THE NEUROPROTECTIVE EFFECTS OF PITUITARY ADENYLATE-CYCLASE ACTIVATING POLYPEPTIDE (PACAP) IN FOCAL CEREBRAL ISCHEMIA

Including some additional related studies

I. INTRODUCTION AND BACKGROUND

I/1. CEREBRAL ISCHEMIA

Cerebrovascular disease, commonly referred to as "stroke", is the third most common cause of death and leading cause of chronic disability in western countries. In Hungary, 40 000 patients are hospitalized with this diagnosis per year, and more than 20 000 deaths occur due to stroke and its complications (Nagy 1999). Although a great deal of progress has been made in identification of risk factors, in understanding the pathophysiology and in diagnostic strategies, the managent of human stroke still remains a major clinical problem.

One of the main causes of stroke is the occlusion of the middle cerebral artery (MCA) or its branches. The therapeutic interventions that are known to be effective in human stroke are very limited. Dozens of new candidate therapeutic substances have been demonstrated to exert neuroprotection against ischemic brain damage in animal models of stroke. However, most of these agents are effective in animals only when administered either before or shortly after the onset of ischemia, which limits their clinical usefulness (Dyker and Lees 1998).The toxic side effects and the inability to cross the blood-brain barrier (BBB) are the major other reasons that most of the therapeutic agents proven to reduce the infarct size in experimental animals have failed to ameliorate human stroke outcome (Dyker and Lees 1998, Plum 1997).

Almost all animal models of focal ischemia primarily involve unilateral MCA occlusion (MCAO). The two basic classes of insult are the permanent and temporary ischemia. There are numerous different techniques used to occlude the MCA (rev.: Lipton 1999). The most commonly used methods are the proximal occlusion with or without craniectomy. In the latter case, an intraluminal suture is inserted through the

carotid arteries to the origin of the MCA, before the origin of the lenticulostriate arteries, thereby both basal ganglia and cortical areas are damaged. The evaluation of the ischemic damage is most commonly done by staining brain sections with 2,3,5-triphenyltetrazolium chloride (TTC). The staining action of TTC is based on the presence of intact mitochondrial enzymes, which reduce the tetrazolium salts to a deep red formazan compound. In necrotic tissues there is no staining, while in damaged, but not yet necrotic tissues the staining is pink (Bederson et al. 1986a).

I/2. PITUITARY ADENYLATE CYCLASE ACTIVATING POLYPEPTIDE (PACAP)

Pituitary adenylate cyclase activating polypeptide (PACAP) was first isolated from ovine hypothalami, and occurs in two amidated forms, with 38 and 27 amino acid residues (Miyata et al. 1989, 1990). PACAP is a member of the secretin/glucagon/VIP family, and has 60% sequence similarity with VIP, but its adenylate cyclase stimulating activity has been shown to be 1000-10,000 times greater than that of VIP. The primary structure of PACAP38 is identical among all mammalian species examined, and it also shows marked similarity with lower vertebrates and nonvertebrates, with differences in only 1-4 amino acids (rev.: Arimura 1998). This suggests that the structure of PACAP has remained very conserved throughout phylogenesis and it may reflect its importance in fundamental functions in the nervous system.

Despite the high similarity between VIP and PACAP, the distribution of these peptides is quite different. In human tissues, PACAP38 is the predominant form, and PACAP27 makes up less than 10% of the total PACAP (Arimura et al. 1991). Highest concentration of PACAP is found in the hypothalamus, but other areas of the brain, including cerebral cortex, brainstem, basal ganglia, thalamus, posterior pituitary cranial nerve nuclei also contain PACAP immunoreactive cells (Köves et al. 1990, Ghatei et al. 1993, Arimura 1998). The distribution pattern of PACAP in the human brain is unique: it shows no similarity to the distribution pattern of other neuropeptides in the central nervous system (CNS) (Köves et al. 1991, Palkovits et al. 1995). In the peripheral organs, high concentrations are found in the testis, adrenal medulla and pancreas, but nearly all organs contain detectable amount of PACAP

(Arimura et al. 1991, Köves et al. 1993). The PACAP receptor is G protein-coupled with seven transmembrane domains and also belongs to the VIP receptor family. At least eight subtypes of PAC1-receptors are found, which bind PACAP but not VIP, while VPAC1 and VPAC2 receptors bind both VIP and PACAP with a similar affinity (Arimura 1998, Harmar et al. 1998).

PACAP has been demonstrated to act mainly as a hypophysiotropic hormone, neurotransmitter and neuromodulator (rev.: Arimura 1998). The major regulatory role of PACAP in pituitary cells appears to be the regulation of gene expression of pituitary hormones and/or regulatory proteins that control growth and differentiation of the pituitary glandular cells. These effects appear to be exhibited directly or indirectly through paracrine or autocrine actions (Arimura 1998, Köves et al. 1998). Numerous other effects of PACAP have been reported, both in the central and peripheral nervous systems, as well as in endocrine and other organs. A recent review has summarized the current status of research on the distribution, molecular biology and physiological effects of PACAP (Arimura 1998).

I/3. NEUROPROTECTIVE EFFECTS OF PACAP IN VITRO AND IN VIVO- BACKGROUND FOR THE PRESENT STUDY

Since its discovery, increasing *in vitro* evidence has been accumulated on the neurotrophic and neuroprotective effects of PACAP in various neuronal cell cultures. PACAP receptor-mediated actions have been demonstrated on cell proliferation and survival in developing nervous systems (Waschek 1996). PACAP stimulates the outgrowth of neurits in different cell cultures and the survival of PC12 cells (Deutsch et al. 1992, Hernandez et al. 1995, Tanaka et al. 1996), and it can prevent natural cell death in the spinal cord of the chick embryo and in cultured cerebellar granule cells (Arimura et al. 1994, Canonico et al. 1996). Neurotrophic actions have also been demonstrated in rat cortical neurons (Morio et al. 1996b), in mesencephalic neurons (Takei et al.1998), in dorsal root ganglion neurons (Lioudyno et al. 1998) and in basal forebrain cholinergic neurons (Lindholm et al. 1999).

PACAP has been shown to have neuroprotective effects in various pathophysiological conditions (Arimura 1998, Said 1999). It protects cultured rat cortical neurons against glutamate-induced cytotoxicity (Morio et al. 1996a),

dopaminergic neurons against 6-hydroxydopamine-induced cytotoxicity (Takei et al. 1998) and attenuates glutamate-induced neurotoxicity in hippocampal neurons (Changlin and Yan 1999). Dramatic neuroprotection has been observed in cultured hippocampal neurons at subpicomolar concentrations against a HIV envelope protein, gp120-induced neuronal cell death (Arimura et al. 1994) and in a lipopolysaccharide-induced neurotoxicity model in mixed cortical/glial cultures (Kong et al. 1999). Recently, it has been demonstrated that PACAP promotes the survival of basal forebrain cholinergic neurons *in vitro* and *in vivo* after injury (Lindholm et al. 1999, Skoglosa 1999). These studies indicate that PACAP is neuroprotective against various conditions of neurotoxicity and neuronal injury.

A basic requirement for any *in vivo* neuroprotective substance is the ability to cross the BBB. PACAP has been proven to cross the BBB by a saturable transport system in mice (Banks et al.1993). The percent of the intravenously (iv) injected dose of PACAP38 entering the whole mouse brain is about 0.118%, which is about six times more than that for morphine (Banks et al. 1996). The ability to cross the BBB and the high influx rate have raised the possibility of PACAP being neuroprotective also *in vivo*, by systemic administration.

The CA1 neurons of the hippocampus are highly vulnerable to transient forebrain ischemia and are used as a model for evaluating neuroprotective agents. Neuronal death in CA1 neurons is characteristically a delayed neuronal death, which is protracted in time during the first few days following transient ischemia, providing an interval for rescuing these cells. Based on the very low concentrations needed for neuroprotection in *in vitro* studies, and the efficacy of the passage of PACAP across the BBB, it had been postulated that very small amounts of systemically administered PACAP would be needed to reach the concentration necessary for its neuroprotective effect in the brain. It was found that PACAP38 significantly attenuated neuronal cell death in the CA1 area of the hippocampus by intracerebroventricular administration (Uchida et al. 1996), with maximum cytoprotection at 1 pmol/h over 7 days. The tissue concentrations of PACAP38 reached during and under this condition may approximate 0.1 pM which showed the greatest neuroprotection *in vitro* (Arimura et al. 1994). Significant neuroprotection was also observed by slow iv infusion of PACAP38 at concentrations of 16-160 pmol/h, even when treatment was delayed by 24 hrs. The iv bolus injection prior the slow iv infusion was found to be necessary for the neuroprotective effect, which raises the possibility of the presence of a binding

protein for PACAP38 in the blood (Somogyvari-Vigh et al. 1998). Recently, the binding factor of PACAP38 has been identified in the human plasma as ceruloplasmin (Tams et al. 1999).

I/4. EXPERIMENTAL AIMS

The primary aim of the present study is to describe the neuroprotective effect of PACAP38 in a rat model of focal cerebral ischemia. In order to study the effects of a candidate neuroprotective substance, it is of extreme importance to standardize the method of focal ischemia, since the results obtained by different investigators show high variation. During the attempt to standardize the model, we have found that postischemic hyperthermia is an unavoidable consequence of the filament model, and we have conducted several experiments on the time course and the effects of spontaneous postischemic hyperthermia. We have studied the effects of delayed systemic administration of PACAP38 in a focal ischemia model, and we have investigated this neuroprotective effect under different experimental conditions. We have also studied the passage of PACAP38 across the BBB in the rat and how this passage is affected in focal ischema.

II. MATERIALS AND METHODS

In the present section, materials and methods are described which have been used generally in our experiments. Details and modifications are mentioned in the sections dealing with specific experiments.

Animals

Adult male CD rats weighing 275-300 g were purchased from Charles River Laboratories and were maintained at room temperature, in an alternating 12-hr light and 12-hr dark cycle (lights on at 6 am). The animals were quarantined for at least 7 days before the experiment. Before MCAO, the rats were placed in individual cages and fasted overnight, but allowed free access to water.

Anesthesia

The rats were anesthetized with halothane in a mixture of 70% nitrous oxide and 30% oxygen. Anesthesia was induced with 3% halothane and subsequently maintained with 1% halothane delivered with a face mask.

Body temperature

Body temperature was maintained in the normal range (36.5-37.5°C) with a heating lamp and a heating pad during the operation. Temperature was monitored with a rectal probe and kept in the normal range during the first 6 hrs after MCAO. According to our observations, the body temperature of most of the animals rises 15-20 minutes after MCAO, and remains high (39-39.5°C) for a couple of hours (section III/4). Cooling the animals in the cold room combined with alcohol application on the back of the animals proved to be the only effective way to prevent temperature from rising above normal, which would greatly influence the size of the damaged brain area, and could even obliterate the effects of the tested drugs (Memezawa et al. 1995). Those rats which developed a too high or too low temperature, in spite of attempts to control the body temperature, were excluded from further experiments.

Focal Cerebral Ischemia

The left middle cerebral artery (MCA) was occluded using the intraluminal suture technique described by Longa et al (1989). The left carotid region was exposed through a midline cervical incision. The common carotid artery and the external carotid artery below the origin of the occipital artery were ligated with a 3-0 mm suture. The only extracranial branch of the internal carotid artery, the pterygopalatine branch was also ligated to prevent incorrect insertion of the occluder filament. A 5-cm length of a 4-0 monofilament nylon suture (Ethilon; Ethicon Inc, France), whose tip had been rounded by heating near a flame and coated with poly-L-lysine (Belayev et al. 1996a) (Sigma) was introduced from the carotid bifurcation into the internal carotid artery until a mild resistance was felt (18-19 mm), thereby occluding the origin of the MCA. Following surgery, anesthesia was discontinued and the rats were allowed free access to food and water. Rats were under anesthesia for approximately 10 minutes, including induction of anesthesia and surgical procedure. Recirculation of the MCA was established 2 hrs after MCAO by gentle withdrawal of the suture until the rounded tip reached the insertion point where a slight resistance was felt (under the same anesthetic conditions as surgery).

Neurological Evaluation

The neurological evaluations were carried out 2 hrs after MCAO according to the methods described by Bederson et al. (1986b) and Memezawa et al (1992). Animals with successful occlusion of the MCA showed gait disturbances with circling or walking to the right (corresponding to Grade 3 by Bederson et al.). Rats showing circling also always showed forelimb flexion, thorax twisting, decreased pain reflexes of the right forelimb, and decreased resistance to lateral push. Our preliminary studies are in agreement with those of Memezawa et al. (1992): rats which displayed these latter signs but without circling, did not have a satisfactory, reliable occlusion. Therefore, animals without circling or walking to the right were excluded from further experiments. Including those rats would increase the variance and could lead to misinterpretation of the data (section III/2). Some animals developed convulsive behavior, which would also influence the infarct size independently of the ischemic damage; therefore, those animals were also excluded (section III/2).

Calculation of the Infarct Volume

All rats were decapitated under halothane anesthesia, and brains were removed rapidly and cooled in 98% isoamyl alcohol (Sigma) at minus 20°C for one minute. Two-mm-thick coronal sections were cut with the aid of a brain slicer matrix (Braintree Scientific Inc). Sections were immersed in 2% TTC (Sigma) for 30 minutes, then transferred to 10% phosphate-buffered formalin for fixation. Animals which developed subarachnoideal hemorrhage were excluded from further evaluation.

Brain areas were traced and measured using an image analysis system (NIH Image). Unstained areas were defined as ischemic lesions (Bederson et al. 1986a). The areas of infarcted tissue and the areas of both hemispheres were calculated for each brain slice. An edema-index was calculated by dividing the total volume of the hemisphere ipsilateral to MCAO by the total volume of the contralateral hemisphere (Yanamoto et al. 1996). The actual infarct volume adjusted for edema was calculated by dividing the infarct volume by the edema index. The results are given as infarcted volume as the percentage of the total measured brain volume.

Statistical Analysis

Results are presented as the mean ± SEM. Statistical analysis was conducted with a one-way ANOVA followed by *t*-test for comparisons between groups. A *t*-test with Bonferroni correction was used for comparisons between individual histological sections. $P < 0.05$ was considered statistically significant.

III. STANDARDIZATION OF THE METHOD

(Temporal evolution of the infarct size and effects of postischemic spontaneous hyperthermia and resurgery on the brain damage)

III/1. BACKGROUND

The most commonly used animal model of stroke is MCAO with an intraluminal suture. Although it was first described more than ten years ago (Longa et al. 1989), the optimal experimental conditions are still questions of debate (Schmid-Elsaesser et al. 1998, Petullo et al. 1999). A recent study has pointed out that the numerous modifications reported in the literature indicate that the model has still not been standardized (Schmid-Elsaesser et al. 1998). Some authors have even admitted that it took months of trial and error to achieve a reproducible ischemia (Memezawa et al. 1992), while others have reported that it was impossible to obtain reproducible infarct size with the original filament model (Belayev et al. 1996a, Yanamoto et al. 1998).

The results obtained by different investigators have shown large variations in infarct sizes, which makes it difficult to compare results from different laboratories. The discrepancies have been attributed to a number of factors:

a. Different arterial pattern and strain differences

Anatomic variation of the MCA in the same strain and sex is one of the factors which is thought to be a potential source of variation in infarct size (Fox et al. 1993, Cai et al. 1998). This variation in the arterial pattern leads to differences in the collateral blood flow, which may account for the differences in infarct size. It has been shown that the filament model leads to larger infarct size than the craniectomy model because the filament model interferes with and compromises the collateral circulation, especially in permanent occlusion (Belayev et al. 1997, Herz et al. 1998). There is a considerable difference in infarct sizes between different rat strains (Markgraf et al. 1993, Lipton 1999), which is only partly due to the arterial patterns and can also be attributed to difference in excitatory amino acid release (Herz et al. 1996). Substrain

and vendor diferences have also been demonstrated to lead to differences in infarct size (Oliff et al. 1995, 1996).

b. Age of animals

Contradictory results have appeared concerning the influence of the age of animals on the infarct size. It has been reported that rats over 3-400 g yield to smaller and more inconsistent infarct sizes than younger rats (Longa et al. 1989), which may be due to a more developed collateral system in older rats. Others have reported the opposite: older rats produce significantly larger infarct volumes (Oliff et al. 1995, Sutherland et al. 1996)

c. Technical details

Small changes in the technique can also greatly influence the infarct size. Using two different 4-0 filaments has been reported to significantly influence the infarct volume (Kuge et al. 1995), such as using different coatings on the filament. Suture coated with poly-L-lysin has been found to yield to larger infarct size and smaller individual variation than the uncoated sutures (Belayev et al. 1996a). Silicon coated sutures have also been reported to produce a reliable infarct size (Schmid-Elsaesser et al. 1998).

d. Complications of MCAO

Hemorrhage is a complicating factor in MCAO and may occur spontaneously or during/after reperfusion. A recent study (Schmid-Elsaesser et al. 1998) has reported that subarachnoid hemorrhage is more common than previously believed. It causes further cerebral damage (Quereshi et al. 1999) and therefore animals with hemorrhage must be excluded. Convulsions are mentioned by very few reports as a complicating factor (Tamura et al. 1981, Uchino et al. 1994, Kim et al. 1996), which increases the cerebral metabolism and leads to a type of cell damage that is not related to ischemia (Tamura et al. 1981, Auer and Siesjo 1988). Therefore, animals with convulsive behavior after MCAO must also be excluded from the experimental groups. Premature reperfusion can also account for differences in the infarct size. A recent report (Schmid-Elsaesser et al. 1998) has drawn the attention to inadvertent premature reperfusion in the filament model which may be caused by inappropriate filament tightening or by movements of the animal.

e. Temperature

The protective effect of intra- and postischemic hypothermia in cerebral ischemia is well known (rev.: Barone et al. 1997). Hyperthermia, on the other hand, leads to larger infarct size. Hyperthermia is partly due to the ischemic insult itself, which appears to be more serious in the filament model (Zhao et al. 1994a, Lipton 1999). Temperature control by a heating lamp and pad during MCAO is mentioned in almost every paper using this technique. However, controlling the postischemic hyperthermia is mentioned by very few authors. Based on these observations, it is desirable to control the temperature when studying focal ischemia and effects of candidate neuroprotective drugs. Hyperthermia can not only increase the infarct size, but can also nullify the effects of otherwise neuroprotective drugs (Memezawa et al. 1995, Ginsberg and Busto 1998).

f. Anesthesia

As mentioned previously, postoperative hyperthermia may occur with any type of anesthesia. The most widely used anesthesia in MCAO studies is the halothane anesthesia. Halothane, while anesthesia is lasting, causes hypothermia, and it has even been used by some investigators to reduce postoperative hyperthermia, thus decreasing brain damage in global ischemia (Kuroiwa et al. 1990). Halothane and sevoflurane have been reported to reduce the infarct size also by others (Warner et al. 1993), but this effect seems to be merely due to hypothermia and not the direct effects of halothane (Kuroiwa et al. 1990). Several studies describe the beneficial effects of barbiturate anesthesia on the infarct size although it has not been proven in humans (Scheinberg 1991, Warner et al. 1991). According to these studies it is very important to standardize the anesthesia in order to obtain comparable experimental groups which are not affected by different durations of anesthesia.

g. Neurological scoring

There are numerous different scoring systems to evaluate neurological deficits after MCAO. One of the most widely used is the one by Bederson et al. (1986b) who originally described the scoring system in rat MCAO by craniectomy. Forelimb flexion, decreased resistance to lateral push and circling are examined. The grading is 0-3, with circling being the most severe (Grade 3). They have observed that when circling is present, the other two signs are also always present. They have found

correlation between the infarct size and the neurological deficit. Other studies have attributed only moderate deficit to circling, and designated severe deficit to those animals with falling or no spontaneous walking (Longa et al. 1989). Many other scoring systems have been described, including examination of symmetry of movement, reaction to touch on the trunk and vibrissae, rota-rod test, grid walking test and various memory tests (Yanamoto et al. 1988, Garcia et al. 1995b, Rogers et al. 1997). Memezawa et al. (1992) have observed that only animals with walking or circling in one direction have successful occlusion of MCA, therefore they excluded all animals with no signs of circling.

In summary, most authors believe that examining the neurological signs is important, and deficits show close correlation with the infarct size. Only one study (Wahl et al. 1992) found no correlation between neurological deficits and infarct size, and they never observed circling, only exceptional rolling behavior. Filament insertion by any technique may not result in adequate occlusion of the MCA, therefore, neurological examination is important not only for grading the neurological deficits of the animals, but also for assessing animals with adequate, comparable occlusions of the MCA.

III/2. TEMPORAL EVOLUTION OF THE INFARCT SIZE

According to the above section, it is of extreme importance to standardize the MCAO model. The aim of the following experiments was to obtain the temporal evolution of the infarct size under our experimental conditions, and to describe the time course and effects of postischemic spontaneous hyperthermia, which is one of the major factors influencing the infarct volume.

Methods

MCAO was done according to the methods described in Section II. In order to obtain the temporal evolution of the infarct size, animals with positive neurological signs were sacrificed 4 (n=15), 8 (n=9), 12 (n=14), 24 (n=16), 48 (n=12) or 72 (n=10) hrs

after MCAO. In 15 rats no recirculation was established, these constitute the permanent ischemic animals, which were sacrificed 48 hrs after the occlusion. In the 48-hr transient ischemia group, we also examined the inclusion of animals with questionable neurological signs, i.e.: animals which partially circle or do not circle at all but show other neurological signs (section III/1).

Results and Discussion

Measurements of the damaged brain areas show that infarcted brain volume was 2.2 ∀ 0.66% 4 hrs after MCAO, 6.27 ∀ 2.1% at 8 hrs, 8.2 ∀ 2.24% at 12 hrs, 21.75 ∀ 1.5% at 24 hrs, 22.34 ∀ 1.18% at 48 hrs and 16.14 ∀ 3.2% at 72 hrs (Fig 1). There was no significant difference between the 24-, 48- and 72-hr groups. The variation was the smallest in the 48-hr group. Permanent ischemia resulted in an infarct size of 24.66 ∀ 1.5%.

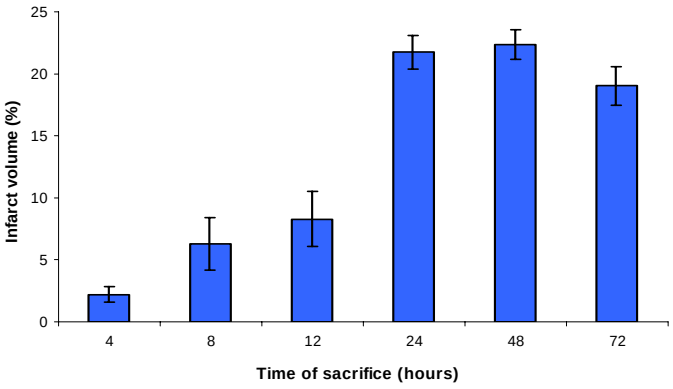


Figure 1
Temporal evolution of infarct volumes. The MCA was occluded for 2 hrs, and the animals were sacrificed either 4, 8, 12, 24, 48 or 72 hrs after MCAO. Infarct volumes are expressed as the percentage of total brain volumes.

These results show that the infarct size gradually increased during the first 12 hrs, and rats seemed to respond very differently to the ischemic insult during this period, as indicatied by the larger standard error. The infarct size nearly reached the maximum at 24 hrs, while at 48 hrs, it was slightly larger, but the difference was not significant. After 48 hrs, the slight decrease may be due to invading normal cells that participate in the healing process, and stain well with TTC (Liszczak et al. 1984). Based on the small variation and maximum infarct size 48 hrs after the insult, we chose to sacrifice the animals at 48 hrs in our further experiments.

If we included animals in the 48-hr transient ischemia group with no definite circling in the evaluation (ie.: started circling but continued to walk in a straight line) (n=21), the infarct volume was 17.9 ∀ 1.64%. If those animals which did not show circling at all (n=26), but exhibited other neurological signs were also included, the average infarct volume was 13.9 ∀ 1.94% (Fig 2).

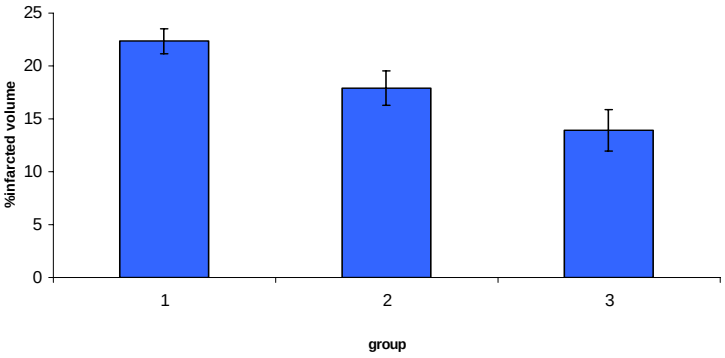


Figure 2
Infarct size at 48 hrs after MCAO in otherwise untreated rats. (1) animals with positive neurological signs, including circling, (2) including animals which circled but were also able to walk in a straight line, (3) including animals which did not show circling but exhibited other neurological signs. Infarct volumes are expressed as the percentage of total brain volumes.

These results show that the selection of the animals to be included in the data collection must be standardized. As it has been pointed out by many investigators, infarct size shows high correlation with the severity of the neurological deficits (section III/1). In our model, if we included only those animals which clearly showed circling behavior and other neurological signs, and did not develop convulsions, we were able to obtain a highly reproducible infarct size with small variation. This is in accordance with the observations of Memezawa et al (1992). As we included more animals that showed questionable circling, the average infarct size decreased with a larger variation. If those animals which did not show circling with other neurological signs were included, the average infarct size was even smaller with an even larger variation (in this group, the infarct size varied from 0% to 28%). Therefore, in further experiments we only included animals with definite circling behavior.

III/3. EFFECTS OF REANESTHESIA AND LIGATION OF THE INTERNAL JUGULAR VEIN 4 HOURS AFTER MCAO ON THE INFARCT SIZE

Based on the temporal evolution of the infarct size, we planned to start administration of PACAP38 through a cannula in the internal jugular vein 4 hrs after MCAO. Therefore, it was of interest to see the effects of reanesthesia and the ligation of the internal jugular vein. Seven animals were reanesthetised 4 hrs after MCAO, and the internal jugular vein was opened and then ligated. The animals were kept under anesthesia for 10 minutes. In the control group (n= 12) no intervention was done after MCAO.

Results and conclusions

The infarct volume in the control group was 22.23 ± 1.53%, in animals where the internal jugular vein was ligated, it was 24.37 ± 4.3% (Fig 3). Although the average infarct volume resulted to be larger with a higher individual variation, the difference was not significant. Therefore, it can be concluded, that reanesthesia for 10 minutes and ligation of the internal jugular vein 4 hrs after MCAO does not significantly influence the infarct size.

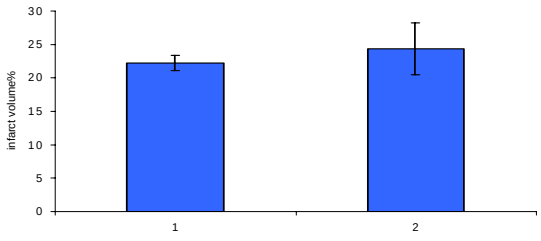


Figure 3
Infarct size 48 hrs post-MCAO in (1) control animals and in (2) animals with ligation of the internal jugular vein. Infarct volumes are expressed as the percentage of total brain volumes.

III/4. TIME COURSE AND EFFECTS OF SPONTANEOUS POSTISCHEMIC HYPERTHERMIA

Background

Body or brain temperature can substantially alter the extent of ischemic damage (section III/1). The neuroprotective effects of intra- and postischemic hypothermia, and the deleterious effects of hyperthermia, are well known (Chen et al. 1991, Morikawa et al. 1992, Barone et al. 1997, Maier et al. 1998). During the operation for MCAO, the core temperature usually falls if it is not controlled. Temperature control with a heating lamp or heating pad in order to avoid intraischemic hypothermia is mentioned by almost all researchers who use the filament technique. However, the control of postischemic hyperthermia is mentioned by very few authors, which could be due to the fact that the occurrence of postischemic spontaneous hyperthermia after MCAO is not a universal observation. Zhao et al. (1994a) described the time course of spontaneous hyperthermia and speculated that this phenomenon is a complication of the filament technique. Schmid-Elsaesser et al. (1998) did not observe similar elevations in the core temperature in a recent reevaluation of this model.

Clinical studies have shown that stroke patients often develop mild hyperthermia or fever which enlarges the infarct size and worsens the outcome of stroke; therefore, maintaining body temperature at normothermic levels is highly recommended (Azzimondi et al. 1995, Reith et al. 1996, Ginsberg and Busto 1998). Numerous studies have demonstrated the deleterious effect of controlled hyperthermia for different durations in both global and focal ischemia. However, the effects of postischemic spontaneous hyperthermia have received little attention in animal research. Since stroke patients often develop hyperthermia, it is reasonable to study the effects of uncontrolled, fluctuating core temperatures after MCAO.

The aim of the present study was to describe the time course of postischemic spontaneous hyperthermia under different experimental setups. Although hyperthermia has been shown to be detrimental even if it is delayed, it is well accepted that events during first few hours have the largest influence on brain damage and the potential therapeutic interventions. Therefore, we monitored the temperature

very closely during the first few hours, and we also examined the effects of spontaneous hyperthermia on the extent and temporal progression of brain damage.

Methods

Experiment 1

In order to obtain the time course of changes in the core temperature after MCAO, temperature was measured with a rectal probe every 10 minutes during the first 2 hrs, every 20 minutes up until 4 hrs, and then at 5, 6, 8, 24, and 48 hrs after MCAO.

Two groups of rats underwent 90 minutes (n=8) or 120 minutes (n=8) transient focal ischemia, and one group (n=8) had a permanent occlusion. These rats showed all the neurological signs 2 hrs after MCAO. To determine whether rats with an incomplete occlusion have similar rises in body temperature, 12 rats which exhibited no circling behavior but showed the other neurological signs after a 2-hr transient ischemia were used (section III/2). The sham-operated control group (n=4) was subjected to all of the steps for MCAO, except that the filament was introduced only to 10 mm, which is not sufficient to occlude the MCA.

Experiment 2

In order to examine the effect of spontaneous hyperthermia on the infarct volume, rats were subjected to 90 (n=10) or 120 (n=9) minutes of transient, or permanent (n=11) ischemia. Core temperature was strictly maintained within the normal range (36.5-37.5°C) during the first 6 hrs after MCAO. A large area of the fur on the animal's back was shaved off, and when the temperature started rising above 37°C, the animals were placed in a cold room (4°C). In addition, alcohol (70%) application on the back of the animals was used in cases of a sudden rise in temperature. As soon as the temperature started falling below 37°C, rats were allowed to return to room temperature. Animals subjected to successful transient or permanent ischemia from Experiments 1 and 2 were decapitated under anesthesia 48 hrs after MCAO and brain damage was measured according to the methods described in section II.

Experiment 3

In order to examine the effect of spontaneous hyperthermia on the temporal progression of brain damage, rats underwent a 2-hr transient focal ischemia under the

same conditions as described above. Animals were sacrificed 4 hrs (n=8) or 12 hrs (n=8) after MCAO without any temperature control. Another group of rats was subjected to the same intervention, but the core temperature was strictly maintained within the normal range, as described in Experiment 2. Fifteen rats were killed 4 hrs, and 14 rats 12 hrs after MCAO. The evaluation of the extent of brain damage was conducted as described in section II. In this experiment, the penumbral area was also measured, which is indicated by the pink TTC staining (Bederson et al. 1986a). At 48 hrs, the demarcation between infarcted and normal tissue (white and red areas, respectively) is quite clear, but at only 4 and 12 hrs after MCAO there is a considerably large area which is pink, and is localized at the margins of the white areas (Bederson et al. 1986a, Cole et al. 1990, Memezawa et al. 1992). The data obtained in this experiment were combined with the data from the animals subjected to a 2-hr transient ischemia in Experiments 1 and 2.

Results

Experiment 1

None of the animals showed hypothermia during and after MCAO. The temporal profiles of the core temperatures after transient and permanent MCAO (Fig. 4a,b,c) showed that temperature started rising 15-20 minutes after MCAO and reached 39-40.5°C in most animals during the first hour. There was a slight decrease following reperfusion at 90 or 120 minutes, but sustained hyperthermia was observed thereafter, during the first 8 hrs. The individual responses in body temperature showed variations in the time of onset of hyperthermia after MCAO and after reperfusion. In animals with permanent occlusion, hyperthermia was observed throughout the first 24 hrs. The temperature decreased in all 3 groups after the first day, and the temperature of most animals returned to normal levels 48 hrs after MCAO.

In animals with an incomplete occlusion (exhibited no circling behavior) of the MCA for 2 hrs, the pattern of changes in core temperature was similar to the above groups (Fig. 4d), although the rise in temperature was more delayed, and temperatures did not reach as high levels as in animals with complete occlusion. The sham-operated animals displayed only a slight increase in temperature, which did not

rise above 38.5°C in any animal (Fig. 4e). The onset of this slight hyperthermia was more gradual than in the ischemic groups.

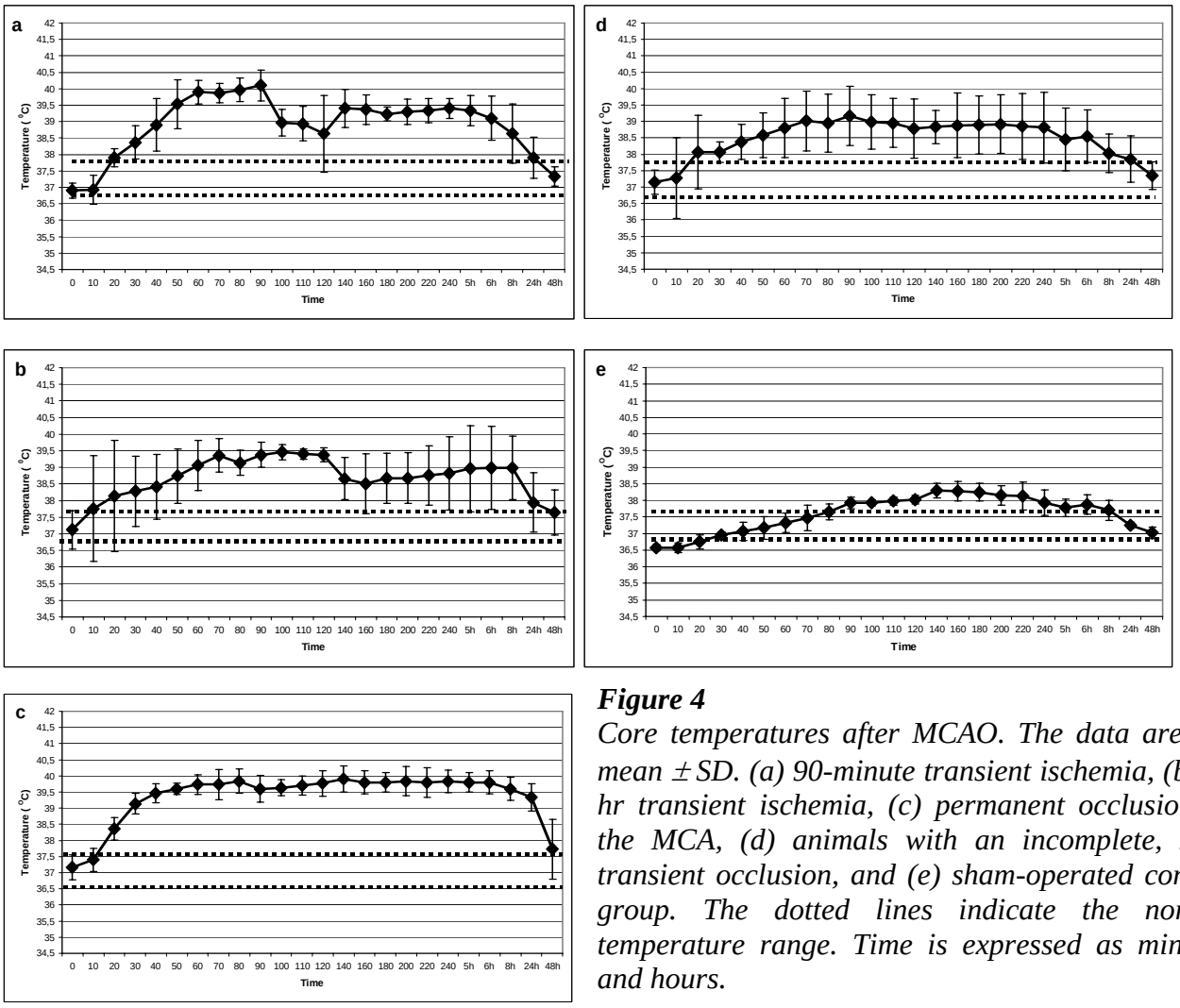
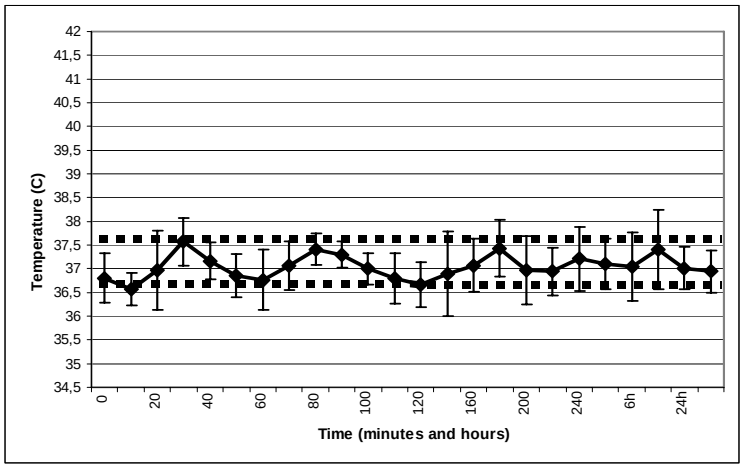


Figure 4

Core temperatures after MCAO. The data are the mean ± SD. (a) 90-minute transient ischemia, (b) 2-hr transient ischemia, (c) permanent occlusion of the MCA, (d) animals with an incomplete, 2-hr transient occlusion, and (e) sham-operated control group. The dotted lines indicate the normal temperature range. Time is expressed as minutes and hours.

Experiment 2

Temperature could be maintained within the normal range after MCAO by controlling it during the first 6 hrs (Fig. 5a). When cooling was discontinued, the animals tended to develop a slight increase in the core temperature but it did not reach 38.5°C, and soon returned to normal levels. One of the animals displayed a core temperature above 39°C in spite of being placed in the cold room throughout the entire 6 hrs. This animal was excluded from the group.



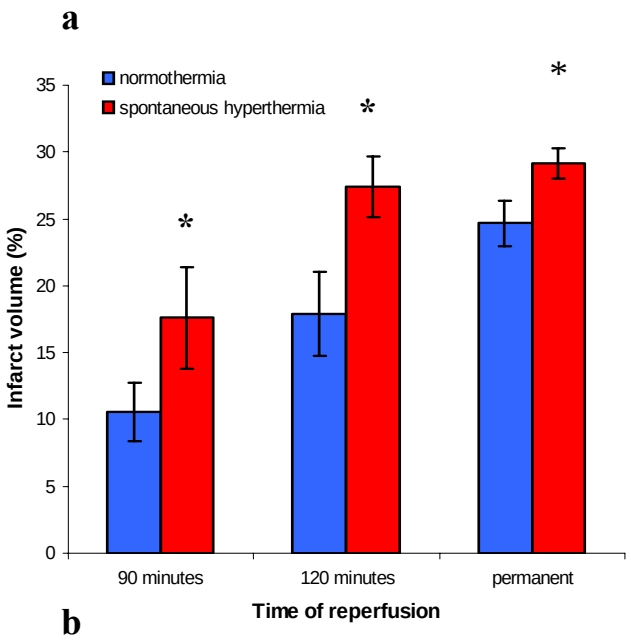


Figure 5
*Spontaneous hyperthermia and the extent of brain damage. (a) Core temperatures in animals under strict temperature control during the first 6 hrs following MCAO. The dotted lines indicate the normal temperature range. (b) Infarct volumes after a 90-minute or 2-hr transient, or after a permanent occlusion of the MCA in normothermic and in hyperthermic animals. Infarct volumes are expressed as the percentage of total brain volumes. *P< 0.05 versus the normothermic groups.*

Measuring the infarct volumes after 90- or 120-minute transient, or permanent ischemia in rats with spontaneous hyperthermia revealed that hyperthermia significantly increased the brain damage in all 3 groups, when compared to the corresponding normothermic control groups (Fig. 5b). The infarct volumes in rats that underwent a 90-minute transient focal ischemia with or without temperature control were 10.6 ± 6.1 or 17.6 ± 6.3%, respectively. This is a 39.9% increase in the infarct volume, which is statistically significant ($P < 0.05$). A similar increase was observed in the groups with a 120-minute transient focal ischemia: infarct volumes were 17.9 ± 6.5% in the normothermic and 27.4 ± 5% in the hyperthermic animals. This is a

34.7% increase ($P < 0.05$). Even animals with a permanent occlusion showed larger infarct volumes when temperature was not controlled: 24.7 ± 5.5% (normothermic) and 29.2 ± 2.9% (hyperthermic), which is a 15.5% increase ($P < 0.05$). A comparison between animals with different reperfusion times revealed that although infarct sizes are increased when animals display hyperthermia, reperfusion 90 minutes after MCAO is more beneficial than reperfusion at 120 minutes, even in animals with postischemic spontaneous hyperthermia ($P < 0.05$). However, reperfusion at 120 minutes is adventageous when compared to permanent occlusion only when temperature is kept normothermic ($P < 0.05$); it is no longer beneficial in hyperthermic animals ($P > 0.05$). Infarct sizes in hyperthermic animals with a 90-minute transient ischemia reached the infarct sizes of normothermic animals with a 120-minute transient ischemia. Infarct volumes of the hyperthermic rats with a 120-minute transient ischemia even exceeded those of the normothermic rats with permanent ischemia.

Experiment 3

The data of measurements of the temporal progression of the brain damage at 4, 12 and 48 hrs are summarized in Fig. 6. When animals are sacrificed before the brain damage has reached its maximum, staining with TTC results in large areas of the brain which are stained pink at the margins of the white (infarcted) areas. These areas are considered to be the penumbra. There was no significant difference ($P > 0.05$) between normothermic and hyperthermic animals at 4 and 12 hrs when the pink and white areas were added together (Fig. 6a). Total damaged brain volumes were 10.2 ± 5.3% and 9.9 ± 4.1% in the 4-hr, and 16.9 ± 4.3% and 18.5 ± 3.5% in the 12-hr normothermic and hyperthermic groups, respectively. A significant difference ($P < 0.05$) was observed only in the animals sacrificed 48 hrs after MCAO: total brain damage was 21.1 ± 6.2% in the normothermic, and 29.1 ± 5.7% in the hyperthermic animals, which is a 27.6% increase. However, the infarcted (white) and penumbral (pink) areas showed significant differences at all time points (Fig. 6b,c). The white areas measured 4 hrs after MCAO showed an almost three-fold (291%, $P < 0.05$) increase in the hyperthermic group compared to the normothermic control group (6.4 ± 3.1% versus 2.2 ± 2%), while the pink areas were decreased by 56.8% ($P < 0.01$) in animals with hyperthermia (3.5 ± 1.8% versus 7.9 ± 3.3%).

The infarcted and penumbral areas at 12 hrs showed similar differences between the groups, although the differences were not as marked. Infarcted (white) volumes were increased by 30.8% ($P < 0.05$) in the hyperthermic group ($8.3 \pm 4.1\%$ versus $12.3 \pm 3.2\%$), and penumbral (pink) volumes were decreased by 30.2% ($P < 0.05$) in the hyperthermic animals ($8.7 \pm 2.2\%$ versus $6.2 \pm 1.9\%$). At 48 hrs the infarct volume was also significantly larger in the hyperthermic animals ($17.9 \pm 6.5\%$ versus $27.4 \pm 5\%$), which is a 34.64% increase ($P < 0.05$). The demarcation between infarcted and normal (stained deep red) tissues becomes quite clear 48 hrs after the ischemic insult; however, the pink areas still showed a significant difference between the normothermic and hyperthermic animals. It was $3.1 \pm 1.9\%$ in the normothermic, and $1.6 \pm 0.6\%$ in the hyperthermic animals, which is a 46.9% decrease ($P < 0.05$).

These results clearly demonstrate that postischemic spontaneous hyperthermia not only increases the infarct volumes measured 48 hrs after transient and permanent ischemia, but also accelerates the progression of brain damage. Reperfusion shows no beneficial effect 2 hrs after MCAO, and penumbral areas become incorporated into the necrotic area at much earlier times in spontaneously hyperthermic rats than in normothermic rats.

Discussion

These results show that under certain experimental conditions, postischemic spontaneous hyperthermia is an inevitable complication of MCAO, and that it increases both the extent and the temporal progression of cerebral damage.

In most cases of brain injuries, brain temperatures have been shown to be slightly different from rectal temperatures, usually exceeding it (Jiang et al. 1991, Mellergard and Nordstrom 1991, Ginsberg and Busto 1998, Maier et al. 1998). However, rectal temperature has been proposed to reflect the changes in brain temperature (Zhao et al. 1994a), or even to be identical in the filament model of MCAO (Jiang et al. 1996), and clinical studies have mostly used core temperature measurements (Przelomski et al. 1986, Azzimondi et al. 1995, Reith et al. 1996); therefore, we found rectal measurements to be appropriate for our study. Although rats can change their temperature within a very short time, we found that

measurements made every 10 minutes closely reflected these changes and no very large fluctuations were ever observed.

In the present study, we found that severe spontaneous postischemic hyperthermia occurs in rats following MCAO. Intraoperative warming of rats is mentioned by almost all investigators using the filament technique of MCAO, however, very few authors have mentioned the postoperative control of temperature. In our experiments, no hypothermia developed during the surgery, which may be due to the low levels and short exposure (10 minutes) to halothane. Clinical studies have drawn more attention to this phenomenon, since stroke patients often develop mild hyperthermia or fever (Przelomski et al. 1986, Azzimondi et al. 1995, Reith et al. 1996, Ginsberg and Busto 1998). Our experimental conditions closely resembled the clinical conditions, because the animals were allowed to wake up and move freely after the 10-minute anesthesia.

Based on the literature, the occurrence of postischemic hyperthermia is not a general observation in rat models of focal ischemia. In our study, we observed that temperatures reached very high postischemic levels. A similar degree of hyperthermia after MCAO in rats has been mentioned by another few investigators (Zhao et al. 1994a, Memezawa et al. 1992, 1995). In their studies, the rise of temperature was observed to be much slower, i. e., the temperature reached the hyperthermic level only after 1-2 hours. We found that the temperature usually rose 20-30 minutes after MCAO, but in some individuals as soon as 15 minutes after anesthesia. This may reflect a difference in the MCAO technique, since according to their descriptions (Memezawa et al. 1992), the animals were under halothane anesthesia for much longer periods (20-30 minutes) than in our experiments. Spontaneous hyperthermia has also been observed following isoflurane anesthesia with the filament technique (P.-E. Chabrier, personal communication). Others have reported no spontaneous hyperthermia in rats (Schmid-Elsaesser 1998). The reason why postischemic hyperthermia is not observed by all investigators may be due to a variety of factors. It has been argued that hyperthermia may be strain related or be correlated with a more severe ischemia (Schmid-Elsaesser 1998). We observed hyperthermia in both the 90-minute and 2-hour transient ischemia as well as in permanent ischemia. Since the elevations in the temperature did not significantly differ among these groups, it could be argued that since all rats in our experiment had elevated core temperature, the rise in temperature is strain related rather than dependent on the duration of the occlusion.

Besides strain differences, small technical details of MCAO have been reported to account for differences in infarct sizes (Kuge et al. 1995, Belayev et al. 1996a, Cai et al. 1998). Similar to the effect on the infarct size, these technical differences along with differences in the duration and type of anesthesia could also explain why others have not found postischemic hyperthermia.

The occurrence of spontaneous hyperthermia in rat models of stroke as well as in stroke patients has been attributed to a variety of systemic complications and central factors (Przelomski et al. 1986, Wass et al. 1995, Kim et al. 1996, Loddick and Rothwell 1996). It has also been suggested that temperature can be an independent component and not an epiphenomenon of other complications in stroke patients (Azzimondi et al. 1995). Inflammatory factors, such as interleukin-1, have been suggested to play a role in the hyperthermic response following MCAO (Loddick and Rothwell 1996). Others have claimed (Uchino et al. 1994, Zhao et al. 1994a) that the rapid development of hyperthermia may not reflect an inflammatory reaction but rather damage to the hypothalamic temperature regulating center. Histological examination has revealed ischemic damage in the hypothalamus in animals subjected to a 2-hour transient focal ischemia and it has been argued that the intraluminal filament interferes with the hypothalamic circulation (Zhao et al. 1994a). Other studies have also showed that the intraluminal thread jeopardizes and interferes with the collateral blood flow (Memezawa et al. 1995, Herz et al. 1998). Coimbra et al. (1996) have argued that the observed hyperthermia after transient global ischemia is not related to the halothane treatment, that it cannot be the result of exogenous pyrogens, and that the temporal evolution of hyperthermia correlates well with the development of neuronal damage. This suggests that the severity of the ischemic insult may determine the release of factors inducing hyperthermia. We found that rats which did not have a satisfactory occlusion of the MCA also showed elevated temperature compared to the sham-operated animals. This also raises the possibility of interference with the collateral circulation, since the filament was advanced to the same distance as in the animals with successful MCAO, only these animals did not show satisfactory neurological signs. According to our observations, these animals did not have satisfactory occlusion of the MCA (section III/2).

In the present study, we found that spontaneous hyperthermia increased the infarct volume in both permanent and transient MCAO, regardless of the duration of the occlusion. Although we found that the detrimental effect of hyperthermia was not

as prominent in the permanent as in transient occlusion experiments, the increase in infarct volume was still significant. Morikawa et al. (1992) did not find significant effects of temperature in permanent ischemia. Memezawa et al. (1995) have also argued that temperature rises have a greater effect on brain lesions following transient than following permanent ischemia. Others (Chen et al. 1991) have observed increases in infarct volumes also in permanent occlusion. Kim et al. (1996) found exacerbation of the damage by hyperthermia after a 1-hr transient ischemia only when brain temperatures were kept at 40°C for 3 hrs, but not at 39°C.

Although the brain is known to be more vulnerable to changes in temperature in global ischemia (Barone et al. 1997), spontaneous hyperthermia has been reported not to be a major aggravating factor in global ischemia by some investigators (Kato et al. 1991, Colbourne et al. 1993). Others have found that ischemic injury in global ischemia can be attenuated by preventing postischemic hyperthermia by either continuing anesthesia (Kuroiwa et al. 1990), or by treatment with an antipyretic drug (Coimbra et al. 1996). The increase in infarct volume by hyperthermia has been attributed to a variety of factors: neurotransmitter release, free radical formation, cytoskeletal degradation, interleukin-1 formation, blood-brain barrier changes, hyperglycemia, and various other causes (Dietrich et al. 1990, Uchino et al. 1994, Eilers and Bickler 1996, Kil et al. 1996, Loddick and Rothwell 1996, Morimoto et al. 1997, Ginsberg and Busto 1998, Castillo et al. 1999).

The cerebral infarct has been demonstrated to progress from the lateral part of the caudoputamen as the ischemic core towards the cortex, which comprises the ischemic penumbra (Memezawa et al. 1992, Garcia et al. 1995a). TTC staining has been shown to be a very reliable indicator of ischemic damage after 12-24 hrs (Bederson et al. 1986a) when no statistical difference can be shown between damaged brain areas assessed by histological evaluation or TTC staining (Isayama et al. 1991, Memezawa et al. 1992). The border zones between normal and severely damaged tissues were separated by tissue which stained pink with TTC (Bederson et al. 1986a, Memezawa et al. 1995). Although pink staining is not an absolute indicator of cerebral damage, it can be an early indicator of tissue ischemia (Bederson et al. 1986a, Cole et al. 1990). In the present study, we demonstrated that spontaneous hyperthermia significantly accelerates the transformation of ischemic damage from the less severely injured penumbral tissue to cerebral infarction. The total damaged brain volumes measured 4 and 12 hrs after MCAO did not show significant

differences; however, the distributions between penumbral and infarcted volumes were significantly different. This was most marked at 4 hrs, when the size of the infarcted tissue was increased three-fold compared to normothermic animals.

Hyperthermia has been suggested to increase the vulnerability of the ischemic region, especially the marginal zones; therefore, penumbral tissues have been considered to be more temperature sensitive (Chen et al. 1991, Takagi et al. 1994, Kim et al. 1996). Our results reinforce these observations by showing that during the early hours of ischemic damage, penumbral tissues become involved in infarcted areas more promptly if temperature is allowed to rise spontaneously. Similar effects have been described in global ischemia (Dietrich et al. 1990), where hyperthermia has been observed to play an important role in determining the temporal profile of ischemic damage, and hyperthermia converted ischemic cell injury to infarction more rapidly. We have also demonstrated that reperfusion at 2 hrs is beneficial under normothermic conditions, but it is no longer beneficial in hyperthermic animals. Hyperthermia might explain why Memezawa et al. (1992) did not find reperfusion at 2 hrs beneficial, in contrast to others (Kaplan et al. 1991). These results are especially important from the clinical point of view because the penumbra during the first few hours is considered to be the target for therapeutic interventions (Juurlink and Sweeney 1997). However, spontaneous hyperthermia can attenuate or even abolish the effects of neuroprotective drugs (Memezawa et al. 1995), and can shorten the therapeutic window.

IV/1. NEUROPROTECTIVE EFFECTS OF PACAP38 IN FOCAL ISCHEMIA

Methods

Animal preparation, MCAO surgery, neurological evaluation and measurements of brain damage were carried out according to the methods described in section II.

Drug administration

The rats that showed positive neurological signs at 2 hrs after MCAO were randomly divided into control and PACAP-treated groups. Administration of PACAP38 (Polypeptide Laboratories) started 4 (n=20), 8 (n=15) or 12 (n=15) hrs after MCAO. The operative conditions were the same as for the MCAO surgery. A micro-osmotic pump with a pumping rate of 1 μl/hr (Alza Corp) was filled with PACAP38 dissolved in 0.1% bovine serum albumin (BSA) (Sigma). The filling and operational instructions given by Alza were closely followed. Pumps were prepared 12 hrs before intravenous cannulation, and were soaked in physiological saline at 37°C for 12 hrs. The pump was attached to micro-renathane tubing (Type MRE-040) (Braintree Scientific Inc). The tubing was examined after 12 hrs for the presence of bubbles. Those pumps that had bubbles were not used for implantation because of the uncertain fluid conductivity. Three cm of the tubing was inserted into the internal jugular vein so that the tip of the cannula was located in the right atrium. The pump was implanted in the subcutaneous space of the nuchal region. The infusion rate was 160 pmol PACAP38/μl/hr, and an additional bolus injection of 5 nmol/300 μl/rat of PACAP38 was given intravenously (into the penal vein). These doses were chosen based on previous *in vitro* and *in vivo* studies (Uchida 1996). This surgical procedure also took approximately 10 minutes to be completed. Control groups received 0.1% BSA in 0.9% saline as a bolus injection and infusion 4 (n=11), 8 (n=15) or 12 (n=11) hrs after MCAO under the same operative conditions as the PACAP-treated animals.

Measurements of the physiological parameters

In 6 randomly chosen animals in both the vehicle- and PACAP-treated groups, the left femoral artery was catheterized for monitoring the blood pressure, pH, arterial gases, blood glucose level and hemoglobin during and after surgery and after treatments. Blood samples were collected before, 5 min, 30 min, 2 hrs, 6 hrs and 24 hrs after treatments. These animals were not included in further evaluations, because they were subjected to more extensive surgical trauma, a more prolonged anesthesia, and more behavioral stress than the other vehicle- or PACAP-treated animals.

Results

There was no significant difference in body weight, temperature or mortality between the groups. Body temperature rose in each animal 15-20 minutes after MCAO, but it could be maintained within the normal range by cooling the animals. Only one rat showed elevated body temperature in spite of cooling, this rat was excluded from further experiment. One or two rats died in each group, mostly during the first 24 hrs. Premature deaths were mainly caused by cerebral hemorrhage or extreme cerebral edema. Two rats in the 4- and 8-hr PACAP-treated groups, 1 rat in the 4-hr control group, and 3 rats in the 12-hr control group developed convulsions during the first 24 hrs. Hemorrhage, which was not fatal, occurred in 2 rats of the 8-hr and 1 rat in the 12-hr PACAP-treated groups, and 2 rats in the 8- and 12-hr control groups. All animals with convulsions or hemorrhage were excluded from further evaluation. There was no significant difference regarding pH (7.35 – 7.38), PCO_2 (35.2 – 46.1 mmHg), PO_2 (130.0 – 149.5 mmHg), blood glucose concentration (77.0 – 95.5 mg/dl) and hematocrit (37.1 – 43.8%) between the control and PACAP-treated animals. The mean arterial pressure dropped by 30 mmHg following the PACAP bolus injection, but gradually returned to normal levels after 30 minutes.

When the treatment started 4 hrs after the MCAO, the infarct volumes of the PACAP-treated group and the control group were $10.38 \pm 2.02\%$ and $21.13 \pm 4.02\%$, respectively (Fig. 7). This was a 50.88% reduction compared to the control group and was highly significant ($P < 0.01$). In the PACAP-treated group, the unstained areas are located mainly in the lateral part of the caudoputamen and the surrounding cortical

areas, which comprise the core of the infarct (Memezawa et al. 1992, Garcia et al. 1995a). The anterior and medial parts of the striatum, as well as large areas in the cortex remained unaffected when compared to the control group. An analysis of the infarcted areas for each 2-mm coronal section from the control group and the group treated with PACAP38 starting 4 hrs after MCAO (Fig. 8a) showed that the most affected sections were the middle sections (3-5), which represent most of the territory of the MCA. The reduction of the infarct size with treatment starting 4 hrs after MCAO is also most pronounced in the middle sections, whereas the reduction is not significant in the first section. This effect can be clearly seen in the representative photographs of the 6 TTC-stained brain slices from the 4-hr control group and the PACAP-treated groups (Fig. 8b).

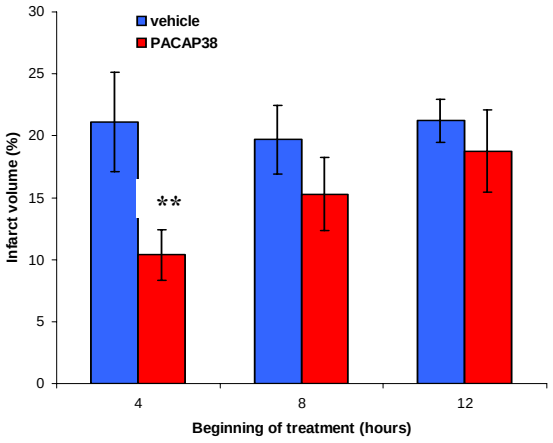


Figure 7
*Infarct volumes after a 2-hour transient focal ischemia. The animals received either PACAP38 or vehicle (0.1% BSA in 0.9% saline) 4 hrs, 8 hrs or 12 hrs after MCAO, and were sacrificed 48 hrs following MCAO. Infarct volumes are expressed as the percentage of total brain volumes. **P < 0.01 compared to the vehicle-treated control group.*

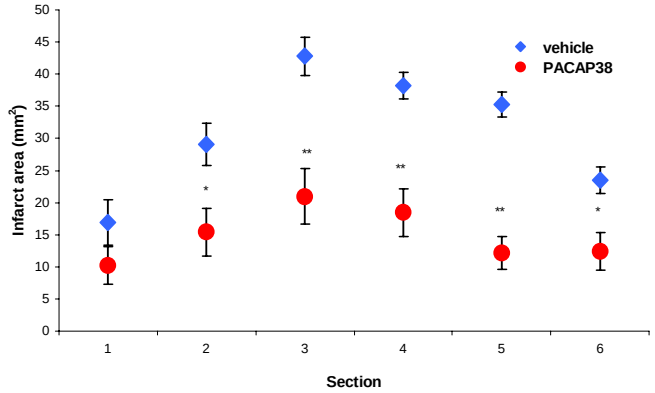


Figure 8a
*Infarcted areas in each of the 6 coronal sections in the anterior-posterior direction from the control and PACAP38-treated groups. The treatment was started 4 hrs after MCAO. *P< 0.05, **P< 0.01.*

When the treatment started 8 hrs after the MCAO, the infarct volumes in the PACAP-treated and in the control groups were as follows: control group: 19.69 ± 2.79%, and the group treated with PACAP38 starting 8 hrs after MCAO: 15.3 ± 2.93% (Fig. 7). This is a substantial (22.3%), but not statistically significant ($P = 0.14$), reduction in infarct volume.

The reduction in the size of the infarct in the group treated with PACAP38 starting 12 hrs after MCAO was also not statistically significant. The infarct size of the PACAP-treated group was 18.75 ± 3.31% whereas that of the control group was 21.22 ± 1.73% (Fig. 7). This is an 11.64% reduction in infarct size which again did not show significance ($P > 0.05$).

Discussion

These results demonstrate that systemic administration of PACAP38 effectively reduces infarct volume in a rat model of focal ischemia when administration begins 4 hrs after MCAO. Infarct volumes still tend to be smaller when treatment starts 8 or 12 hrs after MCAO, although the reductions are not statistically significant.

Premature mortality was evenly distributed among all groups, indicating that the administration of PACAP38 did not contribute to the mortality. Mortalities were mainly due to brain edema or cerebral hemorrhage. Convulsions and subarachnoid hemorrhage can occur as a complication of the MCAO experiments (section III/1). We observed convulsions in some rats during the first 24 hrs, regardless of the treatment and the animals core temperature. Those animals were excluded from further evaluations. The occurrence of hemorrhage also showed no relation to the treatment.

Among the physiological parameters, only blood pressure showed a significant difference between the control and the PACAP-treated animals: the intravenous bolus injection of 5 nmol of PACAP38 caused a prompt fall of the blood pressure, and returned to the preinjection level 30 minutes later. This effect of PACAP has been demonstrated earlier (Arimura 1998), the peptide is well known to relax vascular smooth muscle (Warren et al. 1992). It is possible that intravenous administration of

PACAP38 causes dilation of cerebral arteries and increases cerebral blood flow, which could be beneficial for the treatment of MCAO (Tong et al. 1993). However, this cannot account for the neuroprotective effect observed in this study, because altering the blood pressure and cerebral blood flow 4 hrs after the ischemic insult has no beneficial effects on infarct size (Kuroda and Siesjo 1997, Plum 1997, Dyker and Lees 1998). In addition, our preliminary study showed that an intravenous bolus injection of PACAP38 alone did not alter the infarct size after MCAO. A bolus injection of the peptide was given before the slow infusion because there is a PACAP binding protein in the circulation of rats (Somogyvári-Vigh et al. 1998, Tams et al. 1999). It is possible that binding of PACAP38 to the binding protein would nullify the action of the peptide, and the bolus injection of PACAP38 was used in order to saturate the binding sites of the circulating binding protein.

The present results provide an additional *in vivo* demonstration of the neuroprotective effect of PACAP38. Since its discovery, the neurotrophic and neuroprotective effects of PACAP have been extensively investigated *in vitro* (section I/2). These studies indicate that PACAP is neuroprotective in various pathophysiological conditions, at nanomolar to subpicomolar concentrations.

PACAP has been demonstrated to readily cross the BBB (Banks et al. 1993). This raised the question whether a systemic administration of PACAP would be neuroprotective also *in vivo*. Indeed, its neuroprotective effect has been shown in a rat model of global ischemia (section I/3). In the present focal ischemia model, the same doses were used. Most compounds investigated as possible treatments for stroke in animal models are maximally effective when given as an initial bolus followed by a constant intravenous infusion (Plum 1997). In a recent study, it has been found that such an administration pattern is necessary to obtain neuroprotection with PACAP38 (Somogyvári-Vigh et al. 1998). Our bolus injection of 15 nmol/kg bodyweight is most probably able to saturate the binding protein for PACAP38 in the circulation and to cause a rapid increase in plasma PACAP38 to the level appropriate for neuroprotection (Somogyvári-Vigh et al. 1998).

The exact mechanism for the neuroprotective effect of PACAP38 remains to be studied. According to the studies mentioned in section I/3, it appears to be mediated by direct and indirect actions. The indirect effects seem to be mediated through astrocytes, as has been documented for VIP (Brenneman et al. 1987, 1999). Astrocytes have been shown to react to much lower concentrations of PACAP than

neurons or pituitary cells (Tatsuno et al. 1996a). It has been demonstrated that PACAP stimulates the proliferation of astrocytes, and the production and release of various neurotrophic substances, including interleukin-6 and other chemokines (Gottschall et al. 1994, Tatsuno et al. 1996b, Moroo et al. 1998, Brenneman et al. 1999). *In vitro*, PACAP is neurotrophic at nanomolar to picomolar concentrations when there are no astrocytes in the culture, but at low picomolar to femtomolar concentrations when astrocytes are included (Arimura 1998). According to other observations, VIP and PACAP may exert protective effects through inhibiting proinflammatory factors (Martinez et al. 1998, Soares et al. 1998, Delgado et al. 1999). PACAP has also been shown to inhibit the cyclooxigenase pathway in cerebral microvessels and to inhibit platelet aggregation (Kis et al. 1999a,b). PACAP has also been demonstrated to reduce the lipopolysaccharide-induced secretion of nitric oxide in mixed cortical neuron/glia cultures at very low concentrations (Kong et al. 1999). It is very unlikely that the concentration of PACAP38 in the brain tissue reaches the nanomolar range with intravenous administration of the doses we used. Therefore, it is likely that the neuroprotective effect of PACAP38 *in vivo* involves indirect mechanisms.

The data presented here suggest a promising therapeutic use for PACAP in cerebral ischemia. Among its practical advantages are that it readily crosses the BBB and is effective at extremely low concentrations. Many potential therapeutic agents which are effective in animal models have failed during clinical trials because of their toxicity (Dyker and Lees 1998). PACAP is a naturally occurring peptide, which has not been shown to have toxic side effects at this low dosage (Arimura 1998). More important, the most promising feature of PACAP is the apparently long therapeutic window, since it is effective even when administration is delayed for 4 hrs after ischemia. Several papers have described the time course of neuronal damage after an ischemic event (Auer and Siesjo 1988, Garcia et al. 1995a). Neuronal necrosis progresses from the core of the lesion towards the initially less severely damaged penumbral zone, which contains potentially salvageable neurons (Kuroda and Siesjo 1997). The period available for rescuing penumbral regions is longer than for the ischemic focus, and rescue attempts have been directed mainly toward the penumbra (Juurlink and Sweeney 1997). Although there are large discrepancies between different studies, many authors agree that the window of opportunity for effective therapeutic intervention is only a few hours (Scheinberg 1991, Memezawa et al.

1992ab). Most of the successful attempts to reduce infarct size have occurred when the treatment started either before or very shortly after the onset of ischemia (Scheinberg 1991). The clinical usefulness of such therapeutic agents is very limited due to the time interval between the recognition of the symptoms and the possible beginning of a treatment in stroke patients (Scheinberg 1991, Dyker and Lees 1997). According to recent investigations, the time window for therapeutic intervention may extend far beyond a few hours (Dereski et al. 1993, Marchal et al. 1996, Plum 1997, Shuaib and Breker-Klassen 1997) and the evolution of ischemic injury, especially in the penumbral regions, is more protracted in time than previously believed. A few studies offer some hope that even delayed treatment may have beneficial effects and tissue damage can be influenced long after the ischemic event (Zhao et al. 1994b, Zhang et al. 1996, Ginsberg and Busto 1998, Markgraf et al. 1998, Nagayama et al. 1998, Aronowski et al. 1999, Chabrier et al. 1999). In our study, PACAP38 reduced infarct size when administration started 4 hrs after MCAO, and infarct volumes still tended to be smaller even if treatment started 8 and 12 hrs after MCAO, although this reduction was not significant. Changing the experimental conditions, or combining PACAP with other neuroprotective substances may result in PACAP being effective after even longer delays.

In summary, systemic administration of PACAP38 in focal ischemia may be a promising therapeutic agent in stroke management. PACAP38 decreased the infarct volume in a MCAO model in rats even when administration began as late as 4 hrs after the onset of ischemia. This phenomenon needs to be investigated further in order to extend the window of therapeutic opportunity.

IV/2. NEUROPROTECTIVE EFFECTS OF PACAP38 IN FOCAL ISCHEMIA UNDER DIFFERENT EXPERIMENTAL CONDITIONS

Background

Although the filament model of MCAO was described more than ten years ago, the optimal experimental conditions and neurological scoring systems are still contradictory in the literature (section III/3). Some widely accepted neuroprotective agents in animals have been shown to be ineffective under certain experimental

conditions or in different species. Hyperthermia is one such condition, which can greatly influence the infarct size and the effects of neuroprotective drugs (Meden et al. 1994, Ginsberg and Busto 1998). MK-801, a glutamate receptor antagonist has failed to reduce the infarct size in spontaneously hyperthermic animals (Memezawa et al. 1995). Another study has reported that MK-801 was ineffective in a thrombotic stroke model under conditions of either normothermic or mildly hypothermic head temperature (Yao et al. 1993). Oliff et al. (1996) have demonstrated that the protection by MK-801 was not equally effective in different rat strains and in rats from different vendors. Isradipine and levemopamil, Ca-channel blockers with promising therapeutic use in stroke (Sauter and Rudin 1990), have also failed to ameliorate brain damage in different animal models of both global and focal ischemia (Bailey et al. 1995, Gomi et al. 1995). Inconsistent results have been obtained also with opiate antagonists, and endothelin receptor antagonists (Obana et al. 1988, McAuley et al. 1996). Therefore, a very thorough investigation of the experimental conditions which affect the evaluation of the neuroprotective drug candidate is required preceding the clinical trials. We examined two common factors which might obscure the otherwise significant neuroprotective effect of PACAP38. In the present study, we report on the effects of PACAP38 on the infarct size following MCAO in moderately hyperthermic and normothermic rats, and on the consequences of including animals with questionable MCAO or convulsions.

Methods

Animal preparation, MCAO surgery, neurological evaluation and measurements of brain damage were carried out according to the methods described in section II.

Temperature control

The temperature of one group of rats was strictly maintained in the normal range (36.5-37.5^{o}C) during the first 6 hrs (section II). In another group of rats, body temperature was only moderately controlled: measurements were carried out every 20 minutes during the first 6 hrs following MCAO, and animals were placed in the cold room when temperature rose above 38 ^{o}C. No fur was shaved and no alcohol was applied.

Neurological evaluation

Neurological evaluations were carried out according to the methods described in section II. Rats which displayed circling or partial circling but walked always to the right side were considered to have satisfactory occlusion of the MCA. Animals which partially circled but were able to walk in a straight line, or did not circle at all but showed other neurological signs, were considered to have an unsatisfactory occlusion (section III/2). Animals were observed very closely during the first 8 hrs following MCAO for convulsions.

Drug administration

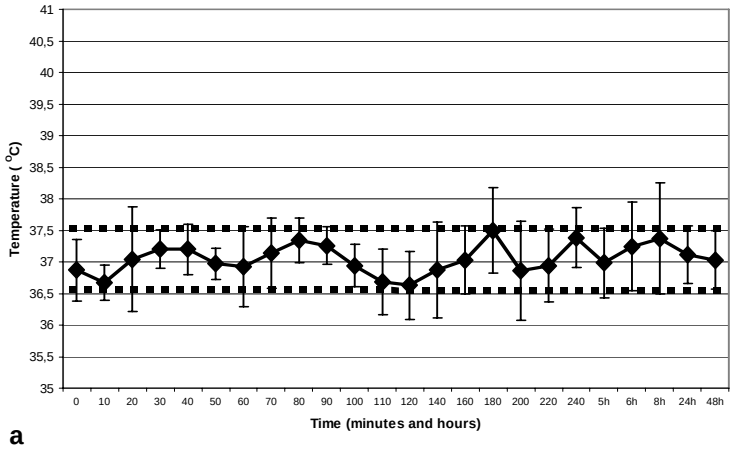
Administration of PACAP38 started 4 hrs after MCAO, according to the methods described in section IV/1. From the normothermic group, animals showing all the neurological signs were treated with PACAP38 (n=10) or the vehicle (n=9). Additional rats, which showed all the neurological signs except for circling, were given PACAP38 (n=7) or the vehicle (n=10). From the moderately hyperthermic group, rats showing all the neurological signs were treated with PACAP38 (n=20) or the vehicle (n=10). Additional rats which showed positive neurological signs without circling were given PACAP38 (n=21) or the vehicle (n=11).

Results

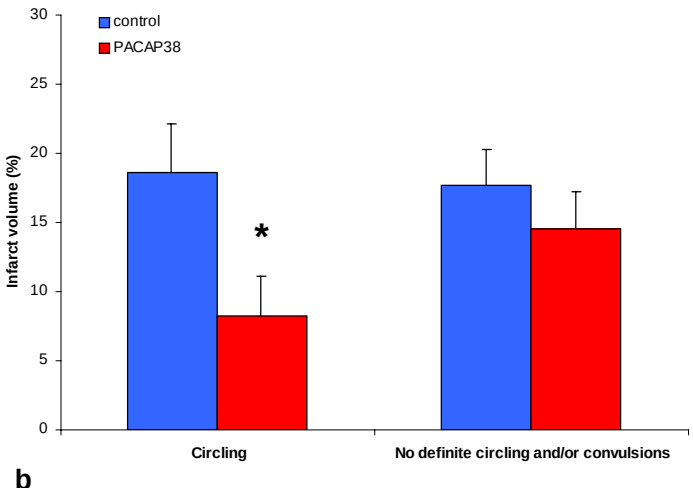
1. Normothermic Animals

By strictly controlling the temperature, the core temperature could be maintained in the normal range during the first 6 hrs following MCAO (Fig 9a). Two animals died in the control group, and only one animal died in the PACAP-treated group during the first 24 hrs. Five animals in the control, and 2 animals in the PACAP-treated group developed convulsion during the first 8 hrs. Four animals developed too high or too low body temperatures for more than 20 minutes in spite of strict temperature control. Including only strictly normothermic animals without convulsions, the infarct volumes in animals with successful occlusion of the MCA were 18.61 ± 3.51% in the control and 8.26 ± 2.81% in the PACAP-treated group (Fig

9b). This is a 55.62% reduction ($P < 0.05$). Including animals without definite circling behavior and/or convulsions resulted in infarct volumes of 17.71 ± 2.53% in the vehicle-treated animals and 14.53 ± 2.64% in the PACAP-treated animals (Fig 9b). There was no significant difference ($P > 0.05$) between these latter two groups.



a



b

Figure 9
*Normothermic animals. (a) Core temperatures after MCAO under strict temperature control during the first 6 hrs following MCAO. (b) Infarct volumes after a 2-hr transient focal ischemia in vehicle- and PACAP-treated animals. Infarct volumes are expressed as the percentage of the total brain volume. *P< 0.05 versus control group.*

2. Moderately Hyperthermic Animals

In the animals with moderate temperature control, body temperatures rose above 38°C 30-40 minutes after MCAO even though the animals were placed in the cold room (Fig 10a). Body temperatures remained above normal in most cases during the 6-hr observation, although extreme rises in temperature (above 39°C) could be prevented. Six of the 21 control animals and 14 of the 41 PACAP38-treated animals died prematurely during the first 24 hrs, mainly due to extreme brain edema and/or hemorrhage. The difference in the mortality rates between the control and PACAP-treated animals was not significant, but it was significantly higher than in the

normothermic group. Five animals had convulsions in each group during the first 8 hrs.

When the animals with convulsions and/or no definite circling were excluded, the infarct volumes were 21.13 ± 4.2% in the control and 10.38 ± 2.02% in the PACAP-treated group (Fig 10b). This is a 50.88% reduction ($P < 0.05$). However, including animals with no definite circling behavior and/or convulsions resulted in infarct volumes of 16.71 ± 3.79% in the control animals and 14.89 ± 2.01% in the PACAP-treated animals, which is not a significant difference (Fig 10b).

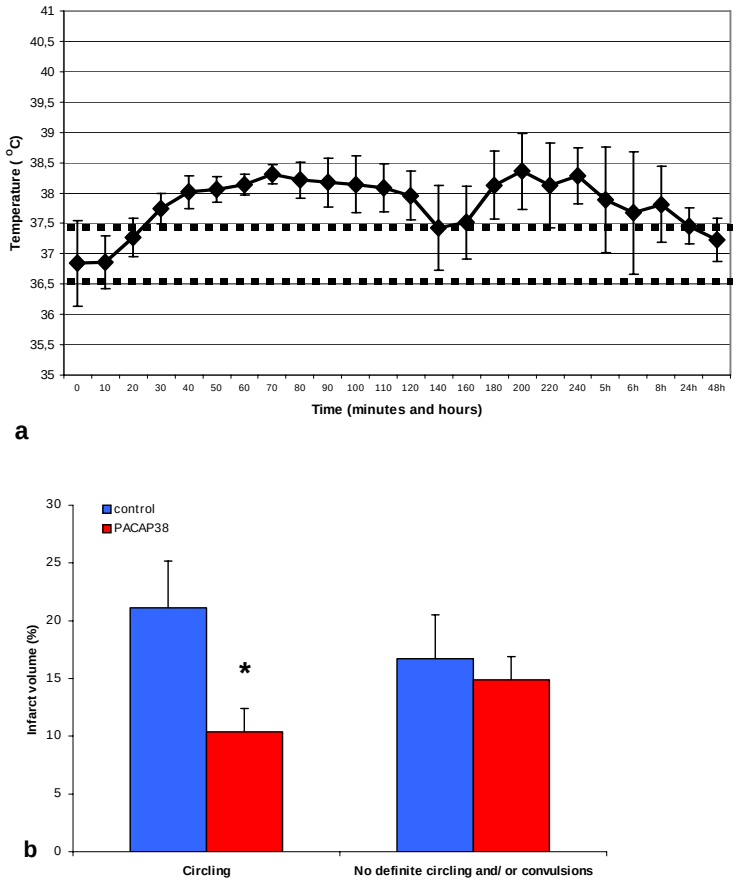


Figure 10
*Moderately hyperthermic animals. (a) Core temperatures under moderate temperature control during the first 6 hrs following MCAO. (b) Infarct volumes after a 2-hr transient focal ischemia.Infarct volumes are expressed as the percentage of the total brain volumes. *P<0.05 versus the control group.*

In summary, it is not necessary to maintain the core temperature strictly within the normothermic range to demonstrate a neuroprotective effect of PACAP in a rat model of focal ischemia. In order to demonstrate a significant neuroprotection with PACAP, it is necessary to exclude animals without the critical neurological sign used for assessing an adequate MCAO and those which develop convulsions.

Discussion

In the present study, we showed the neuroprotective effects of PACAP38 in a model of transient focal ischemia produced by MCAO in normothermic and moderately hyperthermic rats. Infarct sizes were slightly larger in the moderately hyperthermic groups when compared to the normothermic animals. PACAP38 was almost equally effective under both conditions: it reduced infarct sizes by more than 50%. However**,** if the animals without definite circling behavior and/or with convulsions were included in the sample, the neuroprotective effect of PACAP38 could not be demonstrated.

Stroke patients often develop hyperthermia during the postinfarction period, which is usually slight or moderate; extreme rises in body temperature are rare, and are mainly associated with more severe systemic complications (section III/4) Experimental animals may also present with postischemic spontaneous hyperthermia, which can influence the size of the infarct**,** as also shown in this section and section III/4, and may modify the effectiveness of neuroprotective drugs (Memezawa et al. 1995). According to our previous observations, sustained spontaneous hyperthermia is always present in rats after MCAO under our experimental conditions (section III/4). The animals can be maintained within normothermia by very strict temperature control during the first 6 hrs following MCAO**.** This control can be achieved by placing the animals in the cold room with the fur on their back shaved off and by applying alcohol as needed. Simply placing the animals in the cold room without shaving off their fur on their back and without additional alcohol application results in moderate hyperthermia, which rarely exceeds 38.5-39°C. Therefore, we considered these moderately hyperthermic animals to be appropriate to study the effectiveness of PACAP38 under conditions which more closely resemble the mildly hyperthermic condition of stroke patients**.** We found PACAP38 to be almost equally effective in the normothermic and in the moderately hyperthermic animals.

Although MCAO with the filament technique is a widely used model of stroke, the optimal experimental conditions are still questions of debate (section III/1). Filament insertion by any technique may not result in adequate occlusion of the MCA, which can be responsible for large variations in infarct sizes, and may lead to

misinterpretation of data (Schmid-Elsaesser et al. 1998, Petullo et al. 1999). There are numerous neurological scoring systems to evaluate the postischemic neurological deficits which show a correlation with the infarct size (section III/1). Like most other investigators, we also examine motor deficits such as forelimb flexion, thorax twisting, decreased resistance to lateral push, gait disturbances with circling or walking in one direction, and decreased pain reflexes. We found that animals without circling or walking to the right did not have an adequate, reliable occlusion, while animals with circling always show the other neurological signs. This finding is consistent with the observations of Memezawa et al. (1995). Therefore, instead of attempts to grade the neurological signs, we considered animals with circling to have a satisfactory occlusion of the MCA (Memezawa et al. 1992, 1995). The absence of the typical neurological signs suggests that either the MCA was not adequately occluded, or a more highly developed collateral circulation protected the tissue perfused by the MCA from ischemic damage.

A few reports have mentioned convulsions as complicating factors in MCAO (section III/1). We observed convulsions in a few animals in both the hyperthermic and the normothermic groups. It was not the aim of the present study to find a correlation between the occurrence of convulsions and hyperthermia or neurological signs. We excluded animals with convulsions from the evaluation, because seizures can lead to a type of cell damage that is not related to ischemia (section III/1). Including these animals increases the heterogenity of the brain damage in both the control and the PACAP-treated groups. Consequently, the difference in the infarct sizes between the control and PACAP-treated groups may cease to be significant.

In conclusion, when animals were carefully selected according to the presence of typical neurological signs and the absence of convulsions after MCAO, PACAP38 significantly reduced the infarct size by more than 50% in both normothermic and moderately hyperthermic animals, even when administration was delayed by 4 hrs after the insult.

V. PASSAGE OF PACAP38 ACROSS THE BLOOD-BRAIN BARRIER IN NORMAL AND ISCHEMIC BRAIN

Background

A basic requirement for any candidate neuroprotective substance for treatments of stroke is to cross the BBB and to reach sufficient therapeutic levels within the brain (He et al. 1993). The BBB is a structural and functional barrier between cerebral capillaries and brain parenchyma, and composed of non-fenestrated endothelial cells connected with tight junctions and lined with a continuos basement membrane. The BBB restricts free passage of large lipophobic molecules from blood to the CNS and *vice versa*. Peptides and polypeptides may cross the BBB by simple diffusion or by transport systems. Passage can be unilateral from blood to brain or from brain to blood, or bilateral. By way of these specialized transport system, the BBB regulates the interactions between peptides and the CNS (Banks and Kastin 1987) Therefore, the BBB is not a rigid, static barrier but a dynamic interface between the circulatory system and the CNS (Rapaport 1976, Banks and Kastin 1987, Pan and Kastin 1999b, Pan et al. 1999).

The normal function of the BBB is altered by ischemia. Increased BBB permeability is associated with severe ischemic injury, occurring with some delay after the insult (Belayev et al. 1996b, Salaki et al. 1991). In general, after ishcemic brain injury, there are dynamic changes in the permeability of the BBB. Therefore, the entry of neuroprotective agents into the brain will depend on both the extent of BBB disruption and the substance studied.

PACAP has been shown to cross the BBB with a saturable transport system in mice (Banks et al. 1993). The percent of the intravenously injected dose of PACAP38 entering the whole mouse brain is about 0.118% when expressed as %iv/g, which is about six times more than the %iv/g for morphine (Banks et al. 1996). In the present experiment, we examined the passage of PACAP38 across the BBB in rats, and how this passage is affected by focal ischemia.

Methods

Radiolabeling of PACAP38 and Albumin

Synthetic PACAP38 (Polypeptide Laboratories, Torrance, CA) was labeled with ^{125}I (NEN Life Science Products, Boston, MA) by the lactoperoxidase method and purified on reversed phase HPLC on a Vydac C18 column (Rainin Instrument Co, Inc, Woburn, MA), with a 60-minute linear gradient of 10 to 60% acetonitrile in 0.1% trifluoroacetic acid. The specific activity of the peptide was about 500 µCi/µg, as assayed by the self-displacement method of immunoassay (Gottschall et al. 1990) Human serum albumin was labeled with ^{99m}Tc using a kit (Amersham Health Care, San Antonio, TX).

Multiple Time-Regression Analysis of Entry into the Brain

The rate of entry into the brain of ^{125}I-PACAP38 and ^{99m}Tc-albumin was measured. The right carotid artery and the penal vein of the animals were exposed. Rats were given an iv injection into the penal vein of 500 µl of physiological saline containing 0.1% BSA, 5 x 10^6 cpm of ^{125}I-PACAP38, and 10^7 cpm of ^{99m}Tc-albumin.

The carotid artery was severed at 1, 2, 3, 4, and 5 minutes after the iv injection and whole blood was collected and centrifuged at 4˚C at 500 g for 10 minutes. Immediately after the collection of blood, the rats were decapitated and the brain was removed. Serum and brain samples were obtained and the right and left hemispheres were weighed separately. The serum and the brain hemispheres were assayed for the radioactivity of ^{125}I and ^{99m}Tc simultaneously.

To correct for the decrease of ^{125}I-PACAP38 during iv circulation secondary to plasma clearance, exposure time was calculated as an integral value of serum radioactivity over time divided by serum radioactivity at a given time (Patlak et al. 1983). The brain/serum ratio of radioactivity (µl/g) was plotted against exposure time. The slope of the linear regression line represents the influx rate, and the intercept at time 0 is the initial volume of distribution of ^{125}I-PACAP38 in the brain for each group.

Capillary Depletion Study to Determine the Compartmental Distribution

The anesthetized rats received an intrajugular injection of about 3 µCi of ^{125}I-PACAP38 and 4 µCi of ^{99m}Tc-albumin in 300 µl of lactated Ringer's solution

containing 1% BSA, and were decapitated at 1, 3, and 9 minutes immediately after blood was collected from the right carotid artery. The cerebral cortex was harvested and weighed (n=4 at each time point). The cortex was homogenized in a glass homogenizer in the capillary buffer and mixed thoroughly with 26% of dextran to enable effective pelleting of the capillaries of the brain (Guiterrez et al. 1993) An aliquot of the homogenate was centrifuged at 5,400 g for 15 minutes at 4˚C. The supernatant and pellet were separated carefully and the radioactivity was measured in a gamma counter. The effectiveness of the separation has been confirmed by an assay for the endothelial marker γ-glutamyl transpeptidase as previously reported (Guiterrez et al. 1993) The ratios of radioactivity of ^{125}I-PACAP38 in the supernatant (parenchyma) or pellet (capillary) over serum were calculated, and the contamination of the vascular component was further corrected by subtraction of the ^{99m}Tc-albumin ratios.

Permeability of the BBB in Different Brain Regions

The permeability of various brain areas to ^{125}I-PACAP38 and ^{99m}Tc-albumin was measured in control rats and between 4 and 48 hrs after MCAO. MCAO surgery was carried out according to the methods described in section II. Control rats were anesthetized and the carotid area was exposed but no occlusion was performed.

Permeability was assessed at 4, 6, 24 and 48 hrs after MCAO. Every group included 6-8 experimental and 2-3 control rats. The rats were anesthetized, and the right carotid artery was exposed. ^{125}I-PACAP38 and ^{99m}Tc-albumin in saline/0.1% BSA were injected iv into the penal vein and the carotid artery was severed 3 minutes after the iv injection. Serum and various brain areas were collected, weighed, and the levels of radioactivity were measured. The tissue/serum ratios of radioactivity (μl/g) were calculated, and the group means were reported with their standard errors.

Statistical Analysis

Means are reported with standard errors in repetitive measures. Statistical comparisons between groups were performed with one-way analysis of variance. Tukey's test was used for *post hoc* analysis to determine which areas were

significantly different. Differences were considered significant when $P < 0.05$. The GraphPad Prism program was used for the multiple-time regression analysis.

Results

Multiple Time-Regression Analysis

The brain/serum ratios of radioactivity for ^{99m}Tc-albumin and ^{125}I-PACAP38 were plotted against exposure time. For ^{99m}Tc-albumin, there was no linear regression. For ^{125}I-PACAP38, there was a linear regression (n=10, $P < 0.05$). The influx rate for ^{125}I-PACAP38 was 6.5 ± 2.33 µl/g min, and the initial volume of distribution in the brain was 102.7 µl/g (Fig 11).

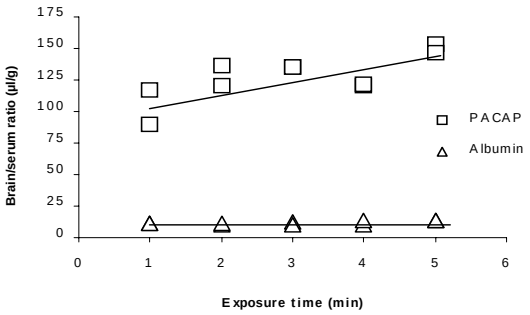


Figure 11
Uptake rates of ^{125}I-PACAP38 and ^{99m}Tc-albumin into intact rat brain. ^{125}I-PACAP38 enters the brain rapidly after an iv injection with an influx rate of 6.5 µl/g-min. In contrast, there was no significant entry of albumin (0.02 µl/g/-min).

Capillary Depletion Study

At 1, 3, and 9 minutes after iv injection in the rats, there was a significantly greater amount of ^{125}I-PACAP38 in the brain parenchyma compared with the capillaries (Fig. 12) (1 min: $F(1,6) = 8.80$, $P < 0.05$; 3 min: $F(1,6) = 26.48$, $P < 0.005$; 9 min: $F(1,6) = 25,25$, $P < 0.005$).

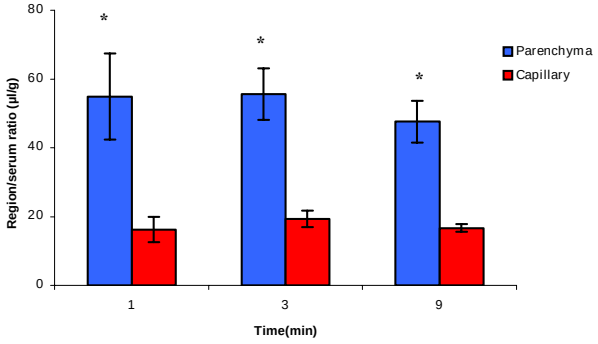


Figure 12
*Brain parenchyma or brain capillary/serum ratios vs. exposure time for ^{125}I-PACAP38 after correction for contamination of the vascular component by subtraction of the ^{99m}Tc-albumin ratios. Each point represents the mean ±SEM (n = 4/group.) * $P < 0.005$ compared the parenchyma to the vasculature.*

Permeability of the BBB after MCAO

The effects of left MCAO on the entry of ^{99m}Tc-albumin into both hemispheres are shown on Figure 13. On the side with transient occlusion, there was a significant decrease at 4 hrs ($P < 0.01$). In the contralateral hemisphere, the decrease in the entry of albumin was significant at 4, 6, and 24 hrs ($P < 0.05$). Comparisons between the left and right hemispheres, however, were not significant.

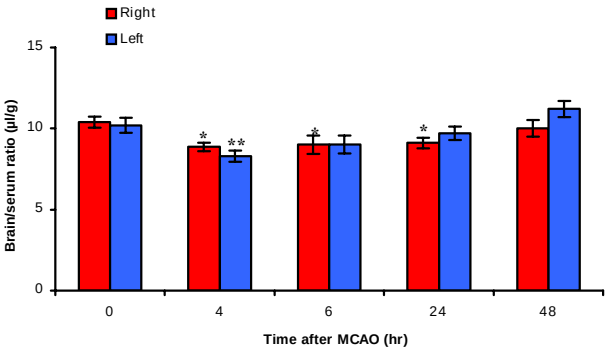


Figure 13
*Uptake of ^{99m}Tc-albumin into the intact (right) and damaged (left) hemisphere after 2 hrs of transient MCAO at different time points. Each point represents the mean ±SEM *P < 0.05, ** P < 0.01 compared to the ipsilateral hemisphere of the control group.*

The entry of PACAP38 was 7–14 times greater than that of albumin at each time and also showed a transient decrease after MCAO, with a maximal decrease of 36% on the damaged hemisphere. The nadir was reached at 6 or 24 hrs after occlusion, later than that seen for albumin. We calculated the differential permeability, expressed as the ratios of ^{125}I-PACAP38 over ^{99m}Tc-albumin. As seen in Figure 14, there was a trend for an increase soon after reperfusion followed by a significant decrease at 24 hrs after MCAO ($P < 0.05$ for the right hemisphere, and $P < 0.01$ for the left). There was no significant difference between the two hemispheres at any time, and each showed about a 20% increase at 4 hrs and a 45% decrease at 24 hrs. At 48 hrs the differential permeability returned to the baseline.

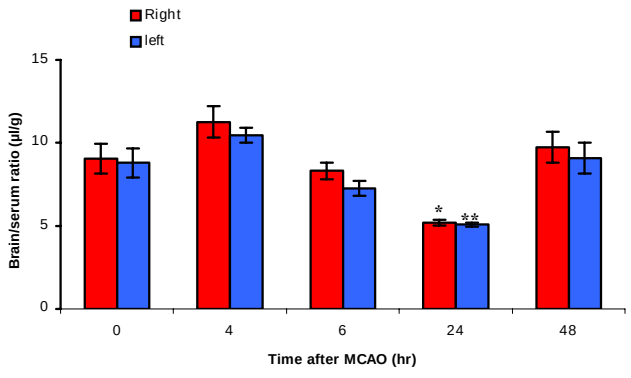


Figure 14
*Uptake of ^{125}I-PACAP38/ ^{99m}Tc-albumin into the right (intact) and left (damaged) hemisphere after 2 hours of transient MCAO at different time points. Each point represents the mean ±SEM (n = 6-8/group.) * P < 0.05, ** P < 0.01 compared to the ipsilateral hemisphere of the control group.*

To distinguish the selective susceptibility of the cortex, striatum, and the rest of the hemisphere, the differential permeability in each of the divided regions was plotted in Figure 15. In general, the temporal changes in all regions followed a similar pattern.

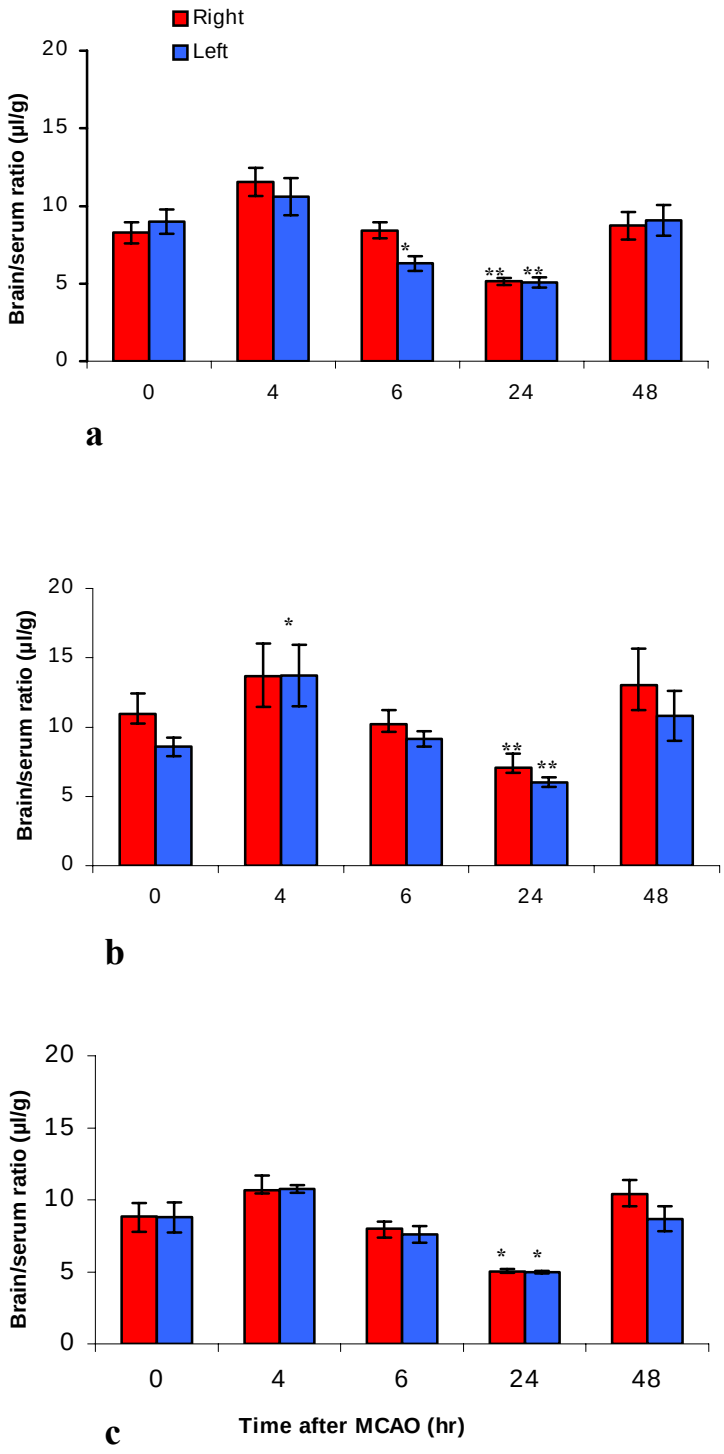


Figure 15
*Distribution of ^{125}I-PACAP38/ ^{99m}Tc-albumin uptake between the intact (right) and damaged (left) part of the cortex (a), striatum (b) and the remaining portion of the hemisphere (c) after 2 hours of transient MCAO at different time points. Each point represents the mean ±SEM * P < 0.05, ** P < 0.01 compared to the ipsilateral hemisphere of the control group.*

Discussion

In the normal rat, PACAP38 entered the rat brain rapidly after a bolus injection, shown by an influx rate of 6.5 µl/g-min. This is even higher than that seen in mice (Ki of 2.86 ± 0.57 µl/g-min) where a saturable transport system across the BBB has been shown (Banks et al. 1993). In comparison, epidermal growth factor, which also has a rapid and saturable entry, has an Ki of about 2.0 µl/g/min (Pan and Kastin 1999a, Pan et al. 1999), whereas most neurotrophins have influx rates ranging from 0.2 to 0.8 µl/g-min (Pan and Kastin 1999b). By contrast, ^{99m}Tc-albumin included in the same injection did not significantly penetrate the BBB, indicating that the albumin detected in the brain is confined to the cerebral vasculature, and that PACAP38 did not alter the vascular space.

In addition to the high influx rate, PACAP38 also had a high initial volume of distribution in the brain (102.7 µl/g). This may indicate that PACAP interacted with the cerebral vasculature or had a significant entry during the first several minutes, as seen with brain-derived neurotrophic factor (Pan et al. 1998). We, therefore, compared the compartmental distribution of PACAP38 shortly after an iv injection by separating the accumulation of PACAP38 in brain parenchyma vs. vasculature. Figure 12 shows that PACAP38 in brain parenchyma was significantly higher even at 1 minute ($P < 0.05$) after an iv injection. In addition, a previous study using HPLC proved that most of the PACAP38 entering brain was intact at 5 minutes (Banks et al. 1993). Therefore, PACAP38 is a promising candidate for providing neurotrophic support to the CNS after systemic delivery.

Left MCAO followed by reperfusion 2 hrs later caused a dynamic change in the entry of albumin from blood to brain, reflected by the ratios of radioactive albumin in brain over blood [(cpm/g)/(cpm/µl)]. There was a transient decrease in the accumulation of albumin at 4 hrs on both sides of the brain, the ipsilateral side showing a more prominent decrease. However, the decrease of albumin entry into the contralateral hemisphere seemed to be more long-lasting, and did not return to baseline until 48 hrs later. We speculate that these changes are initially related to the cessation of blood flow to the distribution of the left MCA, followed by global changes that lead to hypoperfusion of the whole brain, such as systemic hypotension,

release of vasoactive substances, or redistribution of blood flow (Nagasawa and Kogure 1989, Slater et al. 1977). We did not observe disruption of the BBB during the study, as assessed by any of the ratios of albumin entry after an iv injection. This is consistent with the study by Gotoh et al. (1985), showing that an increase in the permeability to albumin is not significant until 3 days after the ischemic event. Our results are different from those of Hatashita and Hoff (1990), who found that the albumin transfer index increased at 12 hrs and peaked at 72 hrs. Discrepancies between this report and our observation are probably related to differences in the animal models. In their experiments, occlusion was introduced by the transretroorbital approach, without reperfusion. The slight reduction rather than increase in BBB permeability to albumin most likely resulted from reduced capillary perfusion (Betz et al. 1994).

The alterations of PACAP38 entry into brain after MCAO and reperfusion followed a different temporal course than that of albumin. The maximal decrease of PACAP38 entry occurred between 6–24 hrs after MCAO, much later than that seen for albumin. This could not be explained by the delay in reperfusion, because once the cerebral blood flow in the ischemic areas was restored, PACAP38, about two-thirds the size of albumin, should have more readily penetrated the partially disrupted BBB.

We next calculated the differential permeability, expressed as the ratio of PACAP38 entry vs. albumin entry. The values initially increased at 4 hrs, followed by a significant decrease at 24 hrs. If there were two inert substances crossing the BBB by simple diffusion, the differential permeability would have remained the same when the barrier was completely disrupted, and increased when there was increasing restriction in diffusion secondary to the restoration of the barrier function, so that the smaller substance passed the barrier more easily. Therefore, the differential permeability could serve as a sensitive marker for partial disruption of the BBB (Ziylan et al. 1984). Early and partial disruption of the BBB has been shown by a fluorescent quantitative method (Belayev et al. 1996b). However, the time course of fluctuation of differential permeability in our study does not correlate well with the Evans Blue fluorescence. Most likely, the changes of differential permeability of PACAP/albumin is attributable to the dynamic changes of the transport system of PACAP38 across the BBB.

We noticed that the decrease in the entry of albumin and PACAP was present in both hemispheres, and the differential permeability in different structures in the ischemic core and penumbral region was similar. It is possible that MCAO triggered secondary events, such as the release of vasoactive substances, that changed cerebral perfusion and the permeability of the BBB in general. Again, the changes of PACAP38 entry suggested specific interactions of this bioactive peptide with the BBB. It was previously reported that lesions impairing the BBB do not entirely abolish the saturable transport system for substances that are actively involved in the pathological processes (Banks et al. 1998, Pan and Kastin 1999b). After spinal cord injury by transection (Pan et al. 1997) or by compression (Pan et al. 1999), the permeability of the BBB is increased distal to the lesion, whereas the transport system for tumor necrosis factor α (TNF) is upregulated. Similarly, experimental autoimmune encephalomyelitis causes transient breakdown of the BBB as well as enhances the blood-to-CNS entry of TNF (Pan et al. 1996). For PACAP38, there seems to be a down-regulation of the transport system. After spinal cord injury by transection in the lumbar region in the mouse, there is a prolonged depression of the influx of PACAP38 into the brain and spinal cord cephalic to the lesion, followed by an increase 1 week later. These changes are related to alterations of the saturable transport system and inhibitable by excess unlabeled PACAP38 (Banks et al. 1998). In the current study, we showed that the entry of PACAP38 decreased after reperfusion following MCAO. The decrease was not explained by general alterations of cerebral blood flow or disruption of the BBB. Thus, this study provides another example that the BBB is actively involved in the pathophysiology of CNS disorders and regulates the availability of bioactive substances from blood.

In summary, PACAP38 had a rapid influx after an iv injection in the rat and was available to the brain parenchyma. Unilateral MCAO was associated with an altered function of the PACAP38 transport system of the BBB, leading to a transient, global decrease in the influx of PACAP38.

VI. APPENDIX

PRELIMINARY STUDIES FOR FURTHER INVESTIGATION OF THE NEUROPROTECTIVE EFFECTS OF PACAP IN OTHER MODELS OF NERVOUS INJURY

- PACAP IN THE EARTHWORM

Introduction and background

The annelid nervous system is a favorite model in invertebrate neuroscience. It has long been known that if pieces of the nerve cord or the cerebral ganglion of the earthworm are removed, morphological as well as functional regeneration takes place (Aros and Vigh 1962, Koritsanszky and Hartwig 1974, Cornec et al. 1987). This unique capacity of the earthworm nervous system to regenerate provides an excellent model for studying mechanisms of, and substances involved in neural regeneration. (Yogev et al. 1991, Lyckman and Bittner 1992, Todorov et al. 1992, Krause et al. 1994, Lore et al. 1999).

It has recently been demonstrated that PACAP may be involved in axonal regeneration following peripheral nerve injury and traumatic brain injury (Zhang et al. 1996, Lioudyno et al. 1998, Skoglosa et al. 1999). In order to examine the possible role of PACAP in an invertebrate model of nervous regeneration, the first step was to demonstrate the occurrence of PACAP in tissues of the earthworm. Based on the extremely conserved structure of PACAP throughout phylogenesis (section I/2), and the numerous vertebrate-like peptides demonstrated in annelids (Dhainaut-Courtois et al. 1985, Curry et al.1989, Al-Yousuf 1990, Satake et al. 1999), we assumed that PACAP might also occur in oligochaete worms**.** It has been found that PACAP shows marked similarity to human PACAP27 even in a nonvertebrate, protochordate species (McRory and Sherwood 1997). Besides these results, only scarce studies have been published on the function of PACAP-like substances in invertebrates (Zhong 1995, Zhong and Pena 1995), and little is known about the occurrence of this peptide in lower animals. First we mapped the distribution of PACAP38-like immunoreactivity using immunohistochemistry, which was followed by radioimmunoassay (RIA), HPLC and bioassay studies. In the present section, these recent findings are summarized without the intention of detailed description.

Methods

For immunohistochemical purposes, adult specimens of 3 earthworm species, *Eisenia fetida, Lumbricus terrestris* and *Lumbricus polyphemus* were used. Serial cryostate sections were stained according to the peroxidase-antiperoxidase method, using PACAP38 antiserum (Peninsula), which was followed by silver intensification in some of the sections.

For RIA purposes, cerebral ganglia, subesophageal and ventral nerve ganglia were collected from 30 animals (*Lumbricus polyphemus*). Different parts of the alimentary tract (foregut, stomach, midgut and hindgut) were removed from 5 animals, gonads and metanephric tubules from 10 worms. Tissues were processed for RIA and assayed for PACAP27 and 38 using a highly specific RIA procedure (Arimura et al. 1991). PACAP27 antibody was generated against synthetic PACAP27 and recognizes PACAP27 but not PACAP38. The PACAP38 antiserum was generated against synthetic PACAP(24-38) and recognizes PACAP38 as well as peptides containing the epitope of PACAP(24-38), but not PACAP27. The lyophilised extracts from the cerebral ganglia and ventral cord were purified, concentrated and then subjected to reverse-phase high performance liquid chromatography (RP-HPLC).

The biological activity of the HPLC fractions were determined by their ability to accumulate extracellular cAMP in pituitary cell cultures (Tatsuno et al. 1991). Pituitary cells from adult female CD rats were cultured for 3 days, then the dried HPLC fractions dissolved in the media were added to the cells. After 3 days incubation, cAMP was determined by RIA (Gottschall et al. 1988).

Results

As demonstrated by immunohistochemistry, the distribution pattern of PACAP38-like immunoreactivity was similar in all 3 species studied. The cerebral ganglion contains numerous immunoreactive cells in the dorsal cell mantle, and fibers are intensively stained in the central neuropil. The subesophageal and ventral cord ganglia also displayed immunoreactive cells and fibers, although the number of cells was much less. In the peripheral nervous system, immunoreactivity was found in the enteric nervous system, in epidermal sensory cells and in the clitellum (Figs 16, 17).

Figure 16

Distribution of PACAP38-like immunoreactivity in the CNS of annelids. (a) Dorsal immunoreactive cells in the cerebral ganglion of Lumbricus polyphemus (arrows). (b) Cerebral ganglion of Eisenia fetida: immunoreactive cells along the dorsal cellular mantle (arrows), immunoreactive fibers in the central neuropil (star) at the originating part of the connective (arrowhead). (c) Centromedial cells in the caudal part of the cerebral ganglion in Lumbricus polyphemus (arrows). (d) Ventromedial immunoreactive cells in the subesophageal ganglion of Eisenia fetida (arrows). (e) ventromedial immunoreactive cells in the ventral cord ganglion of Lumbricus terrestris (arrows). (f) Immunoreactive fiber network in the central neuropil of the ventral cord ganglion of Lumbricus terrestris (star). Bars represent 50 micrometers in a,b,d,e,f, and 75 micrometers in c.

Figure 17

PACAP38-like immunoreactivity in the enteric system, epidermis and clitellum of annelids. (a) Stomatogastric ganglionic cells in Lumbricus polyphemus (arrows). (b) Immunoreactive fibers in the pharynx of Eisenia fetida (arrowheads). (c) An immunopositive epithelial cell in the pharyngeal wall of Lumbricus polyphemus (arrow). (d) Epidermal sensory cells in Lumbricus terrestris (arrows). (e) Ventral ganglion in the clitellum of Lumbricus terrestris: ventromedial (arrow) and lateral (arrowheads) cells and an intensely stained central neuropil (stars). (f) Subepithelial cells in the clitellar part of the intestines of Lumbricus terrestris (arrows). Bars represent 50 micrometers on a-e and 100 micrometers on f.

As measured by RIA, the amount of endogenous PACAP showed a marked topographical variation in the tissues of the earthworm (Table 1). Cerebral ganglion and ventral cord contained the highest concentration of PACAP. The concentration of PACAP27 was 10-15 times higher than that of PACAP38. The subesophageal ganglion had significantly less PACAP27 and 38 compared to other parts of the CNS. The foregut also contained a substantial amount of PACAP27 and 38, and the PACAP27 content was about 3 times higher than PACAP38. The gizzard, midgut and hindgut had about the same level of both PACAP residues and the concentration was significantly less than in the foregut. The nephridial system also contained a significant amount of PACAP27 and 38 comparable to the foregut. The concentration of the two residues was about the same in this system. In the reproductive system, the PACAP38 content was 10 times higher than the PACAP27. The serial dilutions of the various extracts yielded a displacement curve parallel to that of synthetic PACA38 (Fig 18) and PACAP27 in respective RIA (Fig 19).

Table 1. *Tissue content of immunoreactive PACAP 27 and 38*

Tissues	PACAP27	PACAP38
Cerebral ganglia	14.42 ± 1.22	1.51 ± 0.24
Subosephageal ganglia	0.73 ± 0.43	0.13 ± 0.02
Ventral cord	17.12 ± 2.52	0.95 ± 0.21
Foregut	2.14 ± 1.21	0.72 ± 0.06
Gizzard	0.03 ± 0.01	0.06 ± 0.01
Midgut	0.19 ± 0.04	0.01 ± 0.01
Hindgut	0.31 ± 0.06	0.32 ± 0.04
Excretory system	1.17 ± 0.32	0.82 ± 0.22
Seminal vesicle	0.18 ± 0.04	0.32 ± 0.03

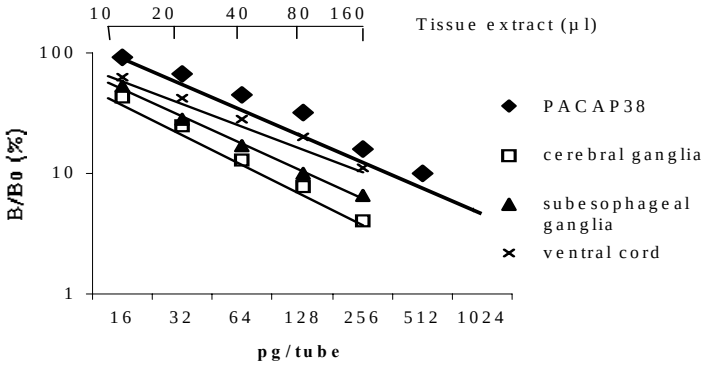
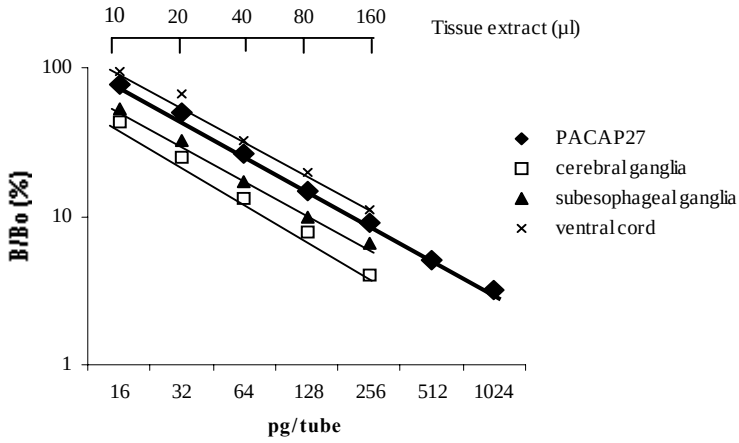


Figure 18
Dose response curve for PACAP38 standard and for Lumbricus polyphemus tissue extracts. The dilution curves of various tissue extracts are almost parallel with synthetic PACAP38.



Experiments are reported in ng/mg protein. Mean ± SEM (n = 4)

RP-HPLC analysis was employed to chara immunoreactivity in the earthworm nervous system. HPLC fr RIA for PACAP27 and PACAP38 using PACAP antisera w site. PACAP27 immunoreactivity was assayed by the PACAP which recognize PACAP27 but not PACAP38. The extrac which corresponds to synthetic PACAP27 (Fig 20a). Using t (88111-3) which could detect PACAP38 and peptides co

Figure 19
Dose response curves for PACAP27 standard and for Lumbricus polyphemus tissue extracts. The dilution curves of various tissue extracts are parallel with synthetic PACAP27.

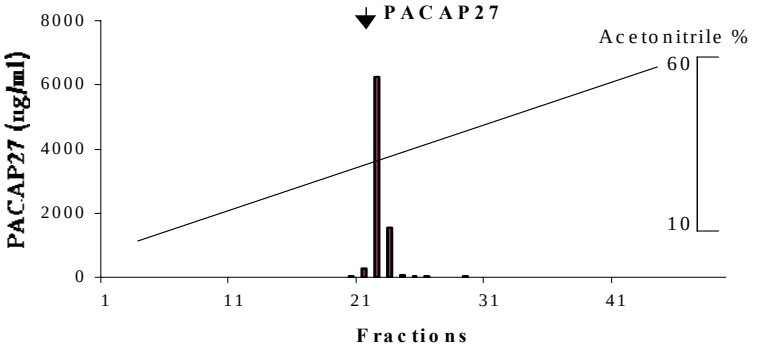
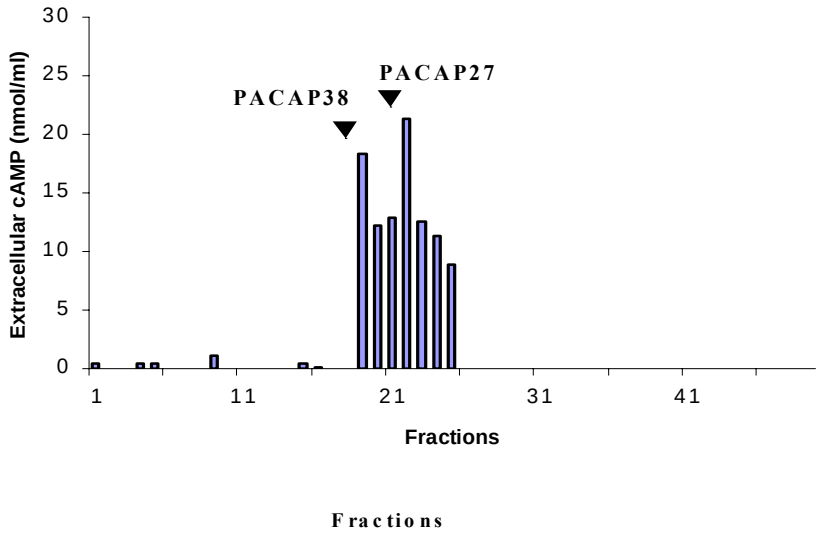


Figure 20
RP-HPLC elution profile of PACAP-li in Lumbricus poly-

PACAP24-38, but not 27, showed a single peak in fraction 19 co-eluted with synthetic PACAP38 (Fig 20b).

The fractions of the cerebral ganglia and ventral cord showed two distinct bioactive peaks (Fig 21), which were co-eluted with PACAP38 and PACAP27 respectively. This indicates that the PACAPs extracted from the earthworm have adenylate cyclase activating activity.



b

Figure 21
RP-HPLC elution profile of PACAP bioactivity. The bars show the bioactivity from the same RP-HPLC fractions as those shown in Fig. 20, expressed in terms of ability to stimulate cAMP production in rat pituitary cultures. The arrows indicate the retention times of synthetic PACAP38 and PACAP27.

Summary

Our present results provide evidence that PACAP-like peptides occur in oligochaete worms. This is the first detailed description of PACAP-like immunoreactivity in an invertebrate species. Serial dilutions of the extracted tissues showed a high degree of structural homology to PACAP27 and 38 standards suggesting a similar amino acid sequence to human PACAPs. The extract of these tissues showed a distinct bioactivity as measured by cAMP accumulation from cultured rat anterior pituitary cells. Based on the distribution pattern it can be suggested that these peptides play a role in nervous transmission and/or modulation, and in other functions of the gastrointestinal,

reproductory and excretory systems. Our results provide the basis for future studies on the role of PACAP in neuroregeneration of the earthworm.

VII. SUMMARY OF NEW FINDINGS

1. We have shown that postischemic spontaneous hyperthermia is an inevitable consequence of middle cerebral artery occlusion in rats under certain experimental conditions. This spontaneous hyperthermia not only increases the infarct size, but accelerates the incorporation of penumbral tissues into the necrotic area. This is most pronounced during the first 4 hours, which is the main target period for therapeutic interventions. Also, reperfusion after 2 hours is no longer beneficial in hyperthermic animals.

2. We have demonstrated the neuroprotective effects of PACAP in focal ischemia. It was found that PACAP reduced the infarct size by more than 50%, even when given 4 hours after the onset of ischemia. This effect is not influenced by moderate hyperthermia.

3. We have demonstrated that PACAP crosses the blood-brain barrier in rats. This passage is altered after middle cerebral artery occlusion: all regions of the brain show a selective increase in the passage of PACAP38 across the blood-brain barrier 4 hours after the occlusion, which is not related to generalized changes in the permeability. A significant decrease in the passage is observed 6 and 24 hours after MCAO, but it returns to baseline levels 48 hours after occlusion.

4. We have shown that PACAP occurs in Oligochaeta by immunohistochemistry and RIA. PACAP27 seems to be the major form in the nervous tissue, and PACAP38 in the reproductive system, although all tissues contain detectable levels of both forms. The HPLC extracts from tissues show biological activity.

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IX. Abbreviations

BBB	Blood-brain barrier
BSA	Bovine serum albumin
CNS	Central nervous system
MCA	Middle cerebral artery
MCAO	Middle cerebral artery occlusion
TTC	Triphenyl tetrazolium chloride
iv	intravenous

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XI. LIST OF PUBLICATIONS

(USED AS BASIS FOR THE PRESENT THESIS)

Reglődi D, Somogyvári-Vigh A, Vigh S, Kozicz T, Arimura A (2000): Delayed systemic administration of PACAP38 is neuroprotective in transient middle cerebral artery occlusion in the rat. ***Stroke*** 31: 1411-1417

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