size and the severity of behavioral deficits were related in rats with proximal MCA-O. These results, however, contrast with those reported by Duverger, Lecoffre & MacKenzie, (1985) and by Wahl and co-workers (1992), who did not find such a correlation. We estimated the relationship between the severity of behavioral impairments based on ranks of severity of MCA-O induced deficits over strains (from the first experiment) and estimates of

the cortical infarct volumes from additional rats of the same strains that had undergone proximal MCA-O.

First experiment: effects of proximal unilateral MCA-O in eight strains of rats

Material and Methods

Subjects

Male Brown Norway (BN) rats (University of Limburg, Maastricht, The Netherlands), Fischer 344 (F344) rats [CDF(F-344)/CrlBR; Charles River WIGA AG, Sulzfeld, Germany], Long Evans (LE) rats (LE/Mol; Møllegaard APS, LI. Skensved, Denmark), Lewis (LEW) rats (LEW/Mol; Møllegaard APS, LI. Skensved, Denmark), and Sprague Dawley (SD) rats (Mol:SPRD; Møllegaard APS, LI. Skensved, Denmark), Spontaneous Hypertensive Stroke-Prone (SHR-SP) rats (BAYER AG, Bayer Research Center, Wuppertal, Germany; SP-strain derived from the SHR-Okamoto strain), Wistar (WISW) rats [BOR:WISW SPF(Cpb), Winkelmann, Borchen, Germany; recently, this strain has been renamed HsdCpb:Wu], and Wistar Kyoto (WKY) rats (WKY/Mol; Møllegaard APS, LI. Skensved, Denmark) were used. The animals were housed four to six in standard Makrolon® type IV cages under an artificial 12 hour light/12 hour dark regimen (lights on from 7:00 to 19:00) in a temperature (ca. 21.5°C ± 0.5°C)- and humidity (50%)-controlled animal room. Water and food were available ad libitum. Before testing, the animals were transferred to the experimental room where they were housed throughout the entire testing period. Housing conditions were similar to those in the animal room.

Unilateral middle cerebral artery occlusion (MCA-O)

Under general anesthesia (chloral hydrate, Fluka Chemie AG, Buchs, Switzerland; 400 mg/kg i.p.) the MCA of 10 rats per strain was occluded unilaterally according to the standard surgical procedure described by Bederson et al. (1986) with minor modifications. The left temporo-parietal region of the head was shaved and the skin was disinfected and opened between the orbit and the external ear canal. After a midline incision was made, the temporal muscle was divided and pulled aside with surgical hooks to free the lateral aspect of the skull. The facial nerve, major facial arteries and veins, the lateral eye muscle, the intra- and extraorbital lacrimal glands, and the zygomatic bone were left intact. Under a surgical microscope a small burr hole was drilled directly under the zygomatic arc, 1-2 mm rostrally to its caudal origin. After careful opening of the dura, the exposed MCA and its branches were permanently occluded between the olfactory tract and the inferior cerebral vein by micro bipolar electro-coagulation (Bipolator 50, Fischer MET GmbH, Freiburg, Germany). To avoid recanalization, the occluded vessels were removed. The operation area was covered with a small piece of sterile absorbable gelatine sponge (Marbagelan®, Behringwerke AG, Marburg, Germany). Muscle and skin wounds were closed with tissue glue (Histoacryl®, B. Braun Melsungen AG, Melsungen, Germany). As it has been reported that hypothermia might be neuroprotective in animal models of ischemia (Green et al., 1992; Hunter, Green & Cross, 1995; Barone, Feuerstein & Withe, 1997; Corbett, Nurse & Colbourne, 1997) and cortical impact injury (Dixon et al., 1998), the body temperature was monitored during surgery and was maintained between 36.5 and 37.5°C by using a heating pad. The animals recovered from anesthesia, lying on a heating pad and covered with some layers of tissue. In earlier, unpublished experiments we had monitored both rectal temperature and subdural temperature and

found that brain hypothermia did not occur when the body temperature was maintained in this range. After recovery from anesthesia, the rats were returned to their home cage.

Behavioral tests

One to 5 days before MCA-O and precisely 2 days thereafter, the behavioral state of the animals was evaluated. Effects of the MCA-O were assessed by measurement of body weight and by rating the severity of deficits in a series of simple behavioral tests (grasping reflex, walking initiation, circling behavior, C-shaped lateral bending of the body, forelimb and hindlimb flexion, paw test, and corneal reflex). At least 1 hour before behavioral testing started, the rats were transported from the animal vivarium to the testing room. The entire battery of tests was administered three times in close succession under normal illumination between 9:00 and 12:00 a.m.

Vocalization, urination, defecation: for each test battery series, whether a rat vocalized, urinated, or defecated was registered separately. When a rat vocalized, urinated or defecated at least once during at least one series of tests, a score of 1 was given; otherwise, the score zero was given.

Grasping with hindpaws: the rat was held in one hand, with thumb and index finger around the chest, immediately under the forelegs. Then, the experimenter gently touched the soles of the hindpaw with the index finger of the other hand, first on the side ipsilateral to the MCA-O, then on the contralateral side. Grasping was scored as zero (no behavioral abnormality on this test). When the rat failed to grasp, the score was 1. Scores were given separately for the ipsi- and contralateral side.

Walking initiation and circling: this task was adapted from Whishaw, O'Connor, and Dunnett (1985). The rat was placed on a horizontal platform into the center of two concentric circles with diameters of 20 cm and 60 cm. The latency to initiate walking, defined as a forward movement of one body length or an at least 180° turn of the body, was registered. In addition, the latency to move out of the inner and outer circles with all four legs was scored. The observation was terminated as soon as the rat left the outer circle, or when 60 seconds had elapsed, whichever event occurred first. We also counted the number of ipsilateral and contralateral 180° turns of the body.

C-shaped lateral bending of the body: the rat was gently lifted by its tail and was held about 15 cm above the table surface. We observed whether the animal showed C-shaped bending of the body to the ipsilateral side and whether it showed fore- and hind-limb flexion (see below). The absence of C-shaped bending was scored as zero, a score of 1 was given when this impairment was slightly present, whereas severe C-shaped bending of the body was scored as 2. Wahl and co-workers (1992) observed similar abnormal postures in MCA-occluded rats, which they called *thorax twisting*, whereas Barth and Schallert (1987) observed a similar abnormality in superior colliculus and in lateral hypothalamus-lesioned rats in their *hang-tail test*. They called this postural abnormality *trunk flexion.*

Forelimb and hindlimb flexion: at the same time we observed whether the animal showed fore- and hindlimb flexion (called *forelimb and hindlimb clasping,* respectively, by Bederson et al., 1986; Obana, Pitts & Nishimura, 1988; Whishaw, O'Connor & Dunnett, 1985). The absence of forelimb flexion was scored as zero (i.e. the rat extended its forelimbs toward the table and thus showed no behavioral abnormalities on this test). The presence of forelimb flexion was scored as 1. The same scoring procedure was applied to assess ischemia-induced hindlimb flexion. The absence of hindlimb flexion was scored as zero (i.e. the rat stretched its hindlimb caudally, parallel to its body), its presence was scored as 1. Scores of zero thus indicate that the rat showed no behavioral abnormalities.

Corneal reflex: to measure the corneal reflex, a rat was held in one hand, with thumb and index finger around the chest, immediately behind the forelimbs. Then, a nylon thread (length 50 mm, diameter 0.35 mm) was gently passed across the cornea. Care was taken to avoid touching the eyelids.

Paw test: this test was originally designed to evaluate the cataleptic effect of neuroleptic drugs. The procedure described by Ellenbroek et al. was followed (Ellenbroek et al., 1987; Vrijmoed-de Vries, Tönissen & Cools, 1987; Ellenbroek & Cools, 1988). In short, the test apparatus consists of a frame made of polyvinyl chloride (PVC) in which two holes for the forelimbs and two holes for the hindlimbs were drilled. In addition, there was a V-shaped opening for the tail. The rat was held in one hand, with thumb and index-finger around the chest, immediately under the forelegs. First, its hindlimbs and then its forelimbs were placed into the holes of the test apparatus. The rat was then released, and the latency (in s) to retract the fore- and hind-limbs contralateral to the MCA-O from the holes was registered.

Feeding behavior: the rat was given a few sunflower seeds, a favorite food, in its home cage, and feeding behavior was observed for 1 minute (Brito & Brito, 1990). No behavioral abnormalities were apparent (score zero) when a rat held a sunflower seed with both forepaws. If a rat used only the ipsilateral forepaw, 1 was scored. A 2 was scored when a rat appeared to be unable to pick up a seed with its paw(s), i.e. whenever it tried to pick up a sunflower seed with its mouth without using its paw(s). This test was given only once at the end of a testing session, i.e. when the three series of tests had been completed.

Statistical evaluation

For each test separately, the sum of scores over the three passages through the battery of tests within a session was determined and analyzed. In all tests based on ratings, the absence of behavioral abnormalities was scored as zero. Thus, the higher the scores were, the more pronounced were the behavioral problems. The maximum value in the tests based on ratings was 3, indicating severest behavioral impairments, except for 'C-shaped lateral bending of the body', where the severest deficiency was scored as 6. The scores for the behavioral tests were analyzed by a two-way analysis of variance (ANOVA) with the factors Strain and the repeated measures factor Testing session (behavior before versus after the MCA-O).

For the measures representing ratings, we assessed the effects of the occlusion by using the difference scores between the raw pre- and post-occlusion measurements, which were analyzed by nonparametric Kruskal-Wallis analysis. The parametric ANOVAs and the non-parametric Kruskal-Wallis analyses yielded highly similar results. Therefore, only the results of ANOVAs will be reported. In addition, *t*-statistics were used to test the hypothesis that the difference scores (post-occlusion session minus pre-occlusion session) of particular strains deviated from zero, i.e. whether the occlusion affected the measure under consideration.

Rank correlations across strains: it is conceivable that within a particular test the variables measured are correlated. In particular, the three measures of walking initiation, i.e. latency to move one body length, latency to leave the inner and outer circles might be correlated. The same might be true for the two measures fore- and hindlimb flexion, and for the two measures latency to retract the fore- and hindleg contralateral to the MCA-O from the holes of the paw test apparatus. We assessed this by using the Kendall rank correlation coefficient τ on the ranked strain means. When behavioral measures are correlated, they probably provide a measure for the same underlying trait or characteristic (Walsh & Cummins, 1976).

Results

During the pre- as well as the post-occlusion testing session there were strain differences for all variables measured, except vocalization and contralateral corneal reflex. Here, only the repeated measures analyses (or alternatively, the analyses on the difference scores) with respect to effects of the Testing session and to Testing session by Strain interactions will be considered and discussed. It should be remembered that effects of Testing session cannot be detected if ranked scores are considered. The difference scores for raw data (post-occlusion minus pre-occlusion scores) provide the best estimate for general effects of the factor Testing session.

Body weight and physical condition

After MCA-O, the body weight of rats of the eight strains decreased (Testing session: $F_{1,72} = 1279.64$, $p < 0.01$) to a similar extent (Testing session by Strain interaction: $F_{7,72} = 1.74$, n.s.). The general physical condition 2 days after surgery was reasonable. Physical examination revealed that the wounds were properly closed.

Emotionality-related measures

Vocalization, urination, defecation: neither vocalization, nor defecation or urination were affected by the MCA-O. It should be noted, however, that *t*-tests on the difference scores per strain revealed that defecation scores decreased for the LE, LEW, and WKY strains (see Table 1).

Behavioral deficits

Grasping with hindpaws (Fig. 1, 1st row, left panel): the MCA-O reduced or abolished the grasping reflex of the contralateral hindpaw in all strains (see Table 1). The ipsilateral hindpaw, on the other hand, was differently affected by the occlusion in the eight strains (Difference scores for Strain: $F_{7,72} = 6.55$, $p < 0.01$). This reflex was disturbed in the SHR-SP and WISW rats, whereas there were no statistically reliable differences between the pre- and post-occlusion testing sessions in the other six strains (see Table 1).

Walking initiation: the *latency to move one body length* (Fig. 1, 1st row, center panel) increased after MCA-O (Testing session: $F_{1,72} = 39.16$, $p < 0.01$), but differently in individual rat strains (Testing session by Strain interaction: $F_{7,72} = 13.38$, $p < 0.01$). There was a statistically reliable increase in the latency to move one body length in the F344, SHR-SP and WISW rats only.

The same was true for the *latency to leave the inner* and *latency of leave the outer circle* (inner circle, Testing session: $F_{1,72} = 67.51$, $p < 0.01$; Testing session by Strain interaction: $F_{7,72} = 5.86$, $p < 0.01$, see Fig. 1, 1st row, right panel; outer circle, Testing session: $F_{1,72} = 51.32$, $p < 0.01$; Testing session by Strain interaction: $F_{7,72} = 5.43$, $p < 0.01$, see Fig. 1, 2nd row, left panel).

Correlations: the three measures of walking initiation, i.e. latency to move one body length, latency to leave the inner and outer circles, were correlated across strains (Kendall rank correlation coefficient: $\tau = 0.89$, $p < 0.01$).

Circling behavior: although ipsi- and contralateral circling will be discussed separately, the results for ipsilateral circling should be considered in relation to the results for contralateral circling.

Before MCA-O, the strains differed with respect to ipsilateral circling (Strains: $F_{7,72} = 3.29$, $p < 0.01$), with LE, SD, WISW and WKY rats, in particular, making few ipsilateral turns. After surgery, the number of ipsilateral turns decreased, whereas the number of contralateral turns increased (see below) (Testing session: $F_{1,72} = 5.00$, $p < 0.05$; Testing session by Strain interaction: $F_{7,72} = 2.73$, $p < 0.05$).

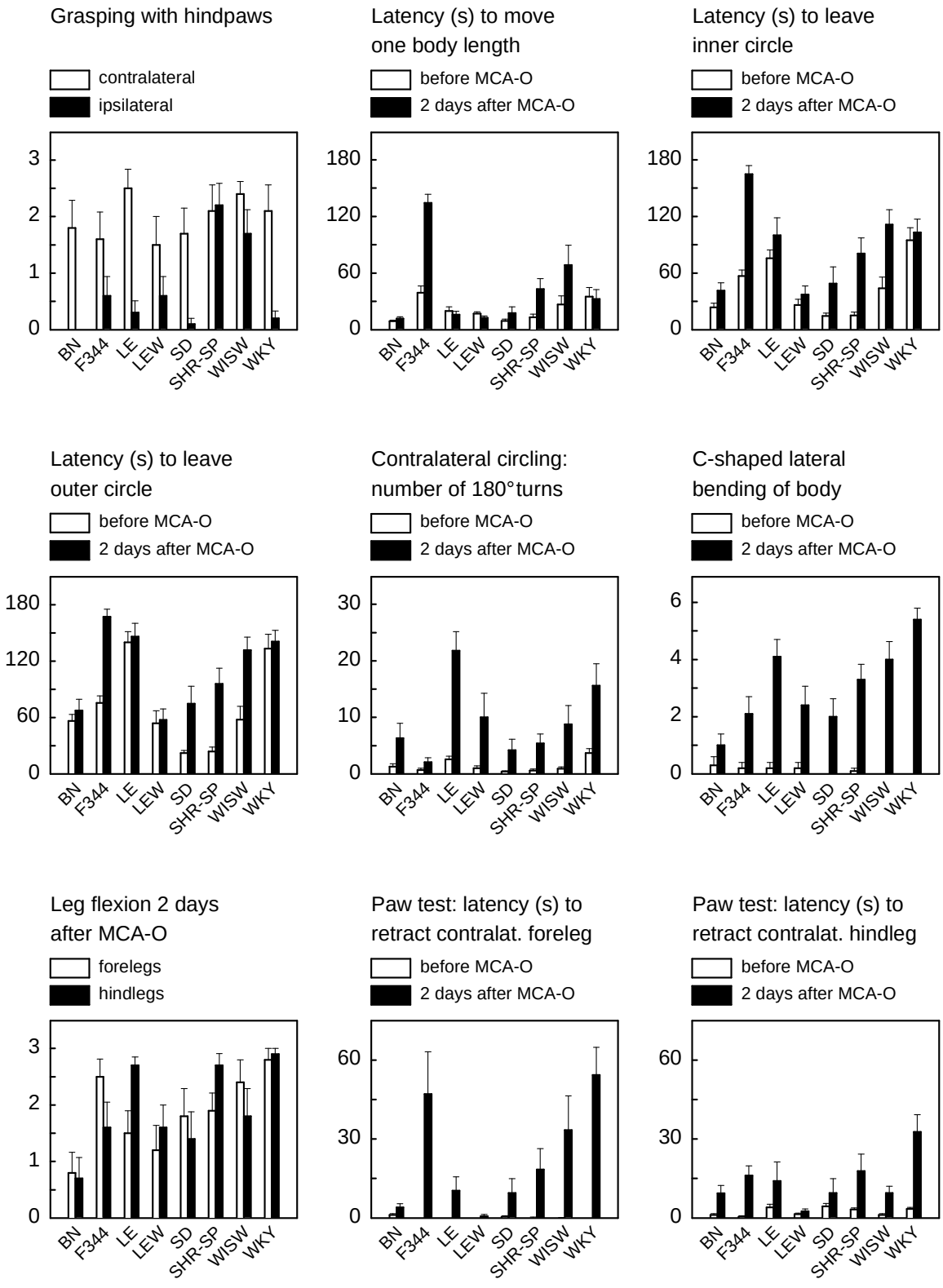

Figure 1. *Behavioral effects of unilateral occlusion of the left proximal MCA in rats of eight different strains. The means and standard errors of the means for grasping reflex of the ipsi- and contralateral hindpaw (first row, left panel), latencies to move one body length (first row, center panel), latencies to leave inner circle (first row, right panel), latencies to leave outer circle (second row, left panel), contralateral circlings (second row, center panel), C-shaped bending of body (second row, right panel), flexion of the forelegs and hindlegs (3rd row, left panel), latencies to retract the contralateral foreleg (third row, center panel), and latencies to retract the contralateral hindleg from the paw test apparatus (third row, right panel) are depicted.*

The Testing session by Strain interaction was most likely caused by the fact that after the occlusion rats of all strains except the BN rats reduced the number of ipsilateral circlings to near zero.

Very few contralateral 180° turns (Fig. 1, 2nd row, center panel) were made during the pre-occlusion testing session. The rat strains differed on this measure (Strain: $F_{7,72} = 6.43$, $p < 0.01$). Contralateral circling increased after surgery (Testing session: $F_{1,72} = 54.43$, $p < 0.01$), but to a different extent for the different strains (Testing session by Strain interaction: $F_{7,72} = 3.48$, $p < 0.01$). The increase was confirmed by *t*-test for the LE, SHR-SP, WKY and WISW rats (see Table 1).

Table 1. *t-values (df = 9) on difference scores (post-occlusion testing scores minus pre-occlusion testing scores) to evaluate the effects of unilateral MCA-O on body weight, vocalization, defecation, urination, and on a battery of behavioral tests in eight rat strains. All differences were calculated on raw data. Per rat strain we tested whether the difference scores deviated from zero (i.e. whether the MCA-O affected the measure under consideration). Note that for some measures statistically reliable differences were detected by the individual t-tests, despite the fact that ANOVAs did not indicate main effects of the factor Testing Session, or of interactions of this factor with the factor Strain. Therefore, the results from this table should not be interpreted without taking into consideration the outcomes of the appropriate ANOVAs.*
Abbreviations used: BN, Brown Norway; F344, Fischer-344; LE, Long Evans; LEW, Lewis; SD, Sprague Dawley; SHR-SP, Spontaneous Hypertensive Stroke-Prone; WISW, Wistar (Winkelmann); WKY, Wistar Kyoto
*$^{+}$ $p < 0.01$; * $p < 0.05$*

		Rat strain							
Measure		BN	F344	LE	LEW	SD	SHR-SP	WISW	WKY
1	Body weight	23.50^{+}	14.41^{+}	12.42^{+}	7.06^{+}	16.66^{+}	17.86^{+}	10.23^{+}	20.85^{+}
Emotionality-related measures									
2	Vocalization	-	-	-	-	-	-	-	-
3	Defecation	-	-	-4.39^{+}	-2.59^{+}	-	-	-	-2.69*
4	Urination	-	-	-	-	-	-2.75*	-	-
Behavioral deficits									
5	Ipsilateral grasping	3.67^{+}	3.36^{+}	7.32^{+}	3.00^{+}	3.79^{+}	4.58^{+}	10.85^{+}	4.58^{+}
6	Contralateral grasping	-	-	-	-	-	5.66^{+}	4.02^{+}	-
7	Latency to move one body length	-	10.35^{+}	-	-	-	3.00*	2.73*	-
8	Latency to leave inner circle	-	14.96^{+}	-	-	-	3.66^{+}	4.45^{+}	-
9	Latency to leave outer circle	-	15.70^{+}	-	-	3.07*	3.94^{+}	3.92^{+}	-
10	Ipsilateral circling	-	-2.45*	-	-	-	-	-2.33*	-4.29^{+}
11	Contralateral circling	-	-	5.78^{+}	-	-	2.76*	2.32*	2.94*
12	C-shaped lateral bending of body	-	3.14*	6.09^{+}	3.09*	3.16*	6.00^{+}	6.32^{+}	13.50^{+}
13	Forelimb flexion	-	8.13^{+}	3.74^{+}	2.71*	3.67^{+}	6.04^{+}	6.00^{+}	14.00^{+}
14	Hindlimb flexion	-	3.54^{+}	17.68	4.00^{+}	2.94*	9.30^{+}	3.67^{+}	29.00^{+}
15	Ipsilateral corneal reflex	-	-	-	7.32^{+}	-	-	-	-
16	Contralateral corneal reflex	-	-	-	-	-	-	-	-
17	Paw test: latency to retract forepaw	2.33*	2.93*	-	-	2.38*	2.28*	2.57*	5.18^{+}
18	Paw test: latency to retract hindpaw	2.58*	4.27^{+}	-	-	-	-	3.20^{+}	4.50^{+}

C-shaped lateral bending of the body: before the MCA-O, rats did not show C-shaped lateral bending of the body (Fig. 1, 2nd row, right panel), but after MCA-O C-shaped bending of the body of differing severity was seen depending on the rat strain (Difference scores for Strain: $F_{7,72} = 6.69$, $p < 0.01$). BN rats appeared to be unaffected by the occlusion (see Table 1).

Forelimb and hindlimb flexion: the MCA-O caused fore- and hindlimb flexion in all strains except the BN strain (Difference scores for Strain for forelimb flexion: $F_{7,72} = 3.69$, $p < 0.01$; for hindlimb flexion: $F_{7,72} = 4.05$, $p < 0.01$). Note that the pattern of behavioral deficits for these two variables was different over strains (Fig. 1, 3rd row, left panel).

Correlation: the two measures fore- and hindlimb flexion were correlated across strains (Kendall rank correlation coefficient: $\tau = 0.69$, $p < 0.05$).

Corneal reflex: the MCA-O did not affect the corneal reflex of the contralateral eye, whereas it impaired the reflex of the ipsilateral eye of the LEW strain (Difference scores for Strain: $F_{7,72} = 7.86$, $p < 0.01$) (see Table 1). The ipsilateral eye of these animals was not as open as the contralateral eye.

Time to retract the contralateral fore- and hindlimbs from the paw test apparatus: before occlusion, the rats retracted their forelimbs from the holes of the paw test apparatus as soon as they were released. After the occlusion, there were clear strain differences in the latency to retract the forelimb (Testing session by Strain interaction: $F_{7,72} = 5.49$, $p < 0.01$, Fig 1, 3rd row, center panel). In particular, the mean summed latency to retract the contralateral forelimb exceeded 30 seconds in the F344, WISW and WKY rats. No effects were seen in the LE and LEW rats. In the BN rats, the effect was small, but was confirmed statistically (see Table 1).

The effects of the MCA-O on the time to retract the contralateral hindlimb (Fig. 1, 3rd row, right panel) were less severe than for the forelimbs. Again the effect was different over strains (Testing session by Strain interaction: $F_{7,72} = 2.84$, $p < 0.05$). The LE, LEW, SD, and SHR-SP rats appeared to be unaffected (see Table 1).

Correlation: the two measures latency to retract the fore- and hind-limbs contralateral to the MCA-O from the holes of the paw test apparatus were correlated across strains (Kendall rank correlation coefficient: $\tau = 0.89$, $p < 0.01$).

Feeding behavior: before surgery, all rats picked up the sunflower seeds, and held them with both forepaws. After surgery, most of the rats either did not approach the food, or did not pick up the seeds. Therefore, reliable data could not be collected for this test, and further statistical evaluation was omitted.

Second experiment: effects of proximal and distal unilateral MCA-O in LE, LEW, and SHR-SP rats

Material and Methods

Subjects

Male LE, LEW, and SHR-SP rats were used. The suppliers were the same as in the first experiment. Housing conditions were same as described for the previous experiment.

Surgery

Twenty rats per strain were randomly assigned to one of two conditions: ten animals received a unilateral MCA-O close to its origin (proximal occlusion: Shigeno et al., 1985; Shiraishi & Simon, 1989), and ten other rats received more distal occlusions (Bederson et al., 1986; Shigeno et al., 1985). The first and second experiment were run in parallel, the rats receiving the proximal occlusions were taken from the first experiment.

Behavioral Tests

The protocol for the behavioral tests was identical to that of the first experiment. A blind procedure was applied. The experimenter did not know which occlusion method had been applied in the individual animal.

Statistical evaluation

Scoring was as in the first experiment. These scores were analyzed by a two-way ANOVA with the factors Strain (LE, LEW, SHR-SP), Occlusion site (proximal versus distal occlusion) and the repeated measures factor Testing session (behavior before versus behavior after occlusion).

For measures representing ratings, effects of the testing session (i.e. of the occlusion) were estimated by using difference scores between the raw pre- and post-occlusion measurements. These difference scores were evaluated statistically by an Occlusion site by Strain ANOVA. In addition, *t*-statistics were used to test the hypothesis that the difference scores (post-occlusion session minus pre-occlusion session) of particular Occlusion site by Strain groups deviated from zero.

Results

Strain differences were observed for all variables measured, except with respect to vocalization and to the contralateral corneal reflex, during the pre- as well as the post-occlusion testing session. Here, only measures are considered and discussed which showed a main effect of the factor Occlusion site or which were differentially affected by this factor (as indicated by Occlusion site by Strain and/or Testing session interactions). Effects of Testing session cannot be detected if ranked scores are considered. Instead, the difference scores on raw data (post-occlusion minus pre-occlusion scores) provide the best estimate for effects of the factor Testing session.

Body weight and physical condition

After surgery, the body weight in all Occlusion site by Strain groups decreased (Testing session: $F_{1,52}$ = 463.83, $p < 0.01$). The decrease was more pronounced in the rats with the proximal occlusion (Testing session by Occlusion site interaction: $F_{1,52}$ = 66.40, $p < 0.01$). The general physical condition 2 days after surgery was reasonable. Physical examination revealed that the wounds were properly closed.

Emotionality-related measures

Vocalization, urination, defecation: neither vocalization, nor urination was affected by the MCA-O, irrespective of the occlusion sites. Defecation scores decreased after the two types of occlusion (Difference scores for Occlusion site: $F_{1,52}$ = 4,94, $p < 0.05$), the reduction being greater in the rats that had undergone proximal occlusion (compare also Table 2).

Behavioral deficits

Walking initiation: the *latency to move one body length* (Fig. 2, upper left panel) was different between strains (Testing session by Strain interaction: $F_{2,52} = 13.60$, $p < 0.01$), and differently affected by the occlusion site (Testing session by Occlusion site interaction: $F_{1,52} = 9.02$, $p < 0.01$). There appeared to be an increase particularly in the rats with distal occlusion. *t*-tests on difference scores (post-occlusion testing scores minus pre-occlusion testing scores), however, confirmed this increase for the SHR-SP rats only, irrespective of the occlusion site (see Table 2). In contrast, the *latency to leave the inner* and the *latency to leave the outer circle* were not differentially affected by the site of the occlusion.

Circling behavior (Fig. 2, upper center panel): contralateral circling was slightly increased after proximal, but not after distal MCA-O (Testing session by Occlusion site interaction: $F_{1,52} = 26.75$, $p < 0.01$). This effect was different for the three rat strains, as indicated by a Testing session by Strain by Occlusion site interaction ($F_{2,52} = 4.26$, $p < 0.05$). Before MCA-O, LE rats already showed some contralateral circling (Strain by Occlusion site interaction during the pre-operation testing session: $F_{2,52} = 6.56$, $p < 0.01$), which became more pronounced after proximal MCA-O.

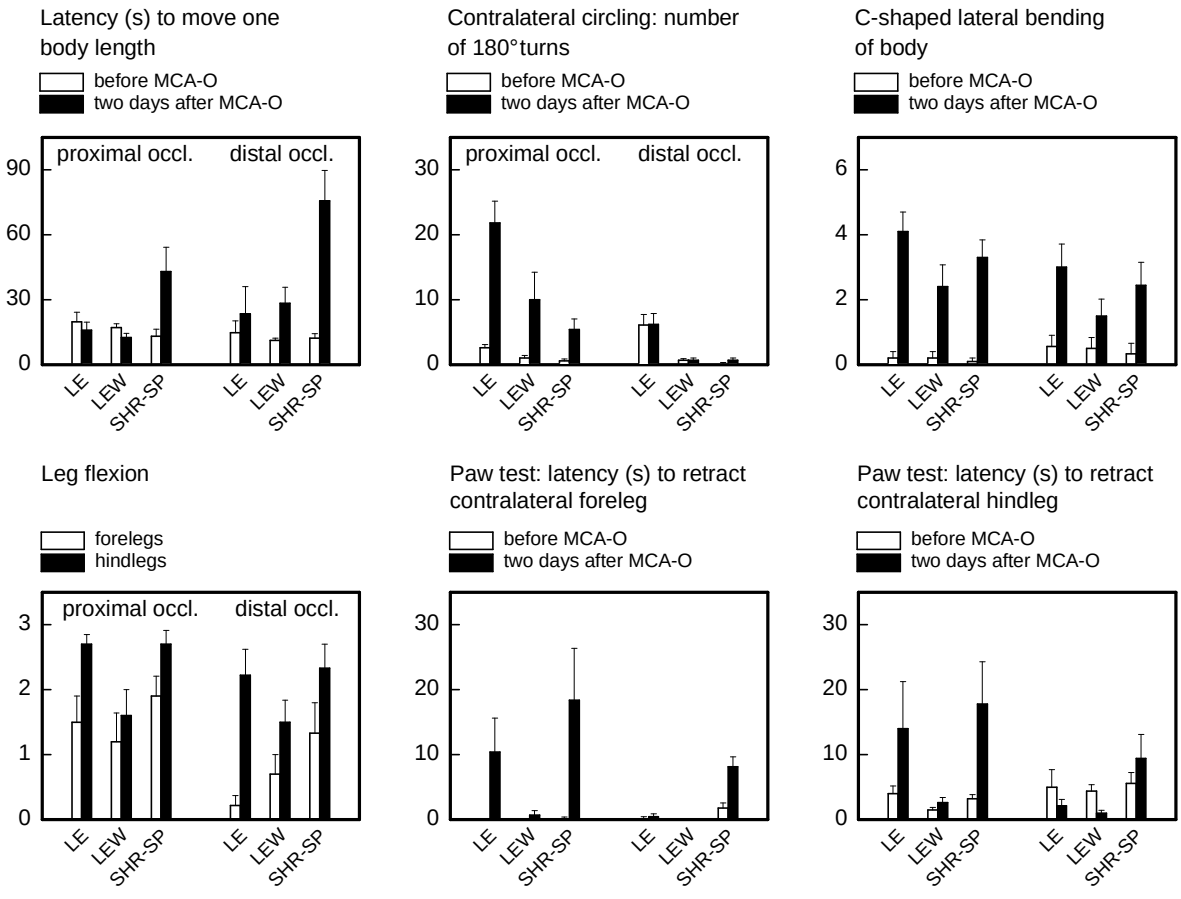

Figure 2. *Behavioral effects of unilateral occlusion of the left proximal or distal MCA in rats of three different strains. The means and standard errors of the means for latencies to move one body length (upper left panel), contralateral circlings (upper center panel), C-shaped bending of body (upper right panel), flexion of the forelegs and hindlegs (lower left panel), latencies to retract the contralateral foreleg (lower center panel), and latencies to retract contralateral hindleg from the paw test apparatus (lower right panel) are depicted.*

C-shaped lateral bending of the body: proximal and distal MCA-Os induced C-shaped bending of the body to a different degree (Difference scores for Occlusion site: $F_{1,52} = 5.94$, $p < 0.05$; Fig. 2, upper right panel); the proximal occlusion induced C-shaped bending more severe than the distal one did.

Forelimb and hindlimb flexion: forelimb flexion was induced by both proximal and distal MCA-O, the flexion being more pronounced after proximal occlusion (Difference scores for Occlusion site: $F_{1,52} = 6.98$, $p < 0.05$; Fig. 2, lower left panel). Hindlimb flexion was induced to a similar extent by proximal and distal MCA-O.

Table 2. *t-values on difference scores (post-occlusion testing scores minus pre-occlusion testing scores) to evaluate effects of unilateral MCA-O on body weight, vocalization, defecation, urination, and on a battery of behavioral tests in three rat strains. Rats had undergone either proximal or distal MCA-O. All differences were calculated on raw data. We tested whether the difference scores within Occlusion Site by Strain groups deviated from zero.*
Note that for some measures statistically reliable differences were detected by the individual t-tests, despite the fact that ANOVAs (see text) did not indicate main effects of the factor Testing Session, or of interactions of the factor Testing Session with the factors Occlusion Site and/or Strain. Therefore, the results from this table should not be interpreted without taking into consideration the outcomes of the appropriate ANOVAs.
*$^{+}$: $p < 0.01$; *: $p < 0.05$*

		Rat strain					
		LE	**LEW**	**SHR-SP**	**LE**	**LEW**	**SHR-SP**
Measure		**Proximal MCA-occlusion**			**Distal MCA-occlusion**		
1	Body weight	12.42^{+}	7.06^{+}	17.86^{+}	6.73^{+}	5.80^{+}	10.66^{+}
Emotionality-related measures							
2	Vocalization	-	-	-	-	-	-
3	Defecation	-4.39^{+}	-2.59*	-	-	-	-
4	Urination	-	-	-2.75*	-	-2.71*	-
Behavioral deficits							
5	Ipsilateral grasping	7.32^{+}	3.00*	4.58^{+}	3.16*	4.15^{+}	2.29*
6	Contralateral grasping	-	-	5.66^{+}	-	-	5.38^{+}
7	Latency to move one body length	-	-	3.00*	-	2.37*	4.72^{+}
8	Latency to leave inner circle	-	-	3.66^{+}	-	-	5.07^{+}
9	Latency to leave outer circle	-	-	3.94^{+}	-	-	4.87^{+}
10	Ipsilateral circling	-	-	-	-2.96*	-	-
11	Contralateral circling	5.78^{+}	-	2.76*	-	-	-
12	C-shaped lateral bending of body	6.09^{+}	3.09*	6.00^{+}	3.65^{+}	2.37*	2.80*
13	Forelimb flexion	3.74^{+}	2.71*	6.04^{+}	-	2.33*	2.83*
14	Hindlimb flexion	17.68^{+}	4.00^{+}	9.30^{+}	5.55^{+}	3.77^{+}	3.79^{+}
15	Ipsilateral corneal reflex	-	7.32^{+}	-	-	5.07^{+}	-
16	Contralateral corneal reflex	-	-	-	-	-	-
17	Paw test: latency to retract forepaw	-	-	2.28*	-	-	3.41^{+}
18	Paw test: latency to retract hindpaw	-	-	-	-	-5.35^{+}	-

Time to retract the contralateral fore- and hindlimb from the paw test apparatus: even before MCA-O, SHR-SP rats were slower to retract their forelimbs from the holes of the paw test apparatus than rats of the other two strains (Fig. 2, lower center panel) in the distal occlusion group (Occlusion site by Strain interaction: $F_{2,52} = 3.45$, $p < 0.05$). After surgery, the time to retract the contralateral foreleg increased most in the SHR-SP rats (see Table 2; Testing session by Strain interaction: $F_{2,52} = 5.08$, $p < 0.05$).

The effects of the occlusions on the latency to retract the contralateral hindlimb (Fig. 2, lower right panel) were similar to those found for the contralateral forelimb. Again, the effect was most pronounced in rats with proximal occlusion (Testing session by Occlusion site interaction: $F_{1,52} = 5.63$, $p < 0.05$). Note that in the LEW rats with distal occlusion, the latency was reduced by the occlusion (see Table 2).

Feeding behavior: reliable data could not be collected for this test (compare experiment I) and further statistical evaluation was omitted.

Third experiment: strain differences in infarct volume after MCA-O and comparison of the effects of proximal and distal occlusions

Because the survival times in the first two experiments were different (ranging from 0 to 28 days after behavioral testing), we could not compare infarct volumes between strains or between strain by occlusion site groups, as infarct volumes decrease non-linearly with longer survival time (unpublished observations; Persson et al., 1989). Therefore, additional rats underwent the same surgery, but their behavior was not assessed. These animals were killed 7 days after MCA-O to make sure that the determination of cortical and striatal infarct volumes were not biased by ischemia-induced early cerebral edema. The brains were processed histologically to determine infarct volumes in the cortex and the caudate/putamen.

Material and Methods

Subjects

Male rats of the BN (n = 7), F344 (n = 5), LE (n = 6), LEW (n=6), SD (n = 6), SHR-SP (n= 6), WISW (n =7) and WKY (n = 5) strain were used. The rats received proximal MCA-O as described in the first experiment. In addition, male LE (n = 5), LEW (n = 7) and SHR-SP rats (n = 7) received distal occlusion of the MCA, as described in the second experiment. Suppliers were the same as in experiments 1 and 2.

Histological evaluation of the brain damage caused by proximal and distal middle cerebral artery occlusion

The rats were decapitated 7 days after MCA-O. Their brains were rapidly removed and cooled down (between -30° and -40°C) in n-methyl butane (E. Mer ck, Darmstadt, Germany). Serial coronal sections (20-µm thick) were cut throughout the infarct (standard distance of 500 µm) with a cryostat microtome (Microm Laborgeräte GmbH, Walldorf, and Reichert-Jung, Leica Vertrieb GmbH, Cologne, Germany). Slide-mounted tissue sections were stained with cresyl fast violet. The volume of the cortical and striatal infarct was determined with a computer-assisted image analysis system (Optimas, BioScan Inc., Edmonds, WA, USA). Infarct volumes are expressed in mm^3 ± SEM.

Statistical analysis

A) *Strain comparison:* infarct volumes were analyzed by an analysis of variance with the factor Strain, followed by post-hoc Least Significant Difference (LSD) comparisons.

B) *Effects of proximal versus distal occlusions:* an analysis of variance with the factors Strain and Occlusion site was performed. The analysis was supplemented with post-hoc LSD comparisons between the six Strain by Occlusion site groups.

C) *Exploratory analysis of the relationship between behavioral deficits and cortical infarct volume:* we were interested in determining the relationship between the volume of the infarcted area and the occlusion-induced behavioral deficits. Unfortunately, behavioral data and data on the infarct volumes were not available from the same rats. However, all but the SD and WISW are inbred strains which means that the genotype in six of the eight strains is exactly specified. Consequently, the results from different studies in which the same genotypes are used can be readily compared (Russell & Gibson, 1972).

The sums of ranks of the different strains might provide the best estimate of the 'true' ranking of the effects of the unilateral MCA-O on behavioral measures. Two groups of measures can be distinguished: the first group (vocalization, defecation, urination) might be related to emotional reactivity. The second one might reflect behavioral impairments (see also Tables 1 and 2: measures 5 to 18). For both clusters, the sums of ranks were determined per strain (from the second group of measures, however, measures 10, 15 and 16 were omitted, because they were not affected by the MCA-O). These sums were ranked over strains, and the relationship between the ranked infarct volumes and the ranked sums of ranks of the two clusters was determined separately by Kendalls rank correlation coefficient τ.

Results

Strain comparison: the infarct volumes in cortex and striatum of rats of the eight strains are shown in Figure 3, left panel. The cortical infarct volumes differed between strains ($F_{7,41} = 27.31$, $p<0.01$). Post hoc analysis confirmed that the infarct volumes were greatest in SHR-SP and LE rats, and smallest in WKY and LEW rats. No strain differences were found for the infarct volumes in the striatum ($F_{7,41} = 1.04$, n.s.).

Effects of proximal versus distal occlusions: the infarct volumes of the six Strain by Occlusion site groups are given in Figure 3, right panel. Strain differences for the cortical infarct volumes were confirmed ($F_{2,31} = 124.49$, $p < 0.01$). The proximal occlusions produced, on average, bigger infarcts than the distal occlusions did ($F_{1,31} = 20.93$, $p < 0.01$). There was, however, a Strain by Occlusion site interaction ($F_{2,31} = 3.93$, $p < 0.05$), indicating that the differences in infarct volume produced by the two occlusions were different in the three strains used. Post-hoc analysis confirmed that the proximal occlusion induced greater infarct volumes in the cortices of the SHR-SP rats only, whereas the infarctions produced by proximal and distal occlusions were similar in the LE and the LEW rats.

The proximal MCA-O induced a bigger infarction in the caudate/putamen than the distal occlusion did ($F_{1,31} = 49.03$, $p < 0.01$). This effect was similar in the three rat strains, as neither strain differences ($F_{2,31} = 1.26$, n.s.) nor Strain by Occlusion site interactions ($F_{2,31} = 1.47$, n.s.) were found.

Relationship between behavioral deficits and volume of the cortical infarct: the relationship between infarct volume and the index for emotional reactivity was $\tau = 0.357$ (p-value for n = 8, estimated via Z-

approximation of associated p-values: p > 0.1, n.s.). This lack of correlation might have been expected because the MCA-O did not affect emotionality related measures.

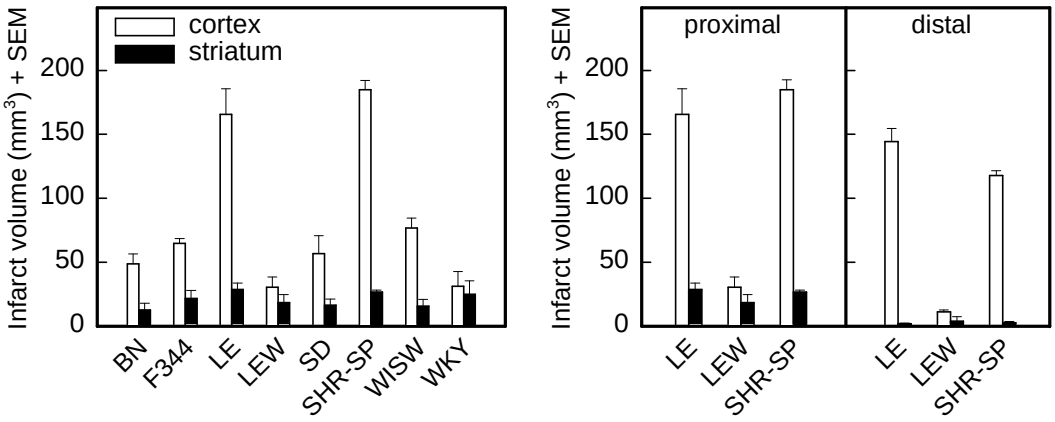

Figure 3. *Cortical and striatal infarct volumes (mm^3) of eight different rat strains (left panel) after proximal MCA-O, and of three different rat strains after either unilateral proximal or distal MCA-O (right panel) are depicted as means and standard errors of the means (SEM).*

There was no relationship between cortical infarct volume and behavioral deficits (which was calculated from the ranked sum of ranks over measures 5 to 18; measures 10, 15 and 16 excluded; compare Tables 1 and 2; τ was 0.286, with p > 0.1, n.s.). From the ranked sum of ranks over measures 5 to 18 (measures 10, 15 and 16 excluded), we conclude that the effects induced by the MCA-O were most profound in the SHR-SP, the WISW and WKY rats, and least severe in the BN, SD and LEW rats. Different rank orders of the severity of impairments over strains, of course, might be found when other subsets of the measures are considered.

Discussion

Three experiments were performed to investigate: first, whether MCA-O has different effects on sensorimotor functions of different rat strains; secondly, whether proximal MCA-O produces more severe behavioral dysfunctions than distal occlusions; and thirdly, whether infarct volume and degree of behavioral impairments are related.

MCA-O did not affect emotionality-related measures (i.e. vocalization, defecation, and urination). This result is consistent with the finding of Tominaga and Ohnishi (1989), who observed that the time spent on the light and dark sides of a two-compartment passive avoidance apparatus during a 3-minute adaptation period was not affected by MCA-O in SD rats. An increased or decreased amount of time spent in the dark compartment would have been an index of increased or decreased fear or emotionality (van der Staay, Kerbusch & Raaijmakers, 1990).

Experiment 1 confirmed that the pattern of sensorimotor malfunctions induced by proximal unilateral MCA-O are highly strain dependent. MCA-O impaired performance in the tests grasping with

hindpaws, latency to move one body length (walking initiation), latency to leave the inner and outer circles, contralateral circling, C-shaped lateral bending of the body, fore- and hindlimb flexion, and the time to retract the contralateral fore- and hindlimbs from the paw test apparatus. Correlation analysis across strains confirmed that measures were related within particular tests. Since the three measures of walking initiation were highly correlated across strains, one might well dispense with two of them (Walsh & Cummins, 1976) and use only the first measure. The same holds true for the limb-flexion test, where measuring either fore- or hindlimb flexion would be sufficient, and for the paw test, where it would suffice to measure either the latency to retract the fore- or the hindpaw.

Interestingly, the MCA-O affected grasping with hindpaws not only on the contralateral side, which was expected, but also at the ipsilateral side. This may be due to enlargement of the ipsilateral hemisphere by cytotoxic edema with a subsequent increase in intracerebral pressure (Persson et al., 1989) during the acute phase after infarction, i.e. the first days after MCA-O.

The most severe impairments were seen in the WISW, SHR-SP, and WKY rats (generally considered as the normotensive control strain for the SHR and the SHR-SP strain). These three strains are Wistar-derived. By contrast, the BN rats showed only mild behavioral deficits after the MCA-O. Especially in the WKY strain, the severe disruption of behavior after MCA-O contrasts with the estimated infarct volume, which was relatively small in this strain (compare experiment 3).

Strain differences in the cerebrovascular anatomy, for example the degree of branching of the MCA, the diameter and distribution of cerebral vessels and of the collateral supplier system, might explain the great variety of behavioral effects of MCA-O seen in the different strains. Breuer and Mayevsky (1992), for example, described differences between the vascular pattern in different lines of gerbils belonging to the species *Meriones unguiculatus* and *Meriones tristrami*. The anatomical patterns of blood vessel distribution in the brains of gerbils are genetically determined. Barone and colleagues (1993) found that the vascular anatomy at the level of the posterior communicating arteries differs in various mouse strains. They hypothesized that these differences are related to the sensitivity of mouse strains to cerebral ischemia. In rats, branching of the MCA of the Sprague Dawley strain was found to be different from that of the SHR strain (Shiino, 1989). Non-systematic observations in the rats strains used in the present study support the notion of pronounced strain differences in the branching of the MCA.

Moreover, Coyle and Jokelainen (1982) described numerous inter-arterial anastomoses between the middle cerebral artery and the anterior cerebral artery (ACA) in Wistar rats. Comparing WKY and SHR rats, Coyle, Odenheimer, and Sing (1984) found fewer collateral vessels between the ACA and the MCA of SHR rats. In a study comparing inter-arterial anastomoses of Sprague Dawley rats and Wistar rats from Simonsen Laboratories, and of Sprague Dawley rats from Taconic Laboratories and Charles Rivers Laboratories, respectively, fewer anastomoses were found in the rat strains from Simonsen Laboratories than of the other two breeders (Oliff, Coyle & Weber, 1997). Although it is conceivable that anastomoses provide alternate routes of blood flow after MCA-O, Oliff, Coyle, and Weber (1997) did not find evidence for the notion that the patterning, or diameter of these inter-arterial connections account for differences in the lesion size between strains and lines of rats. To our knowledge, no information is available with respect to anastomoses between ACA and MCA in the other strains involved in our study.

The second experiment confirmed that an MCA-O close to its origin under the lenticulostriate branch (proximal occlusion: Shigeno et al., 1985; Shiraishi & Simon, 1989) induces slightly more behavioral malfunctions, because of the partial infarction of the caudate/putamen, than a distal occlusion does

(Shigeno et al., 1985). Differential effects of the lesion site were seen in the tests latency to move one body length (walking initiation), circling behavior, C-shaped lateral bending of the body, fore- and hindlimb flexion, and time to retract the contralateral fore- and hindlimb from the paw test apparatus.

Table 3. *Infarcted cortical areas after proximal occlusion of the MCA in eight rat strains. The brains of the rats were removed and photographed before histological processing. The infarcted areas were determined by simple visual inspection of the brains after removal from the skull and by analyzing photographs. The approximate proportion of infarction per area is indicated. The estimations are based on the stereotaxic atlas of the rat brain by Paxinos and Watson (1986).*
Note, that the areas in which infarcts were found were extremely variable within the WISW strain.

	Rat strain							
Cortical area	BN	F344	LE	LEW	SD	SHR-SP	WISW	WKY
Agranular insular cortex, dorsal part	<½	<¼	½	½	½	½	<½	<¼
Agranular insular cortex, posterior part	½	<½	½	>½	1	½	½	-
Agranular insular cortex, ventral part	1	-	½	1	1	½	1	1
Forelimb area	-	-	½	-	-	½	-	-
Frontal cortex, area 1, primary motor cort.	-	-	½	-	-	½	-	-
Frontal cortex, area 3	-	-	1	1	-	1	<¼	-
Gustatory cortex	1	1	1	-	1	1	1	-
Hindlimb area	-	-	½	-	-	½	-	-
Lateral orbital area	-	-	-	-	-	-	-	1
Primary visual cortex, binocular part	-	-	½	-	-	½	-	-
Primary visual cortex, monocular part	-	-	½	-	-	½	-	-
Occipital cortex, area 2, lateral part	-	-	½	-	-	½	<¼	-
Occipital cortex, area 2, mediolateral part	-	-	1	-	-	1	-	-
Occipital cortex, area 2, mediomedial part	-	-	½	-	-	½	-	-
Primary somatosensory cortex	<¼	<½	1	¼	½	1	½	-
Supplementary somatosensory cortex	½	1	1	1	-	1	1	-
Prepiriform cortex (primary olfactory)	-	-	-	-	-	-	-	>½
Perirhinal area	-	-	½	-	-	½	-	-
Temporal cortex, area 1	-	-	1	½	½	1	1	-
Temporal cortex, area 2	-	-	½	-	-	½	-	-
Temporal cortex, area 3	-	-	1	½	1	1	½	-
Olfactory tubercle	-	-	-	-	-	-	-	<½

Histological evaluation of the brain damage caused by proximal and distal MCA-O confirmed that the damage after distal MCA-O nearly exclusively involved cortical areas, the caudate/putamen being spared (Shigeno et al., 1985; Bederson et al., 1986). By contrast, proximal occlusion also affected subcortical areas (i.e. the dorsolateral caudate/putamen) (Shigeno et al., 1985). In the cortex, only the SHR-SP rats showed larger infarcts after proximal compared with distal MCA-O. The occlusion site did not differentially affect the cortical infarct volumes in the LE and LEW rats.

The infarct volumes of SHR and WKY rats are well within the range reported by others (e.g. Benavides et al., 1990). However, in order to evaluate the relationship between infarct size and behavioral deficits, it is also necessary to know which cortical and subcortical areas are destroyed or affected by the MCA-O. An attempt to classify the infarcted areas, based on the rat brain atlas by Paxinos and Watson (1986) of the eight rat strains, is summarized in Table 3.

We analyzed the data of the third experiment by using ranked sum scores of ranks of the different neurological scores rather than the sum scores, as other authors have done, to assess the behavioral consequences of focal brain ischemia (e.g. Bederson et al., 1986; Wahl et al., 1992; Garcia et al., 1995; Katsuta et al., 1995), because scoring was different between neurological tests. Our results did not support the notion that *across* strains infarct size is related to the behavioral consequence of the MCA-O. While correlation coefficients do not unravel causal relationships, if there are no reliable correlations between variables, then it is unlikely that the variables are related causally. Thus, our data suggest that sensorimotor malfunctions cannot be predicted from the infarct volume. However, there still may be a relationship between the size and location of the infarcted area and the degree and type of behavioral impairments *within* particular strains, as has been reported for Sprague-Dawley rats (Bederson et al., 1986; Obana, Pitts & Nishimura, 1988; Markgraf et al., 1992; Rogers et al., 1997; Lyden et al., 1997). Others, however, did not find this type of correlation when using this rat strain (Wahl et al., 1992).

In conclusion, our results show that different rat strains are differently affected by MCA-O, that the occlusion site affects the infarct volume, and that there is no simple relationship between the volume of the infarct and the severity of behavioral malfunctions. Wistar-derived rat strains (WISW, WKY and SHR-SP) appear to develop more severe behavioral dysfunctions after MCA-O than other strains do.

4.3
Unilateral middle cerebral artery occlusion does not affect water-escape behavior of CFW1 mice*

Abstract

Male CFW1 mice acquired the standard Morris water-escape task *before* half of the animals received an unilateral occlusion of the middle cerebral artery (MCA). Retention was then measured in one session. In addition, the mice acquired a new platform position during daily training sessions on four consecutive days. In a second experiment, naive male CFW1 mice acquired the water-escape task *after* surgery. At the end of the fifth session, a probe trial was given. In both experiments the control group consisted of mice that had been sham-operated: their MCA was exposed surgically, but was left intact. Even though the MCA-occlusion-induced infarcts in the CFW1 mice covered the cranial part of the dorsomedial cortex (destroying substantial areas of the primary somato-sensory cortex and smaller parts of the primary motor cortex) and part of the striatum, disabling behavioral impairments in the Morris water-escape task were not observed. Surgery *per se*, however, seemingly had disruptive effects on water-escape behavior.

Introduction

A widely used method to induce stroke in rodents consists of transient or permanent occlusion of the middle cerebral artery (MCA) (Rogers et al., 1997). These rodent models of stroke are considered to be of particular relevance with respect to human stroke (Katsuta et al., 1995). The permanent MCA occlusion (MCA-O) results in a consistent focal cerebral infarct (Tamura et al., 1985), which has been reported to cause neurological dysfunctions (e.g. Bederson et al., 1986; Wahl et al., 1992; Rogers et al., 1997), and deficits in learning and memory, assessed in, for example, inhibitory or passive avoidance tasks (e.g. Tamura et al., 1985; Yamamoto et al., 1991; Hirakawa et al., 1994) and in spatial discrimination tasks (e.g. Markgraf et al., 1992; Okada et al., 1995a,b; Kumon et al., 1996; Smith et al., 1996). However, the effects of MCA-O on cognitive functioning have not yet been reported for mice.

Using CFW1-mice, we have observed that MCA-O has no apparent effect on the behavior of mice in the home-cage. Within a few hours after surgery the mice were able to resume their normal eating pattern, to move around and to climb without any visible deficits. Because of the apparent normality of sensorimotor functions seen after MCA-O, we performed two experiments to assess whether occlusions affect performance in a complex learning and memory task.

* This chapter is based on the publication: van der Staay, F.J., Stollenwerk, A., Horváth, E. & Schuurman, T. (1992). Unilateral middle cerebral artery occlusion does not affect water-escape behavior of CFW1 mice. *Neuroscience Research Communications*, **11**(1), 11-18.

The water-escape task in a circular pool was used (Morris, 1984). This task assesses spatial learning and memory and is sensitive to the effects of naturally occurring and experimentally induced impairments in brain functions in rodents. The standard Morris water-maze task measures predominantly spatial reference memory (RM; Mundy, Barone & Tilson, 1990). The reference memory holds trial-independent information (Barnes, 1988b) about, for example, the position of the escape platform in the water tank, which the animal is required to learn. The task cannot be solved by using olfactory, visual or kinesthetic cues (Hagan et al., 1983). In addition, the task is acquired relatively rapidly and there is no need to apply deprivation procedures (Lamberty & Gower, 1991a).

The Morris maze has been used with rats to assess, for example, impairments due to normal aging (e.g. Aitken & Meaney, 1989; see also Chapter 2), hippocampal lesions (Whishaw, 1987), fimbria-fornix lesions (Spruijt et al., 1990) nucleus basalis magnocellularis (nbm) lesions (Mundy, Barone & Tilson, 1990), ablations of the frontal (Fantie & Kolb, 1990; Kolb, Sutherland & Whishaw, 1983), or parietal cortex (DiMattia & Kesner, 1988; Kolb & Walkey, 1987), complete hemidecortication (Kolb & Tomie, 1988), and permanent MCA-O (Okada et al., 1995a,b; Smith et al., 1996).

Compared with the enormous number of Morris water escape studies with rats as subjects, few studies have been performed using mice (e.g. Denenberg et al., 1991; Lamberty & Gower, 1991a). Wehner, Sleigh, and Upchurch (1990), using DBA and C57Bl mice and 11 recombinant inbred strains derived from these two parental lines, found extreme differences in the capability of the strains to acquire the spatial Morris maze problem. The question addressed in the first experiment was therefore whether CFW1 mice are able to learn to escape onto an invisible platform in the place version of the water-escape task (Morris, 1984). We then investigated whether unilateral occlusion of the MCA affects the retention of the water-escape behavior that had been acquired before surgery. In addition, the acquisition of a new position of the escape platform (reversal learning) after MCA-O was assessed. In a second experiment, we studied the effects of MCA-O on the acquisition of the water-escape task in naive mice.

First experiment: effects of MCA-O on the retention and reversal of a water escape task in CFW1 mice

Material and Methods

Animals

Twenty male CFW1 mice, weighing 34.7 ± 0.5 grams (mean ± SEM), were supplied by Winkelmann (Borchen, Germany). The animals were housed in groups of ten in standard Makrolon type III cages. They were kept under an artificial 12 hour light/12 hour dark regimen (lights on from 7:00 to 19:00) in a temperature (ca. 21.5℃) and humidity (50%) control led animal room. Water and food were available ad libitum. Before testing, the animals were transferred to the experimental room where they were housed throughout the entire testing period. Housing conditions were similar to those in the animal room.

Surgery

Surgery was performed according to Welsh et al. (1987) with minor modifications. Briefly, mice were anesthetized with chloral hydrate (400 mg/kg i.p.). During surgery body temperature was monitored and maintained between 37℃ and 38℃ with a warming pad. On the left side of the head the skin was opened vertically between the orbit and the external ear canal. The dorsal and caudal margins of the temporalis muscle were detached with scissors and partially removed. The remaining part was folded forwards so that the upper lateral aspect of the skull became visible. The facial nerve, the eye muscles, and the zygomatic bone were left intact. The MCA was exposed under an operating microscope. After the dura had been opened, the MCA and its branches were occluded by microbipolar electrocoagulation, followed by the removal of the occluded vessels. In sham-operated mice the MCA was exposed, but not occluded. The temporalis muscle and the skin were closed with tissue glue (Histoacryl, Braun-Melsungen, Melsungen, FRG) and the wound was treated with bacteriostatic powder (Marfanil/Prontalbin, Bayer, Leverkusen, FRG). After recovery from anesthesia mice were returned to their home cage.

Apparatus

The water tank consisted of a circular gray tub (diameter 77 cm; depth 27 cm) filled with water (22℃) to a depth of 16.5 cm. The escape platform was a solid gray cylinder (diameter 8 cm) submerged 1.0 cm below the surface of the water. The water was made opaque, by adding milk powder, so that the mice could not locate the submerged platform by visual cues. The water tank was situated in a room illuminated by white fluorescent strip lights and by daylight through a window. Abundant extra-maze cues were provided by normal laboratory facilities, including desks, computer equipment, a second water tank, the presence of the experimenter, and by a radio on a shelf that was playing softly, providing background noise.

Behavioral testing

Acquisition of the water-escape task: training consisted of releasing the mouse, facing the wall of the tank, in one of four start locations (north, east, south, or west). Each start position was used once in a series of four trials; the order was determined at random for every subject. The position of the platform (quadrant west) was held constant throughout the acquisition phase. A mouse that escaped onto a platform was allowed to stay there for 10 seconds, before the next trial started. If a mouse failed to escape onto the platform within 60 seconds, it was put onto the platform by the experimenter and was allowed to stay there for 10 seconds. The next trial was then started.

The mice received eight trials in close succession per day (massed trials). They were run on this schedule for eight days (to a total of 64 acquisition trials). All testing was done between 9:00 and 14:00. The mice were matched on their mean escape latencies during the last four trials of the eighth acquisition session, and one of the animals from each matched pair was randomly chosen to receive unilateral occlusions of the MCA (MCA-group). The other animal underwent sham-surgery (sham-group). The sham-group consisted of 8 animals; the MCA-group consisted of 9 animals.

Retention after MCA-O: all mice were allowed to recover from surgery for two days. Then retention of the water-escape behavior was assessed with eight massed trials during one session.

Reversal learning after MCA-O: the procedure was identical to that followed during acquisition and retention sessions. The escape platform, however, was moved from the west quadrant to the east quadrant. Starting on the day after retention testing, the mice were trained to escape onto the invisible escape platform with eight massed trials on each of four consecutive days.

Histological verification

The mice were decapitated seven days after MCA-O. The brains were rapidly removed and frozen in n-methylbutane at -40°C. Coronal sections (20-µm thick) were cut through the infarcted area with a standard distance of 300-µm with a cryostat microtome (Leitz, Wetzlar, FRG). Slide-mounted tissue sections were stained with cresyl fast violet. Cortical and striatal infarct volume was determined with a computer-assisted analyst system (Optimas, BioScan Inc., Edmonds, WA, USA).

Statistical analysis

The development of the body weights after surgery were evaluated by an analysis of variance (ANOVA) with the repeated measures factor Days after surgery (day zero, i.e day of operation, and days 4 and 7 after operation), and the factor Occlusion (sham occlusion vs. MCA-O).

For each mouse and each session of eight trials, the latencies to escape onto the platform and the number of quadrant entries were averaged (Lalonde & Joyal, 1991). These data were used for statistical evaluation. Analyses were performed separately for the eight acquisition sessions, the retention session, and the four reversal sessions.

The rate of acquisition was analyzed by a repeated measures ANOVA over the eight acquisition sessions. The effects of occlusion on retention performance were analyzed by an Occlusion (sham occlusion vs. MCA-O) by repeated-measures factor Sessions (pre-surgery matching session vs. first session after surgery) ANOVA.

The effects of the occlusion on switching to the reversal problem were evaluated by an Occlusion by Sessions (retention session vs. first session of reversal learning) ANOVA, with repeated measures on the factor Sessions.

Finally, differences between occlusion groups on the rate of acquisition of the reversal problem were evaluated by an Occlusion by Sessions (first to fourth session of reversal learning) ANOVA. Again, Sessions was considered as a repeated measures factor. Differences between sham-occluded and MCA-occluded rats during all phases of the experiment were evaluated per session by *t*-statistics.

Results

Histology

In most animals the infarct involved the cranial part of the dorsomedial cortex (including substantial areas of the primary somatosensory cortex and smaller parts of the primary motor cortex), and part of the caudate/putamen. In comparison with animals that were sacrificed two days after occlusion, the infarct area was smaller after seven days (unpublished observations) because of shrinkage of revascularized necrotic tissue infiltrated by glia. This precluded reliable determination of the infarct volume in the present study. An estimate for the acute effects of the occlusion might be derived from data of mice that underwent the same surgical procedure, but which were sacrificed two days after the MCA-O (n = 71). Their average infarct volume (± SEM) was 29.7 mm^3 (± 1.6). Occlusion of the MCA never damaged the hippocampus, a structure critically involved in spatial discrimination learning.

Five of the twenty mice did not complete all phases of the experiment and consequently were not included in the statistical analyses. One mouse was not able to swim long enough to reach the platform. Two mice did not stay on the platform. Instead, they tried to jump to the edge of the water tank, or jumped into the water as soon as they were put onto the platform. One sham-operated mouse

died during surgery; another animal from this treatment group was eliminated from the study after histological evaluation because it suffered from a severe inflammation that destroyed large regions of the ipsi- and contralateral brain.

Body weight of the mice

The two groups had similar weights ($t_{13} < 1.0$, n.s.; see Fig. 1) before operation. The weights (grams ± SEM) at operation (day 0) and on days 4 and 7 after surgery were 35.3 ± 1.1, 33.0 ± 1.3, and 32.0 ± 1.2 grams, respectively, for the six remaining sham-operated mice, and 34.3 ± 0.7, 31.6 ± 1.3, and 31.7 ± 1.2 grams, respectively, for the nine MCA-occluded animals. Weights decreased after surgery (Days after operation: $F_{2,26} = 20.6$, $p < 0.01$). Both groups were similarly affected by the operation (Occlusion by Days after operation interaction: $F_{2,26} < 1.0$, n.s.).

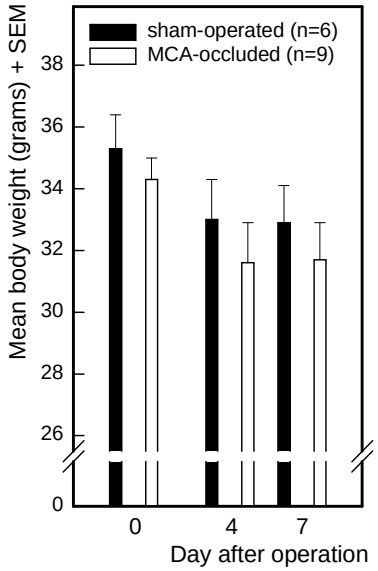

Figure 1. *Mean body weights (grams) and standard errors of the means (SEM) at operation (day 0) and on days 4 and 7 after the operation, for six sham-operated and nine MCA-occluded CFW1 mice.*

Acquisition of the water-escape task: there was a decrease in the escape latencies (Sessions: $F_{7,98} = 8.0$, $p < 0.01$) and the number of quadrant entries (Sessions: $F_{7,98} = 4.8$, $p < 0.01$) over the eight acquisition sessions (Fig. 2)

Matching and retention testing after occlusion: the combined matching on escape latencies and quadrant entries produced highly similar groups with respect to both variables (both $t_{13} < 1.0$, n.s.).

The mice showed a marginal increase in escape latency during the retention session compared with during the eighth acquisition session (Sessions: $F_{1,13} = 3.6$, $0.10 > p > 0.05$). The operation-induced impairment was similar for both the sham-operated and the MCA-occluded group (Occlusion by Sessions interaction: $F_{1,13} < 1.0$, n.s.). Surgery had a clear disruptive effect on the number of quadrant entries. The number of quadrant entries was increased after the operation (Sessions: $F_{1,13} = 9.2$, $p < 0.01$) to a similar extent in both groups (Occlusion by Sessions interaction: $F_{1,13} < 1.0$, n.s.).

Reversal of the water-escape task: compared with the retention session, the escape latencies were marginally higher when the first session of the reversal problem was given (Sessions: $F_{1,13} = 3.3$,

0.10 > p > 0.05). The marginal increase in escape latencies was similar for both the sham-operated and the MCA-occluded groups ($F_{1,13}$ < 1.0, n.s.). There was no increase in the number of quadrant entries on the first reversal session in comparison with the retention session, nor did MCA-O have an effect (all $Fs_{1,13}$ < 1.0, n.s.).

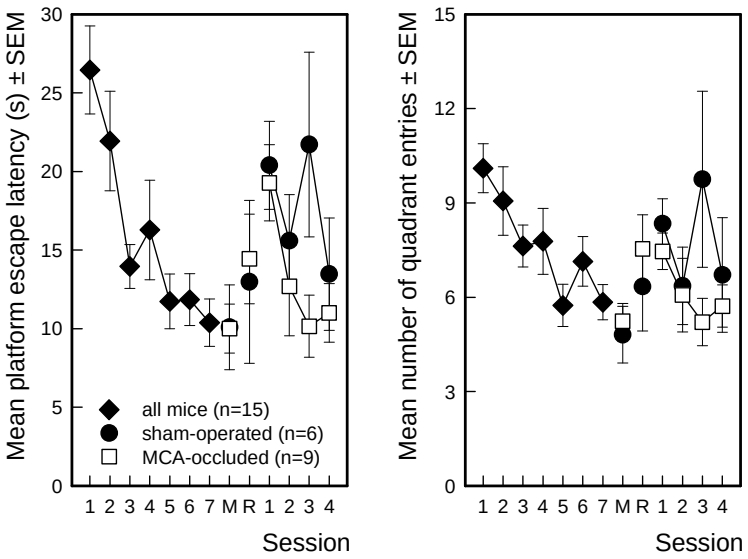

Figure 2: *Session means ± standard errors of the means (SEM) for platform escape latencies in s (left panel) and number of quadrant entries (right panel) of CFW1 mice that were trained to find a submerged platform. After an acquisition phase of eight sessions, the mice were assigned to a sham-operation or a MCA-O group after matching. Results are depicted for the matching session ('M' = eighth acquisition session), the retention session after surgery (assigned 'R'), and for the four acquisition sessions on a reversal problem.*

The impression from Fig. 2 that the escape latencies of the sham-operated mice were, on average, higher than those of the MCA-occluded group was not confirmed statistically (General mean: $F_{1,13}$ < 2.4, n.s.). The mice reduced the time to escape onto the platform in the course of training (Sessions: $F_{3,39}$ = 3.0, p < 0,.05). The rate of acquisition of the reversal problem, however, was similar for both groups ($F_{3,39}$ = 1.6, n.s.).

The results were somewhat different for the number of quadrant entries. The occlusion had no effect on the number of quadrant entries (General mean: $F_{1,13}$ = 2.6, n.s.), and there was no improvement over sessions (Sessions, and Occlusion by Sessions interaction: $Fs_{3,39}$ < 1.4, n.s.).

Second experiment: effect of MCA-O on the acquisition of a water escape task in naive CFW1 mice

Material and Methods

Animals: twenty male CFW1 mice, weighing 30.1 ± 0.3 grams (mean and SEM), were supplied by Winkelmann (Borchen, Germany). The housing conditions were as in the first experiment.

Surgery: the mice were pair-matched for body weight and were assigned to a sham-operated or a MCA-occluded group, using an ABBA rule. The surgery was performed as in experiment 1.

Apparatus: The same apparatus as in the first experiment was used.

Behavioral testing: after surgery the mice were allowed to recover for two days. The training procedure was the same as in experiment 1. In addition, a probe trial (trial no. 41) was given in the fifth acquisition session, approximately 3 hours after completion of the last acquisition trial.

Statistical analysis: effects of the operations on the body weight of the mice were evaluated as in experiment 1. The effects of the occlusion on acquisition were analyzed by an Occlusion (sham occlusion vs. MCA-O) by Sessions (sessions 1 to 5) ANOVA, with repeated measures on the last factor. The effects of the occlusion per session were evaluated by *t*-statistics. Treatment effects on the swimming times per quadrant during the probe trial were assessed by an Occlusion by Quadrant ANOVA (time in clockwise, training, opposite, and counter-clockwise quadrant were considered as levels of the repeated measures factor Quadrant).

Results

Weight of the mice

One sham-operated mouse died shortly after surgery. The two groups had similar weights at operation (t_{17} = -1.7, n.s.; see Fig. 3). The body weights decreased after operation and subsequently recovered (Days after operations: $F_{1,17}$ = 37.4, $p < 0.01$), but the operations did not differentially affect body weights in the two treatment groups (Occlusion by Days after operation interaction: $F_{2,34}$ = 2.7; $0.10 > p > 0.05$).

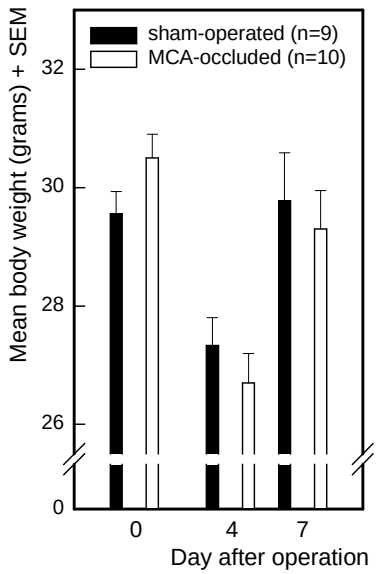

Figure 3. *Mean body weights (grams) and standard errors of the means (SEM) at operation (day 0) and on days 4 and 7 after the operation for nine sham-operated and ten MCA-occluded CFW1 mice.*

Acquisition of the water-escape task

Platform escape latencies (see Fig. 4, left panel): both groups needed, on average, the same time to find the platform (General mean: $F_{1,17} < 1.0$; n.s.). The escape latencies decreased over the five acquisition sessions (Sessions: $F_{4,68}$ = 28.3, $p < 0.01$), but the decrease was similar for both groups (Occlusion by Sessions interaction: $F_{4,68} < 1.0$; n.s.).

Number of quadrant entries (see Fig. 4, center panel): the results were identical to those for escape latencies. No effects of surgery were found on the average number of quadrant entries (General mean: $F_{1,17} < 1.0$; n.s.). Over sessions, the number of quadrant entries decreased (Sessions: $F_{4,68} = 21.4$, $p < 0.01$) similarly for the sham-operated and the MCA-occluded mice (Occlusion by Sessions interaction: $F_{4,68} < 1.0$; n.s.).

Probe trial (see Fig. 4, right panel): the mice spent about 40% of their time in the quadrant where the escape platform had been situated during acquisition, about 30% in the quadrant situated clockwise from the training quadrant, and only about 15% in the adjacent and opposite quadrants, respectively (Quadrants: $F_{3,51} = 16.3$, $p < 0.01$). The treatments did not differentially affect this pattern of quadrant occupancy ($F_{3,51} < 1.0$; n.s.).

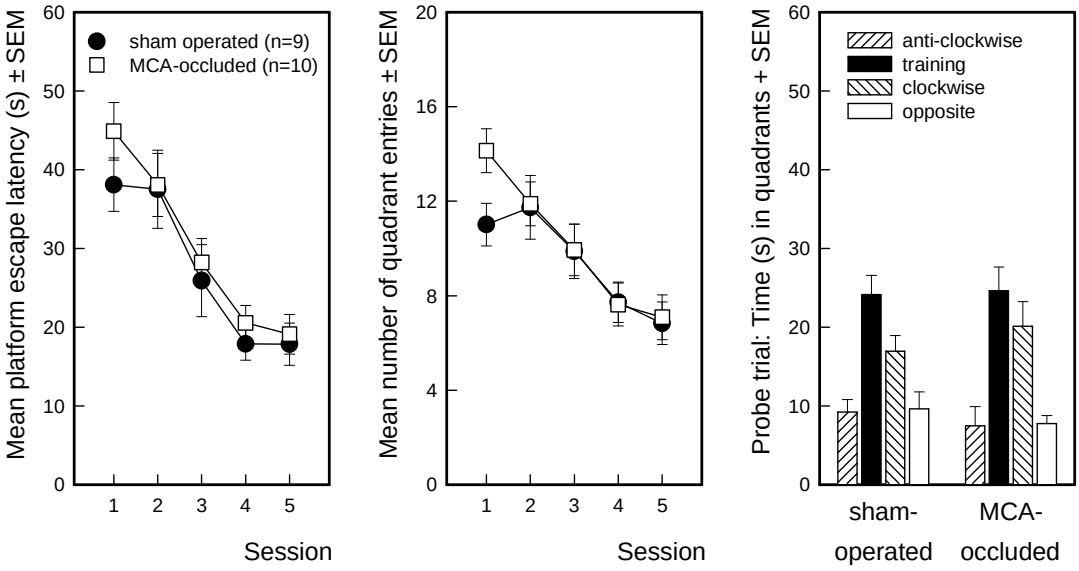

Figure 4: *Session means and standard errors of the means (SEM) for platform escape latencies in s (left panel) and number of quadrant entries (center panel) of naive CFW1 mice, which had been sham-operated or MCA-occluded, during training to find a submerged platform in a Morris water escape task. The right panel shows the mean time (s + SEM) spent by sham-operated and MCA-occluded CFW1 mice in each quadrant of the circular pool during a 1-minute probe trial (trial no. 41).*

Discussion

The first experiment revealed that the water-escape behavior of mice that had already acquired the task before surgery was not differentially affected by the MCA-O. These results contrast with findings that MCA-O can disrupt the retention of a task which has been acquired pre-surgery (Hirakawa et al., 1994). The surgery *per se*, however, might have had a marginal disturbing effect on water-escape performance, with the retention performance being slightly worse than that shown during the matching session. Also, contrary to expectations (e.g. Kolb, Sutherland & Whishaw, 1983) moving the platform to a different quadrant did not reveal any specific MCA-O-induced effects on the efficiency of a mouse's escape behavior. Again, surgery *per se* might have affected the performance of both treatment groups: the mice did not improve their performance over the reversal sessions if the number of quadrant entries, a crude measure for the distance swum to reach the submerged platform, is

considered. This finding contrasts with the escape latencies, a measure frequently used to assess the acquisition of water escape behavior (cf. Hagan et al., 1983; Lalonde & Joyal, 1991; Morris, 1984), which showed a statistically reliable decrease over the reversal sessions.

The performance of the sham-operated mouse that had a severe inflammation in both hemispheres of the brain was indistinguishable from that of the rest of the sham-operated animals (all performance measures of this animal fell within one standard deviation from the group means depicted in Fig. 2). Taken together, these results suggest that the cortical (and striatal) areas affected by the MCA-O do not play a significant role in the spatial orientation of CFW1 mice in the water-escape task. Once the task has been acquired, the marginal or weak impairments found are most likely caused by non-specific effects of the surgery.

The second experiment was performed to investigate whether MCA-O could disrupt the acquisition of water-escape behavior in naive CFW1 mice. Again, there was no differential effect of the MCA-O on cognitive functions. Our results contrast with findings which showed that rats had an impaired performance in passive avoidance tasks after MCA-O (e.g. Yamamoto et al., 1991; Smith et al., 1996), and in the acquisition of spatial discrimination tasks (e.g. radial maze: Okada et al., 1995a,b), including the Morris water escape task (Markgraf et al., 1992; Kumon et al., 1996; Smith et al., 1996)

The learning curves for both measures, escape latencies and number of quadrant entries, were similar in the sham- and in the MCA-occluded mice. This was also true for the degree of spatial bias towards the platform position, as measured during the probe trial. The mice improved their escape performance in the course of training, and their acquisition rate was similar to that seen in the first experiment during the acquisition before occlusion.

Comparison of the acquisition curves of both experiments shows that the mice that learned to locate and to escape onto the submerged platform *after* surgery, had longer escape latencies and made more quadrant entries (i.e. swam further before finding the platform) than the mice in the first experiment. This observation suggests that the operation *per se* affected escape behavior. This interpretation, however, needs confirmation from a study that includes both a sham-occluded and an untreated control group.

We conclude that the water escape behavior of CFW1 mice is unaffected by unilateral MCA-O. This corroborates earlier unsystematic observations at our laboratory that the behavior of mice from this strain in the home cage appears to be normal after occlusion. It cannot be excluded, however, that the unilateral lesioning produced small, unnoticed neurological impairments. In rats, for example, Andersen, Andersen, and Finger (1991) found neurological deficits after unilateral MCA-O in only a small subset of the battery of examinations they performed (see also Chapters 4.1, and 4.2).

Cortical lesions produced by ablation have consistently been found to reduce the performance of rats in Morris water-escape tasks (e.g. DiMattia & Kesner, 1988; Fantie & Kolb, 1990; Kolb, Sutherland & Whishaw, 1983; Kolb & Tomie, 1988; Kolb & Walkey, 1987). However, less brain tissue is damaged after MCA-Os than after removal by aspiration techniques. Although the somato-sensory and the motor cortices were affected by the occlusions (albeit to a variable degree) in our experiments, the size of the resulting lesions might have been insufficient to produce behavioral deficits (compare Moran et al., 1984), perhaps because the sensorimotor cortex in rodents is more medial than that of humans (Robinson, 1981), and consequently, is less damaged than is patients suffering from MCA-O induced stroke. Alternatively, there might have been a rapid recovery of function, so that deficits in spatial discrimination learning and retention in the water maze in CFW1 mice were not observed. More mouse

studies, eventually including different strains, are needed to investigate whether MCA-O affects cognitive performance in mice, as has been shown in rat studies.

4.4

Repeated acquisition of a spatial navigation task in mice: effects of spacing of trials and of unilateral middle cerebral artery occlusion*

Abstract

The working memory version of the Morris water escape task, the repeated acquisition task, consists of trial pairs in which an animal is started twice from the same start position. Animals have mastered this task when they need less time to find the platform in the second of the two trials. In the present study, male C57BL mice were trained on this task with massed, spaced, or spaced delay trials in which there was a 90-minute delay between the first and second trials of a pair. The mice trained with spaced trials learned the repeated acquisition task, whereas the mice trained with massed or spaced delay trials were not consistently able to do so.

When the mice had reached a stable baseline performance, the middle cerebral artery (MCA) was occluded or the mice were sham-operated. Then, the effects of the MCA-occlusion (MCA-O) on the performance in the repeated acquisition tasks were studied. MCA-O hardly affected the performance in this task, irrespective of the spacing condition of the trials, although surgery *per se* seemed to have a transient disruptive effect.

Introduction

The Morris water escape task (Morris, 1984) is one of the most frequently used experimental paradigms to assess disturbances of cognitive functions as a consequence of aging (e.g. Aitken & Meaney, 1989), specific brain lesions (e.g. Kolb, Sutherland & Whishaw, 1983; Kolb & Walkey, 1986; Whishaw, 1987; Mundy, Barone & Tilson, 1990; Denenberg et al., 1991), and experimentally induced infarcts (e.g. van der Staay et al., 1992; see Chapter 4.3), and to evaluate the properties of potential cognition enhancing compounds (e.g. Vincent & Sepinwall, 1992; Pierce et al., 1993; Pitsikas, Brambilla & Borsini, 1993). The standard water escape task, in which an animal is required to localize a submerged platform, measures predominantly spatial reference memory (RM; Mundy, Barone & Tilson, 1990). RM holds trial-independent information (Barnes, 1988b) about, for example, the position of the escape platform in the water tank.

Repeated acquisition procedures, unlike standard water escape tasks, are designed to assess an additional memory component, namely working memory (WM) (Whishaw, 1987, 1995; van der Staay & de Jonge, 1993). Within a daily training session of the repeated acquisition paradigm, each of four start

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positions (situated in the northern, eastern, southern, or western quadrant of the pool) is used randomly in every series of four trial pairs. Thus, a rat or mouse is randomly started from each of the four starting positions on both trials of a pair. From one trial pair to the next, or from one daily training session to the next, the escape platform is positioned in a different quadrant. Successful repeated acquisition is demonstrated when subjects have shorter latencies to find the platform during the second trial of a pair than during the first trial (one trial learning), i.e., when they show an improved WM performance. It has been shown that aged rats perform this task less well than young rats do (e.g. van der Staay & de Jonge, 1993; Frick et al., 1995), and that sleep deprivation impairs the RM, but not the WM component of this task in rats (Youngblood et al., 1997).

Permanent occlusion of the middle cerebral artery (MCA) in rats or mice is used as an animal model to investigate the pathophysiology of focal cerebral ischemia (Welsh et al., 1987), to screen and characterize putative neuroprotective substances (e.g. Gotti et al., 1990; Hara et al., 1991; Yamamoto et al., 1991; Park & Hall, 1994), and to assess ischemia-induced behavioral and neurological disturbances (e.g. Tamura et al., 1985; Bedersen et al., 1986; Markgraf, et al, 1992; Whishaw, 1995; van der Staay, Augstein & Horváth, 1996a,b). It has been reported that middle cerebral artery occlusion (MCA-O) in rats disrupts the acquisition of the standard, i.e. RM, version of the Morris water escape task. The degree of impairment, however, appears to be variable, ranging from a mild impairment of the acquisition (e.g. Markgraf et al., 1992) through severe trasiently retarded acquisition (e.g. Kumon et al., 1996) to complete failure to acquire this task (e.g. Shinoda, Matsuo & Toide, 1996).

Although there have been many studies in which rats were used with the Morris water escape task, there have been relatively few in which mice were used (e.g. Sweeney et al., 1988; Himori et al., 1990; Wehner, Sleigh & Upchurch, 1990; Lamberty & Gower, 1991b; Whishaw, 1995; Cohn, MacPhail & Paule, 1996). Using mice as subjects, we found that MCA-O did not affect learning in a standard Morris water escape task, and that MCA-O after the Morris task had been acquired also had no effect on the retention performance (van der Staay et al., 1992). Because C57BL mice readily learn to escape onto the submerged platform in the standard Morris task (e.g. Whishaw, 1995; Klapdor & van der Staay, 1996), we decided to assess the effects of MCA-O in this strain, using the WM (i.e. repeated acquisition) version of the Morris water escape task. This task is more difficult than the standard water escape task for rats and mice (Petrie, 1995). To experimentally manipulate the degree of difficulty of this task, three versions were used: a massed trials version consisting of four trial pairs per daily session; a spaced version, in which only one trial pair was given per session; and a spaced delay version, in which only one trial pair was given per session but there was a 90-minute interval between the first and the second trials of the trial pair.

The first aim of the present study was to investigate whether male C57BL mice are able to acquire the repeated acquisition task. The second aim was to assess whether manipulating the training conditions, i.e. the temporal spacing of the trials within trial pairs, differently affect the rate of learning and the performance level reached. For the repeated acquisition task in the Morris water tank, no such investigations have as yet been published (Cohn, McPhail & Paule, 1996). The third aim was to study whether unilateral MCA-O affects retention performance in the three versions of the task. We hypothesized that the most difficult version of the task would be most sensitive to the disruptive effects of MCA-O.

Material and Methods

Animals

Fifty-five C57BL mice (C57BL/6J/Ola/Hsd, supplied by Harlan UK Limited, Bicester, United Kingdom), weighing 20 ± 2 grams were used. They were randomly assigned to six experimental groups (see Table 1). The animals were housed in groups of ten in standard type III Makrolon™ cages. Prior to the experiment, the animals were allowed to adapt to our animal facilities for at least one week. They were kept under constant temperature (21℃) and humidity (50%), with an artificial 12-h light/dark cycle (on: 7.00 p.m.), and had free access to food and water.

Table 1. *Summary of the experimental design. The assignments of male C57BL mice to the different spacing of trial pairs in the repeated acquisition task, and to the occlusion of the middle cerebral artery (MCA-O) or to the sham operation are depicted. In addition, the number of acquisition sessions pre-surgery, and the number of trial pairs per acquisition session are shown.*
Abbreviations used: SD, spaced delay; S, spaced; M, massed

Group assignment pre surgery	Task version	Surgery	Group assignment post surgery	n	Acquisition sessions	Trial pairs per session
SD	spaced trials,	MCA-O	SD-MCA	12	16	1
	90-min delay	sham	SD-sham	8	16	1
S	spaced trials,	MCA-O	S-MCA	9	16	1
	no delay	sham	S-sham	7	16	1
M	massed trials,	MCA-O	M-MCA	11	10	4
	no delay	sham	M-sham	8	10	4

Apparatus

Testing took place in a gray circular tub of Polyethylene with slightly sloping walls (∅ 73 cm at the top, ∅ 66 cm at the bottom, 54 cm height). During the sessions the tub was filled up to 37.5 cm with tap water (21℃), and a gray platform (∅ 7.3 cm, 37 cm high) was placed in the middle of a quadrant (either north, east, south, or west). The behavior of the mice in the Morris task was scored manually. A video camera mounted above the center of the pool provided a picture of the pool on a TV monitor. On the monitor the swimming pool was divided into four equal quadrants which were further divided into a 4 * 4 matrix of squares. The data were recorded with and stored in a PC equipped with an appropriate program. Movements of a mouse were scored by pressing the cursor key corresponding to the appropriate quadrant. Line crossings within a quadrant were scored by pressing the corresponding cursor every time a mouse crossed a line with its whole body. We calculated three measures from the raw data: escape latency, line crossings, and swimming speed.

Behavioral testing

Acquisition: the groups ‘spaced delay’ (SD) and ‘spaced’ (S) had one trial pair per day for 16 days (schematically depicted in Fig. 1). Each mouse was started twice from the same position, with the

platform being in the same location. For the group SD there was a delay of 90 minutes between the first and second trials of a trial pair, whereas for group S both trials were in close succession. The animals of the 'massed' (M) group were tested in four no-delay trial pairs per day for 10 days (= 40 trial pairs). During the four trial pairs a mouse was randomly started from each of the four possible directions north, east, south, and west, and the escape platform was randomly placed into the center of each of the four quadrants.

The animal was allowed to stay on the platform for 15 seconds, before the next trial (groups S and M) or the inter-trial interval (group SD) started. If a mouse failed to escape onto the platform within 60 seconds, it was put onto the platform by the experimenter and was allowed to stay there for 15 seconds before the next trial or the inter-trial interval started. After completion of the last trial of a session (groups S and M), or after completion of both the first and second trials of a session (group SD) the mouse was placed in a padded drying cage under a red heating light. When its fur was dry it was moved back into the homecage.

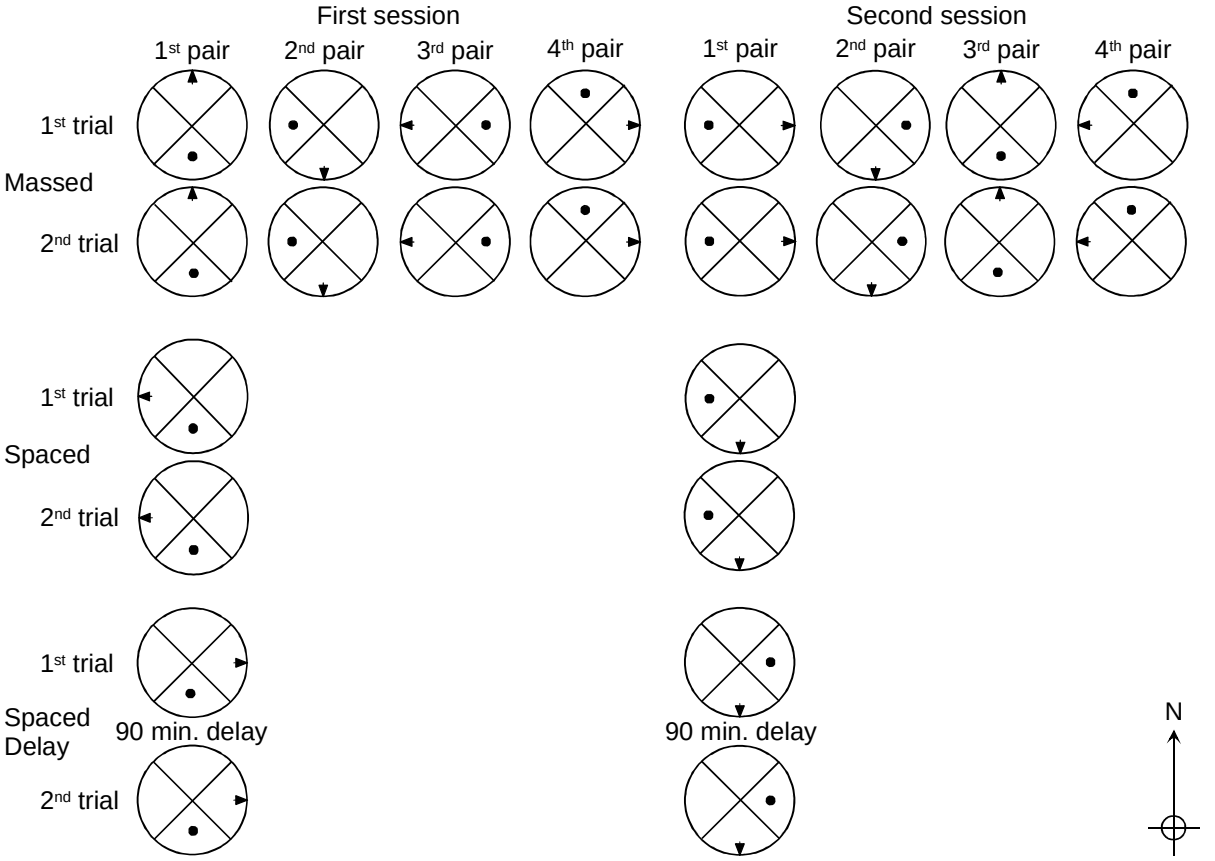

Figure 1. *Schematic overview of the training procedures in the repeated acquisition task. Examples for the first two sessions are depicted. The animals of the 'massed' group were tested in four no-delay trial pairs per daily session. The groups 'spaced' and 'spaced delay' had one trial pair per session. Each mouse was started twice from the same position, with the platform being in the same location. For group 'spaced' both trials were in close succession, whereas for the group 'spaced delay' there was a delay of 90 minutes between the first and second trials of a trial pair. The start position at the rim of the pool is marked by an arrow, the platform position in the center of a quadrant is shown by '●'.*

Post-surgery testing: testing was resumed four days after surgery. The testing procedures were as during the acquisition of the task.

Middle cerebral artery occlusion: surgery was performed according to Welsh et al. (1987) with minor modifications. Briefly, the mice were anesthetized with chloral hydrate (400 mg/kg i.p.). During surgery, the body temperature was maintained between 37°C and 38°C with a warming pad. On the left side of the head the skin was opened vertically between the orbit and the external ear canal. The dorsal and caudal margins of the temporalis muscle were detached with scissors and partially removed. The remaining part was folded forward so that the upper lateral aspect of the skull became visible. The facial nerve, the eye muscles, and the zygomatic bone were left intact. The MCA was exposed under an operating microscope. After the dura had been opened, the MCA and its branches were occluded by microbipolar electrocoagulation, followed by the removal of the occluded vessels. In sham-operated mice the MCA was exposed, but not occluded. The temporalis muscle and the skin were closed with tissue glue (Histoacryl, Braun-Melsungen, Melsungen, FRG) and the wound was treated with bacteriostatic powder (Marfanil/Prontalbin, Bayer, Leverkusen, FRG). After recovery from anesthesia the mice were returned to their home cages.

Histological verification: the mice were decapitated three (group M), four (group SD), or five (group S) weeks after surgery. The brains were rapidly removed and frozen in n-methylbutane at -40°C. Coronal sections (20-µm thick) were cut through the infarcted area with a distance between slices of 300 µm with a cryostat microtome (Leitz, Wetzlar, FRG). Slide-mounted tissue sections were stained with cresyl fast violet.

Statistical analysis

Three measures were analyzed:

- the time to escape onto the platform, i.e. the escape latency (Morris, 1984),
- the total number of line crossings during a trial, i.e. the distance traveled, and
- the number of crossings divided by the escape latency, i.e. the swimming speed (crossings * s^{-1}).

Effects of the temporal distribution of trials on the learning curves: the learning curves of mice trained with the different spacing conditions were analyzed across the first ten daily sessions. Only the first trial pair per session was considered (there was only one trial pair per daily session in the spaced and the spaced delay condition, whereas 4 trial pairs were given in the massed condition), i.e. each trial pair in the analysis stands for one acquisition session. In order to investigate whether the version of the task affected acquisition differently, an analysis of variance (ANOVA; Winer, 1971) was performed with the factors Spacing (spaced vs. spaced delay vs. massed), and the two repeated measures factors Trial pairs (first trial pair of sessions 1 to 10), and Trials Within Pairs (first vs. second trial). These analyses were complemented with post-hoc comparisons using Fisher's LSD test.

To analyze whether similar performance levels were reached in the three spacing conditions at the end of acquisition, and whether the random assignment of the mice to undergo sham lesioning or MCA-O had produced similar groups within spacing conditions, an ANOVA with the factors Spacing (spaced vs. spaced delay vs. massed), Lesion (MCA-O vs. sham operation), and the two repeated measures factors Trial pairs (first trial pair of sessions) and Trials Within Pairs (first vs. second trial) was performed on the four daily sessions before surgery (sessions 13 to 16 for the spaced and the spaced delay condition, and sessions 7 to 10 for the massed condition).

Effects of the MCA-O: the effects of the MCA-O were assessed by an ANOVA with the factors Spacing (spaced vs. spaced delay vs. massed), Lesion (MCA-O vs. sham operation), and the three repeated measures factors Surgery (pre lesion vs. post lesion), Trial Pairs (first trial pair of sessions), and Trials Within Pairs (first vs. second trial) on the four daily sessions before surgery (sessions 13 to 16 for the spaced and the spaced delay condition, and sessions 7 to 10 for the massed condition) and on the four daily sessions after surgery.

In addition, the post-operation performance was evaluated separately with an ANOVA with the factors Spacing (spaced vs. spaced delay vs. massed), Lesion (MCA-O vs. sham operation), and the two repeated measures factors Trial pairs (first trial pair of sessions) and Trials Within Pairs (first vs. second trial) on the four daily sessions after surgery.

Results

Histology

Small areas of infarcted tissue were obvious on the surface of the brains, the areas being smaller the longer after surgery they were examined. The damage induced by occlusion of the MCA, however, could not be quantified reliably, because infarct volumes decrease non-linearly over the course of time (unpublished data; Chiamulera et al., 1993). According to Chiamulera and co-workers (1993) the decrease in infarct volume after MCA-O might be due to *"phagocytic activity, which leads to a gradual elimination of necrotic material and oedema reabsorbation"* (p. 257). Figure 2 shows the typical position and size of the infarcted area in a C57/BL mouse, determined one week after MCA-O. The infarcts are predominantly restricted to the neocortex.

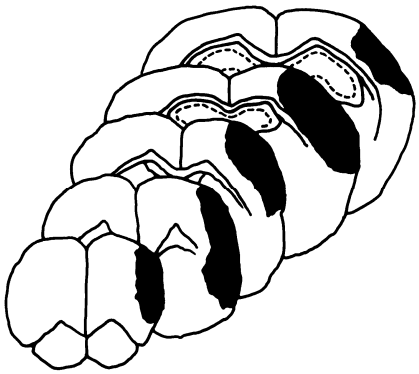

Figure 2. *Typical infarction induced by occlusion of the middle cerebral artery in male C57BL mice. The distance between slices was 300 µm. The drawing is based on slide-mounted tissue sections stained with cresyl fast violet. To increase the visibility of the infarcts, the original slices were scanned and turned into gray scale graphics. The scans were inverted, contrast and brightness were adjusted, and a sharpening filter was applied.*

Effects of the temporal distribution of trials on the learning curves

Swimming speed: in general, the swimming speeds in the second trials of a pair were higher in the spaced (S) and massed (M) conditions than in the spaced delay (SD) condition (General mean: $F_{2,52}$ = 7.29, $p < 0.01$; data not shown). There were complex interactions between Spacing, Trial Pairs, and Trials Within Pairs for the swimming speed (Spacing by Trial pairs interaction: $F_{18,468}$ = 6.00, $p < 0.01$; Spacing by Trials Within Pairs interaction: $F_{2,52}$ = 31.50, $p < 0.01$; Trial Pairs by Trials Within Pairs

interaction: $F_{9,468} = 2.13$, $p < 0.05$; Spacing by Trial Pairs by Trials Within Pairs interaction: $F_{18,468} = 2.52$, $p < 0.01$).

Escape latency: because of the complex interactions found for swimming speed, the escape latencies (data not shown) might be biased. In this case, the number of line crossings (i.e., the distance swum) provides an unbiased measure for learning, because it does not depend on the speed with which the mice negotiate the water tank.

Line crossings (see Fig. 3): averaged over the trial pairs, the spacing conditions affected the number of line crossings differently (General mean: $F_{2,52} = 10.02$, $p < 0.01$). The mice trained with spaced and with spaced delay trials swam, on average, longer distances to find the platform than did the mice trained with massed trials.

The mice learned to reduce the distance swum to reach the platform across sessions (Trial Pairs: $F_{9,468} = 18.80$, $p < 0.01$), and the rate of learning was different for the spacing conditions (Spacing by Trial Pairs interaction: $F_{18,468} = 1.71$, $p < 0.05$). The mice trained with massed trials showed a steeper learning curve across sessions than the animals trained with spaced trials.

In general, there was a difference between the first and the second trials of a pair (Trials Within Pairs: $F_{1,52} = 8.47$, $p < 0.01$), i.e. the mice swam a longer distance in the first trial of a pair than in the second one. The spacing conditions did not affect this difference between the trials within trial pairs (Spacing by Trials Within Pairs interaction: $F_{2,52} < 1.00$, n.s.).

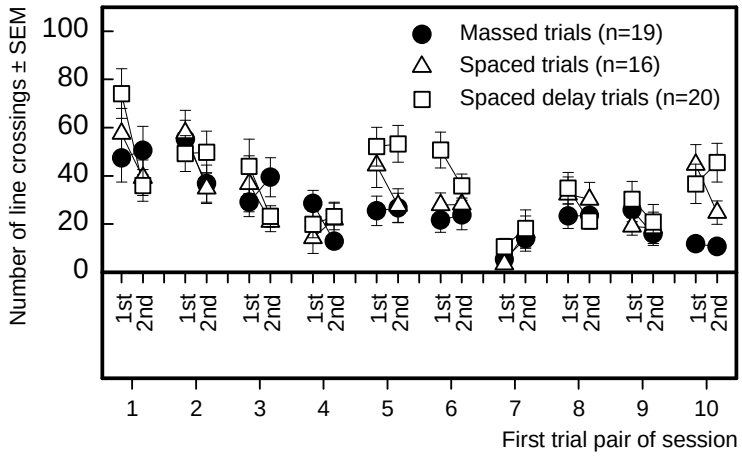

Figure 3. *The number of line crossing during the first and second trial of session 1 to 10 of mice, trained with either massed trials, spaced trials, or spaced delay trials, are depicted as means ± standard errors of the means (SEM).*

Across sessions, the difference between the trials of a pair changed differently for the spacing conditions (Spacing by Trial Pairs by Trials Within Pairs interaction: $F_{18,468} = 1.72$, $p < 0.05$). In general, the mice in the spaced condition swam a shorter distance in the second trial of a trial pair than in the first one. The mice trained with massed or spaced delay trials, by contrast, showed somewhat less congruent performance from trial pair to trial pair. In only half of the sessions did mice reach the platform by swimming a shorter route in the second trial of a pair. These trial pairs were evenly spaced over the training sessions.

Performance on the last four sessions prior to surgery

There were no differences in the mean number of line crossings in the last four sessions pre-surgery (Spacing: $F_{2,49}$ = 1.46, n.s.; Lesion: F1,49 < 1, n.s.), nor were there interactions between the factors Spacing and Lesion ($F_{2,49}$ < 1, n.s). Note that in this analysis the factor Lesion refers to the assignment of rats to either the sham or the MCA-O groups within spacing conditions, and is included to test whether the performance of these groups was similar before the operations.

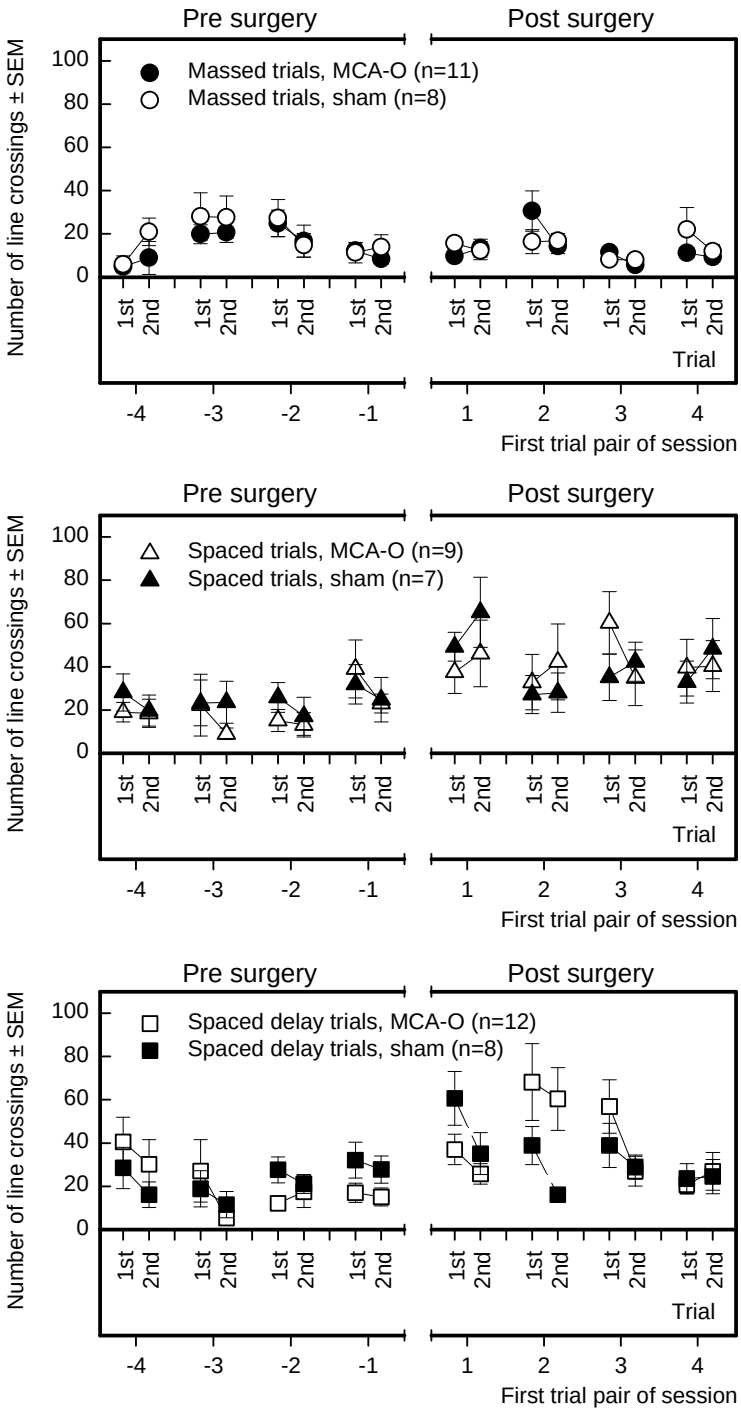

Figure 4. *Performance in the first trial pair of the four last sessions prior to surgery (-4, -3, -2, -1) and of the four sessions following surgery (1, 2, 3, 4). The means ± standard errors of the means (SEM) of the number of line crossings are depicted for the groups trained with massed trials (upper panel), spaced trials (center panel), or spaced delay trials (lower panel).*

However, spacing differently affected the change in the number of line crossings across the four last pre-surgery sessions (Spacing by Trial Pairs interaction $F_{6,147} = 3.86$, $p < 0.01$). This interaction most probably was due to a slight, inverted U-shaped, change in the number of line crossings in the groups trained with massed trials, whereas no such effect was seen in the groups trained with either spaced, or spaced delay trials. During the last pre-surgery sessions, the number of lines crossed decreased from the first to the second trials of a pair (Trials Within Pairs: $F_{1,49} = 7.12$, $p < 0.01$). This difference between the first and second trials of a pair was not affected by any of the other factors. This means that the performance level of the groups was similar before surgery.

Effects of the MCA-O (see Fig.4)

Pre- versus post-surgery comparison: repeated measures analysis on performance in the last four acquisition sessions and in the four post-surgery sessions revealed that MCA-O differentially affected performance (Surgery by Lesion by Trial Pairs interaction: $F_{3,147} = 3.07$; $p < 0.05$). The performance of sham-operated mice trained with spaced or spaced delay trials seemed to be slightly more affected by surgery than that of the MCA-O mice (this effect seemed to be restricted to the second and third training sessions after surgery). The nature of the interaction, however, is not known. There was neither a main effect of the lesion, nor were there any further interactions with the other factors (all Fs with associated probabilities > 0.10).

Thus surgery *per se* affected performance ($F_{1,49} = 29.36$; $p < 0.01$), and this effect was different for the spacing conditions (Spacing by Surgery interaction: $F_{2,49} = 12.92$, $p < 0.01$). The distances mice swam to reach the platform were, on average, longer in the groups trained with spaced or with spaced delay trials, than in the groups trained with massed trials. The learning curves across the first trial pairs of the four post-surgery sessions were differently shaped (Spacing by Surgery by Trial Pairs interaction: $F_{6,147} = 2.97$; $p < 0.01$). Whereas the performance level of the groups trained with massed trials was very stable, this was not true for the other groups of mice, where the performance from session to session was more variable. Surgery itself appeared to disturb WM performance in the groups trained with spaced trials more than in the groups trained with either spaced delay or massed trials (Spacing by Surgery by Trials Within Pairs interaction: $F_{2,49} = 4.03$, $p < 0.05$). In particular, these differences were due to an enhanced number of line crossings in the second trial of a pair in the groups trained with spaced trials (Spacing by Surgery by Trials Within Pairs interaction: $F_{2,49} = 3.72$; $p < 0.05$).

Performance on the four sessions after surgery: in an attempt to elucidate this complex Surgery by Lesion by Trial Pairs interaction further, we assessed the performance of the mice in the four sessions after surgery separately.

There was an effect of spacing of the trials ($F_{2,49} = 46.56$; $p < 0.01$): the mice trained with massed trials swam, on average, less than half the distance swum by the mice trained with spaced or spaced delay trials). The spacing conditions affected the learning curves differently (Spacing by Trial Pairs interaction: $F_{6,147} = 2.39$, $p < 0.05$). The learning curves of the groups trained with massed trials were flat, whereas those of the other groups varied from session to session. The spacing conditions also affected the difference between the first and second trials of pairs differently (Spacing by Trials Within Pairs: $F_{2,49} = 6.95$, $p < 0.01$). The first and second trials of pairs were hardly different in the groups trained with massed trials.

The MCA-O appeared to affect the learning curves differently (Lesion by Trial Pairs interaction: $F_{3,147} = 2.91$, $p < 0.05$). The MCA-occluded mice swam, on average, longer distances than did the sham-operated mice. There was, however, no differential effect on the second trial of a pair (Lesion by Trials

Within Pairs interaction: $F_{1,49} < 1.0$ n.s.). The precise nature of the complex Surgery by Lesion by Trials Within Pairs interaction found in the pre- versus post-surgery comparison, therefore, could not further be elucidated.

Discussion

The standard water escape task in which an animal is required to find a submerged platform predominantly measures spatial reference memory (Mundy, Barone & Tilson, 1990). The reference memory (RM) holds trial-independent information (Barnes, 1988b) concerning, for example, the position of the escape platform. Repeated acquisition procedures are designed to assess an additional memory component, namely WM (Whishaw, 1987, 1995; van der Staay & de Jonge, 1993).

Several authors have shown that rats are capable of mastering repeated acquisition tasks (e.g. Nagahara & McGaugh, 1992; van der Staay & de Jonge, 1993) in massed (van der Staay & de Jonge, 1993), spaced (Whishaw, 1995) and spaced delay conditions (Rashidy-Pour, Motamedi & Motahed-Larijani, 1996). The WM version of the Morris water escape task has been found to be sensitive to, for example, the effects of the aging process (van der Staay & de Jonge, 1993), or to the effects of experimentally induced cognitive deficits (e.g. hypoxia-induced learning decrements, Shukitt-Hale, Stillman & Liebermann, 1996; medial septal area inactivation-induced impairments, Nagahara & McGaugh, 1992; Rashidy-Pour, Motamedi & Motahed-Larijani, 1996).

Effects of the spacing of trials on the acquisition of the WM version of the Morris task

The first aim of the present study was to investigate whether male C57BL mice are able to acquire the repeated acquisition task (Whishaw, 1987, 1995) and whether a different temporal distribution of the trials affects this performance. We found that mice are able to learn the repeated acquisition tasks, as has previously been found for rats (e.g. Whishaw, 1987, 1995; van der Staay & de Jonge, 1993). Irrespective of the spacing of trials, all groups reduced the distance swum to reach the platform across the acquisition sessions: mice were able to use the information from the first trial of a pair to find the platform faster in the second trial of a pair. However, the spacing of trials strongly affected learning.

Successful one-trial learning, i.e. an improved WM performance, was most convincingly shown in the group trained with spaced trials. The group trained with a delay of 90 minutes between the first and second trials swum longer distances to find the escape platform, and the improvement from the first to the second trials of a pair was less consistent than that of mice trained with spaced trials. The mice trained with massed trials showed an improvement from the first to the second trial of a pair up to about the sixth acquisition session, and thereafter no further improvement. Zhou and co-workers (1998) also found that C57BL mice were able to acquire a repeated acquisition task in the Morris water tank, using a massed trials procedure. They changed the platform position once per day and gave 5 pairs of trials from 5 different start position per training session. Due to this methodological difference and to differences in the data analysis, direct comparisons between the results reported by Zhou and colleagues (1998) and ours cannot be performed.

The most successful way to train mice on the WM version of the Morris task is to use spaced trials (one trial pair per day) where both trials are given in close succession. Contrary to our results, Whishaw (1995) did not see an improvement in WM performance in C57BL mice trained with spaced

trials in two daily sessions, each consisting of one trial pair. By contrast, in our study, the mice in both spacing conditions received only one daily session.

Although the mice trained with spaced delay trials did not show a consistent improvement from the first to the second trial of a pair across the first acquisition sessions, their performance did improve, and by the end of acquisition they had reached a performance level similar to that of mice trained in spaced trials without delay. Thus, if a sufficient number of sessions are given, mice might be able to acquire the WM version of the Morris task with spaced delay trials, as has previously been shown for rats (Rashidy-Pour, Motamedi & Motahed-Larijani, 1996). The across-session improvement in finding the escape platform during the first trial of a session might reflect that the mice had developed an efficient strategy to find the submerged escape platform. If this is true, then spatial information about the platform position becomes less important in the course of training.

Comparison of the acquisition curves of the spaced and the spaced delay groups shows the spaced delay condition to be the most demanding condition. In the massed condition, proactive interference might have developed which distorted performance in the second trials in the later phase of acquisition. Alternatively, the good performance level reached already in the first trial of a session might have precluded the possibility for further improvement in the second trial. This explanation is supported by the observation that the final performance level reached by C57BL mice in a standard Morris water escape task (Klapdor & van der Staay, 1996) is similar to that in the repeated acquisition task. In the standard Morris task, C57BL mice made on average about 10 line crossings to reach the escape platform by the end of acquisition, i.e. in the fifth daily session. A similar performance level was reached by the seventh session in the repeated acquisition task.

Young mice seem to have a poorer one trial learning performance in the repeated acquisition task than young adult rats (van der Staay & de Jonge, 1993). Although one must be aware of the restrictions when making comparisons across experiments and species, the question whether the poorer performance of mice reflects predominantly WM deficits, or whether it is caused by deficits in RM or conceptual learning i.e. an inability to acquire the procedural aspect of the task, needs further investigation. The acquisition of one-trial learning depends upon procedural memory (M'Harzi et al., 1987), which in turn might be considered an aspect of spatial RM (Olton, Becker & Handelmann, 1979). In the repeated acquisition paradigm, one might consider the decrease in the distance swum to reach the escape platform over sessions to be an improvement in RM performance.

Effect of MCA-O on the performance of the WM version of the Morris task

In an earlier study we found that unilateral MCA-O in mice does not affect water-escape behavior in the standard Morris task (van der Staay et al., 1992; Chapter 4.3). In the presen study, unilateral MCA-O did not affect the performance of the three WM versions of the task. The performance of sham-operated mice trained with spaced or spaced delay trials seemed to be slightly more affected after surgery than that of the mice with MCA-O. This effect seemed to be restricted to the second and third training sessions after surgery. The precise nature of this complex interaction, however, could not completely be clarified.

A major problem of a focal permanent occlusion of the MCA for behavioral investigations is that it requires craniotomy (Memezawa, 1993; Rogers et al., 1997). This surgial procedure appears to cause behavioral dysfunctions by itself, because the post-surgery performance of both the MCA-occluded and the sham-operated animals was different from the pre-surgery performance. The spacing conditions appeared to be differentially sensitive to surgery. In the massed trial version only minor

differences were seen between pre-surgery and post-surgery performance, whereas the performance of mice trained with spaced and spaced delay trials decreased transiently after surgery. This observation that surgery affects performance *per se,* is in agreement with the results of a navigation study with mice (van der Staay et al., 1992; see Chapter 4.3), where MCA-O did not affect navigation performance in the standard Morris maze procedure (Morris, 1984) and where the operations appeared to impair post-surgery performance in the water-escape task. As in the present study, the effect was transient. However, the performance of the mice trained with spaced trials did not return to the level attained in the last acquisition sessions.

Sensitivity of the task for effects of experimentally induced brain lesions

Previously, the repeated acquisition task has been found to be sensitive to age-related cognitive dysfunctions (e.g. van der Staay & de Jonge, 1993; Frick et al., 1995). Auer and colleagues (1989) reported that the repeated acquisition task in rats is sensitive and specific for hippocampal damage, and recently Hamm and coworkers (1996) successfully used the task to assess the effects of fluid-percussion traumatic brain injury in rats.

An inherent problem of the repeated acquisition task is that different platform positions induce fluctuations in the distances swum over sessions, probably because the degree of difficulty to localize the escape platform can be different for the different platform locations (see also van der Staay & de Jonge, 1993). Moreover, depending on the particular combination of start position and platform location, the shortest (direct) route to reach the platform is also not always the same length. These methodological aspects of the task clearly induce extra variation in the data, which obscure lesion-induced performance deficits.

The sensitivity of the Morris maze task for lesion-induced deficits might depend, among other factors, on the color of the water tank. Paylor and Rudy (1990) reported that the magnitude of impairments induced by cholinergic blockers was much larger in a gray pool than in a white pool. A gray tank was also used in the present study. We did not, however, find convincing evidence in a study with intact C57BL mice that the pool color affected learning (Klapdor & van der Staay, 1996).

Because of the long survival of the mice in the present study, standard histological methods were not suited to quantify the induced infarct. However, when evaluating the damage induced by the occlusion in an independent group of C57BL mice sacrificed one week after lesioning, we found that the infarcted areas were almost exclusively in the cortex, with subcortical areas being spared. However, using rats, Dixon and colleagues (1995) found deficits in Morris water escape task performance in a model of traumatic brain injury which predominantly affected cortical structures, but spared the hippocampus. Their findings indicate that deficits in the Morris water task can occur in animals with an intact hippocampus.

In summary, the mouse strain used in this study is capable of learning the WM version of the Morris water escape task. One-trial learning was most convincingly shown in the spaced condition. The WM performance in the repeated acquisition task in the Morris maze was not affected by occlusion of the MCA. This lack of effect of the occlusion is in accordance with earlier results (van der Staay et al., 1992). Thus, the repeated acquisition task in the Morris water tank appears to be not suited to assess the effects of MCA-O on learning and memory in C57BL mice. Whether this task is sensitive to the effects of other types of experimentally-induced brain lesions, or to cognition disrupting treatments or compounds, as has previously been shown for other spatial repeated acquisition tasks and for operant repeated acquisition tasks (see Cohn, MacPhail & Paule, 1996) remains to be investigated.

4.5
Behavioral effects of stroke, induced by occlusion of the middle cerebral artery (MCA) in rodents: discussion and conclusions

Tamura, Kawai, and Takagi (1997) state that *"(..) to investigate the pathophysiological mechanisms underlying the development of ischemic brain damage, animal models are indispensable as experimental counterparts of human focal cerebral ischemia"* (p. 276). Rats or mice with infarcts, experimentally induced by permanent occlusion of the middle cerebral artery (MCA) are still the most frequently used animal model of focal brain infarcts, although there appears to be a shift toward models in which occlusion techniques are used which allow reperfusion of the infarcted area (e.g. Belayev et al. 1996; Mancuso, Nimura & Weinstein, 1997).

In this Chapter, we performed a series of experiments with rats or mice to study the effects of cerebral infarction, induced by permanent occlusion of the left MCA, on sensorimotor and cognitive functions.

In Chapter 4.1, we tested Wistar Kyoto (WKY) rats with cerebral infarction induced by permanent unilateral occlusion of the MCA and sham-operated rats in a series of simple behavioral tests 2, 16, and 37 days after surgery. In addition, we measured the rats' motility over a 62-hour period, after the third test series. A subset of the tests appeared to be suitable to assess the effects of cerebral infarction, namely, grasping reflex of contralateral hindpaw, circling behavior, forelimb flexion, hindlimb flexion, and latency to fall off a square bridge. Except for the impaired grasping reflex of the contralateral hindpaw, there was spontaneous and complete recovery of function by the third test session, 37 days after surgery. Some of the other tests might not have been sensitive enough to detect the effects of the unilateral MCA-occlusion (MCA-O) on behavior. However, the WKY rats were very inactive in some of the tests, so that reliable scoring of the effects was not always possible. We concluded that a rat strain other than the WKY strain might be more suitable to study the behavioral consequences of MCA-O.

In Chapter 4.2, we performed three experiments to determine whether the pattern of MCA-O-induced sensorimotor impairments in rats is strain dependent, whether proximal (i.e. close to its origin) and distal occlusions (above the lenticulostriate branch) of the MCA affect infarct volume and the behavioral impairments to a different extent, and finally, whether there is a relation between the infarct volume and the behavioral deficits.

The patterns of sensorimotor malfunctions induced by proximal unilateral MCA-O were highly strain dependent. Of the eight strains tested, Winkelmann Wistar (WISW) rats, spontaneously hypertensive stroke-prone (SHR-SP) rats, and WKY rats were most severely affected. By contrast, Brown Norway (BN) rats showed only mild behavioral deficits after the MCA-O. The second experiment confirmed that proximal occlusions induced slightly more behavioral dysfunctions than distal occlusions did. Histological evaluation of the brain damage caused by proximal and distal MCA-O, confirmed that the distal MCA-O damaged nearly exclusively cortical areas and spared the caudate/putamen. However,

we did not find evidence that the severity of the sensorimotor malfunctions can be predicted from the size of the infarct.

These results show that different rat strains are differently affected by MCA-O, that the occlusion site affects the infarct volume, and that there is no simple relation between the volume of the infarct and the severity of behavioral dysfunctions. Wistar-derived strains of rats (WISW, WKY and SHR-SP) appear to develop more severe behavioral dysfunctions after MCA-O than other strains do.

We assessed the effects of MCA-O on learning and/or retention of different versions of the Morris task using mice. In the first experiment of Chapter 4.3, male CFW1 mice acquired the standard Morris water-escape task *before* half of the animals received an unilateral occlusion of the MCA. We then measured retention in one session. In addition, the mice acquired a new platform position during daily training sessions on 4 consecutive days. In a second experiment, naive male CFW1 mice acquired the water-escape task *after* surgery. At the end of the fifth session, a probe trial was given. In both experiments the control group consisted of mice that had been sham-operated: the MCA was exposed surgically but was left intact. Even though the MCA-occlusion-induced infarcts in the CFW1 mice covered the cranial part of the dorsomedial cortex (destroying substantial areas of the primary somatosensory cortex and smaller parts of the primary motor cortex) and part of the striatum, disabling behavioral impairments in the Morris water-escape task were not observed. Surgery per se, however, seemed to have a disruptive effects on water-escape behavior.

We assessed the effects of MCA-O on the retention of the working memory (WM) version of the Morris water escape task, the repeated acquisition task, in Chapter 4.4. This task consists of trial pairs in which an animal is started twice from the same start position. Animals have mastered this task when they need less time to find the platform in the second of the two trials. Male C57BL mice were trained on this task with massed, spaced, or spaced delay trials in which there was a 90-minute delay between the first and second trials of a pair.

As soon as the mice had reached a stable baseline performance, the MCA was occluded or the mice were sham-operated. Then, we studied the effects of the occlusion on the re-learning of the repeated acquisition tasks. The mice trained with spaced trials learned the repeated acquisition task, whereas the mice trained with massed or spaced delay trials were not consistently able to do so, perhaps due to strong proactive interference in the massed trials condition, and because the task was too demanding in the spaced delay condition. MCA-O hardly affected performance during re-learning of this task, irrespective of the spacing condition of the trials, although surgery *per se* seemed to have a transient disruptive effect. The latter observation corroborates the results obtained with CFW1 mice (compare Chapter 4.3, second experiment) showing that surgery transiently affects performance in the standard Morris water escape task.

Sensorimotor deficits

The pattern of sensorimotor impairments was highly strain dependent (Chapter 4.2), varying from deficits on a number of tests in the Wistar derived strains to nearly no deficits at all in the BN strain. MCA-O in all but the WKY strain damaged the somatosensory cortex, albeit to a variable extent (see Table 3, Chapter 4.2). The motor cortex was hardly affected. This finding may provide an explanation for the relatively weak motor impairments seen. In humans, in whom the motor cortex is affected by occlusion of the MCA, hemiparesis is a common symptom (Adams, Victor & Ropper, 1997, p. 790), whereas permanent MCA-O in rats did not cause sustained hemiparesis or hemisensory deficits, which

is probably because the sensorimotor cortex of rats is more medial than it is in humans (Robinson, 1981).

In rats, laterality of the effects of MCA-O has been found. Robinson (1979; Robinson & Coyle, 1980) reported that ligation of the left MCA-O was without effect on spontaneous activity in rats, whereas ligation of the right MCA-O induced hyperactivity which lasted for about 3 weeks after occlusion. These data suggest functional asymmetries in the rat cortex. It is tempting to suggest that occlusion of the right MCA might have induced stronger sensorimotor effects than those seen in our experiments after left MCA-O. However, the findings of Robinson and colleagues were not confirmed by others. For example, Andersen and colleagues (Andersen, Andersen & Finger, 1991) found no increase in spontaneous activity after occlusion of the right MCA.

As expected, functional deficits occur on the side contralateral to the infarct (Bederson et al., 1986; Andersen, Andersen & Finger, 1991; Markgraf et al., 1992). The SHR-SP and the WKY rat had clear impairments of the grasping reflex of the hindpaws ipsilaterally and contralaterally to the hemisphere, where the MCA-O had been induced. We hypothesized that this reflects the indirect effects of edema formation during the first days after the operations, as a result of the surgical procedure *per se* and occlusion of the MCA. Because of the increase in volume on the affected side, the contralateral side becomes compressed. This compression leads to dysfunction of the contralateral brain hemisphere, which causes sensorimotor deficits ipsilateral to the occluded side.

As edema disappears within 1 week of surgery, one should wait at least 5 to 7 days before starting to assess the consequences of MCA-O on behavior to avoid confounding effects of edema formation. On the other hand, selecting a rat strain that shows *ipsilateral* sensorimotor deficits in the first days after MCA-O might serve as a functional model of infarct-induced edema.

Recovery of function after MCA-O

Sensorimotor dysfunction seemed to recover about 1 month after unilateral stroke, induced by occlusion of the left MCA (Chapter 4.1). The only exception was the grasping reflex of the contralateral hindpaw, which at that time was still slightly impaired. A similar recovery of sensorimotor functions has been reported by Yamamoto and coworkers (1988), and by Markgraf and colleagues after permanent (Markgraf et al., 1992) as well as transient MCA-O (Markgraf et al., 1997). As spontaneous and dramatic recovery has also been observed in patients after acute ischemic stroke (Biller et al., 1990), the MCA-O model in rodents seems to have face validity with respect to the recovery processes seen in a subset of stroke patients. In the majority of patients suffering from MCA-O, however, the behavioral impairments are long lasting (Adams, Victor & Ropper, 1997).

Effects on acquisition and retention of the Morris water escape tasks

Focal occlusion of the MCA did not affect learning and memory in the mice. However, surgery *per se* had a transient effect on the re-acquisition of the Morris water escape task (Chapter 4.3, first experiment, and Chapter 4.4.).

The infarcts induced by MCA-O in mice were mainly restricted to cortical regions. No damage, or only slight damage was seen in subcortical regions. Deficits in learning and memory are more likely to occur when larger subcortical areas are affected (e.g. Yonemori et al., 1996). For example, Liang et al. (1997) observed profound and lasting WM, but not reference memory (RM) deficits in gerbils in the eight-arm radial maze task after global ischemia induced by a 6-minute occlusion of the carotid arteries. Up till about 3 weeks after the occlusion, when behavioral testing was terminated, there was no recovery of WM performance. Block and Schwarz (1996) induced global ischemia in rats by four-

vessel occlusion, a techniques introduced by Pulsinelli and Brierley (1979), in which the vertebral arteries were occluded permanently, and the carotid arteries were occluded transiently. The rats which had four-vessel occlusions showed a slowed acquisition of a standard Morris water escape task when tested in the second week after the operations. By the fifth acquisition session they were able to locate the escape platform as efficiently as the sham-lesioned control rats. However, in the probe trial, the bias for the previous position of the escape platform in rats with brain infarcts was weaker than that of the sham-lesioned controls, indicating that they suffered from spatial memory deficits.

Global forebrain ischemia usually damages the hippocampus, particularly the CA1 region (e.g. gerbils: Kuroiwa, Bonnekoh & Hossmann, 1991; rats: Volpe, Waczek & Davis, 1988; Kiyota, Miyamoto & Nagaoka, 1990; Netto et al., 1993; Block & Schwarz, 1996), and the hippocampus is critically involved in spatial orientation performance (e.g. Barnes, 1988b; Okaichi & Oshima, 1990; Jarrard, 1993, 1995; Schwegler & Crusio, 1995; Dusek & Eichenbaum, 1997). Therefore, deficits in (spatial) learning and memory are more likely to be found in models of global ischemic stroke (see review by Nunn & Hodges, 1994), induced by occlusion of the carotid artery, or by double-, triple-, or quadruple-vessel occlusion, where the hippocampus is damaged, than in a model of focal ischemic stroke induced by occlusion of the MCA, where hippocampal integrity is not compromised.

Pattern of blood vessels

The pattern of blood vessels and the pattern for and extent of collateral anastomoses (Oliff, Coyle & Weber, 1997), which might compensate the altered blood supply in the afflicted areas (Coyle, 1975), appear to be highly variable between strains. These differences might partly account for the differences in the severity of the behavioral dysfunctions seen after occlusion of the MCA. It is advisable, when using a particular strain of rats or mice, to investigate and document thoroughly the pattern of blood vessels and anastomoses between the MCA and the anterior cerebral artery (ACA) as part of the validation of the MCA-O model, as is done with other animal models of stroke. This aspect might be even more important for the evaluation of the consequences of transient versus permanent occlusions.

A major problem of focal permanent MCA-O for behavioral investigations is that it requires craniotomy (Memezawa, 1993; Rogers et al., 1997). This surgical procedure appears to cause behavioral dysfunction by itself: the transient effects on Morris maze performance in the sham-operated and the MCA-occluded mice (Chapters 4.3, first experiment, and Chapter 4.4) and the effects on body weight in rats (Chapter 4.1) support this notion. A decrease in body weight in sham-operated rats and MCA-occluded rats, compared with an intact control group, has also been observed by Yamamoto and colleagues (1988). Less traumatic techniques to induce occlusion of the MCA are available (e.g. Memezawa, 1993; Zhang, Chopp & Powers, 1997). These techniques consist of introducing a coated filament or a monofilament into the internal carotid artery. Then, in order to induce embolization, the filament is forwarded to the origin of the MCA and left in place for a given period of time. Reperfusion is started by retracting the filament. MCA-O with reperfusion, i.e. transient occlusion, has been found to cause more damage in subcortical areas and less damage in the cortex than permanent MCA-O (Zhao et al., 1996; Garcia et al., 1997). However, the pattern and severity of cortical and subcortical damage appears to depend, in a complex and not yet well understood manner, on the duration of occlusion and reperfusion (Garcia et al., 1997).

Does permanent focal MCA-O in rodents mimic the behavioral dysfunctions seen in stroke patients?

Comparison of the deficits seen after MCA-O in rats with those typically seen in patients after MCA-O (Adams, Victor & Ropper, 1997, p. 790; cited in Chapter 4.0) reveals a number of similarities and differences. Similar to patients suffering from stroke caused by occlusion of the MCA, rats also have sensorimotor deficits on the side contralateral to the infarct. However, the deficits appear to be mild compared with those seen in patients. Moreover, most sensorimotor deficits were transient and disappeared within a few weeks after the stroke. In contrast, half the patients who survive the stroke suffer from persistent neurological impairments (Gorelick, 1995). In patients, cognitive deficits are also major symptoms (Adams, Victor & Ropper, 1997). In our animal studies, cognitive impairments after MCA-O were virtually absent.

It is questionable whether permanent cessation of the blood supply to the core of the infarct, as produced in the permanent focal MCA-O model, has relevance for most strokes caused by occlusion of the MCA in patients (Hunter, Green & Cross, 1995), because in patients, the thrombus usually disintegrates and reperfusion of the infarcted area occurs. Consequently, transient ischemic insults of the MCA-O (e.g. Sakai et al., 1996; Zhao et al., 1996, Belayev et al., 1996), or combinations of different (permanent and/or transient) occlusion techniques (e.g. Netto et al., 1993; McAuley, 1995; see also Chapter 4.0, Table 1) might provide better models to mimic human infarcts and to assess the resulting *behavioral* deficits.

The permanent MCA-O appears to be a reliable method to induce reproducible brain damage. This makes the model especially useful for assessing the effects of putative neuroprotective compounds which are expected to reduce the infarct volume (e.g. Gotti, et al, 1990; Matsumoto et al., 1996; Ren & Finklestein, 1997).

In summary, permanent focal MCA-O reliably induces brain infarcts in rats and mice. These occlusions cause sensorimotor deficits which, however, usually recover within a few weeks. We never saw clear effects of MCA-O on learning and memory; the results reported in Chapter 4 on sensorimotor deficits and on the failure to produce clear cognitive deficits have been replicated and extended in other studies (e.g. Klapdor-Dulfer, 1996). Therefore, we conclude that permanent focal MCA-O is not a valid model for the complex, long-lasting and *severe behavioral dysfunctions* seen in patients with stroke. Techniques which allow the induction of transient vessel occlusions without craniotomy should be considered when the major aim of the study is to investigate stroke-induced behavioral deficits, their prophylaxis, or their treatment.