Mannitol Derivate Used as a Marker for Voltammetrically Monitored Transport Across the Blood-Brain Barrier Under Condition of Locus Coeruleus Stimulation

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Abstract. 1-Deoxy-1-nitro-D-mannitol (*DN-Man*) was used (femoral vein injection approximate concentration in the blood 30 mmol l^{-1}) in pentobarbital anaesthetized rats as a promising marker detectable by differential pulse voltammetry (*DPV*) to study its transport across the blood-brain barrier (*BBB*) to the extracellular space of the frontoparietal cortex

DN-Man detection limit in *in vitro* calibrations (saline, blood) using *DPV* and carbon fiber microelectrodes was 0 5 mmol l^{-1} with a good linearity (r = 0 996) over the entire tested range (up to 30 mmol l^{-1})

The slow time-course of the rise of *DN-Man* signal ($y = 106/(1 + (17\ 8/t)^3)$) in the cortex confirmed the functional *BBB* state

Electrical stimulation of the locus coeruleus (*LC*) (300 rectangular pulses at a frequency of 100 Hz, 1 mA, pulse duration 0 2 ms) elevated significantly *DN-Man* current in the cortex (to 168 ± 59% of the control, mean ± S D , n = 8) The evoked permeation increase of the *BBB* to *DN-Man* was short-lasting (minutes), and the second LC stimulation (repeated 5 min after the first one) was ineffective This fact was probably due to the reduction of *DN-Man* levels in blood and/or an altered response of microvessels to neurotransmitters

It was shown here that, under carefully controlled surgical and experimental conditions, *DPV* and *DN-Man* might be useful for the monitoring of the regional dynamics of *BBB* transport changes The presented results also support the view that *BBB* transport can be influenced by *LC* neuronal activity

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Key words: Blood-brain barrier — Locus coeruleus — 1-deoxy-1-nitro-D-mannitol — Electrical stimulation — Voltammetry — Rat — Frontoparietal cortex

Introduction

The blood-brain barrier (*BBB*) controls the passage of drugs and metabolites from the blood into the cerebral extracellular space, and therefore plays an important role in maintaining the homeostasis of the brain microenvironment (Bradbury 1979).

An increase in *BBB* permeability can be achieved by experimental manipulations such as osmotic *BBB* disruption (Roman-Goldstein et al. 1994), or induced by mechanical (Bradbury 1979) or electrical insults (Lee and Olszewski 1961, Bradbury 1979) or initiated by pathological processes such as seizures (Bolwig et al. 1977) or inflammation (Juhler and Neuwelt 1989).

To monitor *BBB* transport changes under physiological and pathological conditions a suitable tracer and a reliable method are required.

The tracer should be partially permeable across the *BBB*, chemically inert, non-toxic and have a slow excretion rate. Furthermore it should not cause *BBB* disruption or influence blood pH or undergo metabolic changes. To conform at least some of the aforementioned criteria, we used an electroactive mannitol derivative (*DN-Man*) (1-deoxy-1-nitro-D-mannitol).

An electrochemical method (voltammetry) has been shown to be useful in monitoring the electroactive drug permeation across the *BBB* (Pavlasek et al. 1996, 1997). The merits of voltammetry are as follows: a) It is a discrete measurement probe with a high spatial resolution. b) It has a high temporal resolution with measurements made in "real" time. c) The changes in free drug concentration over time can be measured in individual animals, in striatal synaptosomal preparations (Murgas et al. 1991) and simultaneous measurement on both sides of the *BBB* (blood, brain tissue) is possible.

The intracerebral noradrenergic, cholinergic and serotonergic innervation trigeminal innervation of the cerebral capillaries has been described (Kalaria et al. 1989, Wahl and Shilling 1993). Electrical stimulation of the locus coeruleus (*LC*) augmented *BBB* permeability in parietal cortex to sodium fluorescein (Sarmento et al. 1994) and to water (Raichle et al. 1975). *LC* was found to be involved in the regulation of the *BBB* integrity (Belova and Sudakov 1989).

In order to verify the suitability of *DN-Man* as a marker for voltammetric monitoring of the *BBB* transport functions, a model referred to the involvement of the central noradrenergic control of the *BBB* functions (Sarmento et al. 1994) was used. It included electrical stimulation of *LC*, and its effect on *DN-Man* transport across the *BBB* to the extracellular space within the brain was voltammetrically monitored. The results suggest that *LC* stimulation evokes a transient increase of *BBB* permeation to *DN-Man*.

Materials and Methods

The experiments were carried out on male Wistar rats with an average body weight (w) of 290 ± 30 g The animals were anaesthetized with pentobarbital (Spofa Prague Czech Republic) 5% solution in saline 0 1 ml/100 g w , i p About one third of this dose was added after approximately 40 min (the duration of the experiment did not usually exceed 120 min) All experiments were performed at room temperature the animals were protected against the lowering of their body temperature due to heat loss The femoral vein was cannulated for drug and saline administration and the tail artery was cannulated for blood sampling

The animals were fixed in a stereotaxic apparatus and four small openings were drilled into the skull three for voltammetric electrodes (Fig 1 4) working (W_c) auxiliary (AX), and reference (R) and the fourth one for placement of a stimulating electrode ST (Fig 1B) Incisions in the dura mater were made for the W_c and ST electrodes, AX and R voltammetric electrodes were placed epidurally

The electrochemically treated (Mermet and Gonon 1988) voltammetric electrode W_c (glass micropipette with carbon fibers, Pavlasek et al 1994) was placed in the left frontoparietal cortex (Figs 1 4 1B) with stereotaxic coordinates AP +1 0 L 1 5 V 1 5 (Paxinos and Watson 1982) The AX electrode (Ag wire with 0 5 mm diameter) and the R electrode (Ag/AgCl wire with 0 5 mm diameter) were positioned in the parietal and frontoparietal region of the right hemisphere

Differential pulse voltammetry (DPV, Justice 1987) was used to record electrochemical signals A polarographic analyzer (PA 4, Laboratory Equipment, Prague Czech Republic) with three electrode system (Fig 1A) was used for DPV with the following parameters speed of the linear potential sweep 100 mV s^{-1}, potential limits from −1500 mV to +1200 mV, pulse amplitude 50 mV, pulse duration 60 ms (current sampling 20 ms before the pulse and again 20 ms before the end of the pulse), pulse period 0 2 s The voltammetric signal was drawn with an x y plotter (XY 4106, Laboratory Equipment, Prague, Czech Republic) The interval between consecutive voltammetric recordings was 1 min

Stimulation (Electrostimulator ST-3, Medicor, Budapest, Hungary) of the locus coeruleus (LC, stereotaxic coordinates AP −9 8, L 1 4, V 7 0) (Figs 1B and 2) was performed with a train of 300 rectangular 1 mA pulses (cathodic in the case of the monopolar stimulation) with a frequency of 100 Hz and pulse duration of 0 2 ms Two types of ST electrodes (monopolar, bipolar) were used The bipolar stimulating electrode (Disa electronic, outer diameter 0 46 mm) was used in 5 experiments The monopolar stimulating electrode, used in 3 experiments, was made of insulated nickel-chromium wire (diameter 0 2 mm, the length of the exposed tip was about 1 mm), a silver plate with an area of 25 mm^2 was positioned on the left temporal side of the skull, and served as the indifferent electrode

Histological identification of the LC and the position of the ST were carried

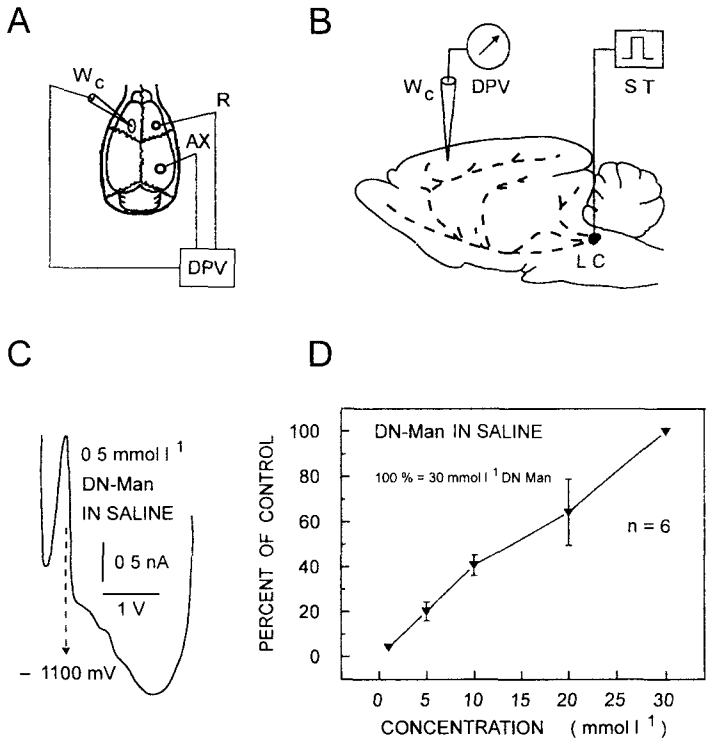

Figure 1. Application of differential pulse voltammetry (*DPV*) to study the neurogenic regulation of the blood-brain barrier (*BBB*) *A*) Experimental setup used for *DPV*. Placement of the working electrode in the left frontoparietal cortex (W_c) of the rat, and localization of the reference (*R*) and auxiliary (*AX*) electrodes on the opposite hemisphere is shown. *B*) A diagram illustrating the noradrenergic projections (dashed lines) of the locus coeruleus (*LC*) to the forebrain. The *DPV* device with W_c and a stimulator (*ST*) with a stimulating electrode positioned in the *LC* is shown. *C*) *In vitro* voltammetric recording of 0.5 mmol l^{-1} 1-deoxy-1-nitro-mannitol (*DN-Man*) in saline. The redox potential of the *DN Man* was about −1100 mV (the dashed line with the arrow). *D*) *In vitro* voltammetric calibrations of the *DN Man* in saline. The height of the *DN Man* peak (current in nA ordinate) for tested concentrations (abscissa) was expressed as percentage of the *DN Man* current in 30 mmol l^{-1} solution (100%). Six measurements with different electrodes were made.

out using light microscopy (Fig. 2). An electrolytic lesion (+5 V D.C. for 10 s) made at the very end of the experiment marked the localization of the *ST* tip. Subsequently, the brain was removed, fixated with 10% formalin and embedded after normal histological processing, in paraffin. 6 μm coronal sections were cut and stained with hematoxylin and eosin.

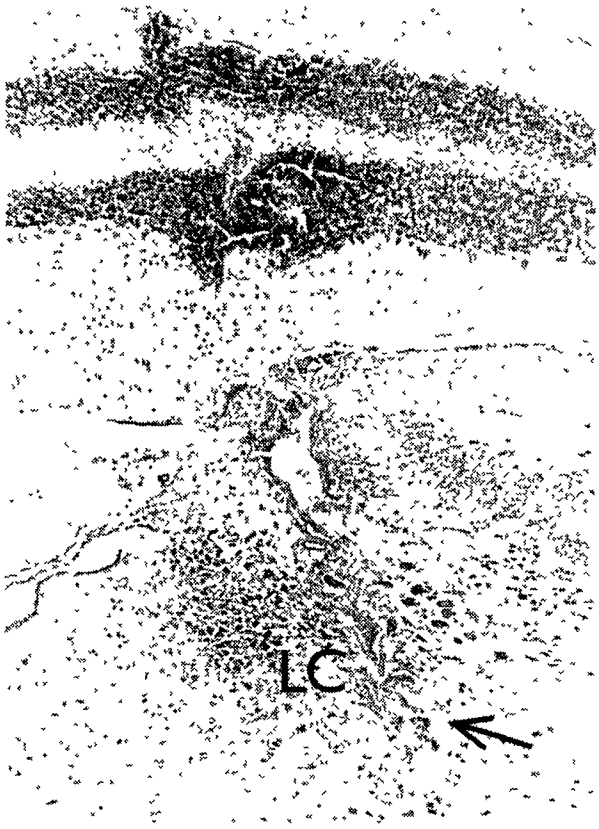

Figure 2. Microphotograph of a coronal section of the brainstem of the rat. Localization of the stimulating electrode tip (arrow) and locus coeruleus (LC). Hematoxylin and eosin staining. Magnification 16×

A mannitol derivative 1-deoxy-1-nitro mannitol (*DN-Man*) (MW 211.2, synthesized by J. Kubala, Bratislava, Slovak Republic), was dissolved in 0.5 ml of prewarmed (37°C) saline shortly prior to its administration via the femoral vein. The concentration of the administered *DN-Man* solution was varied (minimum 0.95 mol l^{-1}, maximum 1.27 mol l^{-1}) so that in every experiment, in spite of the differences in animals' weight, the same *DN-Man* concentration in the blood (30 mmol l^{-1}) was attained, while keeping the injected volume (0.5 ml) constant. The formula used for the calculation of the blood volume (V) was as given by Lee and Blaufox (1985)

$$V(\text{ml}) = (w \times 0.06) + 0.77,$$

where w is weight in grams *DN-Man* injection over a 2 5 min period was followed by infusion of 100 units of heparin in 0 2 ml saline

The experimental protocol in 8 experiments was as follows After finishing surgery and as soon as voltammetric signals stabilized (5–10 recordings at 1 min intervals) the injection of *DN-Man* was started, and the voltammetric measurements continued (at 1 min intervals) until the end of the experiment The *ST* electrode was inserted to the vicinity of the *LC* 31 min after the first *DN-Man* injection In six out of eight experiments, the second injection of the same *DN-Man* dose was applied one minute after *ST* electrode insertion The first stimulation was performed 37 min after the first *DN-Man* injection, in other words, in six experiments with the repeated *DN-Man* administration, stimulation was applied 5 min after the start of the second *DN-Man* injection In each case, stimulation started 5 s before subsequent voltammetric recording The stimulus train was repeated twice at 5 min intervals

To assess *DN-Man* concentration in the blood, three samples (1 ml each) were taken from the tail artery The first one was taken immediately after the surgery (no *DN Man* in the circulation) and a known amount of *DN-Man* was added to it to yield a concentration of 30 mmol l^{-1} The other two samples were taken 10 and 30 min after the first *DN-Man* administration respectively All samples were stored in heparinized test-tubes in a refrigerator, and *in vitro* voltammetric measurements were made in an experimental chamber at the end of the experiment (Fig 3*C*) The volume of each blood sample was immediately replenished by the same volume of the saline infusion over a 2 min period, applied via the femoral vein

The quantification of the electrochemical signals recorded with the *DPV* was performed by measuring the amplitude of the peaks representing the redox current (Fig 4*B b*) Student's *t*-test was used to evaluate the results (arithmetic means and standard deviations are shown)

Results

As observed in *in vitro* measurements with *DPV*, 1-deoxy-1-nitro mannitol (*DN-Man* solution in saline or in blood) gives a clearly separable and stable voltammetric signal with its maximum ranging from −1150 mV to −950 mV The *DN Man* peak in saline (Fig 1*C*) was linearly proportional ($y = A + Bx$, $A = 3\ 8$, $B = 3\ 2$, $r = 0\ 996$) to its concentration within the range of 0 5 mmol l^{-1} (detectable limit) to at least 30 mmol l^{-1} (Fig 1*D*)

Essential for the analysis of the blood-brain barrier (*BBB*) transport is the information about the drug levels on both sides of the *BBB* (circulating blood and nervous tissue) Therefore, the *DN-Man* redox potential in blood samples collected 10 and 30 min following the first i v *DN-Man* injection was measured by *DPV* As tested *in vitro* (*DN-Man* added to a known volume of the blood), there was just

a small lowering (by 4%) of the initial *DN Man* peak amplitude (representing 30 mmol l^{-1} concentration) during the first ten minutes of the calibration (Fig 3*C* open squares) The results obtained from circulating blood were quite different Ten minutes after i v administration of the first *DN Man* dose (expected initial *DN-Man* concentration in circulating blood 30 mmol l^{-1} see Materials and Methods) *DN-Man* peak amplitude attained about twenty percent of that measured *in vitro* (Fig 3*C*, solid squares) Only an insignificant decrease was observed during next 20 minutes

Prior to the *DN Man* injection, three clearly identifiable peaks (1, 2, 3) were present on the voltammetric recordings from the cortex (Fig 3*B*) Peak 1 (Fig 3*B*, *b*) formed at the lower voltage (130 ± 60 mV) corresponds to ascorbic acid, while peak 2 (Fig 3*B*, *c*) with the maximum at a polarization voltage of 490 ± 50 mV represents a catechol-oxidative current (Lane et al 1976 Guadalupe et al 1992 Pavlasek et al 1994) Peak 3 (Fig 3*B*, *a*) with an oxidation potential at 740 ± 40 mV is related to 5-hydroxyindoles and/or their metabolites (Guadalupe et al 1992)

Between 4 and 15 minutes (9 2 ± 4 2 min, $n = 6$) after the first *DN Man* injection, a distinct peak (4) occurred at -1040 ± 50 mV that represented the *DN-Man* redox current (Fig 3*B*, *c*) The data in Fig 3*B* illustrating the time-course of the *DN Man* peak amplitude changes provide information about the *DN Man* transport dynamics across *BBB* in the frontoparietal cortex A maximum of the *DN-Man* current was attained within 30 min after *DN Man* injection The best parameters of the data fit curve with the equation $y = 4/(1+(k/r)^p)$ were $A = 106$ $k = 17\,8$, $p = 3$

Locus coeruleus (*LC*) stimulation (Fig 4 4) was repeated two-times at 5 min intervals The *DN-Man* peak which directly preceded the first stimulation (Fig 4*B*, *a* and Fig 4*C*, time $t_0 + 2$ min) served as a control

The first *LC* stimulation caused a sudden increase in the *DN-Man* peak in the cortex (Fig 4*B*, *a*, *b*, Fig 4*C*) The values ranged from 109% to 279% of the control (168 ± 59%, $n = 8$, $P < 0\,02$, column II in Fig 4*D*) A maximum of the *DN Man* peak augmentation was achieved 2 1 ± 0 6 min ($n = 8$) after the stimulation and a reduction of the *DN Man* peak was observed thereafter

After the second *LC* stimulation, the changes of the *DN-Man* peak amplitude were insignificant (117 ± 23%, $n = 8$, $P > 0\,05$)

In order to exclude the possibility that the observed effects were the result of a change in the electrochemical sensitivity of the working electrode caused by the stimulating current, the effect of stimulation was tested again after the animal's death (i e about 15 min after respiration had stopped) The results shown in Fig 4*D* column III (89 ± 11%, $n = 3$, $P > 0\,05$) rule out this possibility

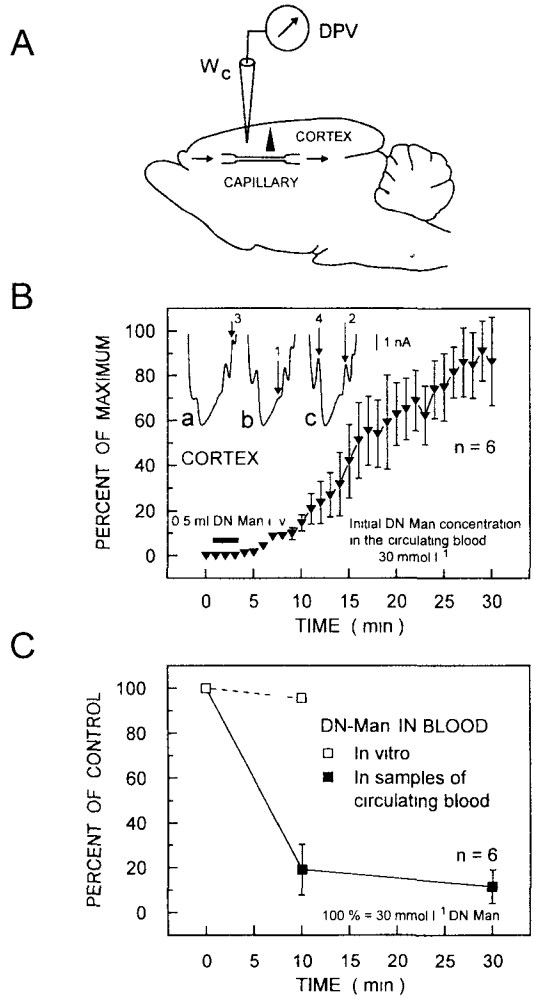

Figure 3. Dynamics of the mannitol derivative (*DN Man*) transport across the blood-brain barrier (*BBB*) in the cortex of the rat monitored with differential pulse voltammetry (*DPV*) (*A B*) and correlated with concentration changes of *DN-Man* in blood (*C*) *A*) Scheme of the drug transfer (solid triangle) from the circulating blood (arrows) across the *BBB* of a capillary into the extracellular space of the cortex The application of the *DPV* method is illustrated (W_c – working electrode in the cortex) *B*) Kinetics of *DN-Man* transport across the *BBB* to the brain The time course of *DN-Man* current changes in the cortex of six rats expressed as percentages of the *DN Man* maximum current (100% maximum current attained within 30 min after i v *DN-Man* administration in each experiment) The initial *DN-Man* concentration in the circulating blood was 30 mmol l^{-1} *a b c* *DPV* voltammograms recorded in the cortex 8 (*a*), 14 (*b*), 30 (*c*) minutes after *DN-Man* administration during one experiment The peak representing the *DN-Man* current

Discussion

Calibrations in saline and in blood samples revealed that 0.5 mmol.l^{-1} *DN-Man* concentration was minimum detectable level and that the relationship between *DN-Man* concentration and *DN-Man* redox peak amplitude was linear and stable (Fig. 3*C*). These results indicated that *DN-Man* transport into the blood cells and its interaction with plasma proteins or with other blood components were weak.

The possibility of local destruction of *BBB* functions caused by the insertion of the voltammetric microelectrode had to be considered. Allen et al. (1992) showed that 20 min after slow (over 2 min) insertion of a microdialysis probe (0.2 mm o. d.), the *BBB* functions were intact. Therefore, we used a slow implantation procedure of the working electrode followed by 10–15 min recovery period before the voltammetric recordings started. The slow time-course of the *DN-Man* redox potential occurrence in the nervous tissue after *DN-Man* injection (Fig. 3*B*) indicates that *BBB* damage with the voltammetric microelectrode was unlikely.

In this study, *DN-Man* concentrations in the circulating blood were much lower (30 mmol.l^{-1}) than the mannitol concentration used for osmotic *BBB* disruption (25% solution, i.e. approximately 1.2 mol.l^{-1}). Moreover, slow administration into the femoral vein was used instead of carotid artery injection. As verified in blood samples, 30 mmol.l^{-1} *DN-Man* negligibly lowered pH (by 0.02 unit); therefore *BBB* opening caused by a pH shift (Oldendorf et al. 1994) was unlikely.

There was a considerable decrease in the *DN-Man* peak in circulating blood, to about 20% of its initial value, during the first ten minutes after the injection. This might reflect *DN-Man* interaction with the vascular bed, its distribution into the extravascular compartments, metabolic transformation and/or elimination. Another mannitol derivative (2,5-anhydro-D-mannitol) is taken up into the liver and rapidly phosphorylated in the rat (Park et al. 1995). It cannot be excluded that a similar mechanism might also account for the *DN-Man* decrease.

In the rat, the brain uptake index (*BUI*) for mannitol yields only a small

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(indicated by 4 above the peak) appeared at −1050 mV The figures above other peaks indicate the respective redox currents of ascorbic acid (1), catecholamines (2), and 5-hydroxytryptamine (3) The calibration represents 1 nA for all voltammograms (*a*, *b*, *c*). *C*) Changes in the *DN-Man* current in the blood. Open squares – redox current of 30 mmol l^{-1} *DN-Man* measured *in vitro* in a blood sample (taken prior to the *DN-Man* injection) immediately after the addition of *DN-Man* to the blood sample (six rats, 100%) and 10 min later (one experiment) Solid squares represent the *DN-Man* current in samples of the circulating blood taken from six rats 10 and 30 min after i.v administration of *DN-Man* and measured *in vitro* at the end of each experiment Values are expressed as percentages of the *DN-Man* redox current of 30 mmol.l^{-1} *DN-Man* in blood samples (first open square). The initial *DN-Man* concentration in the circulating blood was 30 mmol l^{-1}

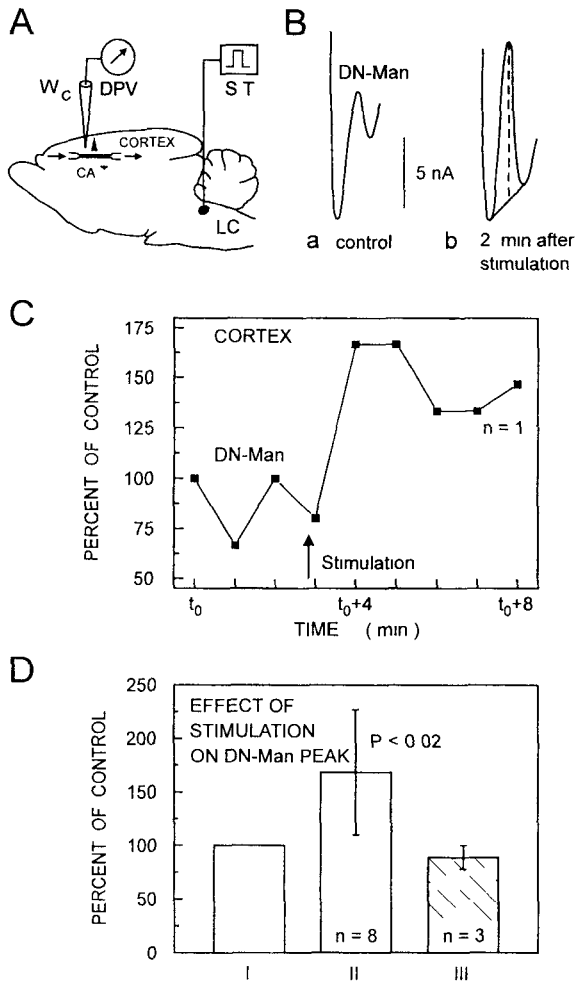

Figure 4. The effect of locus coeruleus (*LC*) stimulation upon the concentration of the mannitol derivative in the rat cortex, studied using differential pulse voltammetry (*DPV*) 4) A schedule showing the experimental layout to study the effect of *LC* stimulation on substrate transfer (solid triangle) from circulating blood (arrows) across the blood-brain barrier of a capillary (*C* 4) into the cortex Note the noradrenergic projections from the *LC* to the cortex indicated by the broken line The *DPV* device with the working electrode placed in the cortex (W_c) and a stimulator (*ST*) with the stimulating electrode positioned in the *LC* are shown *B*) *DPV* voltammogram representing the mannitol derivative (*DN Man*) peak in the cortex 4 min after the second i v administration of *DN Man* (*a*), and the maximal *DN Man* peak reached 2 min after *LC* stimulation, i e 6 min after the second *DN Man* administration (*b*) The dashed line indicates the peak amplitude *C*) The effect of *LC* stimulation on the time-course of the *DN-Man* peak changes in the cortex Ordinate

value (Begley et al 1990), radioactively labeled mannitol (^{14}C-mannitol) crossed the *BBB* very slowly (Daniel et al 1981) and it did not exceed 6 7% of its plasma concentration in the brain cortex (Amtorp 1980) There was a gradual increase in the peak representing the *DN-Man* redox current during the 30 min period after *DN Man* i v administration A slow *DN Man* elevation in the extracellular microenvironment of the cortex might restrict *DN-Man* penetration across *BBB* and its metabolic degradation and/or elimination

The *LC* innervates many brain areas (Moore and Bloom 1978) including the cerebral cortex (Fritschy and Grzanna 1992) To a lesser extent, there are also neurons containing dopamine (Versteeg et al 1976) and 5HT (Palkovits et al 1974) in the *LC* besides noradrenergic cells (Chamba et al 1991) The presence of the enzyme nitric oxide synthase in some *LC* neurons has also been demonstrated (Xu et al 1994) Contacts between the axonal endings of *LC* neurons and the basement membrane of the microvessel wall of the brain capillaries have been confirmed (Rennels and Nelson 1975 Swanson et al 1977)

As proved in a study on rat brain slices (Palij and Stamford 1994), electrical stimulation of the *LC* evokes noradrenaline efflux (verified by pharmacological and electrochemical criteria) As compared to the above authors, in our *in vivo* study the *LC* was stimulated with lower current intensity (1 mA instead of 10 mA) but with larger number of pulses (300 instead of 30)

Our results suggested an increased *DN Man* transport across the *BBB* induced by electrical stimulation of the *LC* There was no difference in *BBB* permeability changes between monopolar or bipolar stimulation (electrode placement was confirmed by histological examination) However, *LC* stimulation elicited a rapid significant increase of *DN Man* concentration in the extracellular space of the cerebral cortex (Fig 4*C*) The dynamic changes of this increase differs from the time-course of the gradual *DN-Man* rise observed after the administration a *DN-Man* dose into the blood circulation (Fig 3*B*) Taken together, our findings concerning *LC* stimulation may reflect the transient character of the *BBB* opening Although the present results do not provide an explanation of the precise mechanism of this phenomenon, we assume that the change of *BBB* permeability is probably associated with alpha-adrenoreceptor stimulation (Preskorn et al 1982), due to activation of the mechanisms responsible for pinocytotic potentiation in the endothelial cell of

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percentages of the *DN Man* control current (*DN Man* peak at t_0 + 2 min was taken as 100%) Abscissa time in min Stimulation is indicated by the arrow Results from one experiment *D*) The effect of *LC* stimulation on the *DN Man* peak in the cortex The first column (I) control (100%) approximately 1 min before stimulation The second column (II) maximal values of *DN-Man* peak after *LC* stimulation (attained between 1 and 3 min after *LC* stimulation) Results from eight experiments Hatched column (III) *LC* stimulation after the animal s death Results from three experiments

the brain capillaries (Sarmento et al 1991)

It could be expected that repeated stimulation might lead to a more pronounced drug level increase in the brain But this was not the case the second stimulation was ineffective This can be explained by a lowering of the *DN-Man* concentration in the blood, altered transport capabilities of the *BBB*, decreased transmitter liberation or by various forms of capillary receptor desensitization (receptor uncoupling receptor affinity changes, down-regulation of receptor numbers) (Sibley and Lefkowitz 1985) Altered sensitivity of the microvessels to neurotransmitters has been observed (Palmer 1986)

According to the experimental data presented herein *DN-Man* seems to be a promising marker for voltammetric monitoring of changes in *BBB* transport Nevertheless, care should be taken when interpreting of the relation between the current and the concentration of the compound The results confirmed that *LC* stimulation increases cerebral vascular leakage for some compounds

Acknowledgements. Support to J P , M H and Cs H by a grant from the Slovak Grant Agency for Science (VEGA 2/1006/97) is acknowledged

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Final version accepted November 11 1998