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ORIGINAL ARTICLE

Omapatrilat: penetration across the blood–brain barrier and effects on ischaemic stroke in rats

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Abstract Omapatrilat (OMA), which simultaneously inhibits the angiotensin-converting enzyme (ACE) and the neutral endopeptidase (neprilysin (NEP)), is widely used in experimental protocols related to hypertension and heart failure. The penetration of OMA across the blood–brain barrier (BBB) and the effects of ACE/NEP inhibition on the recovery from ischaemic stroke have not yet been investigated. Angiotensin (Ang) I injected intracerebroventricularly (ICV) or intravenously (IV) is converted to Ang II by ACE and induces an immediate increase in blood pressure. The pressor responses to OMA administered ICV, orally or IV were studied in male Wistar rats instrumented with an ICV and arterial and venous catheters. OMA infused ICV rapidly appeared in the systemic circulation and more effectively attenuated the systemic than the central pressor responses to Ang I. OMA administered orally (5, 25, 100 μmol/kg body weight) or IV (0.5, 1, 5, 25 μmol/kg body weight) completely abolished increases in blood pressure to IV Ang I up to 2 h after treatment. The pressor responses to ICV Ang I were not altered, indicating that systemically administered OMA does not cross the BBB. To study the effects of ACE and NEP inhibition in the brain on the recovery from ischaemic stroke, OMA was infused ICV over a 5-day period before and 24 h after the occlusion of the middle cerebral artery (MCAO) for 90 min. ICV application of OMA had no effect on infarction volume and marginally improved neurological outcome. We demonstrate for the first time that simultaneous inhibition of ACE and NEP in the brain tissue does not alter the recovery from ischaemic stroke.

Keywords Omapatrilat · Angiotensin-converting enzyme · Neutral endopeptidase · Blood–brain barrier · Focal cerebral ischaemia

Introduction

The inhibitors of the angiotensin-converting enzyme (ACE), the key enzyme of angiotensin (Ang) II biosynthesis, are successfully used in the treatment of hypertension and the prevention of the development of heart failure, cardiac remodelling after myocardial infarction, endothelial dysfunction and albuminuria. Most of the beneficial effects of ACE inhibitors result from the reduced formation of Ang II and inhibition of the bradykinin (BK) degradation (Gohlke and Schölkens 2004). Neprilysin (NEP), also known as neutral endopeptidase (EC24.11), a zinc-dependent, membrane-bound metallopeptidase, is involved in processing and catabolism of a number of vasoactive peptides, including Ang I, natriuretic peptides, BK and adrenomedullin (see Mangiafico et al. 2013 for review). Omapatrilat (OMA), a vasopeptidase inhibitor, which simultaneously inhibits ACE and NEP, showed a powerful antihypertensive efficacy in animal models of human essential hypertension and heart failure and was even more effective in blood pressure control in hypertensive subjects than single ACE inhibitors (Robl et al. 1997; Burnett 1999; Kostis et al. 2004). However, an increased risk for angioedema (2.17 % OMA versus 0.68 % enalapril) prevented

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the approval of OMA for the clinical use. Nevertheless, OMA is still the most widely studied vasopeptidase inhibitor. As a reference and lead compound of this class of drugs, OMA is employed in experimental studies investigating the role of ACE/NEP inhibition on cardiac remodelling and fibrosis or vasomotor responses of blood vessels (Cuculi and Erne 2011; Birner et al. 2012; Mangiafico et al. 2013; Dalzell et al. 2014; von Lueder et al. 2014).

A large number of peptides processed by ACE and NEP, e.g. Ang I, BK, enkephalins, substance P (SP), natriuretic peptides, control multiple brain functions, such as cardiovascular and neuroendocrine regulations and behaviour. The peptides also play an important role in processes occurring in the brain under various pathological conditions, like cerebral ischaemia, inflammation, neurodegeneration and apoptosis. In vivo and ex vivo studies indicate that ACE in the brain can be inhibited after systemic treatment with ACE inhibitors. However, the results are rather equivocal and the penetration of ACE inhibitors through the blood–brain barrier (BBB) remains a controversial topic (Unger et al. 1988; Gohlke et al. 1989). No attempt has been undertaken to study whether systemically administered OMA or other vasopeptidase inhibitors cross the BBB. Therefore, the first aim of the study was to investigate in conscious rats the ability of orally or intravenously (IV) administered OMA to cross the BBB. The second part of the study explores the effects of ACE/NEP inhibition in the brain on the recovery from focal cerebral ischaemia. During the course of the study, it has become apparent that systemically administered OMA does not penetrate across the BBB. To achieve an effective inhibition of ACE and NEP in brain tissue, OMA was infused *intracerebroventricularly (ICV)* by means of osmotic minipumps over a 5-day period before and during 24 h after middle cerebral artery occlusion (MCAO) with reperfusion.

Materials and methods

General remark

The objective of the second part of the current study was to obtain information on the role of brain ACE/NEP in the initiation and regulation of processes associated with focal cerebral ischaemia and to investigate the impact of an ACE/NEP inhibition on stroke outcome. OMA does not penetrate across the BBB, therefore, the mode of ICV application of OMA was chosen. Although ICV infusion of drugs is not a clinically relevant therapeutic approach, the study adhered in the majority of points to the current RIGOR Guidelines for Effective Translational Research (Lapchak et al. 2013; RIGOR. Improving the quality of NINDS-supported pre-clinical and clinical research through rigorous study design and transparent reporting 2012, available from: http://www.ninds.nih.gov/funding/transparency_in_reporting_guidance.pdf) with respect to blinding (see below), randomisation of the treatment groups, inclusion and exclusion criteria, monitoring of cerebral blood flow in each animal, monitoring of arterial blood pressure and heart rate, control of relevant physiological variables in blood, measurement of infarction volume, assessment of neurological deficits, mortality and to appropriate statistical evaluation of the obtained data.

Animals

Male Wistar rats (300–320 g) obtained from Charles River, Sulzfeld, Germany were housed under controlled conditions with respect to temperature, humidity and 12-h light/12-h dark cycle with free access to food and water. All efforts have been made to minimise the suffering of animals. All experimental protocols were approved by the Governmental Committee for the Ethical use of Animals in the German Federal State of Schleswig-Holstein.

Surgical methods

Chloral hydrate (400 mg/kg body weight) injected intraperitoneally was used as anaesthetic for all surgical procedures. For ICV injections, polyethylene cannula (PP 20) was implanted into the left lateral cerebral ventricle. One polyethylene catheter (PP 50) was inserted through the femoral artery into the abdominal aorta and another catheter was inserted into the femoral vein. The catheters were filled with heparinised saline, passed through a subcutaneous tunnel, sealed and secured at the back of the neck. The arterial catheter was used for measurements of cardiovascular parameters and blood collections to determine physiological variables before, during and after focal cerebral ischemia. The venous catheter was used for IV administrations of OMA or vehicle. Experiments were carried out 48 h after the catheter implantation.

Osmotic minipumps (ALZET Model No.2002, Charles River, Sulzfeld, Germany), delivering solutions at a rate of 0.5 μl/h were filled with vehicle or with OMA (6 mmol/l) and implanted subcutaneously 5 days before MCAO as described elsewhere (Dai et al. 1999). Focal cerebral ischemia was induced by occlusion of the middle cerebral artery (MCA) with a 4–0 nylon monofilament for 90 min. Reperfusion was achieved by pulling out the monofilament (Dai et al. 1999). Regional cerebral blood flow (rCBF) was continuously monitored in each rat at one point (1 mm posterior to the bregma, 6 mm from the midline) on the surface of each hemisphere by laser-Doppler-flowmetry (Periflux system 5000, PERIMED AB, Järfälla, Sweden). Abrupt reduction of rCBF by approximately 75 to 90 % indicated a successful occlusion of the MCA. Body temperature was maintained at 37 °C with a

heating pad. Rats with subarachnoid haemorrhage were excluded from the experimental protocol.

General procedures

Recordings of blood pressure and heart rate and determination of physiological variables in blood

All experiments were carried out in conscious, freely moving rats. The arterial catheter was connected to the transducer and the experiments were started when rats were resting and the basal mean arterial pressure (MAP) and heart rate (HR) values were stable. Vehicle or OMA were injected IV through the intravenous catheter connected to an extension catheter with syringe. When OMA was administered intragastricaly, the arterial catheter was connected to the transducer after the treatment. Ang I was injected as a bolus IV at a dose of 50 pmol/kg body weight before and at various time points after IV or oral treatment with vehicle or OMA. For ICV injection, the ICV cannula was connected to an extension catheter (PP 20) with a Hamilton syringe. Ang I (100 pmol) was injected ICV in a volume of 1 μl and flushed with 3 μl of physiological saline. OMA was injected ICV in volume of 1 to 3 μl (depending on the dose, see below) and flushed with 3 μl of physiological saline. Recordings of systolic, diastolic and MAP and HR were carried out via the arterial catheter using a pressure transducer (Smiths Industries Medical System Company, PVB Medizintechnik GmbH Kirchseeon, Germany) and a Gold Brush pressure computer coupled to a Gold brush 2400 recorder. Analogue output signals were digitalised and then processed using a computerised programme (Culman et al. 1997). The changes in MAP in response to IV Ang I are of short duration (2 min) and are expressed as maximal increases (ΔMAP; mmHg). The pressor response to ICV Ang I lasts for about 25 min, and is, therefore, expressed as the maximal increase in MAP and also as area under the curve (AUC; MAP, mmHg×min). The AUC value represents the sum of MAP changes integrated in time, i.e. one number quantitatively characterises the whole response.

The arterial catheter was also used for blood withdrawals (200 μl each) to determine the following blood variables: pH, pCO_2, pO_2, concentrations of glucose, lactate, sodium, potassium, chloride, calcium, hydrogen carbonate and hematocrite, before and during MCAO and reperfusion (RADIOMETER ABL 700 SERIES (Radiometer Medical A/S, DK-2700 Brønshøj, Denmark)).

Neurological deficits

The used neurological grading system used in the current experiments allows a separate assessment of motor and sensory functions (Garcia et al. 1995). Severe impairments in each test are graded 0 or 1, and no observable deficits are graded 3. The neurobehavioural evaluation consisted of the following five tests and scoring: (1) symmetry of the movement of four limbs, (2) forepaw outstretching, (3) climbing, (4) body proprioception and (5) response to vibrissae touch.

Measurement of infarction volume

Twenty-four hour after MCAO, rats were deeply anaesthetised and intracardially perfused with phosphate-buffered saline (PBS; pH 7.4) followed by 4 % paraformaldehyde. The brains were removed, post-fixed overnight and subsequently cryoprotected in 30 % sucrose for 72 h at 4 °C. Coronal sections (40 μm) were cut in a cryostat from the level bregma + 4.0 mm to the level bregma −7.0 mm. Every 20th slice was used for the determination of the infarction size. Fifteen equidistant coronal sections (40 μm) were stained with cresyl violet, and the infarction volume was calculated by multiplying the sum of the areas by the distance between the sections.

Chemicals

Angiotensin I (Sigma, Deisenhofen, Germany) for IV injection was dissolved in physiological saline (stock solution, 500 nmol/l) stored by −20 °C and appropriately diluted before each experiment. Ang I was injected IV as a bolus at dose of 50 pmol in 100 μl/kg body weight. Ang I for ICV injections (100 pmol/μl) also dissolved in physiological saline. In previous experiments, Ang I dissolved either in physiological saline or artificial cerebrospinal fluid had yielded identical results. OMA ([4S-[4(R*),7,10a]]-octahydro-4-[(2-mercapto-1-oxo-3-phenylpropyl)amino]-5-oxo-7H-pyrido[2,1-b] [1,3]-thiazepine-7-carboxylic acid) was provided by Bristol-Myers-Squibb, Princetown, NJ, USA. OMA is almost insoluble in water (0.04 mg/ml at room temperature); however, the solubility of the inhibitor in aqueous solution increases as the pH of the medium increases (due to the ionisation of the carboxylic acid group and the sulfhydryl group). Therefore, OMA for oral application was dissolved in water by neutralisation with a stoichiometric equivalent of NAOH. For ICV administration, OMA was solubilised in saline and NaOH solution was added to obtain a clear solution. The final pH of the OMA solution was 8.0–8.2, solutions of higher pH values may exert harmful effects when injected ICV. The doses of 30 (100) nmol and 10 nmol of OMA were injected ICV in a volume of 3 and 2 μl, respectively, the dose of 3 nmol was injected in 1 μl. The pH value of the vehicle solution was adjusted to 8.0. The osmotic minipumps delivering solutions at a rate of 0.5 μl/h were filled with OMA at a concentration of 6 mmol/l (pH=8.0), i.e. OMA was infused ICV at the dose of 3 nmol/h. Solubility limits have prevented the use of higher doses of OMA in stroke experiments. All other chemical were purchased from Sigma, Deisenhofen or Merck, Darmstadt, Germany.

Experimental protocols

Effects of ICV treatment with OMA on pressor responses induced by ICV and IV Ang I

Initially, the effects of ICV administered vehicle or OMA on baseline MAP and HR were determined (n=7/group). Rats treated ICV with 100 nmol OMA, displayed rotation along the long axis (barrel rotation), motor disturbances and seizures and considerable increases in MAP and HR (Table 1). Therefore, this dose was not used in further experiments. In the following experiments, rats (n=6–8) were treated ICV with vehicle (controls) or with OMA (3, 10, 30 nmol). The pressor responses to Ang I injected ICV were recorded before and then 15 min, 1, 4 and 24 h post-treatment. MAP increases to Ang I (50 pmol/kg body weight) injected IV at the same time points were also recorded to assess the leakage of ICV-injected OMA into the systemic circulation.

Effect of oral treatment with OMA on pressor responses induced by IV and ICV Ang I

Rats were treated orally with vehicle (controls, n=7) or with OMA (n=7–8/group) at doses of 5, 25, 100 μmol/kg body weight. Ang I (50 pmol/kg body weight) was injected IV before and 1, 2, 4, 8 and 24 h after the treatment and the pressor responses were recorded. In the second set of experiments, rats (n=7–8) were treated orally with the same doses of OMA or with vehicle (controls). The blood pressure responses to ICV Ang I were detected before and 1, 2, 4, 8 and 24 h post-treatment.

Table 1 Basal values of mean arterial pressure (MAP; mmHg) and heart rate (HR; b.p.m.) and maximal increases in MAP (ΔMAP; mmHg) and HR (ΔHR; b.p.m.) after intracerebroventricular (ICV) injection of vehicle or various doses of Omapatrilat (OMA)

ICV injection	Vehicle	OMA	OMA	OMA	OMA
Dose		3 nmol	10 nmol	30 nmol	100 nmol
n	7	6	6	6	8
Basal MAP	100±3	104±4	94±3	100±6	96±3
ΔMAP	2±1	1±2	4±1	7±2	27±2***
Basal HR	287±15	332±13	299±8	308±17	268±16
ΔHR	10±3	8±12	15±9	8±14	77±26*

Results are expressed as the means±SEM. ICV injection of 100 nmol OMA induced an immediate increase in MAP and HR

*P<0.05; ***P<0.001, statistical comparison with the vehicle group and with all groups injected with lower doses of OMA, calculated with one-way ANOVA followed by the post hoc Bonferroni adjustment

Effect of IV treatment with OMA on pressor responses induced by IV and ICV Ang I

The data obtained in the first experimental setting has revealed that OMA given orally even at the highest dose does not cross the BBB. To achieve a rapid, high initial increase in OMA concentration in the systemic circulation, the inhibitor (n=6–8/group) was injected IV at doses of 0.5, 1, 5, 25 μmol/kg body weight. Controls (n=7) received vehicle. The blood pressure responses to IV injected Ang I were recorded before and 30 min and 1, 2, 4, 8 and 24 h post-treatment. The effect of 100 μmol/kg body weight OMA on the pressor response to IV Ang I was tested at 8 and 24 h after the IV injection. In the second set of experiments, rats (n=7–8) were treated IV with vehicle or OMA (0.5, 1, 5, 25 μmol/kg body weight) and the blood pressure responses to Ang I injected ICV were recorded prior and 1, 2, 4, 8 and 24 h post-treatment. We have observed that the pressor response to the second ICV Ang I injection was significantly smaller than that to the preceding one (due to desensitisation of angiotensin AT_1 receptors) when the time interval between the two consecutive ICV Ang I injections was 30 min or shorter. In a separate experiment, Ang I was injected ICV 30 min (0.5 h) after IV treatment with vehicle or various doses of OMA (n=6–7). To ascertain the effects of IV-injected OMA on resting values of blood pressure and heart rate, 100 μmol/kg body weight of OMA was slowly injected IV and the MAP, systolic and diastolic blood pressure and HR were monitored over 120 min (vehicle, n=7; OMA, n=8).

Effects of ICV treatment with OMA on neurological outcome and infarction volume after focal cerebral ischaemia

Osmotic minipumps filled with vehicle or OMA (6 mmol/l) were randomly implanted 5 days before MCAO by laboratory technicians (J.B. or B.S.). The induction of the focal cerebral ischaemia (MCAO for 90 min) was carried out by the same person, who was blinded to the treatments (W.S.). rCBF was monitored continuously before and during MCAO in each rat. Only rats, in which the ipsilateral rCBF was continuously reduced to less than 25 % of the baseline at least 30 min after the onset of MCAO were included into the protocol. ICV infusions of vehicle and OMA were then continued for 24 h. Neurological evaluations were carried out 24 h after MCAO in each rat by the same investigator (J.C.), who was not involved in surgery and was blinded to treatment protocols. The brains for the determination of the infarction volume were obtained from rats intracardially perfused 24 h after MCAO (vehicle, n=11; OMA, n=10). The brains were cut in a cryostat and stained with cresyl violet (W.S.). The infarction volume was evaluated by an investigator, who was blinded to the treatment protocols (Y.Z.).

A separate experiment was designed to monitor systolic and diastolic blood pressure and heart rate and to determine

physiological variables before, during and after focal cerebral ischaemia in rats infused ICV with vehicle or OMA 5 days prior to MCAO. On the day of experiment, baseline cardiovascular parameters were recorded (vehicle, $n=10$; OMA, $n=10$) and the basal blood sample (200 μl) was collected for determination of physiological variables (vehicle, $n=8$; OMA, $n=10$), in conscious rats. The cardiovascular parameters were continuously monitored before, during and up to 150 min after the onset of cerebral ischaemia and then 24 h after the ischaemic insult. Blood samples were withdrawn immediately before the surgery, 60 and 120 min after the MCAO and 30 min after the onset of the reperfusion period. In a separate experiment, the pressor response to Ang I (50 pmol/kg body weight) injected IV was detected on day 5 after continuous ICV infusion of vehicle ($n=7$) or OMA (6 mmol/l; $n=6$) to ascertain the leakage of OMA from the ventricular system into the systemic circulation.

Mortality Three rats treated with vehicle and 3 rats treated with OMA died within 24 h after MCAO.

Statistics

Results are expressed as the mean±SEM. The effects of OMA administered ICV, orally or IV on the MAP increases (ΔMAP) to IV and ICV Ang I, the monitoring of MAP, systolic and diastolic blood pressure and HR, the monitoring of rCBF and data on determination of physiological variables in blood were statistically analysed by two-factorial analysis of variance (ANOVA) for repeated measures. AUC data were analysed by one-way ANOVA followed by the Bonferroni adjustment for pairwise comparisons. Statistical significance of differences in neurological deficits between vehicle- and OMA-treated rats exposed to MCAO did not follow the normal, Gaussian distribution (Kolmogorov–Smirnov test) and were, therefore, analysed by the non-parametric Mann–Whitney test. The effects of vehicle or OMA on the infarction size were analysed by the Student's *t* test for unpaired samples (normal, Gaussian distribution). The Bartlett's test suggested that the differences among the SDs were not significant. A sample size of 10 in each group will have 80 % power to detect an effect size of 1325 using a two group *t* test with a 0.050 two-sided significance level.

Results

General remarks

Ang I injected IV or ICV is immediately converted to Ang II, which increases MAP by activating peripheral or brain AT_1 receptors. The pressor responses elicited by equimolar doses of Ang I and Ang II injected IV or ICV are identical. Ang I injected IV induces a short-lasting (2–3 min) increase in MAP, which then rapidly returns to basal value. Changes in HR were small and variable and there were no differences between the vehicle- and OMA-treated groups of rats. The pressor response to ICV Ang I is characterised by a rapid increase in MAP which reaches maximum at 1–2 min and then returns gradually to basal values. The majority of animals reach control, pre-injection values within 25–30 min after Ang I injection. Intravenous or ICV injection of Ang I to conscious rats resulted in highly reproducible pressor responses throughout the protocols.

Effects of ICV treatment with OMA on pressor responses induced by ICV and IV Ang I

Acute ICV administration of OMA at doses of 3, 10 and 30 nmol had no effect on resting MAP and HR (Table 1). However, the dose of 100 nmol OMA considerably increased MAP and HR along with barrel rotation and motor disturbances. ICV injections of Ang I resulted in a highly reproducible increases in the MAP of about 20–25 mmHg. The dose of 3 nmol OMA inhibited the Ang I response only 15 min following the treatment. In rats treated with 10 and 30 nmol of OMA, the pressor response to Ang I (expressed either as a maximum in MAP increase or as AUC) was attenuated up to 1 h after the inhibitor administration but not at later time points (Fig. 1a, b). OMA injected ICV rapidly appeared in the systemic circulation and dose-dependently attenuated the blood pressure responses to IV Ang I. The systemic inhibitory effects of OMA at doses of 10 and 30 nmol were detectable over a 4-h period post-ICV inhibitor administration (Fig. 1c).

Effect of oral treatment with OMA on pressor responses induced by IV and ICV Ang I

Ang I (50 pmol/kg body weight) injected IV in rats treated with vehicle elicited consistent increases in the MAP. All doses of OMA administered orally completely abolished the pressor response to IV Ang I up to 4 h. The pressor response to IV Ang I was significantly attenuated at 8 h and the inhibitory effects were still detectable 24 h after the treatment; the dose of 5 μmol/kg body weight OMA was less effective than the higher doses of the inhibitor (Fig. 2a). OMA administered orally at either dose had not any effect on the pressor response to ICV Ang I at any time point (Fig. 2b, c, only effects of 25 and 100 μmol/kg body weight OMA are shown).

Effect of IV treatment with OMA on pressor responses induced by IV and ICV Ang I

The dose of 100 μmol/kg body weight OMA or vehicle injected IV induced small immediate increases in MAP and HR which did not significantly differ (ΔMAP: vehicle, 6±2 mmHg;

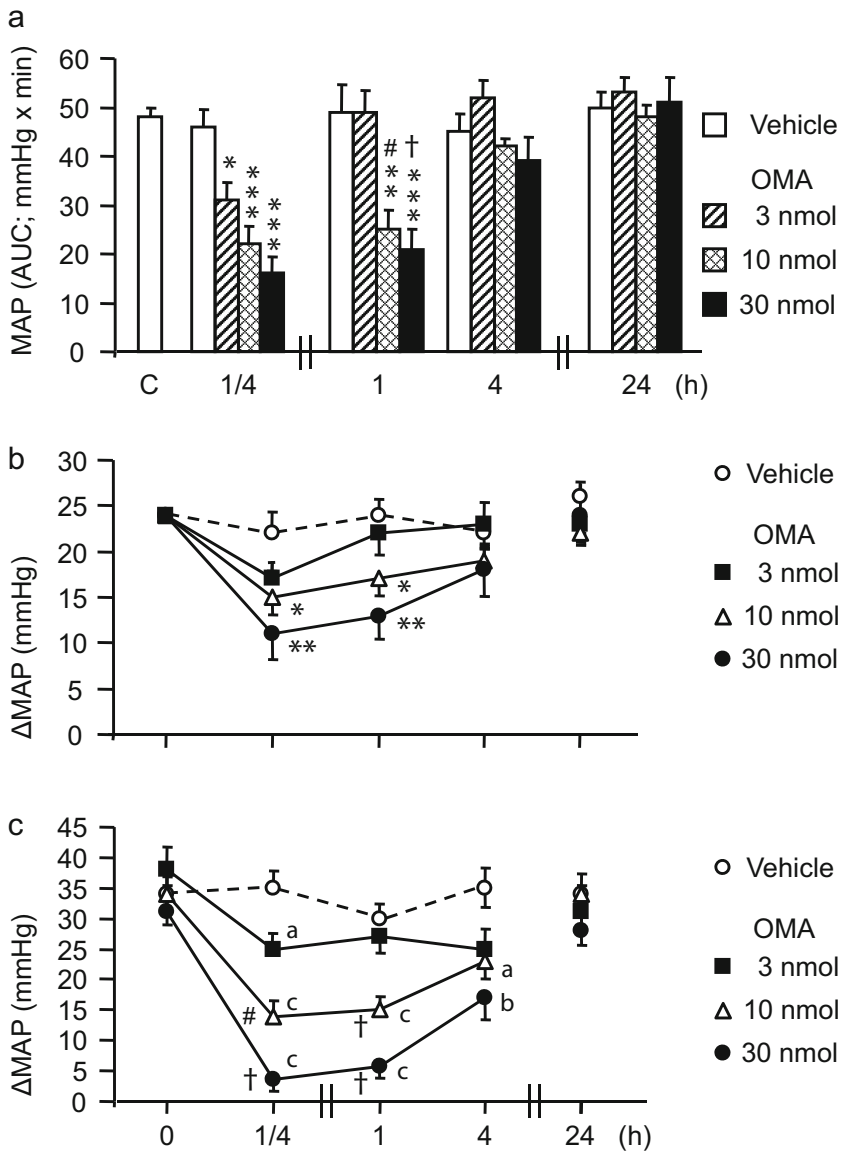

Fig. 1 Effects of Omapatrilat (OMA) injected intracerebroventricularly ICV on mean arterial pressure (MAP) responses to ICV and intravenously (IV) injected Ang I. Results are expressed as the means ±SEM. **a**, **b** OMA inhibits MAP increases induced by ICV Ang I up to 1 h following treatment. MAP values are expressed as AUC (mmHg× min) (**a**) or as maximal increases (mmHg) (**b**). **a** **P*<0.05; ***P*<0.01; ****P*<0.001, statistical comparison with the vehicle-injected group at the given time point; $^{\#}P$<0.05; $^{\dagger}P$<0.001, statistical comparison with the group-treated ICV with 3 nmol OMA (one-way ANOVA followed by the Bonferroni adjustment). **b** **P*<0.05; ***P*<0.01, statistical comparison with the vehicle-injected group at the given time point (two-factorial ANOVA for repeated measures). **c** ICV-injected OMA inhibits dose-dependently pressor responses induced by IV Ang I, expressed as maximal increases in MAP (mmHg). **a** *P*<0.05; **b** *P*<0.01; **c** *P*<0.001, statistical comparison with rats treated ICV with vehicle at the given time point; $^{\#}P$<0.05; $^{\dagger}P$<0.001, statistical comparison with the group treated ICV with 3 nmol OMA at the given time point. Statistical significance of values recorded in all groups at different time points to the control values recorded in the appropriate group at time point 0 are not shown (two-factorial ANOVA for repeated measures)

OMA, 7±1 mmHg; ΔHR: vehicle, 33±13 b.p.m.; OMA, 24±8 b.p.m.). IV injection of 100 μmol/kg body weight OMA had no effects on resting MAP or HR up to 120 min following the treatment (Table 2). The pressor responses to IV Ang I were completely abolished up to 2 h and significantly attenuated at 4 and 8 h following IV injection of either dose of OMA, but not at 24 h. Only the dose of 25 μmol/kg body weight still effectively reduced the MAP response to IV Ang I at this time point (Fig. 3a). The pressor response to ICV Ang I was not altered at any time point following IV injection of OMA at either dose (Fig. 3b, c, only the effects of 5 and 25 μmol/kg body weight are displayed).

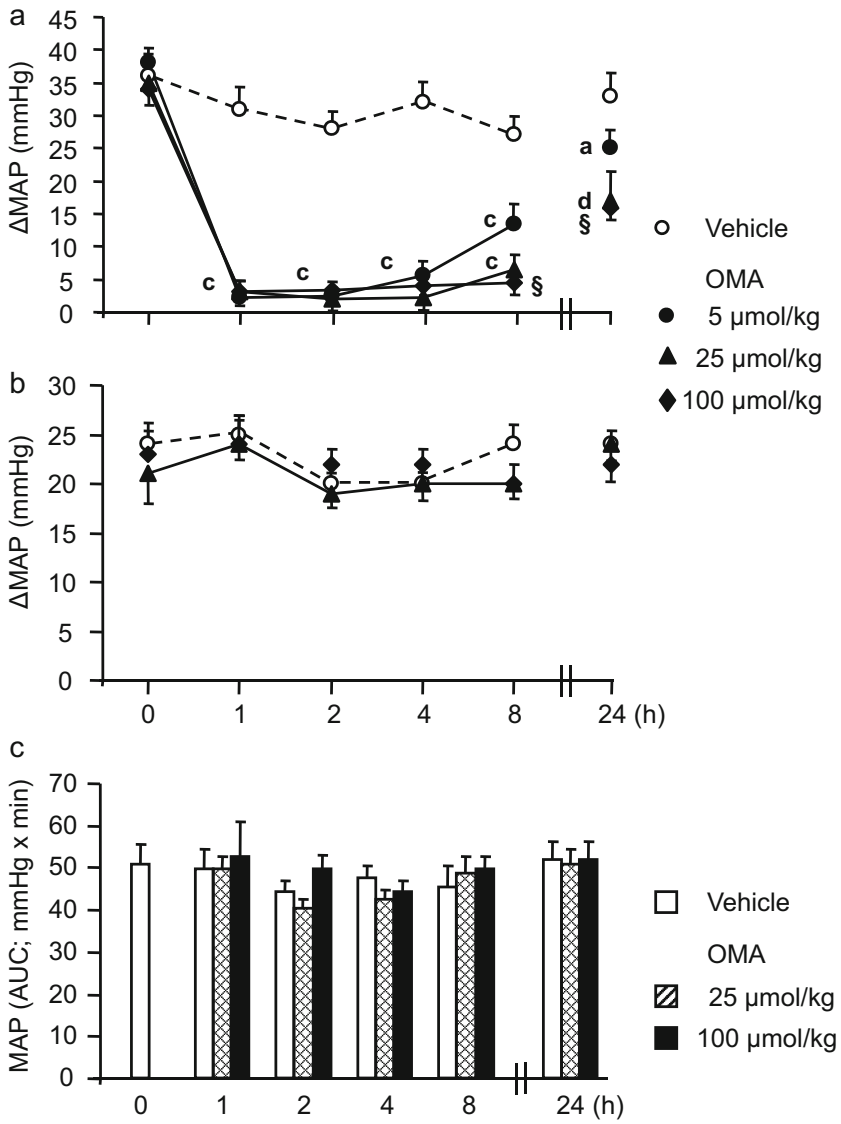

Fig. 2 Effects of oral treatment with various doses of Omapatrilat (OMA) on mean arterial pressure (MAP) responses to intravenously (**a**) and intracerebroventricularly (ICV) (**b**, **c**) injected Ang I. Results are expressed as the means±SEM. MAP values are expressed as maximal increases (mmHg) (**a**, **b**) or as AUC (mmHg×min) (**c**). **c** $P<0.001$, significant reductions of MAP increases to Ang I by all three doses of OMA at 1, 2, 4, and 8 h following oral OMA administration. **a** $P<0.05$; **d** $P<0.001$, statistical comparison with vehicle-treated rats 24 h after treatment; $^{§}P<0.05$; statistical comparison with the value detected in rats treated with 5 μmol/kg body weight OMA at the given time point. Statistical significance of values recorded in all groups at different time points to the control values recorded in the appropriate group at time point 0 are not shown (two-factorial ANOVA for repeated measures). **b**, **c** OMA given orally at either dose had no effect on the pressor responses induced by ICV Ang I at any time point after treatment

Table 2 Time courses of the mean arterial pressure (MAP), systolic (SBP) and diastolic (DBP) blood pressure and heart rate (HR) in rats injected intravenously with vehicle ($n=7$) or Omapatrilat (OMA; 100 μg/kg body weight; $n=8$)

Time (min)		0	5	30	60	90	120
After i.v. injection		Basal					
MAP (mmHg)	Vehicle	91±4	90±3	87±3	88±3	88±3	88±4
	OMA	91±2	93±3	92±4	86±3	91±3	90±4
SBP (mmHg)	Vehicle	121±5	124±4	116±3	120±5	122±2	120±6
	OMA	115±2	121±3	115±4	116±3	116±2	119±3
DBP (mmHg)	Vehicle	81±4	84±4	80±4	81±3	79±2	79±4
	OMA	79±2	85±3	80±4	77±3	78±3	82±4
HR (b.p.m.)	Vehicle	313±14	339±12	330±18	332±20	320±13	315±16
	OMA	310±14	334±15	346±11	342±11	345±13	329±10

Results are expressed as the means±SEM. No significant differences in the MAP, SBP, DBP and HR were detected between vehicle- and and Omapatrilat-treated rats at any time point (two-factorial ANOVA for repeated measures).

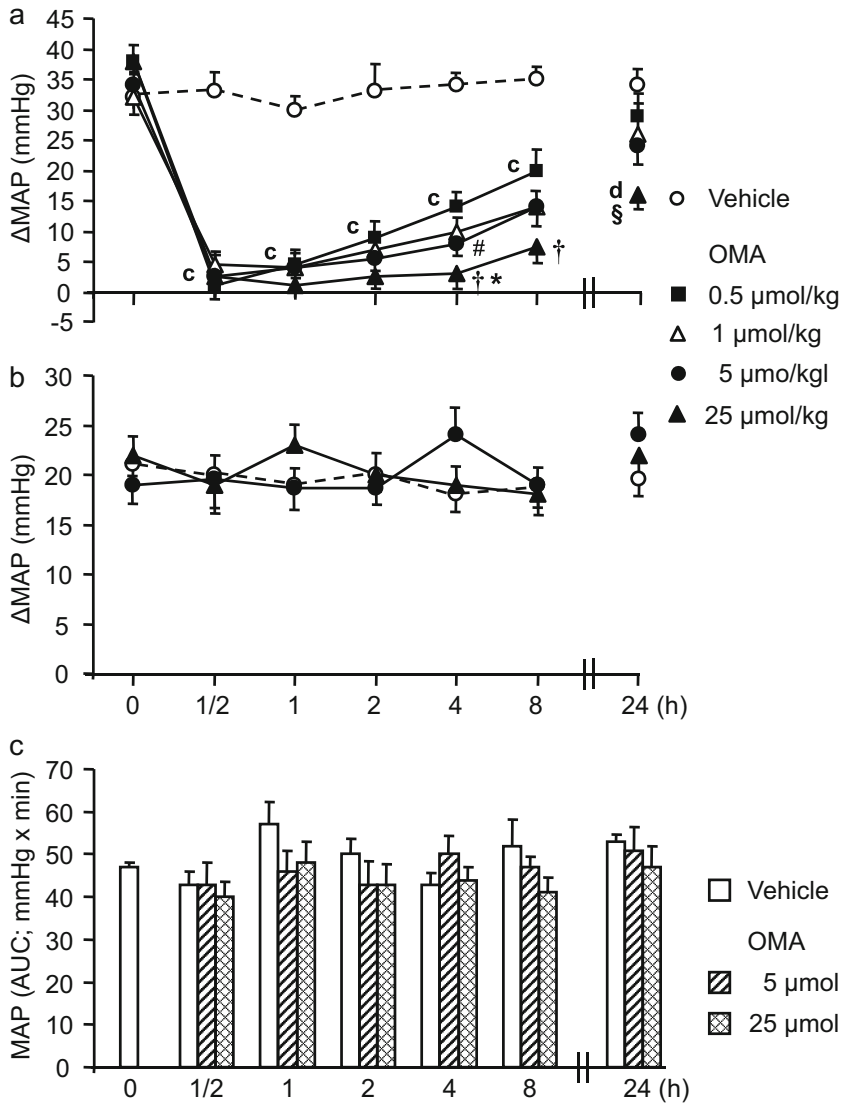

Fig. 3 Effects of intravenous treatment with various doses of Omapatrilat (OMA) on mean arterial pressure (MAP) responses to intravenously (IV) (**a**) and intracerebroventricularly (ICV) (**b**, **c**) injected Ang I. Results are expressed as the means±SEM. MAP values are expressed as maximal increases (mmHg) (**a**, **b**) or as AUC (mmHg×min) (**c**). **c** $P<0.001$, significant reductions of MAP increases to IV Ang I by all four doses of OMA at 30 min, 1, 2, 4, and 8 h following IV treatment. $^{\#}P<0.05$; $^{\dagger}P<0.001$, statistical comparison with the group injected IV with 0.5 μmol/kg body weight of OMA and $^{*}P<0.05$, statistical comparison with the group injected IV with 5 μmol/kg body weight of OMA, 4 h post-treatment. **d** $P<0.001$, statistical comparison with vehicle-treated rats; $^{\S}P<0.05$, statistical comparison of the value detected in rats treated with 0.5 μmol/kg body weight OMA 24 h after treatment. Statistical significance of values recorded in all groups at different time points to the control values recorded in the appropriate group at time point 0 are not shown (two-factorial ANOVA for repeated measures). **b**, **c** OMA injected IV at either dose had no effect on the pressor responses induced by ICV Ang I at any time point after treatment

Effects of ICV treatment with OMA on neurological outcome and infarction volume after focal cerebral ischaemia

The systemic leakage of OMA infused ICV over a 5-day period was negligible, as identical pressor responses to IV injected Ang I (50 pmol/kg body weight) were recorded in vehicle- and OMA-treated rats (ΔMAP: vehicle, 29.0± 3.0 mmHg; OMA, 28.0±3.1 mmHg). rCBF reductions during MCAO were identical in both groups of rats. After the withdrawal of the filament, ipsilateral CBF was restored to approximately 75–85 % of the baseline (Fig. 4a). Resting values for MAP and HR on day 5 after implantation of mini-osmotic pumps were not different between the vehicle- and OMA-treated rats (Fig. 4b, c). Furthermore, no significant differences in MAP and HR values were detected between both groups of rats at any time point during MCAO and the reperfusion period (Fig. 4b, c). A slight but significant decrease in pH values in anaesthetised animals was detected until 150 min after the onset of MCAO. Anaesthesia also induced a decrease in pO_2 and increase in pCO_2 levels and glucose and lactate concentrations (Table 3, significance not shown). Importantly, the values of all physiological variables in blood were

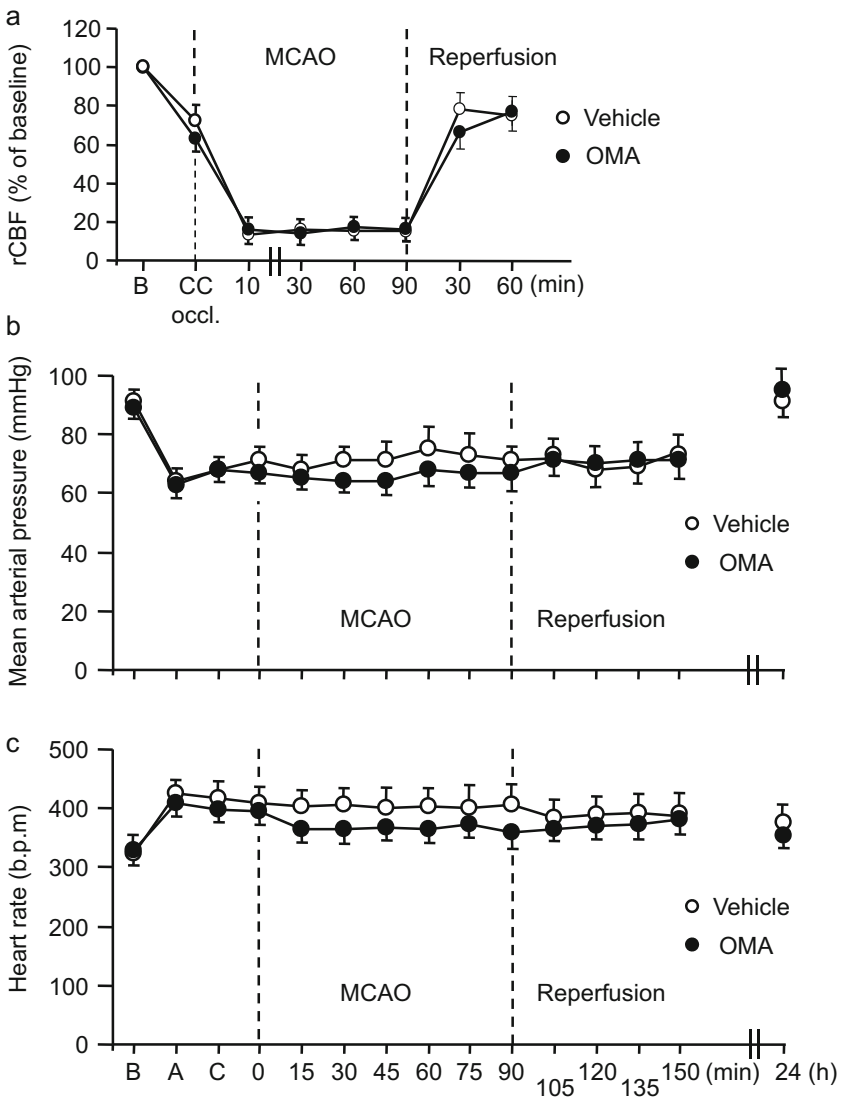

Fig. 4 **a** Changes in the regional cerebral blood flow (rCBF) in the zone of ischaemia during occlusion of the middle cerebral artery (MCAO) for 90 min and during the reperfusion period in rats infused ICV with vehicle or Omapatrilat (*OMA*; 6 mmol/l). rCBF values (the means±SEM) are expressed as the percentage of baseline values recorded before MCAO. No significant differences in rCBF values were detected between the vehicle- (n=21) and OMA-treated (n=21) groups of rats (two-factorial ANOVA for repeated measures). **b** Time courses of the mean arterial pressure (*MAP*) and heart rate (*HR*) in rats treated intracerebroventricularly with vehicle (n=10) or OMA (6 nmol/l; n=10) before and during MCAO and in the reperfusion period. No significant differences in the MAP and HR between the vehicle- and OMA-treated groups of rats were detected at any time point (two-factorial ANOVA for repeated measures). B basal values; A values detected in anaesthetised rats; C values detected immediately after occlusion of the common carotid artery

identical in both groups of rats at any time point (Table 3). The slight reduction in infarct volume by approximately 20 %, detected in rats treated with OMA did not reach statistical significance (Fig. 5a). Twenty four h after MCAO, the rats in both groups suffered from severe neurological deficits and no significant differences in the summation of motor and sensory neurological scores were observed (Fig. 5b). Except of the symmetry of the movement of four limbs, the individual scores of sensory impairments and motor deficits of rats treated with OMA did not differ from those recorded in vehicle-treated rats (Fig. 5c).

Discussion

This study examined the capability of OMA injected ICV to inhibit ACE in the circumventricular organs (CVOs) and periventricular brain structures, the penetration of systemically administered OMA across the BBB and the effect of the inhibitor on the recovery from focal cerebral ischaemia. The main findings are that ICV-injected OMA rapidly appears in the systemic circulation and more effectively inhibits the systemic than the central pressor responses to Ang I, secondly, systemically administered OMA does not diffuse into the brain ventricles and brain tissue and thirdly, central treatment with OMA does not affect the recovery from focal cerebral ischaemia. To our best knowledge, this is the first study reporting on effects of a simultaneous ACE/NEP inhibition in the brain on the outcome after ischaemic stroke.

ICV injection of OMA dose-dependently attenuated the increases in arterial blood pressure induced by centrally (ICV) applied Ang I. Ang I injected ICV is rapidly converted to Ang II by ACE in the CVOs which exhibit high activities of ACE (Saavedra and Chevillard 1982). The cardiovascular response to Ang II is mediated by the release of vasopressin from the posterior pituitary, activation of the sympathetic

Table 3 Physiological variables in arterial blood before, during and after MCAO in rats infused intracerebroventricularly (ICV) with vehicle (n=8) or Omapatrilat (n=10)

Parameter	Treatment (ICV)	B	A	30 min	60 min	120 min
				After the onset of MCAO		
pH	Vehicle	7.42±0.03	7.29±0.01	7.33±0.01	7.36±0.03	7.35±0.01
	Omapatrilat	7.45±0.01	7.29±0.01	7.31±0.02	7.34±0.01	7.34±0.01
pO_2 (mmHg)	Vehicle	80±1.9	73±2.4	79±2.6	85±3.0	87±2.8
	Omapatrilat	86±2.3	78±2.7	85±2.5	90±4.0	91±2.4
pCO_2 (mmHg)	Vehicle	39±1.6	52±3.0	51±3.1	51±4.1	54±3.7
	Omapatrilat	37±1.1	51±1.2	51±3.0	49±4.2	51±4.0
Glucose (mg/dl)	Vehicle	123±7.8	190±20	145±12	133±12	168±17
	Omapatrilat	122±5.8	227±16	160±11	159±12	179±19
Lactate (mmol/l)	Vehicle	1.83±0.35	2.66±0.18	2.22±0.19	2.45±0.20	2.51±0.31
	Omapatrilat	1.56±0.16	3.01±0.41	2.27±0.19	2.46±0.16	2.01±0.19
Na^+ (mmol/l)	Vehicle	140±0.4	139±1.4	138±1.0	138±1.1	137±0.8
	Omapatrilat	143±0.5	139±0.7	138±1.8	140±0.9	138±1.2
K^+ (mmol/l)	Vehicle	4.1±0.3	3.6±0.2	4.0±0.2	4.1±0.1	3.8±0.2
	Omapatrilat	3.8±0.2	3.6±0.1	4.0±0.3	4.0±0.2	3.7±0.2
Cl^- (mmol/l)	Vehicle	107±2.0	109±2.2	107±2.3	107±2.4	105±2.5
	Omapatrilat	109±1.9	110±2.0	111±2.1	108±1.6	109±1.7
Ca^{2+} (mmol/l)	Vehicle	1.20±0.02	1.07±0.03	1.17±0.03	1.14±0.02	1.16±0.04
	Omapatrilat	1.22±0.02	1.11±0.02	1.11±0.03	1.16±0.0.02	1.14±0.02
HCO_3^- (P)	Vehicle	25.7±1.4	24.4±1.6	26.4±1.6	28.1±2.4	29.2±2.3
	Omapatrilat	25.5±1.0	24.0±0.8	24.9±1.0	25.6±2.5	26.9±2.2
Haematocrit (%)	Vehicle	40±2.4	36±3.2	39±2.0	35±2.8	40±0.7
	Omapatrilat	41±2.2	34±2.0	42±2.2	35±4.1	39±1.7

Results are expressed as the means±SEM. No statistically significant differences between vehicle- and Omapatrilat-treated rats were detected (two-factorial ANOVA for repeated measures)

B basal values, *A* values detected in anaesthetised rats immediately before occlusion of the middle cerebral artery (MCAO) for 90 min

nervous system and inhibition of the baroreflex (Haack and Möhring 1978; Hogarty et al. 1992). The pressor response is generated by activation of AT_1 receptors in the CVOs, the subfornical organ (SFO) and the organum vasculosim laminae terminals (OVLT), which lack of the BBB, and the neuronal circuits in the lamina terminalis, a strip of periventricular tissue comprising the anterior wall of the 3rd ventricle (AV3V) (Chai et al. 1987; Mendelsohn et al. 1984). A substantial network of fibres containing Ang II connects the neurones in the CVOs directly or indirectly, via a synaptic relay in the median preoptic nucleus (MnPO), with the vasopressin-secreting neurons in the paraventricular (PVN) and supraoptic (SON) nuclei (Honda et al. 1990; Oldfield et al. 1991; Bains et al. 1992). At early time points after ICV OMA injection, the attenuation of the pressor response to ICV Ang I most probably resulted from a direct inhibition of ACE in the CVOs and consequently reduced conversion of Ang I to Ang II.

Where the CSF interfaces the ependymal cells, the gap junctions provide only limited diffusion restrictions. The lack of the structural barrier enables free exchange of substances between the CSF and interstitial fluid (Johanson et al. 2011; Zheng and Monnot 2012). Providing that OMA injected ICV efficiently diffused from the ventricular system into the adjacent neural tissue, inhibition of ACE would impair the synthesis of Ang II in angiotensin nerve terminals, especially in those localised in the PVN and MnPO, which lie in the close vicinity of the ventricular system. The impaired Ang II synthesis would then result in reduced pressor responses induced by Ang I at later time points. As no reductions in the pressor responses to Ang I were observed, we assume that OMA administered ICV as a single injection rapidly disappears from the ventricular system through the CSF drainage and appears in the circulation (Murtha et al. 2014). The attenuation of the pressor response to Ang I injected IV observed as early as 1 h after ICV OMA application is consistent with this assumption. It should be emphasised that OMA applied ICV inhibited more effectively and for a longer period of time the systemic than the central pressor responses to Ang I.

In the next series of experiments, we studied the penetration of OMA across the BBB and its appearance in the CSF after systemic treatment. As expected, a single dose of OMA administered IV or orally time and dose dependently inhibited the pressor response elicited by IV Ang I. In fact, the cardiovascular response to Ang I was almost completely abolished

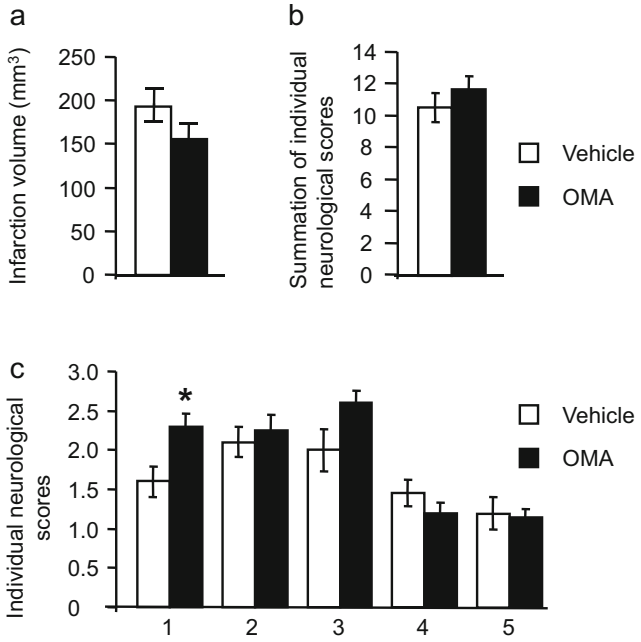

Fig. 5 **a** Infarction volume (the means±SEM) in rats treated ICV with vehicle or with OMA (6 mmol/l) 5 days prior, during and 24 h after MCAO with reperfusion. No significant differences between the vehicle- and OMA-treated groups of rats were detected (Student's *t* test for impaired samples). **b** the summation of individual scores of motor deficits and sensory impairments detected in rats 24 h after MCAO and **c** neurological scores of motor deficits and sensory impairments in rats treated ICV with vehicle or with OMA expressed as the means±SEM. The following tests were evaluated: (1) symmetry of the movement of four limbs; (2) forepaw outstretching; (3) climbing; (4) body proprioception; and (5) response to the vibrissae touch. Rats with better neurological outcome received higher neurological scores. Statistical comparisons with the vehicle-treated group: $*P<0.05$, calculated by the Mann–Whitney test

30–60 min after IV and 1–4 h after oral treatment with the inhibitor. However, OMA administered systemically did not alter the cardiovascular effects of Ang I injected ICV. Even high initial concentrations of OMA in the circulation achieved by IV application of the inhibitor were without any effects. The sensory CVOs, the SFO and the OVLT which are the sites of the conversion of ICV-injected Ang I to Ang II, lack of the BBB. The SFO and OVLT consist of two separated compartments, the hemal milieu and the CSF-dominated milieu. Tight junctions between tanycytic endings and glial cells prevent a direct communication between these two compartments (Krisch et al. 1978, 1987). Although both CVOs exhibit high activities of ACE, the exact localisation of the enzyme within these compartments has not yet been reported (Saavedra and Chevillard 1982). Nevertheless, our data clearly demonstrates that OMA in systemic circulation has obviously no access to ACE in these CVOs.

While the transport of substances across the BBB is strictly limited through the inter- endothelial tight junctions and the underlying basement membrane, the metabolic barrier and transport systems (Persidsky et al. 2006; Engelhardt and Sorokin 2009), the blood-cerebrospinal barrier (BCSFB) is merely formed by tight junctions between epithelial cells (Speake et al. 2001; Johanson et al. 2011). BCSFB allows the passage of small molecules, e.g. peptides and hormones, and low molecular mass substances. OMA circulating in the blood may, therefore be secreted from the circulation into the brain ventricles during the production of CSF in the choroid plexuses. Our findings do not support this assumption, as systemic treatment with OMA did not impair the conversion of ICV injected Ang I to Ang II. The present data together with previous findings (Kubota et al. 2003) provide strong evidence that the BCSFB and the BBB prevent, or, at least to a great extent, limit the penetration of OMA into the brain tissue.

The restricted transfer of OMA between the blood and brain must be reconciled with the multiple functions of different brain peptides processed by the ACE and NEP, of which each subserves distinct central functions. Ang I/II, bradykinin, substance P (SP) amyloid-β peptide are closely involved in a wide variety of pathological processes in the brain, like post-ischaemic inflammation, neurodegeneration and apoptosis. Very recently, Rashid et al. (2014) reported on a sustained upregulation of the membrane-bound endopeptidase, neurolysin (EC3.4.24.16), in different parts of the mouse brain up to 7 days after MCAO with reperfusion. To explore the role of the brain ACE and NEP in the modulation of processes linked to cerebral ischaemia and stroke outcome, we investigated the effects of ACE/NEP inhibition in the brain upon neurologic outcome and infarction volume after MCAO with reperfusion. A single ICV injection of OMA effectively inhibited ACE only for a short period of time. To achieve an effective and steady-state inhibition of ACE and NEP, OMA was continuously infused ICV over a 5-day period before and during 24 h after MCAO. We assume that ICV-infused OMA diffused into the brain interstitial fluid, as (i) a constant concentration of the inhibitor was permanently present in the ventricular system before MCAO, (ii) the lack of a structural barrier between the CSF and the interstitial fluid enables the diffusion of OMA into the brain parenchyma and (iii) disruption of the BBB and other barrier systems, which occurs early after ischaemia/hypoxia, may further facilitate the entry of OMA into the interstitial fluid (Johanson et al. 2011; Zheng and Monnot 2012; Rosenberg 2012). We have convincingly demonstrated that long-term ICV infusion of the same dose of pioglitazone, which does not cross the BBB, effectively ameliorated neuronal injury also in brain areas not localised in the close vicinity to the ventricular system (Zhao et al. 2006; Zuhayra et al. 2011).

ICV OMA did not alter MAP, HR and physiological variables compared with vehicle-treated rats at any time point during MCAO and reperfusion. As ischaemic stroke impairs the central cardiovascular regulation, blood pressure lowering during and after cerebral ischaemia results in a reduction of

cerebral blood flow to the ischaemic area and, consequently, increases infarction volume. The ACE/NEP inhibition did not alter the recovery from ischaemic stroke. The lack of neuroprotection may, on one side, be due to the fact that the used dose of OMA, 3 nmol/h, failed to completely inhibit ACE/NEP in the brain. The poor solubility of OMA in aqueous solutions did not allow the usage of higher doses of the inhibitor. Providing, that sufficient amounts of OMA reached ischaemic brain regions, inhibition of ACE/NEP impaired processing of a variety of peptides exerting diverse actions in different types of cells, including neurones, localised in ischaemic tissue. For instance, decrease in Ang II concentration and angiotensin AT_1 activation may, on one side, alleviate neuronal deficits, reduce neuronal injury due to weakening post-ischaemic inflammation and neuronal cell damage and improve the recovery from ischaemic stroke (Lou et al. 2004). On the other side, diminished angiotensin AT_2 receptor signalling may negatively influence neuronal regeneration and neuronal survival and increase the severity of ischaemic damage (Li et al. 2005; Min et al. 2014). Inhibiting of ACE/NEP increases BK and SP which initiate and regulate a number of pathological processes linked to cerebral ischaemia. BK is an important mediator of inflammatory reactions after ischaemic stroke, including arteriolar dilatation and increased vascular permeability, which considerably contribute to the development of oedema in ischaemic brain. Bradykinin is increasingly produced and released in ischaemic brain parenchyma and, acting at bradykinin B_2 receptors, it promotes neuronal cell death, neutrophil accumulation and oedema formation after ischaemic stroke (Sobey 2003; Ding-Zhou et al. 2003; Gröger et al. 2005; and the references therein). Higher levels of SP also activate neurogenic inflammation and the development of cerebral oedema. Tachykinin receptor antagonists efficiently reduced the BBB opening, cerebral oedema, infarction volume and improved neurological function (Yu et al. 1997; Turner et al. 2011). Considering the great number of diverse and opposing actions mediated by peptides metabolised by ACE and NEP in ischaemic brain, it is not very surprising that treatment with OMA did not exert any beneficial or deleterious effects upon neurological outcome and ischaemic injury after focal cerebral ischaemia.

Our findings also address an important issue about the use of OMA in the prevention and treatment of cardiovascular and neurological diseases. Brain RAS hyperactivity has been implicated in the development and maintenance of hypertension. As OMA does not pass the BBB, the potent and sustained antihypertensive effects of the systemically administered inhibitor are primarily mediated by vascular mechanisms. Treatment of hypertension may also reduce the likelihood and slow the progression of Alzheimer's disease. The inability of OMA to penetrate through the BBB is advantageous as inhibition of brain ACE and NEP may also increase accumulation of amyloid-β peptides (Iwata et al. 2005). Novel ACE/NEP inhibitors, such Ileparil, angiotensin-AT_1 receptor/NEP inhibitors and triple-acting NEP inhibitors (ACE/NEP/endothelin-converting enzyme inhibitors) have been developed and are undergoing clinical evaluation (von Lueder et al. 2014). These novel inhibitors may interfere with the synthesis, degradation or receptor binding of a number of neuropeptides in the brain controlling a wide range of brain functions. Key questions, which need to be answered in future studies, are, whether and to which extent these antagonists penetrate into the brain after systemic treatment and inhibit AT_1 receptors and ACE or NEP, and which are the functional consequences of a long-term alteration in the concentrations and binding of these peptides in brain tissue under pathological conditions.

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