

# Introduction of a pharmacological neurovascular uncoupling model in rats based on results of mice

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## ABSTRACT

Our aim was to establish a pharmacologically induced neurovascular uncoupling (NVU) method in rats as a model of human cognitive decline. Pharmacologically induced NVU with subsequent neurological and cognitive defects was described in mice, but not in rats so far.

We used 32 male Hannover Wistar rats. NVU was induced by intraperitoneal administration of a pharmacological “cocktail” consisting of N-(methylsulfonyl)-2-(2-propynyloxy)-benzenehexanamide (MSPPOH, a specific inhibitor of epoxyeicosatrienoic acid-producing epoxidases, 5 mg kg<sup>-1</sup>), L-NG-nitroarginine methyl ester (L-NAME, a nitric oxide synthase inhibitor, 10 mg kg<sup>-1</sup>) and indomethacin (a nonselective inhibitor of cyclooxygenases, 1 mg kg<sup>-1</sup>) and injected twice daily for 8 consecutive days.

Cognitive performance was tested in the Morris water-maze and fear-conditioning assays. We also monitored blood pressure. In a terminal operation a laser Doppler probe was used to detect changes in blood-flow (CBF) in the barrel cortex while the contralateral whisker pad was stimulated. Brain and small intestine tissue samples were collected *post mortem* and examined for prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) level.

Animals treated with the “cocktail” showed no impairment in their performance in any of the cognitive tasks. They had higher blood pressure and showed cca. 50% decrease in CBF. Intestinal bleeding and ulcers were found in some animals with significantly decreased levels of PGE<sub>2</sub> in the brain and small intestine.

Although we could evoke NVU by the applied mixture of pharmacons, it also induced adverse side effects such as hypertension and intestinal malformations while the treatment did not cause cognitive impairment. Thus, further refinements are still required for the development of an applicable model.

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**KEYWORDS**

barrel cortex hyperaemia, neurovascular uncoupling cocktail, whisker pad stimulation, MS-PPOH, brain prostaglandin E<sub>2</sub>

**INTRODUCTION**

Clinical development of new cognitive enhancer candidate compounds faced 100% attrition rate in the past 15 years, mainly due to lack of efficacy [1, 2]. A major factor of the serial failures is the low translational value of animal models used for predicting human efficacy [3, 4]. Beside the type of the cognitive tasks used for modelling the various human cognitive domains, another critical factor of translational validity is the construct of cognitive deficiency, in other words: how impaired performance is brought about. The frequently used transmitter-specific pharmacological impairments (like e.g. scopolamine- or dizocilpine-induced learning deficits) have inherent limitations by their nature; the connection of their action to a certain disease is often hypothetical; and their so far demonstrated predictive power may also be criticized [5–8].

The role and importance of neurovascular coupling (NVC) in brain activity and cognitive function has come to the forefront of dementia and aging research [9, 10]. Diminished functionality of the neurovascular unit was shown during aging and in various brain disorders [11–13]. Therefore, neurovascular uncoupling may be a translationally valid animal model of human cognitive decline. Pharmacologically induced neurovascular uncoupling with subsequent neurological and cognitive defects was described in mice [14], however, no similar procedure has been reported so far in rats. Transferring the method to rats would extend its applicability to the most preferred species of the animal learning field. Interestingly, while there is a huge literature on various transgenic mouse AD models with reported impaired functional hyperaemia, no such information can be found in rat AD models. Even in the enormously large rat diabetes model literature, few studies specifically investigated NVC in the brain [15–17].

We recently elaborated and established a rat cognitive test battery and testing protocol for more reliable prediction of clinical efficacy [5, 18]. According to the protocol, several cognitive tasks representing different cognitive domains (for example, five-choice serial reaction time task (5-CSRTT) for attention, a cooperation task for social cognition [19], Morris water maze paradigm for spatial memory [20], pot-jumping exercise for procedural memory [21]) were taught to the same cohort of rats. Hereby we created a population with „widespread knowledge” and the effect of a particular impairment method on the various cognitive functions could be simultaneously measured in this population. These impaired states served then as the target of potential cognitive enhancer treatments in a “clinical trial-like”, vehicle controlled, blind, randomized experimental design [22–24]. Neurovascular uncoupling could be integrated into this testing protocol as a distinguished, particularly useful impairing method resulting in a highly relevant model of age- and dementia-related cognitive deficits.

Therefore, the objective of the present study was to establish a pharmacologically induced neurovascular uncoupling (NVU) method in rats as a translationally valid animal model of human cognitive decline.

In the mouse study of Tarantini et al. [14] the authors induced neurovascular uncoupling with the following 7 days long pharmacological treatment:



- N-(methylsulfonyl)-2-(2-propynyloxy)-benzenehexanamide (MS-PPOH), a specific inhibitor of epoxyeicosatrienoic acid (EET)-producing epoxidases; 20 mg/kg/day applied via subcutaneously implanted Alzet osmotic minipump;
- L-NG-nitroarginine methyl ester (L-NAME), nitric oxide (NO) synthase inhibitor, 100 mg/kg/day dissolved in drinking water;
- Indomethacin, a nonselective inhibitor of cyclooxygenases, 7.5 mg/kg per day, p.o.

Neurovascular uncoupling was checked at the end of the treatment period by measuring the impairment of functional hyperaemia in the barrel cortex in response to electrical stimulation of the contralateral whisker pad. In a separate group of mice, changes in extracellular glucose concentration in the barrel cortex (as a metabolic surrogate of cerebral functional hyperaemia) were also measured in the whisker-stimulation paradigm.

The above pharmacological treatment caused circa 2/3 decrease in the evoked cerebral functional hyperaemia measured by both methods without affecting baseline cerebral blood flow (CBF) and somatosensory evoked potentials. This neurovascular uncoupling was accompanied by impaired learning performance in assays of spatial memory (Y-maze place recognition and elevated plus-maze transfer paradigms) and recognition memory (novel object recognition (NOR) test). In addition, defects were observed in motor coordination (rotarod) and forelimb grip strength, but not in gait parameters. Sensorimotor function (adhesive removal test) and olfactory ability and gustatory motivation (buried food retrieval test) were also unaltered.

In the present paper we report on our first attempt to bring about neurovascular uncoupling in rats. Previous studies have indicated that inhibition of NO, EETs and prostaglandins can block functional hyperaemia responses in the rat as well [25, 26]. However in these studies the inhibitory compounds were applied in an acute surgical setting either as brain superfusion or intracerebroventricularly. Thus, the current study was a dose-finding and treatment protocol establishing experiment aiming to ensure the tolerability of the pharmacological treatment while maintaining its efficacy. L-NAME and MS-PPOH may have hypertensive effects [27, 28]; for example, L-NAME caused ~40 mmHg increase in blood pressure already at a dose of 40 mg/kg/day after one week administration in drinking water. The third component of the combination, indomethacin is well known for its ulcerogenic effect [29]. Another challenge is a formulation issue: the required amount of MS-PPOH for a 20 mg/kg/day dose for rats cannot be dissolved in a 200  $\mu$ L osmotic minipump. Therefore we applied these substances as an intraperitoneally (ip.) administered “cocktail”. To assess the cognitive capabilities of the animals, we used Morris water-maze and fear conditioning paradigms. Blood pressure was monitored in a non-invasive way (tail-cuff method), while neurovascular uncoupling was measured by the impairment of functional hyperaemia in the barrel cortex in response to mechanical stimulation of the contralateral whisker pad. Last, we measured *post mortem* prostaglandin E2 levels in the brain and small intestine. The former served as a kind of verification that the cocktail did exert its cerebral effect.

## MATERIALS AND METHODS

### Animals

For this study, we used altogether 28 male Hannover Wistar rats (TOXI-COOP, Budapest, Hungary) weighing 200–225 g at the start of the experiments. Animals were housed in groups



of three in polycarbonate cages (1,376 cm<sup>2</sup> area, 16 cm height, each provided with two cardboard tubes and one wooden brick) under reversed light-dark cycle (dark phase from 16:00 to 4:00). Animals had restricted food access: the amount of the food (commercial pellet rat feed R/M–Z + H produced by SSniff Spezialdiäten GmbH, Soest, Germany) was 45 g for three rats and it was supplied at least 2 h before the end of the dark phase. Drinking water was available ad libitum. The animals were intensively handled before and during the experiments. Housing and all procedures carried out on animals were authorized by the regional animal health authority in Hungary (Pest County Government Office, resolution number PE/EA/00808-7/2021, issued on 17th August, 2021) and conformed to the Hungarian welfare legislation and the EU 63/2010 Directive.

## Experimental design

Animals were tested in the Morris water-maze and fear conditioning paradigms on days 5–7 and 4 and 8 of the treatment period, respectively. Blood pressure of the animals was monitored by a tail-cuff method on days -1, 2, 5 and 7. On the last day, neurovascular coupling was measured in the barrel cortex by laser Doppler recording in a non-recovery operation. Brain and small intestine tissue samples were collected post mortem and processed for prostaglandin E2 level measurements (Fig. 1).

## Treatment

For inducing neurovascular uncoupling the following drugs were used: 5 mg kg<sup>-1</sup> MS-PPOH, 10 mg kg<sup>-1</sup> L-NAME, 1 mg kg<sup>-1</sup> indomethacin, injected i.p. twice daily (at 6 and 18 o'clock) for 8 consecutive days. The compounds were administered in a “cocktail” which was prepared daily as follows: first the proper amount of MS-PPOH (Eötvös Loránd Research Network – Research Centre for Natural Sciences, Budapest, Hungary) was dissolved in 45% hydroxy-propyl-β-cyclodextrin (Cylolab Ltd., Budapest, Hungary) in an ultrasonic water-bath (10 min, 40 °C). Then indomethacin (Sigma-Aldrich Co., St. Louis, USA) was added and sonicated for further 10 min. Indomethacin did not dissolve but formed a fine suspension which did not sediment for a few hours. Last, L-NAME (Merck KGaA, Darmstadt, Germany) was added to the solution which dissolved immediately. Control animals received 45% β-cyclodextrin solution. The dosing volume was 2 mL kg<sup>-1</sup>.

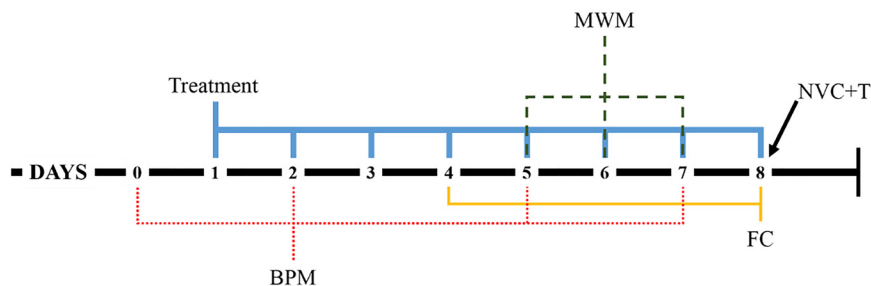


Fig. 1. Timeline of the experiment. BPM – Blood pressure measurement (Days 0, 2, 5, 7), MWM – Morris water-maze (Days 5, 6, 7), FC – Fear-conditioning (Days 4, 8), NVC+T – Neurovascular coupling measurement and termination of animals (Day 8)



## Non-invasive blood pressure measurements

Blood pressure of the animals was measured in a 4-channel CODA<sup>®</sup> apparatus (High Throughput System – Noninvasive Blood Pressure System, Kent Scientific Corp., Torrington, USA) working with a pair of tail-cuffs (occlusion cuff and volume pressure recording cuff). Each recording consisted of 1 acclimation cycle (10 measurements) and 2 regular cycles ( $2 \times 15$  measurements). The measured variables were: systolic, diastolic, and mean blood pressure and heart rate. Prior to analysis, we filtered out every unrealistic data, then we excluded any measurement where the measured value lay outside the mean  $\pm$  2SD range. After this, we calculated a revised mean for each variable.

Each animal had a few habituating sessions about a week before the experiment. In the experiments themselves 4 blood pressure measurement sessions were run: the first was performed one day prior to the start of treatment (baseline, day -1), the other three on days 2, 5 and 7.

## Cognitive tasks

**Fear-conditioning.** The test device was a sound-proof shocking chamber ( $26 \times 26 \times 30$  cm) (Ugo Basile, Gemonio, Italy) in which the behaviour of the animals was recorded with an infrared video camera controlled by the software EthoVision v13.0 (Noldus, Wageningen, Netherlands). The experiment consisted of an acquisition and 24 h later a retention trial. Length of each session was 5 min. During the acquisition trial, the rats received 5 mild foot shocks (0.6 mA, 1 s) as unconditional stimulus at 60 s interval. The shocks were preceded by a compound conditional stimulus consisting of a continuous sound (65 dB, 3 kHz) and a flickering light (1 Hz) for 10 s, where in the last second, the unconditional and conditional stimuli were presented simultaneously. During retention trials, the animals received the same conditional stimuli, in the absence of the foot shock. The main variable was the animals' freezing time during the session.

**Morris water-maze.** The apparatus was a black circular pool (diameter 190 cm, depth 60 cm) filled with water (39 cm,  $23 \pm 1$  °C) and containing a non-visible round escape platform (10 cm diameter) placed 0.5 cm below the water surface. The platform was located in the south-east (SE) quadrant of the pool, 40 cm from the edge of the pool. On the wall of the experimental room extra-maze cues were placed to facilitate the orientation during swimming. At the start of a trial the rat was placed into the pool at one of the four possible start points (North, East, West or South rotated in a systemic manner) and had 3 min to find the hidden escape platform. When the animal didn't find it, it was gently guided to the platform. Rats were allowed to spend 30 s on the platform then were taken out, dried with a cloth and placed in their home-cage. The interval between the trials was 30 min. Escape latency was measured and swimming path was recorded by Smart v3.0 video tracking system software (Panlab, Barcelona, Spain). Every animal participated in three sessions, each consisting of three trials.

## Measurement of neurovascular coupling

The animals were anaesthetized with  $1.5 \text{ g kg}^{-1}$  intraperitoneal injections of urethane prior their surgeries. If it was necessary, smaller doses of additional injections were delivered. The depth of



anaesthesia was checked by the absence of tail-pinch and pedal reflexes. The rats were then fixated in a stereotaxic frame (Stoelting, Wood Dale, IL, USA) and laid on a preheated bench (37 °C) (Supertech Instruments, Pécs, Hungary). We applied lidocaine (Egis, Budapest, Hungary) on their heads as local anaesthesia. Next, a midline incision on the skin was made and the surface of the skull was cleaned. This was followed by the localization of the left barrel cortex (stereotaxic coordinates: 3.3 mm posterior to bregma and 4.5–5.0 mm lateral from the midline [30]), above which we opened an approximately 3 × 3 mm wide cranial window using a dental drill, while avoiding the penetration of the dura mater. The window was filled with artificial cerebrospinal fluid (aCSF – standard composition: 126 mM NaCl; 1.8 mM KCl; 1.25 mM KH<sub>2</sub>PO<sub>4</sub>; 2.4 mM CaCl<sub>2</sub> × 2H<sub>2</sub>O; 26 NaHCO<sub>3</sub>; 12 mM glucose; MgSO<sub>4</sub> × 7H<sub>2</sub>O 1.3 mM) and a laser Doppler probe (OxyFlo Probe, Oxford Optronix Ltd. – ADInstruments, PowerLabs) was immersed in the liquid, at a sheer angle.

The probe measured the changes in the cerebral blood-flow in the barrel cortex, while the contralateral whisker pad was manually stimulated with a brush at an approximately 2 Hz frequency. Data were recorded with LabChart 6 software (version: 6.2.1).

We recorded the change in perfusion units (PFU) as a function of time. PFU is an arbitrary variable where a 5 mV difference was converted as a 1 PFU shift.

The measurement was performed as follows: first, we placed the probe in its designated position and checked for increase in blood flow in response to whisker pad stimulation. If no distinguishable change was observable, we repositioned the probe (preferably in the lateral direction from the midline) in search for proper feedback. After finding a stable signal, we established a steady 1-2 min-long baseline. From here on, we applied the following recording protocol: 2 contralateral stimulations of 30 s duration followed by an ipsilateral “control” stimulation of 15 s length each separated by 3 min rest period. This sequence was repeated four times with 3 min intervals in between.

The mean of the 15 s long baseline immediately preceding stimulation was subtracted from the mean of the values during stimulation and this value was considered the amplitude of the signal. The signal amplitudes of the 8 contralateral stimulations as well as those of the four ipsilateral stimulations (the latter gave the aspecific signal) were averaged. Then the aspecific amplitude average was subtracted from the specific amplitude mean, and the difference provided the characteristic coupling parameter (increased blood flow) of the individual.

## Tissue sampling

After completing the stimulation protocol, the animals were decapitated, the brain was removed and the barrel cortex was dissected from the left hemisphere. Tissue samples were weighed, placed into Eppendorf vials and stored at –80 °C until further processing.

Simultaneously, the animals’ small intestines were removed and measured in length. From the proximal end, approximately 10% was discarded and next 10% was taken as tissue sample. The samples were cut open in longitudinal direction, washed and checked for signs of ulceration or malformation. Lastly, the samples were frozen and also stored at -80 °C for later evaluation.

## ELISA measurement of PGE2 levels

The levels of PGE2 in the small intestine and barrel cortex were determined by ELISA (Cayman Chemical, Ann Arbor, MI, USA, - Prostaglandin E<sub>2</sub> ELISA Kit (Monoclonal), Item No. 514010).



Briefly, barrel cortex and small intestine tissues were homogenized in precooled 100% ethanol containing 10  $\mu\text{M}$  indomethacin, and centrifuged at  $1,500\times g$  for 15 min at 4  $^{\circ}\text{C}$ . Ethanol was evaporated from the supernatants using a vacuum centrifuge, then the residues were resolved in assay buffer and used for determination of PGE2. Values were normalised to the weight of the tissue.

## Statistical evaluation

For statistical analysis we used STATISTICA software (version: 13.5.0.13). For comparison of the results of the two groups (Vehicle vs. Cocktail) in each cognitive test, and on their blood pressure levels, we employed Repeated Measures ANOVA followed by Newman-Keuls *post-hoc* test. For analysing the cerebral blood flow changes and ELISA results, Student's *t*-test for independent samples was used.

One cocktail-treated animal died on day 3 of the treatment (after 5 injections), with no particular symptoms (14 g (6%) weight loss compared to the initial weight). Autopsy showed no visible alteration, and the bowel was not ulcerated. Thus, 13 control and 14 drug-treated animals constituted the final sample size.

## RESULTS

No significant changes were caused by the treatment in the performance of rats either in the Morris water-maze (Fig. 2) or the fear conditioning paradigm (Fig. 3).

However, as a side effect, the treatment elevated the blood pressure in the animals (Fig. 4), and caused alterations (inflammation, bleeding and ulcers) in the small intestine in 8 out of 14 rats.

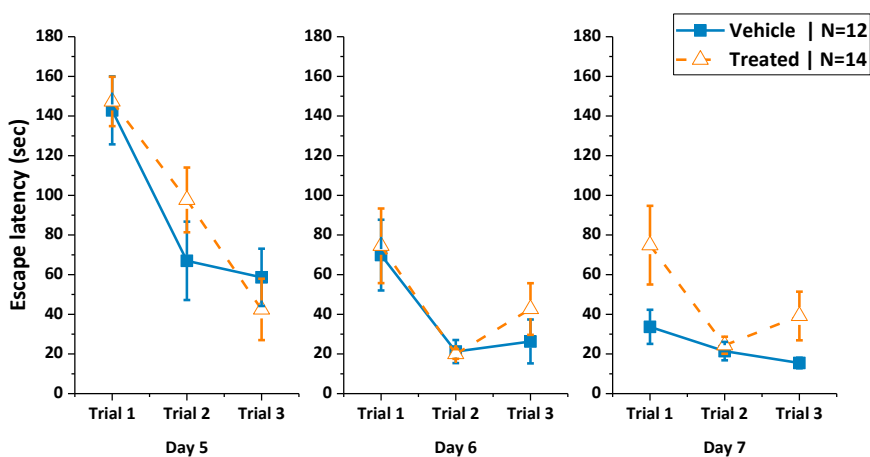


Fig. 2. Learning performance in the Morris water-maze. Mean  $\pm$  s.e.m. values are shown. No significant changes were caused by the “cocktail” treatment (repeated-measures ANOVA)





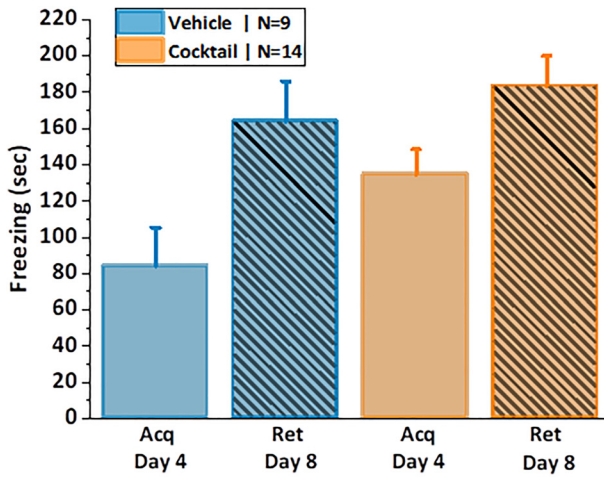


Fig. 3. Freezing levels measured in fear-conditioning sessions. Mean  $\pm$  s.e.m. values are shown. No significant changes were caused by the “cocktail” treatment (repeated-measures ANOVA). Acq – Acquisition session on day 4, clear pattern. Ret- Retention session on day 8, striped pattern

Nevertheless, the treatment caused 54% inhibition in the induced hyperaemia in the barrel cortex when the contralateral whisker pad was stimulated (Fig. 5).

PGE2 levels were significantly lower in the barrel cortex and small intestine compared to control tissue samples (Fig. 6).

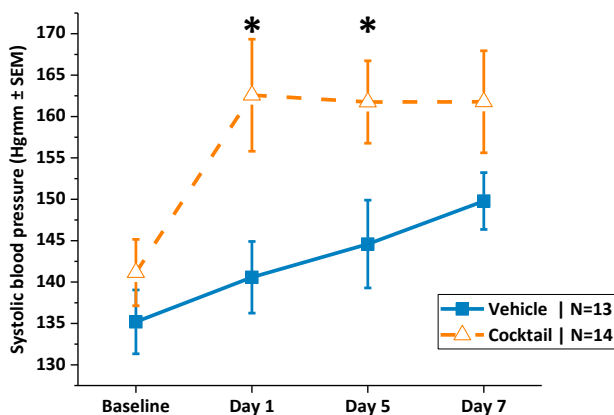
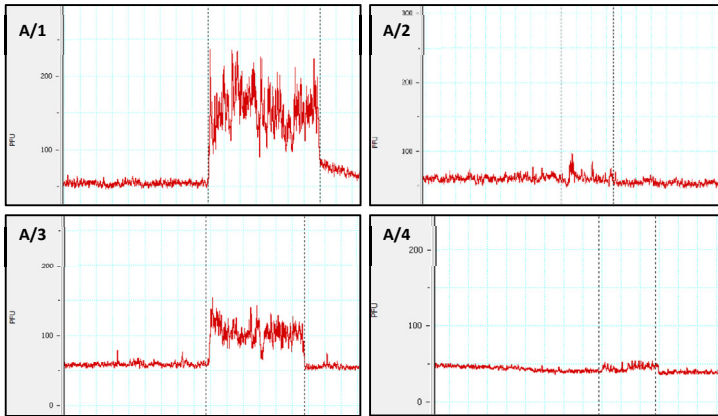


Fig. 4. Systolic blood pressure of the animals in the course of the “cocktail” treatment. Mean  $\pm$  s.e.m. values are shown. Repeated-measures ANOVA: effect of treatment:  $F_{(1, 25)} = 7.405$ ,  $P < 0.05$ ; effect of day:

$F_{(3)} = 8.244$ ,  $P < 0.001$ ; interaction:  $F_{(3, 75)} = 1.903$ , ns. Significance level: \*  $P < 0.05$







B

CBF (PFU)	Contralateral	Ipsilateral	Overall
Vehicle (N=13)	117.8 ± 23.91	3.3 ± 2.21	<b>114.5 ± 24.00</b>
Cocktail (N=14)	56.6 ± 14.87	4.1 ± 1.91	<b>52.5 ± 14.45</b>
		% change	<b>-54.1*</b>

Fig. 5. Changes in CBF. Graphs A/1-4 show screenshot examples of the relative CBF changes measured during stimulation of the contralateral (A/1 and A/3) and ipsilateral (A/2 and A/4) whisker pad in a control (A/1 and A/2) and a treated animal (A/3 and A/4). CBF is expressed in perfusion units (PFU). Table B shows the means of changes in each hemisphere and the overall change in CBF, in regard to the groups, \*:  $P < 0.05$  ( $t$ -test)

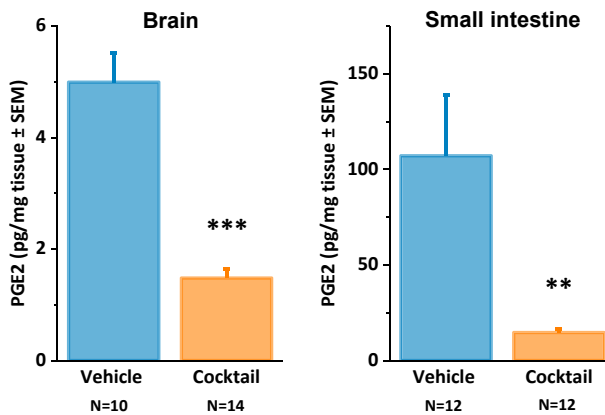


Fig. 6. Changes in prostaglandin E<sub>2</sub> levels in barrel cortex and small intestine tissues. Mean ± s.e.m. values are shown. Two-tailed independent  $t$ -tests. Brain:  $t = 7.272$ ,  $df = 22$ ,  $P$ -value  $< 0.0001$ . Intestine:  $t = 2.888$ ,  $df = 22$ ,  $P$ -value  $< 0.01$ . Significance levels: \*  $P < 0.05$ , \*\*  $P < 0.01$ , \*\*\*  $P < 0.001$



## DISCUSSION

Eight days long treatment with a pharmacological cocktail containing MS-PPOH, L-NAME and indomethacin induced neurovascular uncoupling in rats. However, despite the substantial decrease in the applied doses compared to those used in the mouse study [14], the treatment cocktail was still toxic: it caused ulcers and bleeding in the intestine and hypertension. (We cannot conclude whether the single death in the cocktail-treated group was treatment-related or not.)

Intestinal effects may primarily be attributed to indomethacin while hypertension to the high dose of L-NAME. However, we may presume some kind of synergism among the substances, as L-NAME may aggravate the ulcerogenic effect of indomethacin [29], while indomethacin causes sodium retention [31], thereby it may increase the salt sensitive hypertensive effect of L-NAME [32–34]. MS-PPOH was reported to increase  $\text{Na}^+$  excretion [35, 36], thus it may damp the effect of indomethacin. For achieving a viable model, further dose reduction is needed to avoid such toxicity and it should involve all the three compounds. In a stepwise approach it could be a further halving.

Abolishing hypertension would not only mean eliminating an unwanted side effect but also getting rid of a possible confounding effect of hypertension on NVC. In humans, the effect of elevated blood pressure on NVC is brain region dependent: in some areas functional hyperaemia is larger, in other regions it is smaller in hypertensive patients than in normotensive individuals [37]. Also, one year long antihypertensive treatment results in region-dependent increase or decrease in hyperaemia [38]. In animals, the effect of hypertension on NVC is also controversial [39]. For example, phenylephrine induced hypertension does not result in altered NVC in mice [40], while in the angiotensin II induced hypertension model (either in the acute or the chronic one) the observed decrement in NVC is independent of the hypertension itself [40–42]. In the spontaneously hypertensive Wistar-Kyoto rat strain (SHR) NVC is not impaired at 10 weeks of age, but 20 and 40 week old rats do show decreased NVC [43, 44]. These results suggest that prolonged, chronic hypertension may be detrimental on NVC [39, 45, 46], but short term hypertension may not have a direct effect on it. Thus, we think that the hypertension observed during our relatively short, 8 days long treatment may not have played a significant role in the measured uncoupling.

Although neurovascular uncoupling was detected in the barrel cortex, no impairment in cognitive performance was observed in the study. Both learning assays were of the aversive type that may be less vulnerable to impairing interventions. In contrast, in the mouse study cognitive deterioration was observed in non-aversive paradigms such as Y-maze place recognition, elevated plus-maze transfer and novel object recognition tests [14]. All of these paradigms are short-term, consisting of an acquisition and a retention trial and two of them are simple recognition memory test without active learning like in the Morris water-maze. The plus-maze transfer test has some mild aversivity (height and open space) but it is still much less aversive than either the fear conditioning or the Morris water-maze. In these three mouse tasks there is no stake for the animals, that is, nothing harmful happens if they do not learn or remember. The context of these assays is much less salient (incentivizing) for the subjects than the unavoidable footshocks in the fear conditioning or the immersion into water in the water-maze. Therefore, these assays may be more sensitive to a learning-disrupting intervention. Our findings in a recent study, applying another cognitive defect model, support this assumption: we found that



intracerebroventricularly injected streptozotocin caused memory impairment in the NOR test but not in the Morris water-maze task [47]. In future development of the neurovascular uncoupling model we would apply reward-motivated thus presumably more easily inhibitable learning assays, like, for example, NOR, fixed ratio 1 lever-press training, delayed T-maze alternation. However, the above discussed dose reduction may pave the way for a longer (e.g. 3–4 weeks) dosing regimen, which may result in impairments even in the aversive learning tasks. Additionally, longer treatment would allow to carry out more and/or longer lasting cognitive tests in the experimental period.

A further amendment would be the standardization of the laser Doppler measurement protocol as there may be large interindividual differences in the detected signals, so a smaller signal does not necessarily indicate uncoupling. Due to the nature of the protocol, self-controlled design cannot be applied, that is, a sufficiently large sample size (around 15–20) would be desirable.

Last, using additional biomarkers, for instance, measuring epoxygenase products in the brain, would also contribute to the establishment and validation of the model.

In summary, it appears that a pharmacological neurovascular uncoupling model can be developed in the rat, but substantial amendments, modifications and refinements in the protocol and methodology are still needed to establish a standardized, industrially usable model.

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