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Challenges in Developing a Rat Model of Pharmacologically Induced Neurovascular Uncoupling

PhD thesis

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List of abbreviations

5C/5CSRTT: 5-choice serial reaction time task
A β : amyloid-beta
b.i.d.: bis in die/twice a day
BP: blood pressure
CA1: cornu ammonis 1
CBF: cerebral blood flow
Coop: cooperation
COX: cyclooxygenase
CTA: Conditioned taste aversion
EET: epoxyeicosatrienoic acid
Exp: experiment
fMRI: functional magnetic resonance imaging
fUS: functional ultrasound
FC: fear conditioning
GFAP: Glial fibrillary acidic protein
HP β CD: hydroxypropyl- β -cyclodextrin
icv.: intracerebroventricular
ip.: intraperitoneal(ly)
L-NAME: L-NG-nitroarginine methyl ester
LP: lever press
MS-PPOH: N-(methylsulfonyl)-2-(2-propynyloxy)-benzenhexanamide
MWM: Morris water maze
NO: nitric oxide
NOR: novel object recognition
NOS: nitric oxide synthase
NVC: neurovascular coupling
NVU: neurovascular uncoupling
PBS: phosphate buffered saline
PFU: perfusion unit
PGE₂: prostaglandin E₂
PJ: pot jumping
T: treatment
TS: touchscreen
VGLUT1: vesicular glutamate transporter type 1
YM: Y-maze

1. Introduction

1.1. The neurovascular unit

The neurovascular unit is a multicellular structure that facilitates the tight coupling between vascular and neural activity in the brain, regulating cerebral blood flow (CBF) in response to metabolic demands, termed neurovascular coupling (NVC) (1–3). NVC ensures that localized increases in neuronal activity trigger rapid adjustments in blood supply, matching oxygen and nutrient delivery to metabolic needs (1–4). This process involves coordinated interactions among neurons, astrocytes, pericytes, microglia and vascular endothelial cells, which collectively detect synaptic activity and translate it into vasodilation or -constriction (1,3–5) (Fig 1). Neurovascular uncoupling (NVU) refers to a disruption of this relationship between neuronal activity and blood flow in the brain. NVU occurs when this mechanism fails, leading to mismatches between metabolic demand and blood supply, which can impair brain function. It is often associated with conditions like stroke, epilepsy, Alzheimer's disease, traumatic brain injury and old age.

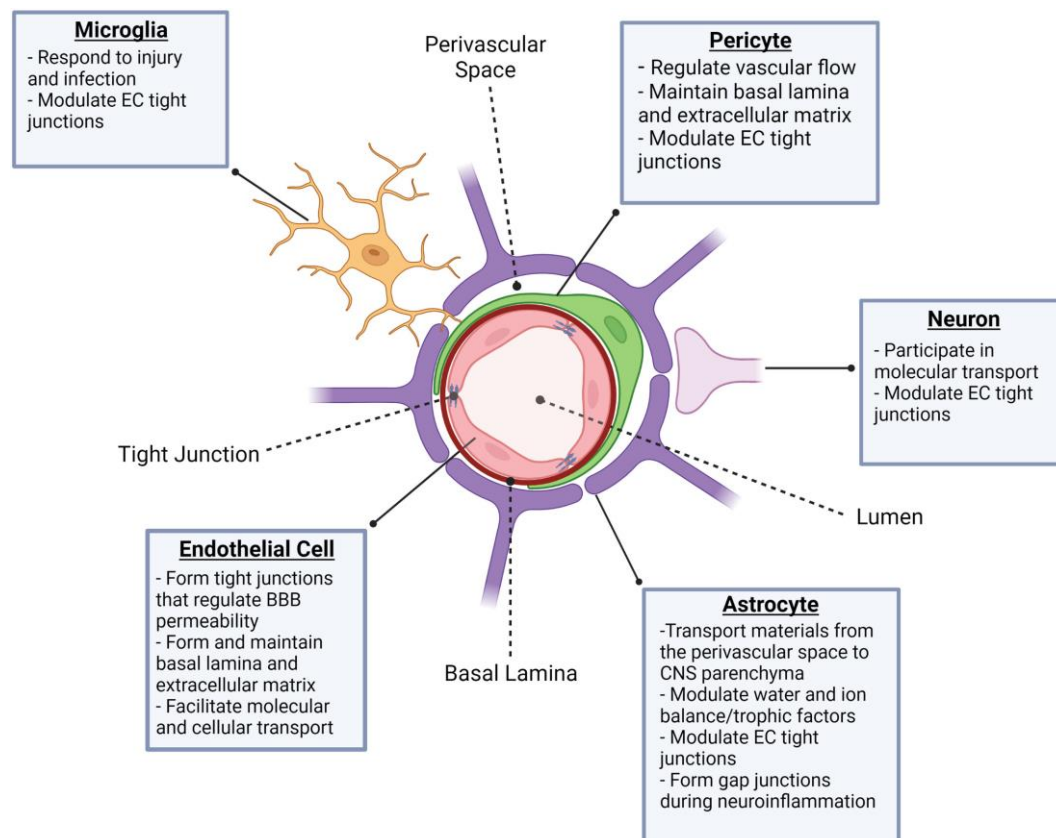


Figure 1. Cellular components of the neurovascular unit (from Smith et al.(6)). For the function of the components see the description below.

Components of the neurovascular unit:

Endothelial cells form the inner lining of brain capillaries and are interconnected by tight junctions, constituting the structural basis of the blood-brain barrier, which restricts paracellular diffusion (7–9). Endothelial cells regulate molecular exchange by expressing transporters, furthermore, they secrete vasoactive factors like nitric-oxide (NO), epoxyeicosatrienoic acids (EETs) and prostaglandins which modulate the vascular tone (1–3,10–12).

Pericytes, mural cells embedded in the basal lamina, regulate the capillary diameter via contractile proteins, thus controlling the CBF. Beyond their role in regulating vascular tone, pericytes maintain blood-brain barrier integrity by stabilizing endothelial tight junctions through platelet-derived growth factor receptor- β signalling. This pathway promotes pericyte-endothelial adhesion and suppresses endothelial transcytosis, reducing leakiness (4,13,14).

Astrocytes are critical components of the neurovascular unit, with their endfeet processes ensheathing brain capillaries, forming a close association with the vasculature (15). Additionally, astrocytes release vasoactive signals, including NO, prostaglandins, potassium ions, and arachidonic acid metabolites, which play a key role in mediating neurovascular coupling (1,3,10,11,13,16–18).

Neurons, particularly glutamatergic and GABAergic subtypes, actively influence vascular tone through various signalling mechanisms (19). NO released by neurons induces vasodilation, enhancing blood flow to active brain regions (13). Vasoactive intestinal peptide secreted by interneurons (20) and noradrenaline released from locus coeruleus projections (21) have both been implicated in neurovascular signalling, although their specific contributions to cerebrovascular regulation remain to be fully established.

Microglia, the resident brain immune cells, survey the neurovascular unit to detect and respond to injury or infection. Upon activation, they release pro-inflammatory cytokines and reactive oxygen species, which can either protect or damage the neurovascular unit depending on the context (22).

The **basal membrane** and surrounding **extracellular matrix** form a structural scaffold that supports the neurovascular unit (23,24). Beyond providing mechanical stability, the extracellular matrix regulates cell signalling through interactions with integrins and other adhesion molecules, influencing cell migration, proliferation, and barrier function.

1.2. Diseases linked to neurovascular unit dysfunction and diminished functional hyperaemia

Dysfunction of the neurovascular unit is associated with several neurological disorders, each characterized by distinct yet overlapping pathological mechanisms that impair CBF regulation. Among these, Alzheimer's disease, aging, hypertension-related small vessel disease and diabetes mellitus represent conditions where neurovascular unit dysfunction plays a central role in disease advancement.

Alzheimer's disease is characterized by early cerebrovascular dysfunction, which contributes to disease progression (25). Even before cognitive symptoms manifest, patients exhibit reduced CBF, particularly in memory-critical regions such as the hippocampus (26,27). This chronic hypoperfusion arises from multiple interdependent mechanisms. Amyloid-beta ($A\beta$) peptides induce pericyte contraction, leading to capillary constriction and impaired microcirculation (5). Simultaneously, reactive oxygen species generated by $A\beta$ accumulation might damage endothelial cells, reducing NO bioavailability and impairing vasodilation. This leads to oxidative stress that impairs blood vessel function and can damage the blood-brain barrier. The resulting reduction in blood flow creates a cycle of ongoing damage to both blood vessels and neurons (28–30). Astrocytes, which normally mediate neurovascular coupling, become dysfunctional as $A\beta$ disrupts their calcium signalling, impairing the release of vasoactive mediators such as prostaglandins and potassium. Over time, this persistent mismatch between neuronal energy demand and blood supply can accelerate synaptic failure and neurodegeneration, creating a cycle where worsening functional hyperaemia might lead to cognitive decline.

Even in the absence of apparent neurological disease, **aging** itself leads to progressive neurovascular deterioration (31). Endothelial cells exhibit reduced NO production, impairing vasodilatory capacity, while pericytes are gradually lost, increasing capillary fragility (32–35). Astrocytes, which normally support neuronal and vascular function,

shift toward a pro-inflammatory reactive state, further disrupting neurovascular coupling (36,37). These age-related changes contribute to the subtle cognitive decline observed in otherwise healthy older adults and increase susceptibility to neurodegenerative diseases such as Alzheimer's disease and Parkinson's disease.

Beyond the primary pathology under investigation, other systemic conditions such as **chronic hypertension** and **diabetes mellitus** promote cerebral small vessel disease and direct vascular injury, impairing NVC through arteriolosclerosis, pericyte loss, capillary rarefaction, advanced glycation end-product accumulation, and oxidative stress, leading to reduced vascular compliance, blunted functional hyperaemia on fMRI, increased blood-brain barrier permeability, and heightened risk of vascular cognitive impairment and dementia (31,38–42).

These disorders afflict a significant portion of the population, creating substantial mental, economic, and social burdens. To advance our understanding of the underlying mechanisms behind this functional deterioration, there is an urgent need for improved disease modelling that more accurately replicates these pathological states related to the loss of functional hyperaemia.

1.3. Lack of NVU targeting approach in antidementia drug research

The pace of developing novel cognitive enhancers has been slow, with a notable absence of new mechanisms advancing to late-stage clinical trials for dementia over the past two decades (43–45). The current therapeutic landscape for Alzheimer's disease is still dominated by symptomatic treatments developed decades ago, specifically cholinesterase inhibitors (donepezil, rivastigmine, and galantamine) and the NMDA receptor antagonist memantine (46,47). Yet their benefits are limited and do not halt disease progression.

The recent approval of monoclonal antibodies targeting A β , such as lecanemab (2023) and donanemab (2024), for early Alzheimer's disease (48–51) represents a shift toward disease-modifying approaches. However, their clinical benefits are modest, and side effects like amyloid-related imaging abnormalities compromise their wider applicability. The specific effects of these therapies on neurovascular function are not fully elucidated.

While numerous molecular pathways implicated in NVU have been identified—including NO signalling (18,52), astrocytic endfoot regulation (53–55), and pericyte-mediated capillary constriction (56–58)—progress toward clinical translation has been limited. For instance, despite evidence linking endothelial NOS dysfunction to impaired vasodilation (59–62), no endothelial NOS-targeted therapies have advanced considerably. Similarly, while pericyte degeneration is an established feature of NVU, the translation of pericyte-stabilizing strategies from preclinical models to human clinical trials for dementia has not yet been realized (63–65).

Other emerging strategies target upstream mechanisms like mitochondrial dysfunction and neuroinflammation. Compounds such as MitoQ, a mitochondrial antioxidant, have shown potential in reducing oxidative stress and neuroinflammation in animal models of central nervous system injury and disease (66–69), and NLRP3 inflammasome inhibitors are being explored for their dual effects on neuroinflammation and vascular damage (70,71). However, these approaches have not proven their clinical utility yet.

A fundamental cause of the scarcity of late-stage clinical candidates targeting the components of NVC may be the lack of predictive preclinical models of NVU useable for drug evaluation. Many existing models may not adequately recapitulate the complex neurovascular pathophysiology of human dementia. Therefore, the development of a well-validated and translationally relevant animal model of NVU is a critical requisite for efficiently testing and de-risking future therapeutic candidates, whether they target NVU directly or their efficacy is simply contingent upon an intact neurovascular system.

1.4. The need for preclinical NVU models

Preclinical research in dementia has been largely dominated by genetic models of amyloidosis (e.g., double or triple-transgenic Alzheimer's disease model mice, or the Five Familial Alzheimer's Disease Mutations (5xFAD) model) (72–75) or tauopathy (e.g., P301S, rTg4510 mouse models) (76–80). While invaluable for elucidating the mechanisms of protein aggregation and related toxicity, these models are not optimized for studying NVU. Their primary objective is to recapitulate complex genetic disease states, which often include vascular components as secondary consequences, rather than to provide a clean, inducible system for screening therapeutics that target cerebrovascular

function directly. Consequently, drug efficacy readouts from these complex models cannot be easily interpreted in the context of NVU.

Similarly, chronic hypoperfusion models (e.g., bilateral carotid artery stenosis) (81) induce sustained vascular insufficiency but model a specific, often irreversible, cerebrovascular pathology. While highly relevant for studying vascular dementia and small vessel disease, these surgical models do not offer the temporal control needed to study the acute, dynamic, and potentially reversible nature of neurovascular coupling itself.

Recent efforts have sought to improve model validity by incorporating aging and comorbid vascular risk factors. Reviews of vascular cognitive impairment models highlight the use of aged rodents (82), and human studies of hypertension–amyloid interactions (83) provide rationale for models combining hypertension (e.g., spontaneously hypertensive rats) with amyloid deposition to represent mixed dementia pathology (83,84). While these approaches provide valuable platforms for studying complex, multifactorial disease progression, they can be time-intensive, variable, and less suited for the high-throughput screening of compounds designed to directly target neurovascular pathways.

The field lacks a standardized, pharmacologically tractable model that can reliably induce a state of NVU for the primary purpose of evaluating candidate drugs aimed at restoring cerebral haemodynamics.

Given the proven relevance of neurovascular uncoupling to human cognitive decline, a pharmacologically induced model in a translationally relevant species like the rat holds significant promise for drug discovery. Such a model would not seek to replicate the full spectrum of human dementia, but rather to establish a highly relevant and controlled system for studying the functional consequences of NVU and for screening potential cognitive enhancers. A study done by Tarantini et al. (85) established this proof-of-concept in mice, inducing NVU through a 7-day pharmacological regimen inhibiting 3 key vasodilatory pathways: EET, NO and prostaglandin production.

- EETs, these bioactive lipid mediators are well-known for their vasodilatory effects, acting on vascular smooth muscle to promote relaxation and enhance blood flow. EETs are produced from arachidonic acid by epoxygenase enzymes.

N-(methylsulfonyl)-2-(2-propynyloxy)-benzenhexanamide (**MS-PPOH**) is a selective inhibitor of these enzymes. 20 mg/kg/day was applied of the compound via subcutaneously implanted osmotic minipump.

- NO is a key signalling molecule in the cardiovascular system, exerting potent vasodilatory effects by activating soluble guanylate cyclase in vascular smooth muscle cells. NO also plays a critical role in neurovascular coupling, where it acts as a mediator between neuronal activation and subsequent increases in cerebral blood flow. L-NG-nitroarginine methyl ester or **L-NAME**, is a widely used inhibitor of NOS, the enzyme responsible for synthesizing NO from L-arginine. Inhibition of NO production with L-NAME leads to vasoconstriction and increased vascular resistance. L-NAME was dissolved in drinking water, 100 mg/kg/day.
- Prostaglandins are lipid compounds with diverse physiological roles, including the modulation of vascular tone. Certain prostaglandins, such as Prostaglandin E₂, promote vasodilation by relaxing vascular smooth muscle. In the context of neurovascular coupling, prostaglandins are known to participate in the regulation of cerebral blood flow in response to neuronal activity. **Indomethacin** is a nonsteroidal anti-inflammatory drug, that inhibits cyclooxygenase (COX) enzymes, specifically COX-1 and COX-2, which are responsible for converting arachidonic acid into prostaglandins. Suppressing COX activity by indomethacin leads to diminished vasodilation and increased vascular resistance. Indomethacin was administered orally, 7.5 mg/kg/day.

This treatment resulted in an approximately 2/3 decrease in evoked cerebral functional hyperaemia and significant impairments in spatial memory, recognition memory, and motor coordination (85). However, no other mice model of pharmacologically induced NVU had been reported since, nor a similar, validated procedure had been established in rats.

1.5. The rodent test battery system applied by our group serving as the framework to incorporate a rat NVU model

Our research team has developed an advanced cognitive test battery for rats designed to evaluate potential cognitive enhancers with improved translational validity (86–90). In this paradigm, experienced rats are trained across multiple cognitive tasks that model distinct human cognitive domains, such as attention (five-choice serial reaction time task, 5CSRTT) (91), social cognition (cooperation task) (92), spatial memory (Morris water maze, MWM) (93), procedural memory ("pot jumping" task) (94), and visual memory (pairwise discrimination) (95). This approach mimics the baseline cognitive abilities seen in human patients, providing a more relevant model than studies using naïve animals and one or two "fashionable" cognitive tasks (e.g. novel object recognition or Morris water maze) (86–89,96,97).

Moreover, to maintain motivation and cognitive performance, the animals are kept under controlled food restriction, a regimen shown to enhance health, improve cognitive function (98–101), slow aging, and reduce mortality compared to ad libitum feeding (102–106). This also ensures consistent engagement in food-rewarded behavioural tasks. A key advantage of this system is the ability to longitudinally assess the effects of cognitive impairment methods and potential treatments within the same animal cohort. Following impairment induction, the system enables the systematic evaluation of putative cognitive enhancers in a controlled experimental framework, incorporating randomization, double-blind procedures, and vehicle-treated comparison groups to minimize bias and enhance reproducibility.

The choice of impairment method is crucial, as it must exhibit strong construct validity for modelling clinical cognitive deficits. While neurotransmitter-targeting agents (e.g., scopolamine, MK-801) have been widely used, their predictive value has been questioned (107,108), and our own studies have yielded inconsistent results with these compounds (109–111). Since NVU is known to impair cognition in patients, we sought to implement it as an impairment method with a stronger neurobiological rationale. The pharmacologically induced NVU model, previously established in mice (as detailed in Section 1.4.) (85), provides a mechanistically grounded method for inducing cognitive deficits that is ideally suited for this purpose.

2. Objectives

The primary aim of this doctoral work was to establish a pharmacologically induced NVU model in rats using MS-PPOH, L-NAME, and indomethacin, and validate this protocol in a translationally relevant population of aged, experienced rats assessed within our cognitive test battery. In doing so, to create a novel platform for evaluating prospective therapeutics on NVU function and its cognitive sequelae as a model of human cognitive decline.

With this aim in mind, the specific objectives of this study were:

1. To establish NVU-inducing pharmacological doses that effectively disrupt neurovascular coupling while minimizing undesirable side effects.
2. To identify cognitive assays within our test battery that are sensitive to NVU-induced impairment.
3. To validate the NVU model in a translationally relevant population by testing its effects in aged, cognitively experienced rats.
4. To optimize treatment protocols through iterative experimentation, adjusting dosages and administration methods based on observed tolerability and efficacy.

These objectives were addressed through a series of experiments, which included dose-finding studies in young naïve rats (Experiment 1 and 2), cognitive testing in aged, experienced rats (Experiment 3), and investigations into the combined (or singular) effects of NVU-inducing compounds (Experiment 4 and 5).

3. Methods

This part along with the “Results” of this doctoral work was mainly taken from our published papers (112,113).

The design of the NVU experiments (Exp 1-4) followed the same scheme. It involved a drug-treated and a vehicle-treated group and consisted of three phases: 1. baseline measurements, 2. treatment period with continued testing of the animals, 3. terminal surgery for neurovascular coupling measurement followed by ex vivo assays.

Treatment of animals was done by separate people from those who performed the cognitive assays, and physiological measurements. The latter were unaware of what were the animals treated with (blinding).

All procedures carried out on animals were authorized by the regional animal health authority in Hungary (Pest County Government Office, resolution number PE/EA/785-5/2019 and PE/EA/00808-7/2021 and conformed to the Hungarian welfare legislation and the EU 63/2010 Directive on the protection of animals used for scientific purposes. All procedures were carried out in accordance with the ARRIVE guidelines and the principles of the 3Rs (Replacement, Reduction, and Refinement).

3.1. Housing of animals

Animals were housed in polycarbonate cages (1,376 cm² area, 16 cm height) under reversed light-dark cycle (light phase from 4 pm to 4 am). They were kept in a temperature-controlled room maintained at 20–23 °C with relative humidity ranging from 60 to 75 %. Drinking water was available ad libitum. Cardboard tubes and aspen bricks were available as environmental enrichment. Animals were intensively handled before and during the experiments. Housing density and food access (commercial pellet rat feed (R/MZ + H, SSniff Spezialdiäten GmbH, Soest, Germany) varied by experiment.

3.2. Exp 1: Dose-finding and protocol optimization in young, naïve Wistar rats.

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

3.2.1. Treatment

For inducing neurovascular uncoupling, the following drugs were used: 5 mg/kg MS-PPOH, 10 mg/kg L-NAME, 1 mg/kg indomethacin, injected ip. twice daily (at 6 am and 6 pm) for 8 consecutive days (*see* Fig 2). The compounds were administered in a cocktail which was prepared daily as follows: first the proper amount of MS-PPOH (prepared at Eötvös Loránd Research Network – Research Centre for Natural Sciences, Budapest, Hungary) was dissolved in 45 % hydroxy-propyl- β -cyclodextrin (HP β CD, Cyclolab 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 a 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 % HP β CD solution. The dosing volume was 2 ml/kg.

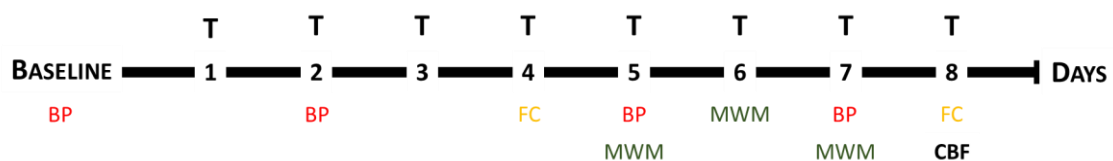


Figure 2. Timeline of Exp 1. All interventions are indicated by corresponding abbreviations on days when they were performed. BP: blood pressure measurement, MWM: Morris water maze, FC: fear conditioning, CBF: cerebral blood flow measurement, T: treatment.

3.2.2. Cognitive assays

Fear-conditioning

The test device was a sound-proof shocking chamber (26×26×30 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 a Retention session. Length of each session was 5 minutes. During the Acquisition trial, the rats received 5 mild foot shocks (0.6 mA, 1 sec) as unconditional stimulus at 60 seconds 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 seconds, 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. Measurements were carried out on Day 4 (Acquisition) and 8 (Retention) (*see* Fig 2).

Morris water maze

The apparatus consisted of a black circular pool with a diameter of 190 cm and a depth of 60 cm, filled with water at 40 cm depth and maintained at 23±1 °C. A hidden round black escape platform, 10 cm in diameter, was submerged 0.5 cm below the water surface and located in one of the four quadrants (south-east, south-west, north-east, north-west), 40 cm from the pool's edge. Extra-maze cues were positioned on the walls of the experimental room to aid in orientation during the swim trials. At the beginning of each trial, the rat was placed into the pool at one of four starting points (North, East, West, or South, systematically rotated) and given 3 minutes to find the hidden platform. Each rat was allowed to remain on the platform for 30 seconds before being removed, dried with a cloth and returned to its home cage. Each animal underwent three trials a day with 20-minute intertrial interval between them. Escape latency was recorded, and the swimming path was tracked using Smart v3.0 video tracking system software (Panlab, Barcelona, Spain). Every animal participated in three sessions (Day 5-7, *see* Fig 2), each consisting of three trials.

3.2.3. Cerebral blood flow measurement

Collecting data of altered CBF was the same in Exp 1 to Exp 4. On the last day of each experiment, following any remaining cognitive tasks, the animals were anesthetized with intraperitoneal injections of urethane at a dose of 1.5 g/kg prior to surgery. The depth of anaesthesia was verified by the absence of tail-pinch and pedal reflexes. The rats were then positioned in a stereotaxic frame (Stoelting, Wood Dale, IL, USA) and placed on a preheated bench set to 37 °C (Supertech Instruments, Pécs, Hungary). Lidocaine (Egis, Budapest, Hungary) was applied locally on their shaved heads. A midline skin incision was made, and the skull surface was cleaned. The left barrel cortex was located using stereotaxic coordinates (3.3 mm posterior to bregma and 4.5–5.0 mm lateral from the midline (114)), and an approximately 3 mm wide cranial window was created with a dental drill, avoiding penetration of the dura mater. The window was filled with artificial cerebrospinal fluid (with a standard composition of 126 mM NaCl, 1.8 mM KCl, 1.25 mM KH_2PO_4 , 2.4 mM $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 26 mM NaHCO_3 , 12 mM glucose, and 1.3 mM $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$), and a laser Doppler probe (OxyFlo Probe, Oxford Optronix Ltd. – ADInstruments, PowerLabs) was lowered in the liquid. This probe measured changes in cerebral blood flow in the barrel cortex while the contralateral whisker pad was manually stimulated with a brush at approximately 2 Hz. Data were registered using LabChart 6 software (ADInstruments, Dunedin, New Zealand, version 6.2.1). Changes in perfusion units (PFU) were recorded over time, with a 5 mV difference corresponding to 1 PFU change. The procedure involved positioning the probe, stimulating the whisker pad, and adjusting the probe position if no notable blood flow change was detected. Once a stable signal was obtained, a 2-minute baseline was established. The recording protocol included two 30-second contralateral stimulations (see example in Fig 7b and Fig 9b) and one 15-second ipsilateral "control" stimulation, each separated by 3 minutes of break. This sequence was repeated four times with 3-minute intervals between each. The mean of the 15 s long baseline period preceding stimulation was subtracted from the mean of the stimulation period to determine signal amplitude. The amplitudes of the eight contralateral stimulations and the four ipsilateral stimulations (aspecific signals) were averaged. The average ipsilateral amplitude was subtracted from the contralateral amplitude mean, yielding the coupling parameter (increased blood flow) characteristic for each individual.

After the CBF measurement- still under the influence of anaesthesia - animals were swiftly decapitated and prepared for tissue sample harvest.

3.2.4. Blood pressure measurement

The animals' blood pressure was measured using a 4-channel CODA[®] apparatus (Non-Invasive Blood Pressure System; Kent Scientific Corp., Torrington, USA), which utilizes two tail cuffs: an occlusion cuff and a volume pressure recording cuff (*see* Fig 3). The animals were placed in holding tubes and the cuffs were put on their tails. The tubes then were placed on a warmed platform and were covered with a black insulating cloth. Each recording session included 40 inflating – releasing cycles of blood pressure measurement. The variables measured were systolic, diastolic, and mean blood pressure, blood flow, and heart rate. Measurement cycles were categorized by the equipment itself as 'failed' or 'OK' but even from the 'OK' set we filtered out any unrealistic data and excluded values that fell outside the mean $\pm$ 2 standard deviations. We then calculated a revised mean for each variable and assigned it to the subject as its measured value on the given day.

Each animal underwent a habituation session 7-9 days before the experiment. Prior to the measurements, the transparent restriction tubes were placed inside the animals' home-cages, in order to help them acclimate being inside of these plastic instruments.

Blood pressure measurements for Exp 1 were done on Day 2, 5 and 7 (*see* Fig 2).

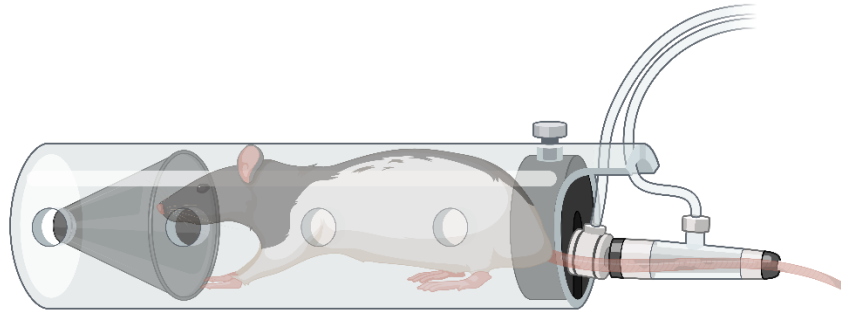


Figure 3. Visual representation of a rat in a restrictive tube during a blood pressure measurement. This image was made with BioRender.

3.2.5. Tissue collection and sampling

After completing the CBF measurement, the animals were decapitated, the brain was removed, and the barrel cortex was dissected from the left hemisphere. Tissue samples for ELISA tests were washed with phosphate buffered saline (PBS, 137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, and 1.8 mM KH₂PO₄, pH 7.4, in 1:10 dilution), weighed, placed into Eppendorf tubes, snap-frozen in liquid nitrogen and stored at -80 °C until further processing. The animals' small intestines after removal were measured in length. From the proximal end, approximately 10 % was discarded and the next 10 % was taken as tissue sample. The samples were cut open in longitudinal direction, cleaned of faeces and checked for signs of ulceration or malformation. Lastly, the samples were washed in PBS, froze and stored at -80 °C for later evaluation.

3.2.6. PGE₂ level determination

The levels of PGE₂ in the small intestine and barrel cortex were determined by ELISA (Cayman Chemical, Ann Arbor, MI, USA, - Prostaglandin E₂ ELISA Kit (Monoclonal), Item No. 514010). Briefly, barrel cortex and small intestine tissues were homogenized in

precooled 100 % ethanol containing 10 μ M indomethacin and centrifuged at 1500 $\times$ g for 15 min at 4 °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.

3.3. **Exp 2:** *Further dose refinement in young, naïve Wistar rats with expanded cognitive testing.*

For this study, we used 17 male Hannover Wistar rats (Toxicop, Budapest, Hungary) weighing 155-215 g and being 2 months of age at the start of the experiment. Four animals were kept in a cage, except one cage containing five. Food was available ad libitum prior to treatment but restricted during treatment (15 g/rat supplied at least 2 h before the end of the dark phase).

3.3.1. Treatment

For inducing NVU the following drugs were used: 2.5 mg/kg MS-PPOH, 0.5 mg/kg indomethacin, 5 mg/kg L-NAME, injected ip. for 9 consecutive days, twice per day (at 6 am and 6 pm) except Day 9 when only one injection was applied in the morning. The compounds were administered in a “cocktail” which was prepared daily as follows: first the proper amount of MS-PPOH was dissolved in 30 % HP β CD in an ultrasonic water-bath (40 min, 24 °C). Then indomethacin was added and sonicated for further 50 min. Last, L-NAME was added to the solution which dissolved immediately. Nine animals were treated with this cocktail, while 8 control rats received 30 % HP β CD solution. The dosing volume was 2 ml/kg.

Treatment allocation

Based on individual numbering, the first two animals served as controls and the next two as treated across two cages, while in two additional cages this allocation was reversed. The last animal (in the cage with five) was assigned to the treated group. This constituted a balanced allocation rather than true randomization, resulting in nearly identical baseline body weights between groups. Separate persons performed the injections and the

measurements; those who did the latter were not aware of which treatment the animals received.

The timeline of the experiment is shown in Fig 4.

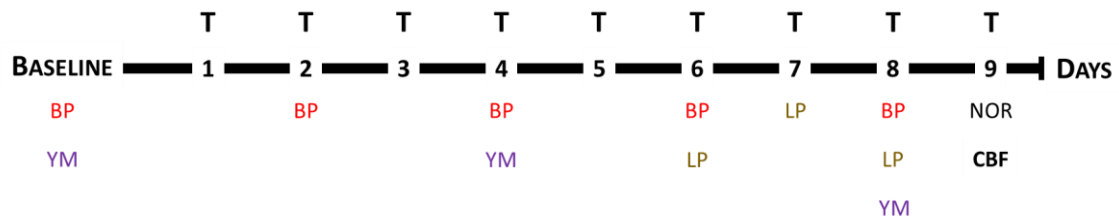


Figure 4. Timeline of Exp 2. All interventions are indicated by corresponding abbreviations on days when they were performed. BP: blood pressure measurement, YM: Y-maze alternation test, LP: lever press learning, NOR: novel object recognition assay, CBF: cerebral blood flow measurement, T: treatment.

3.3.2. Cognitive assays

Y-maze alternation test

The Y-maze used to assess short-term working memory was a symmetrical, dark grey plastic apparatus with three identical arms (45x16x29.5 cm) arranged at 120-degree angles and connected at the centre. Each arm was accessible through an arched opening in a partition wall. The maze was placed in a room with moderate lighting (20 lx at surface level).

At the beginning of each trial, the rat was placed in the designated “entry” arm (arm #1), and its movements were recorded for 10 minutes using a ceiling-mounted video camera. The video feed was displayed on a computer monitor (Record-It!, v1.0, Panlab, Harvard Apparatus, Barcelona, Spain) outside the experimental room, where the experimenter manually logged arm entries in real-time in an Excel document. After each trial, the animal was returned to its cage, and the maze was cleaned with 10 % alcohol.

The collected data were analysed using an Excel macro to determine the total number of entries and the percentage of spontaneous alternation. Spontaneous alternation was

defined as an entry into the least recently visited arm (e.g., 1-2-3-1), with the percentage calculated as:

$$\text{Spontaneous alternation \%} = ((\text{number of spontaneous alternations}) / (\text{total entries} - 2)) \times 100.$$

Animals' performance within the maze was observed at baseline (about a week before the treatment) and on Treatment Day 4 and 8 (*see* Fig 4).

Lever-press training

Animals performed this task from Treatment Day 6 to 8 (*see* Fig 5) in a 30×24×21 cm Skinner box (MedAssociates, VT, USA) equipped with a lever and a magazine at the opposite wall of the chamber. During this short period rats had to learn that pressing the lever would result in getting a pellet in the magazine. If the animals pressed the pedal, the feeder light turned on and remained lit until the delivered pellet was eaten. After a 5-seconds-long intertrial interval the lever was activated again (indicated by a light stimulus above it) until the next lever-press which resulted in the next pellet delivery. The first measurement on Day 6 was 20 minutes long, the subsequent sessions the next two days lasted for 30 minutes. The number of rewards obtained during the sessions was the outcome variable.

Novel object recognition

This assay was conducted on Treatment Day 9, right after the last injection (*see* Fig 4) as described in Gáspár et al (36). Animal behaviour was observed in a 48×48×42 cm box lined with bedding material and recorded using a video camera system (Record-It!, v1.0, Panlab, Harvard Apparatus, Barcelona, Spain). The experiment included an acquisition trial and a retention trial. During the acquisition trial, the rats were allowed to explore two identical objects placed in the box for 3 minutes. Following an 80-minute delay, the retention trial was conducted, where one of the original objects was replaced with a different one (novel object), and the rats again had 3 minutes to explore them. The objects used were a plastic bottle filled with gravel and a glass bottle filled with a blue dye solution. Both objects were equally assigned as familiar or novel. The primary measurement was the exploration time for each object. Recognition memory was assessed

using the discrimination index, calculated as: $DI = (t_{\text{new object}} - t_{\text{old object}}) / (t_{\text{new object}} + t_{\text{old object}})$. According to predefined criteria, rats that spent less than 10 seconds by exploring the objects or examined only one of the two objects during any trial were excluded from the analysis.

3.3.3. Cerebral blood flow measurement

CBF was measured in the same mode as described in Exp 1 on Day 9

3.3.4. Blood pressure measurement

Baseline blood pressure measurement was carried out at 4-6 days before the day of the first treatment. During the treatment, blood pressure was monitored on Days 2, 4, 6 and 8 (*see* Fig 4).

3.3.5. Tissue collection and sampling

Barrel cortices, hippocampi and upper part of small intestine were collected after the CBF assessments in the same manner as described in Exp 1.

3.3.6. 11,12-EET level determination

For the measurement of 11,12-EET, BlueGene ELISA Kits were used (11,12-Epoxyeicosatrienoic Acid ELISAs kit, BlueGene Biotech, China, Catalogue number: E02E0540). For homogenization, 0.02 mol/l PBS (pH 7.0-7.2) was added to the barrel cortex and hippocampus tissues according to the dilution ratio (see homogenization protocol of kit E02E0166 (115) (description is the same as in kit E02E0540, our own setup, which is not available on the manufacturer's site at the time this dissertation was written)) and crushed by metal ball crushing. If the tissue samples were larger, this step was also preceded by pulverisation in a mortar using liquid nitrogen. The sample was then centrifuged for 15 minutes. The collected supernatants were stored in a freezer at -80 °C until evaluation. The obtained amount of supernatants of some animal samples were insufficient for testing it in one well of the ELISA kit, so some samples within groups were pooled or left out.

3.4. Exp 3: Validation in aged, experienced Long-Evans rats to assess translational relevance

We conducted this study with 25 experienced male Long-Evans rats (Janvier Labs, France) weighing 270-490 g and being 27.5 months of age at the start of the experiment. Animals were kept in groups of three (or two if previous cage-mates died). They had restricted food access throughout their life: 45 g for three rats (proportionally varied if needed), with food supplied at least 2 h before the end of the dark phase.

It is important to stress that *experienced* in this context refers to animals that, prior to this experiment, underwent an intensive cognitive training regimen—completing at least one daily cognitive task on weekdays—resulting in over two years of accumulated testing experience. As outlined in the introduction, such subjects provide a translationally relevant model for preclinical studies investigating cognitive dysfunction, as their extensive training history better reflects complex cognitive profiles observed in humans.

The timeline of the experiment is shown in Fig 5.

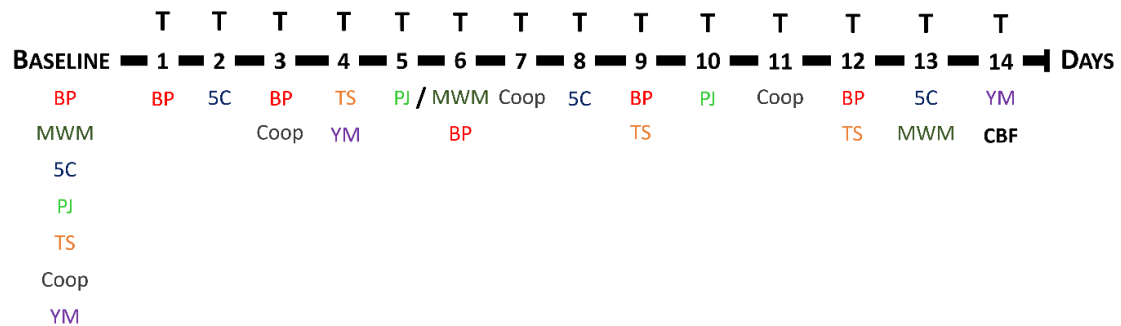


Figure 5. Timeline of Exp 3. All interventions are indicated by corresponding abbreviations on days when they were performed. BP: blood pressure measurement, MWM: Morris water maze, 5C: 5CSRTT, PJ: pot jumping test, TS: touchscreen pairwise visual discrimination, Coop: cooperation task, YM: Y-maze alternation test, CBF: cerebral blood flow measurement, T: treatment. “/” indicates that half of the animals did PJ on day 5 and MWM on day 6, while the other half was tested in reverse order.

3.4.1. Treatment

For 14 consecutive days (*see* Fig 5) the animals were treated ip. with the presumed uncoupling cocktail (n=12) or vehicle (n=13) twice a day (at 6 am and 6 pm) except on Days 1 and 14 when they only received one injection in the evening and in the morning, respectively. The starting dosages were set to 5 mg/kg for MS-PPOH, 7 mg/kg for L-NAME and 0.7 mg/kg for indomethacin.

The compounds were administered in a “cocktail” which was prepared daily as follows: first the proper amount of MS-PPOH was dissolved in 45 % HP β CD in an ultrasonic water-bath (40 min, 24 °C). Then indomethacin was added and sonicated for further 50 min. Last, L-NAME was added to the solution which dissolved immediately. Control animals received 45 % HP β CD solution. The dosing volume was 2 ml/kg.

The animals were divided into seven cohorts whose treatments and behavioural experiments started with a day shift. In the first treated cohort, after the first four injections we observed that both the vehicle- and cocktail treated animals were sleepy and not motivated to work in the cognitive assays, therefore we reduced the cyclodextrin concentration to 30 %. After their tenth injection, because of overt toxicity (*see* Results), we needed to decrease the doses of the components to 2.5 mg/kg MS-PPOH, 5 mg/kg L-NAME and 0.5 mg/kg indomethacin, which were dissolved in 20 % HP β CD + 1 % NaHCO₃. The changed solvent resulted in a faster dissolution time through ultrasound sonication (10 min for MS-PPOH and additional 20 min for indomethacin) and a pH=8.0-8.1 of the “cocktail” solution that was tolerable for the animals, as the pH of the peritoneal fluid is between 7.5 and 8. In case of animals that started to lose weight intensively, drug injections were suspended until they gained weight again. See the detailed treatment and exact number of injections in Table 1.

Table 1. Number of injections and cumulative doses used for the different cohorts of rats during the treatment period in Exp 3. - In the second column the numbers of rats are shown treated with vehicle or “cocktail”. In the 3rd, 4th and 5th column the number of injections of the used vehicle are indicated for the given rats. In the last three columns the cumulative doses of the given substances are shown. The ‘/’ signs in the cells separate the values for different animals. CD stands for hydroxypropyl- β -cyclodextrin.

vehicle		45 % CD	30 % CD	20 % CD + 1 % NaHCO ₃	Cumulative dose (mg/kg)		
dose of MS-PPOH/ L-NAME/ Indomethacin		5-7-0.7 mg/kg		2.5-5-0.5 mg/kg	MS-PPOH	L-NAME	Indomethacin
Cohort A	2, vehicle	4	6	16			
	2, cocktail	4	6	8	70	110	11
Cohort B	4, vehicle	2	6	18			
Cohort C	2, vehicle		6	20			
	1, cocktail		6	12	60	102	10.2
Cohort D	1, vehicle		4	22			
	2, cocktail		4	18/22	65/75	118/138	11.8/13.8
Cohort E	2, vehicle			26			
	2, cocktail			21/26	52.5/65	105/130	10.5/13
Cohort F	4, cocktail			21/24/	52.5/60/	105/120/	10.5/12/
				26/26	65/65	130/130	13/13
Cohort G	2, vehicle			26			
	1, cocktail			20	50	100	10

Treatment allocation

Animals were assigned to vehicle- and drug treated groups in a balanced manner based on their performance in baseline trials (*see below*). Learning parameters from all the

assays were taken into account with the help of an in-house made excel macro. Separate persons performed the injections and the learning assays. Those conducting assays were originally unaware of treatment allocation, however, due to toxicity-induced weight loss, blinding could not completely be maintained.

3.4.2. Cognitive assays

Morris water maze

Description of the experimental set-up is the same as in Exp 1.

Animals learned the task at their age of 3.5-4 months during 4 daily sessions with the platform fixed in the south-east quadrant. Afterwards, they received monthly maintenance training sessions in which the location of the platform was rotated around the four quadrants from session to session. For the current experiment, escape latency values from the last two maintenance sessions preceding the treatment period were averaged for each animal and the obtained mean was considered as baseline performance and used in the group allocation process. During the treatment period MWM task was performed on Day 5/6 and 13 (*see* Fig 5) with different platform locations (north-west and north-east, respectively).

Cooperation paradigm

Social learning was assessed in a cooperative task as described in Gáspár et al. (97). Pairs of animals participated in the task, which was carried out in a Skinner box measuring 30×24×21 cm (MedAssociates, VT, USA). The opposite walls of the chamber were equipped with one nose-poke module, one lever and one magazine for each. The two animals were separated by a dividing fence in the midline of the chamber. A trial started with lights turning on in the nose-poke modules for a maximum of 15 seconds. Any of the animals could initiate the task by continuously nose-poking the module for 3 seconds, which then activated the lever on the opposite side for 15 sec. If the other animal pressed the lever within this period, a reward pellet was delivered for both rats and a new trial started. An omission response was recorded if the rats failed to perform a nose-poke or lever press. Incorrectly timed or out-of-sequence responses resulted in a 5-second

timeout. Learning performance was characterized by the percentage of rewarded trials. Each daily test session lasted 20 minutes.

Animals started to learn cooperation at 6.5-7 months of age. Within 2 months training, most pairs acquired the task, and after that they took part in weekly maintenance sessions. For establishing a baseline for the current experiment learning performance values in the last 3 sessions preceding the treatment period were averaged, and these means were also used for treatment allocation. Cooperation tasks were run on Treatment Day 3, 7 and 11 (see Fig 5).

5-choice serial reaction time task

The 5CSRTT apparatus featured a test box measuring 31×35×34 cm (TSE Systems, Bad Homburg vor der Höhe, Germany). Task was based on method described in Gáspár et al (97). The boxes included five nose-poke modules on the back wall and a magazine on the front wall. During the task, following a 5-second inter-trial interval, a 1-second light stimulus appeared in one randomly chosen nose-poke module, prompting the animal to nose-poke into the indicated hole. A response was considered correct if the animal nose-poked into the designated hole during the stimulus or within 5 seconds afterward (limited hold). Correct responses were rewarded with a pellet delivered into the magazine, and nose-poking into the magazine initiated the next trial. An incorrect response occurred if the animal nose-poked into any hole where the stimulus was absent. An omission was recorded if the rat did not nose-poke within the limited hold period. Incorrect responses and omissions resulted in a 5-second time-out punishment, during which the house light was turned off. After the time-out, the house light was turned back on, and the rat could start the next trial by nose-poking into the magazine. A premature response was recorded if the animal nose-poked into any hole during the inter-trial interval, also resulting in a time-out punishment. Each daily test session lasted 20 minutes. The measured outcomes included the percentages of omission, correct, and premature responses, and accuracy. Definitions for each were the following formulas:

$$\text{correct \%} = (\text{correct responses} / (\text{omissions} + \text{correct responses} + \text{incorrect responses})) \times 100$$

$$\text{omission \%} = (\text{omissions} / (\text{omissions} + \text{correct responses} + \text{incorrect responses})) \times 100$$

$premature \% = (premature\ responses / number\ of\ trials) \times 100$

$accuracy \% = ((correct\ responses / (correct\ responses + incorrect\ responses)) \times 100).$

Animals began to learn 5CSRTT in the first half year of their lives and acquired the skills to perform the abovementioned task in an over 1-month-long training period. Afterwards they performed weekly maintenance tests. For the current experiment, the baseline was determined by averaging data from the last three sessions preceding the treatment. For treatment allocation the correct and premature response ratio was used from these averages. During treatment 5CSRTT was carried out on Day 2, 8 and 13 (*see* Fig 5).

Pot jumping

The method was based on the study of Ernyey et al. (94). In the Morris water maze tank, twelve flowerpots (16 cm high and 10 cm wide at the bottom) were positioned upside down to form a circle. The distance between the centres of adjacent pots increased gradually from 18 to 46 cm in a counterclockwise direction. The tank was filled with water to a depth of 6 cm to prevent rats from climbing off the pots. During each session, the animals were placed on the starting pot, which was the closest to the next pot. For three minutes, the rats could move freely on the pots while their behaviour was observed and recorded with a video camera system. The key outcome parameters were the longest distance jumped between pots and the number of passes.

Pot jumping started around the age of 3.5-4 months. Maintenance sessions were carried out weekly. Baseline was determined by the last two sessions prior to treatment. For treatment allocation two variables, longest distance travelled, and number of passes were used. Pot jumping was assessed on Day 5/6 and 10 (*see* Fig 5).

Touchscreen - Pairwise visual discrimination

The experiment was conducted in a touchscreen apparatus (Campden Instruments Ltd., Lafayette, IN, USA, cat. no. 80604) according to the method in Gáspár et al (97). Each box featured a touch screen at the front and a magazine at the back. The touchscreen could be split into two sections with a cover panel. Subjects were trained to distinguish between two images (one correct and one incorrect) that appeared randomly in the left or right

window of the touchscreen. Half of the animals got Image1 as a correct image, for the other half the Image2 was correct. Selecting the correct image with a nose-poke resulted in a pellet reward, while choosing the incorrect image caused a 5-second timeout during which the house light turned on. The appearance of the two images for the next trial was triggered by the subjects entering and exiting the food magazine. Each daily test session lasted 20 minutes, and the ABET II touch v2.15 software recorded the number of completed trials as well as correct and incorrect responses.

Similarly to 5CSRTT, animals learned the touchscreen task in the first half year of their lives and acquired the skills to perform the final stage described above in an over 1-month-long training period. Later they performed maintenance tests weekly, as well. For the current experiment, the baseline was derived from averaging data from the last three sessions preceding the treatment. For treatment allocation the correct response ratio was used. After the start of the treatment the touchscreen task was conducted on Day 4, 9 and 12 (*see* Fig 5).

Y-maze alternation test

Experimental set-up was the same as described in Exp 2.

Measurements were carried out three times: between 8-11 days before the treatment period (baseline), on Day 4 and Day 14 right after the last injection (*see* Fig 5).

3.4.3. Cerebral blood flow measurement

CBF was measured in the same mode as described in Exp 1 on Day 14

3.4.4. Blood pressure measurement

After 3 habituation measurements within two weeks preceding the treatment period, baseline was recorded on Day 1 prior to the first injection. Additional measurements were carried out on Day 3, 6, 9 and 12 (*see* Fig 5).

3.4.5. Tissue collection and sampling

Brain hemispheres (excluding the cerebellum), upper part of small intestine and both kidneys were collected after the Doppler-probing of the brain, in the same manner as described in Exp 1.

Samples for histological analysis were put inside tissue cassettes, placed into 4 % paraformaldehyde then within 48 hours into 70 % alcohol. Later these were dried and embedded in paraffin wax in preparation for tissue slicing and staining (HistoCore Arcadia C - Cold Plate and HistoCore Arcadia H - Heated Paraffin Embedding Station, Leica, Wetzlar, Germany).

3.4.6. PGE₂ and 11,12-EET level determination

For the measurement of PGE₂ and 11,12-EET levels, the same methods were used as described in Exp 1 and Exp 2, respectively.

3.4.7. Section preparation and staining

Kidney

Samples embedded in paraffin blocks were cut with a microtome (RM2245 Semi-Automated Microtome, Leica, Wetzlar, Germany), then these sections were mounted onto glass slides (Apex Superior Adhesive Slide) in a water bath (Leica HI1210 - Water Bath, Wetzlar, Germany) and finally dried on a heating pad (Leica HI1220 - Slide dryer, Leica, Wetzlar, Germany). After complete de-paraffinization and rehydration, staining was done via Harris haematoxylin and counterstaining via eosin. Histological alterations were scored by type and according to their extension in the section (see Table 4). These scores were then summed up for each individual to get a variable for quantifying between group difference.

Brain

Six brain samples were randomly chosen from each group for immunostaining. Brains were immersion-fixed in 4 % paraformaldehyde in 0.1 M (PBS), embedded in paraffin

and 6 µm thick tissue sections were cut mounted on pre-coated glass slides (StarFrost). Shortly after deparaffinisation and rehydration, the sections were pre-treated with 0.3 % Triton-X 100 for 1 h at 22–24 °C to enhance the penetration of antibodies. Non-specific immunoreactivity was blocked by incubating our specimens in a cocktail of 5 % normal donkey serum (Jackson, Cambridge, United Kingdom), 2 % bovine serum albumin (Sigma, Darmstadt, Germany) and 0.3 % Triton X-100 in PBS for 1 h at 22–24 °C. Sections were incubated for (16 h at least at 4 °C) to select combinations of the primary guinea pig antibodies anti-vesicular glutamate transporter type 1 (VGLUT1, 1:5000, Synaptic Systems, Göttingen, Germany) and anti-glial fibrillary acidic protein (GFAP, raised in rabbit, 1:2000, Synaptic Systems, Göttingen, Germany) diluted in PBS to which 0.1 % NDS and 0.3 % Triton X-100 had been added. After extensive washing, immunoreactivities were determined by carbocyanine 2 or 3-coupled secondary donkey antibodies (1:200 [Jackson], 2 h at 22–24 °C). Glass-mounted sections were cover-slipped with glycerol/gelatine (Sigma, Darmstadt, Germany). Sections were inspected and images acquired on a 780LSM confocal laser-scanning microscope (Zeiss, Jena, Germany) at 20x primary magnification with emission spectra for each dye were limited as follows: Cy2 (505–530 nm) and Cy3 (560–610 nm).

3.5. Exp 4: *Isolation of MS-PPOH's effects in young, naïve Long-Evans rats*

Subjects in this experiment were 16 male Long-Evans rats (Janvier Labs, France, 380–460 g, 6.5-months-old) housed four per cage. Food access was restricted: 60 g/cage supplied at least 2 h before the end of the dark phase.

3.5.1. Treatment

In Exp 4, MS-PPOH was administered intraperitoneally for 9 consecutive days, twice daily (at 5 am and 5 pm) except on Days 1 and 9 when only one injection was given in the evening and in the morning, respectively (*see* Fig 6). The dosage was 5 mg/kg, the molecule was dissolved in 20 % HPβCD and was administered in 1 ml/kg volume. Odd-numbered animals were allocated to the control groups, even-numbered rats to the

MS-PPOH group. This resulted in less than 1 % baseline weight difference between groups. Separate persons performed the injections and the assays; those who did the latter were not aware of which treatment the animals received.

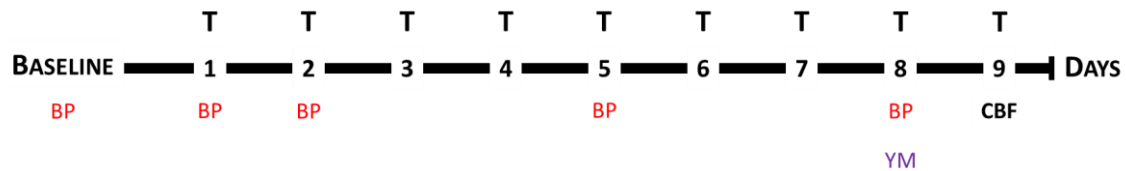


Figure 6. Timeline of Exp 4. All interventions are indicated by corresponding abbreviations on days when they were performed. BP: blood pressure measurement, YM: Y-maze alternation test, CBF: cerebral blood flow measurement, T: treatment.

3.5.2. Cognitive assay

Y-maze alternation test

The test was performed the same way as described in Exp 1. on Treatment Day 8 (see Fig 6).

3.5.3. Cerebral blood flow measurement

CBF was measured in the same mode as described in Exp 1 on Day 9.

3.5.4. Blood pressure measurement

After 3 habituation measurements within a week preceding the treatment period, baseline was recorded on Treatment Day 1 prior to the first injection. Further measurements were carried out on Day 2, 5 and 8 (see Fig 6).

3.5.5. Tissue collection and sampling

Brain hemispheres (excluding the cerebellum), upper part of small intestine and kidneys were collected after the CBF measurements.

Tissue samples for ELISA tests were preserved the same way as detailed in Exp 1.

3.5.6. 11,12-EET level determination

ELISA measurements for EET levels and kidney tissue staining (6 samples from each group) were done as detailed in Exp 2 and 3, respectively.

3.6. **Exp 5:** *Conditioned taste aversion (CTA) testing in aged rats to investigate possible gastrointestinal malaise effects*

To investigate the possible adverse gastrointestinal effect of the treatment cocktail we performed a supplementary conditioned taste aversion (CTA) experiment. First, in order to validate the CTA method in our hands, we previously carried out a CTA test using the standard positive control, lithium-chloride.

We conducted this study with 12 naïve male Hannover Wistar rats (Toxicoop, Budapest, Hungary) weighing 240-260 g and being 2-2.5 months of age at the start of the experiment.

On Day 1, water was withdrawn from the group-housed animals for a 20-hour water deprivation period. On Day 2, animals were separated into individual cages, and two water bottles were introduced (for 4 hours) to measure baseline water consumption and to assess drinking patterns for potential side preferences. Day 3 involved taste aversion conditioning: animals got access to two bottles containing 0.3 % saccharin solution. An hour later the animals were ip. administered 1.2 ml LiCl solution (30 mg/ml, Reanal Laboratory Chemicals Ltd., Budapest, Hungary) or saline. Afterwards, the saccharin bottles were available for an additional hour. On Days 4 and 5, animals were presented with a saccharin solution-containing and a water-containing bottle (Table 2). Fluid consumption was measured for four hours to determine saccharin preference: $(=\text{saccharin solution consumption} / (\text{saccharin solution} + \text{water consumption}) \times 100)$.

Table 2. Design of the LiCl validation test. Daily breakdown of procedures and key actions that took place.

Day	Procedure	Key Actions
1	Water deprivation	Initiate fluid restriction
2	Baseline water intake measurement	Record normal water consumption
3	Conditioning phase	Saccharin access → LiCl/vehicle injection
4-5	Aversion test	Free choice: Saccharin vs. water

For the main CTA experiment we used 11 aged male Hannover Wistar rats (Toxicop, Budapest, Hungary) weighing 410-550 g at 27-27.5 months of age.

For the treatment we used 5 mg/kg MS-PPOH, 1 mg/kg indomethacin and 10 mg/kg L-NAME dissolved in 20 % cyclodextrin + 1 % NaHCO₃. Control animals received 20 % HPβCD. Animals were treated ip. The experiment spanned six days (Table 3), during which animals were individually housed and subjected to controlled feeding and fluid intake measurements. Food pellets were provided at 15 g per animal daily, with uneaten food weighed on conditioning and test days (Days 3–6). Animals were randomly allocated to the two treatment groups. Blinding was not feasible because rats required immediate treatment after the first drinking measurement, as the treatment was embedded in the measurement procedure carried out by a single experimenter.

Water deprivation (20 hours) began on Day 1, followed by a four hour-long baseline water consumption measurement on Day 2 to establish individual drinking patterns. Conditioning was conducted on Days 3 and 4. Animals were provided with two bottles of 0.3 % saccharin solution (Reanal Laboratory Chemicals Ltd., Budapest, Hungary), available for 2 hours. On Day 3, after 1 hour of saccharin consumption, subjects were treated with either the cocktail or vehicle solution. Thereafter, the saccharin solution was accessible for an additional hour. On Day 4 the same treatment preceded the saccharin access and subsequently, the bottles were provided for two hours. On Days 5-6, animals

were provided with a bottle of saccharin solution and a bottle of water for a period of 4 hours to evaluate saccharin preference. Solution consumption was monitored by periodic bottle weighing, and residual food weight was recorded. Saccharin preference was determined as the following:

$$\text{saccharin preference} = \text{saccharin solution consumption} / (\text{saccharin solution} + \text{water consumption}) \times 100$$

Table 3. Design of the CTA experiment. Daily breakdown of procedures and key actions that took place.

Day	Procedure	Key Actions
1	Water deprivation	Initiate fluid restriction
2	Baseline water intake measurement	Record normal water consumption
3–4	Conditioning phase	Saccharin access → ,cocktail'/vehicle injection
5–6	Aversion test	Free choice: Saccharin vs. water

3.7. Statistical evaluation

In the NVU experiment(s) results obtained with variables that were repeatedly measured (cognitive performance, blood pressure) were evaluated by repeated measures analysis of variance (ANOVA). Post-hoc Newman-Keuls test in Exp 1 and Duncan test in Exp 2-4 was carried out when either a significant treatment effect or a significant interaction was calculated. Results of assays performed at a single time point (CBF, ELISA, immune staining) were evaluated by unpaired t-test. Mann-Whitney U-test was applied for the histological scores of the kidney tissue staining. Saccharin preference in the CTA test was evaluated by unpaired t-test. All results are presented as mean ± SEM. Differences were considered statistically significant at $p < 0.05$.

4. Results

4.1. Exp 1: *Dose-finding and protocol optimization in young, naïve Hannover Wistar rats*

Exp 1 was designed to establish whether the triple cocktail could induce neurovascular uncoupling in rats, using doses scaled down from the mouse protocol. We assessed CBF response, cognitive performance, blood pressure, and tissue biochemistry. The results confirmed the sought-after effects but revealed dose-limiting side effects.

No significant changes were caused by the treatment in the performance of rats either in the Morris water maze or the fear conditioning paradigm (*see* Fig 7c, d).

However, as a side effect, the treatment significantly elevated blood pressure in the animals (Treatment: $F(1,25) = 7.405$, $p < 0.05$; Time: $F(3) = 8.244$, $p < 0.001$) (Fig 7b), and caused alterations (inflammation, bleeding, ulcers) in the small intestine in 8 out of 14 rats.

Nevertheless, the treatment caused 54 % inhibition in the induced hyperaemia in the barrel cortex when the contralateral whisker pad was stimulated ($p < 0.05$ (t-test), *see* Fig 7a).

PGE₂ levels were significantly lower in the barrel cortex ($t(22)=7.272$, $p < 0.001$) and small intestine ($t(22)=2.888$, $p < 0.01$) compared to control tissue samples. (*see* Fig 7e, f).

One cocktail-treated animal died on Day 3 of the treatment (after 5 injections), with no particular symptoms (14 grams (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.

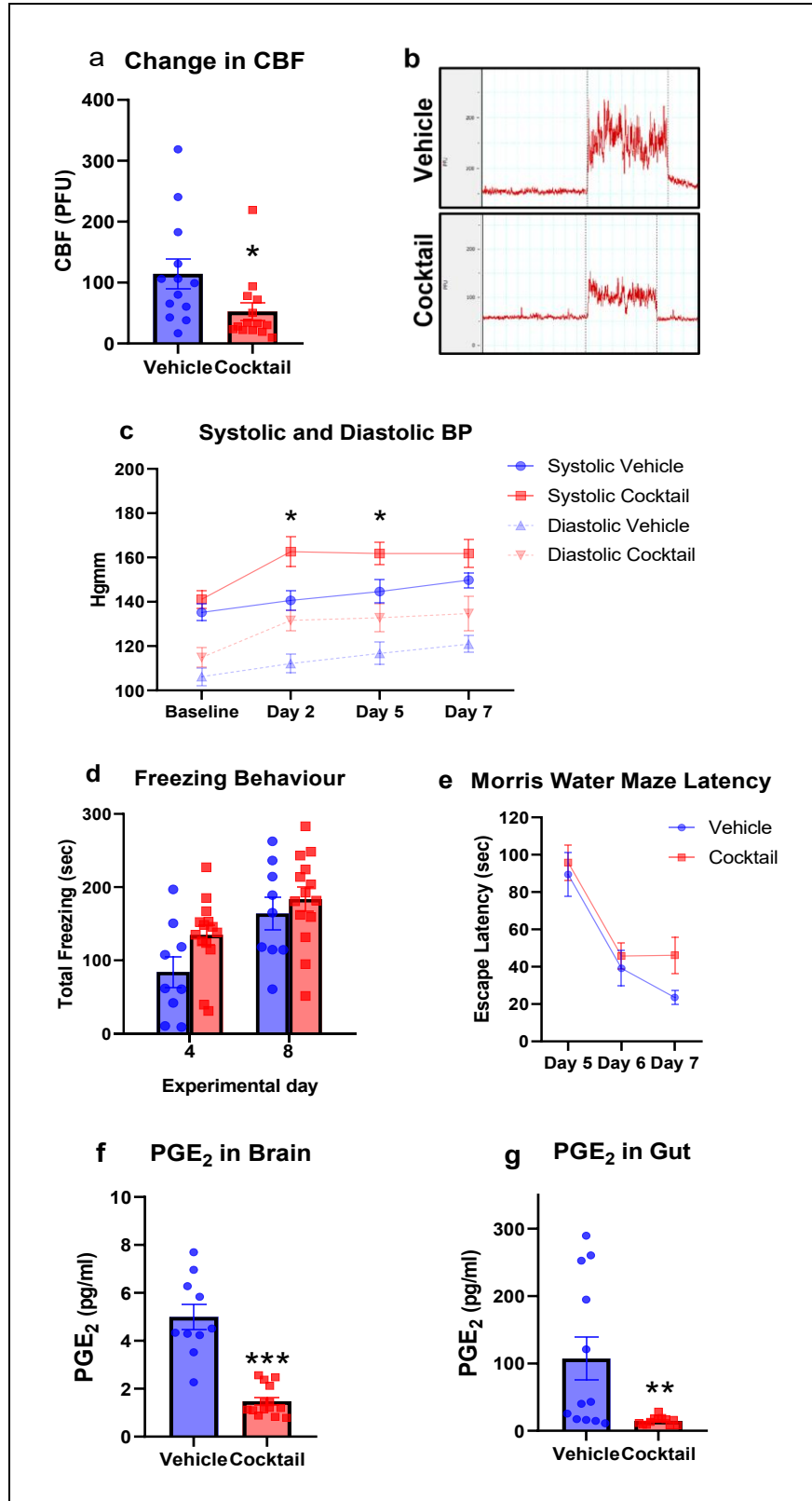


Figure 7. CBF reduction, blood pressure elevation, and PGE₂ target engagement in Exp 1. **a)** Increase in CBF in the barrel cortex in response to whisker stimulation (mean $\pm$ SEM). **b)** exemplary measurement recordings, showing change of CBF (PFU) during a

stimulation period. The vertical dotted lines mark the start and end of such periods, outlining 30 seconds of recordings; 'vehicle': vehicle-treated animal's recording, 'cocktail': cocktail-treated animal's recording. **c)** Systolic and diastolic blood pressure over the course of vehicle or cocktail treatment (mean $\pm$ SEM). In the systolic measurement *: $p < 0.05$ vs 'vehicle', post-hoc Newman-Keuls test after repeated measures ANOVA. **d)** Freezing time in fear conditioning (mean $\pm$ SEM). The first measurement on Day 4 and the second on Day 8 were the Acquisition (Acq) and Retention (Ret) sessions, respectively. **e)** Escape latency in the Morris water maze task (mean $\pm$ SEM). **f-g)** Changes in PGE₂ levels in the brain and small intestine, respectively (mean $\pm$ SEM). ** and ***: $p < 0.01$ and $p < 0.001$, respectively, vs 'vehicle', t-test.

4.2. Exp 2: Further dose refinement in young, naïve Wistar rats with expanded cognitive testing

Given the toxicity observed in Exp 1, we halved all doses to improve tolerability while expanding the cognitive battery. However, this dose reduction also eliminated the NVU effect, highlighting the narrow therapeutic window. We measured CBF, working memory, blood pressure, and EET levels to assess whether lower doses could maintain efficacy.

There was no significant difference between groups in the mean change of CBF in the barrel cortex in response to the stimulation of the contralateral whisker pad (Fig 8a). We also did not see a significant difference in the systolic (see Fig 8c) or the diastolic blood pressure in the two groups although an overall tendency of increase can be seen in the cocktail treated group. This group also showed a significantly lower heart rate (see Fig 8d) compared to controls ($F(1,15)=13.24$, $p < 0.005$). Post-hoc analysis (Duncan test) revealed a significant difference between the two groups on Day 2 ($p < 0.05$). There was no significant difference in EET levels neither in hippocampus nor in barrel-cortex samples (see Fig 8e, f). No significant difference was observed in the Y-maze test (see Fig 8b). Unfortunately, very few animals produced valid data in the lever press and NOR assays, so the outcome of these tests was not evaluable.

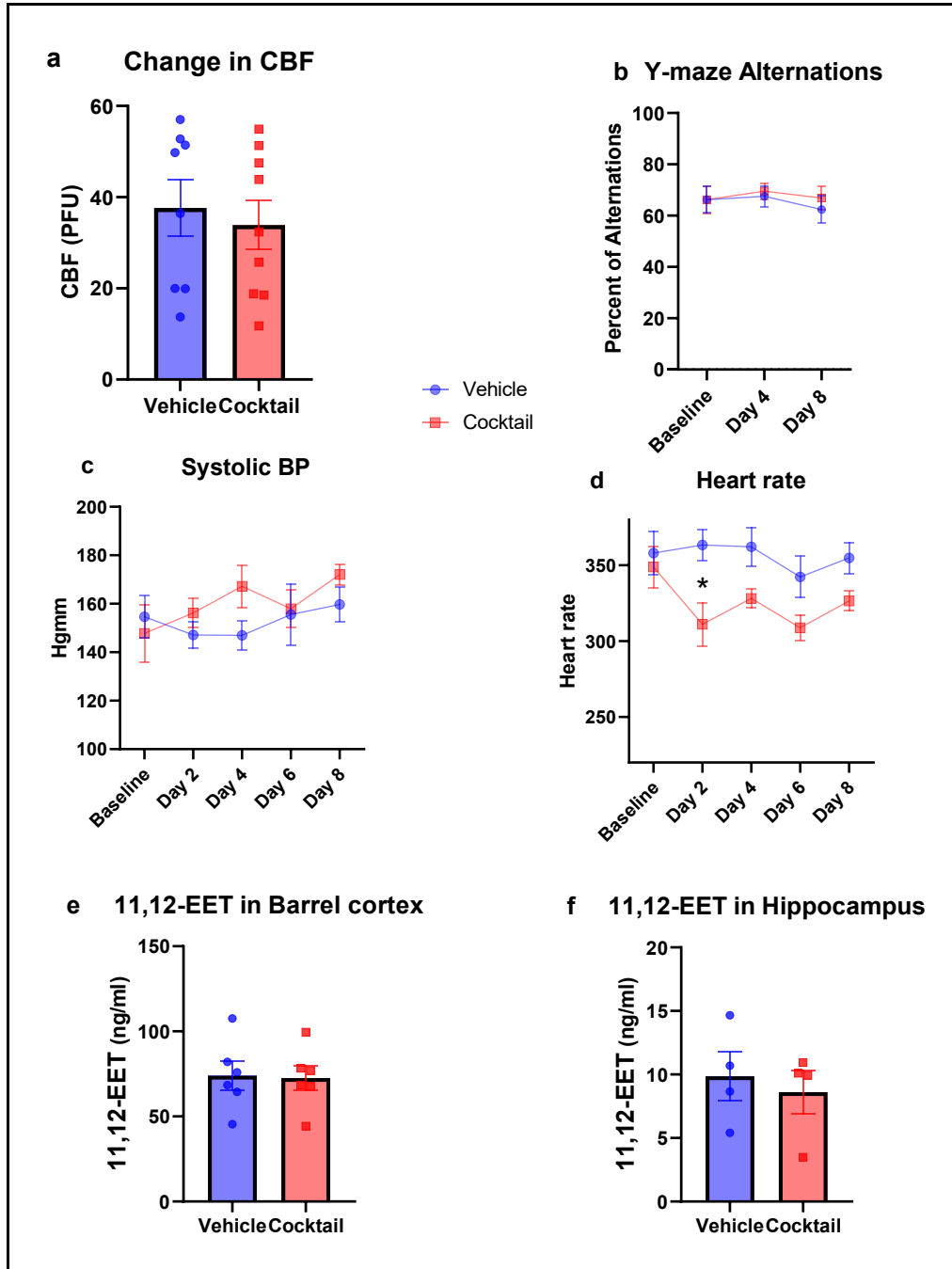


Figure 8. No CBF reduction, cognitive impairment, or EET changes at halved doses in Exp 2. **a)** Increase in CBF in the barrel cortex in response to whisker stimulation (mean $\pm$ SEM). **b)** Percent of alternations in the Y-maze (mean $\pm$ SEM). **c) and d)** Systolic blood pressure and heart rate, respectively, over the course of vehicle or cocktail treatment (mean $\pm$ SEM). *: $p < 0.05$ vs 'vehicle', post-hoc Duncan test after repeated measures ANOVA. **e) and f)** 11,12-EET levels in the barrel cortex and hippocampus (respectively) (mean $\pm$ SEM).

4.3. Exp 3: Validation in aged, experienced Long-Evans rats to assess translational relevance

Exp 3 tested the cocktail in our most translationally relevant population: aged, cognitively experienced rats. We used intermediate doses and adjusted formulations during the experiment to manage appearing toxic effects. Despite these challenges, we observed transient attentional deficits, though CBF reduction was non-significant. Biochemical analysis confirmed PGE₂ reduction, but EET levels remained unchanged. Histology revealed severe nephrotoxicity in treated animals.

The uncoupling cocktail only caused a non-significant, 28 % decrease in the hyperaemia induced by whisker stimulation and measured in the barrel cortex (*see* Fig 9). In three vehicle-treated and 3 cocktail-treated animals we could not detect measurable signals in response to whisker stimulation; thus, they dropped out from this measurement.

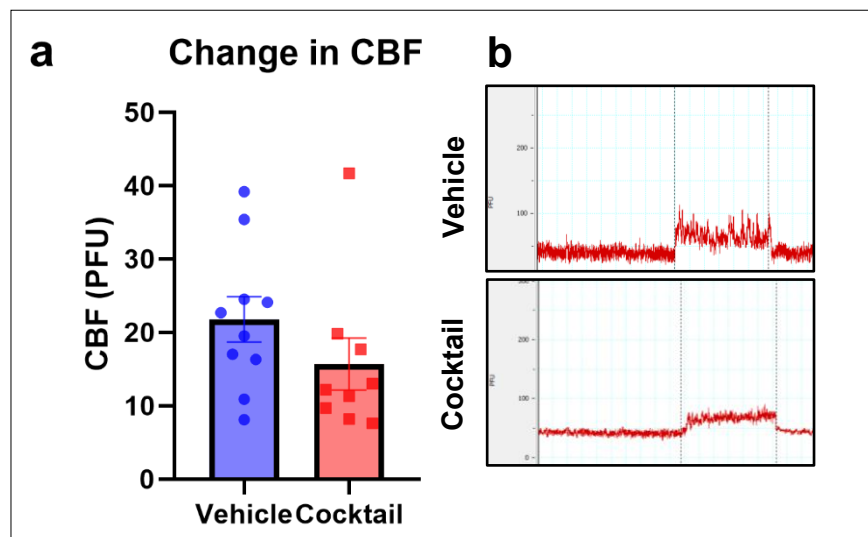


Figure 9. CBF response: non-significant 28% reduction in aged rats in Exp 3. and **a)** increase in CBF in the barrel cortex in response to whisker stimulation (mean $\pm$ SEM) **b)** exemplary measurement recordings, showing change of CBF (PFU) during a stimulation period. The vertical dotted lines mark the start and end of such periods, outlining 30 seconds of recordings; ‘vehicle’: vehicle-treated animal’s recording, ‘cocktail’: cocktail-treated animal’s recording.

The most conspicuous effect of the treatment was the decreased food intake and the consequent weight loss in the cocktail-treated group (Fig 10a). As we strived to take all the cocktail treated animals over the course of the experiment this outcome necessitated to introduce breaks in the series of injections at certain timepoints to avoid too much decrease in the body mass and let the animals to recover weight. As a result, animals in the cocktail treated group received varying cumulative doses of the components: between 50 and 75 mg/kg of MS-PPOH, 100 to 138 mg/kg of L-NAME and between 10 and 13.8 mg/kg of indomethacin. Table 1 shows the exact number of cocktail injections and the cumulative dose of the cocktail components which each rat received. Even with these precautionary measures the decrease in body mass by the end of treatment was 7.8 % (32 g) in the cocktail treated group while it was only 2.8 % (12 g) in the vehicle treated group; the difference was significant (t-test, $t(23)=-3.066$, $p < 0.01$).

There were not consistent changes in the blood pressure and heart rate of the animals (Figure 10b-d). No significant effects were detected in systolic blood pressure, while a significant Time*Treatment interaction was found for diastolic blood pressure ($F(4,92)=2.912$, $p < 0.05$), due to a substantially lower value in the vehicle-treated group on Day 3. ANOVA showed a significant Treatment effect in heart rate measurements ($F(1,23)=2.912$, $p < 0.01$); however post hoc analysis only revealed significant difference between the two groups on Day 6.

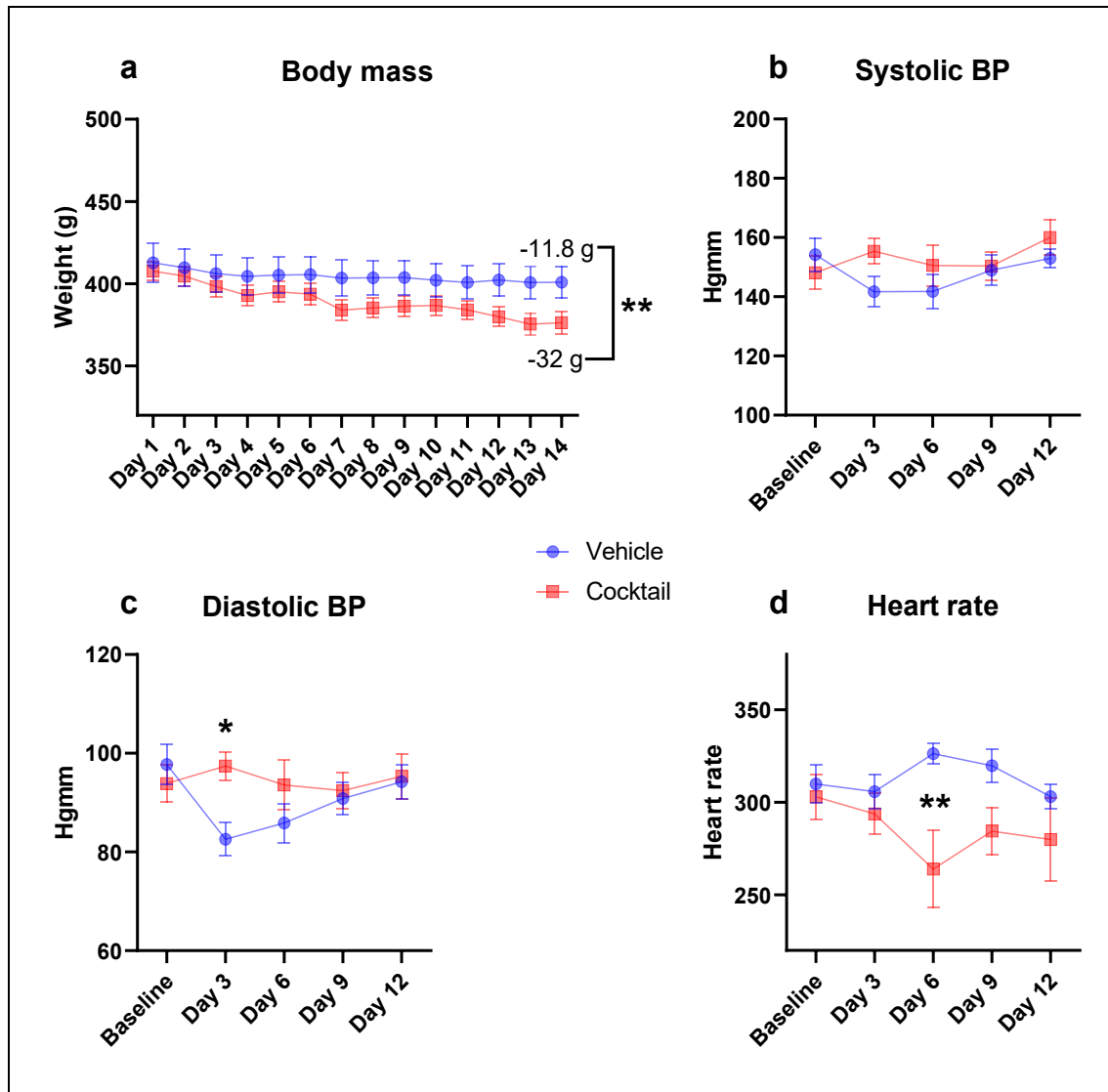


Figure 10. Significant weight loss in treated animals with minimal blood pressure changes in Exp 3. **a)** Change in body mass of the animals during the treatment period (mean $\pm$ SEM). **: $p < 0.01$ vs 'vehicle', t-test. **b), c) and d)** systolic, diastolic blood pressure and heart rate, respectively, over the course of vehicle or cocktail treatment (mean $\pm$ SEM). * and **: $p < 0.05$ and $p < 0.01$.

PGE₂ levels were significantly lower in the 'Cocktail' group both in the brain ($t(22)=-2.954$, $p < 0.01$) and in the small intestine ($t(17)=-3.991$, $p < 0.001$) (Fig 11a, b). On the other hand, no significant alteration was caused by treatment in the case of 11,12-EET levels, in neither sample type (Fig 11c, d).

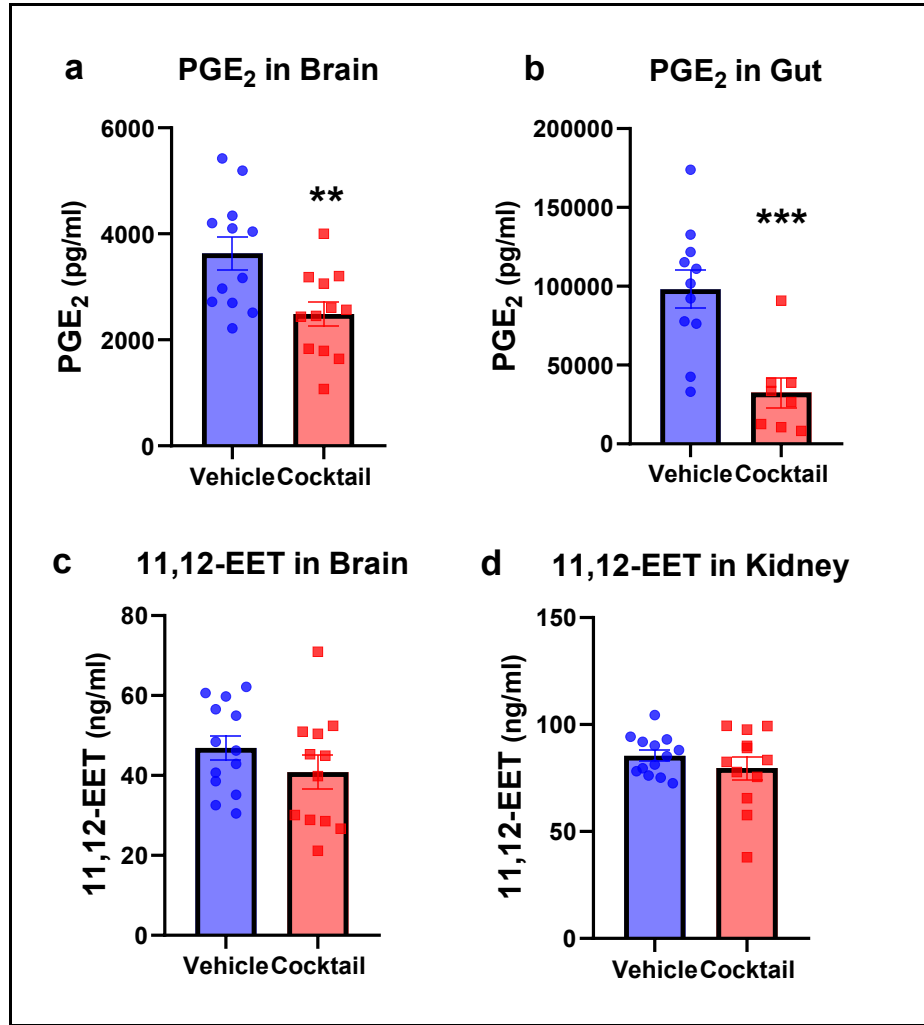


Figure 11. PGE₂ reduced in brain and intestine; 11,12-EET unchanged in brain and kidney in Exp 3. **a) and b)** PGE₂ levels in the brain and small intestine, respectively (mean ± SEM). **c) and d)** 11,12-EET levels in the brain and kidney, respectively (mean ± SEM). ** and ***: $p < 0.01$ and $p < 0.001$, respectively, vs 'vehicle', t -test.

Among the cognitive tasks, significant group differences were only observed in the 5CSRTT measurements (Fig 12a, b). Significant Treatment effects were detected in the percent correct responses ($F(1,23)=6.947$, $p < 0.05$), percent omission responses ($F(1,23)=4.2524$, $p=0.0507$) and accuracy ratio ($F(1,23)=7.242$, $p < 0.05$). In addition, a significant Time effect occurred in the percent correct and omission responses ($F(3,69)=18.902$, $p < 0.001$ and $F(3,69)=23.286$, $p < 0.001$, respectively), and a significant Time x Treatment interaction in the accuracy ratio ($F(3,69)=4.149$, $p < 0.01$).

Correct response rate and accuracy ratio were significantly lower, whereas omission rate was higher in the cocktail-treated group on Day 8.

In MWM, cocktail-treated rats showed a notably higher latency on Day 3 than vehicle-treated rats, but ANOVA did not reveal significant treatment effect or interaction (Fig 12d). In the other variables, such as percentage of correct responses in the pairwise discrimination task (Fig 12c), total arm entries (Fig 12e) and spontaneous alternation percentage (Fig 12f) in the Y-maze, percentage of successful trials in the cooperation task (Fig 12g), and longest distance (Fig 12h) and number of jumps (Fig 12i) in the pot jumping test, no notable group difference could be observed.

However, in all assays a decrease in performance from baseline was observed at the first measurement occasion of the treatment period in both groups. This decrease was significant in the 5CSRTT correct % and omission % responses (Fig 12a), Y-maze arm entries (time effect $F(2,46)=55.215$, $p<0.001$; Fig 12e) and spontaneous alternations (time effect $F(2,46)=5.298$, $p<0.01$; Fig 12f), and pot jump longest distance (time effect $F(2,46)=25.291$, $p<0.001$; Fig 12h) and number of jumps (time effect $F(2,46)=27.683$, $p<0.001$; Fig 12i) variables.

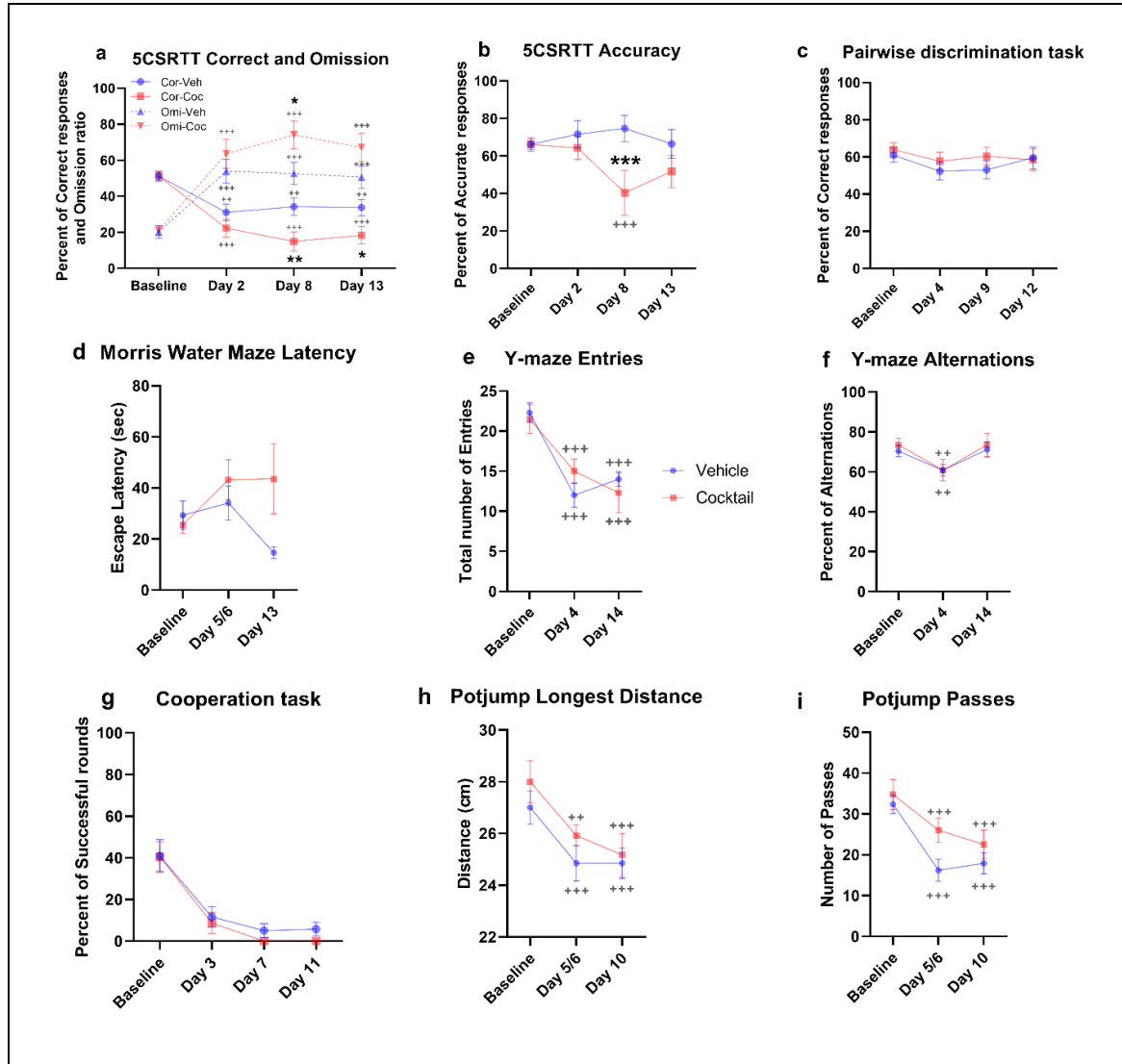


Figure 12. Transient attentional deficits in 5CSRTT with no other cognitive impairments in Exp 3 over the course of vehicle or cocktail treatment. **a)** Correct response percentage and omission percentage in the 5CSRTT task. **b)** Accuracy ratios in the 5CSRTT task. **c)** Correct response rate in the pairwise discrimination task. **d)** Escape latency in the Morris water maze task. **e) and f)** Number of entries and percent of alternations, respectively in the Y-maze. **g)** Percentage of successful trials in the cooperation task. **h) and i)** Longest distance covered and number of passes in the pot jump test. Mean $\pm$ SEM values are shown. *, ** and ***: $p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively, vs 'vehicle'; +, ++ and +++: $p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively, vs 'Baseline'; post-hoc Duncan test after repeated measures ANOVA

For assessing eventual morphological changes caused by the treatment, we investigated the hippocampus, a vulnerable archicortical brain area (Fig 13).

Treatment with the cocktail did not change the density of GFAP⁺ astrocytes in the CA1 region (663.34 ± 52.04 [cocktail] vs. 599.91 ± 51.59 [vehicle], number of astrocytes / 1 mm^2 , $p = 0.42$). The area covered by the processes of individual GFAP⁺ astrocytes were similar in both groups (863.79 ± 46.91 [cocktail] vs. 853.27 ± 43.78 [vehicle], μm^2 , $p = 0.86$). Treatment caused no changes in VGLUT1 fluorescent intensity in the stratum radiatum of the CA1 region (60.44 ± 4.31 [cocktail] vs. 67.19 ± 3.86 [vehicle], arbitrary units, $p = 0.25$).

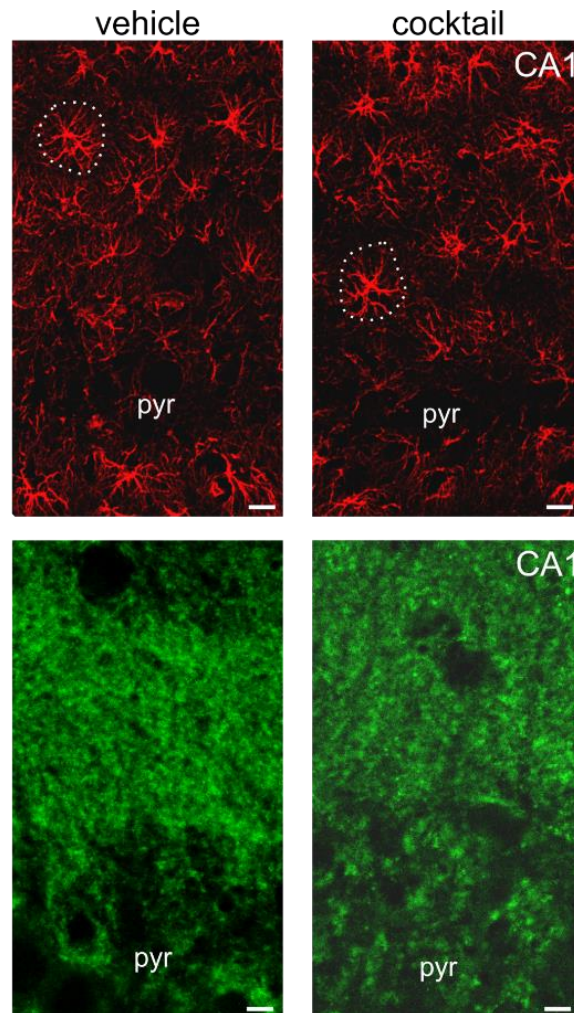


Figure 13. Brain histology: no changes in astrocytes or synaptic terminals. GFAP-immunoreactive astrocytes (red) and VGLUT1-immunoreactive terminals in the CA1 region of the hippocampus. Dashed lines in top images surround the fields which are

covered by the processes of individual astrocytes. Abbreviations: CA1 cornu ammonis 1 region, pyr=pyramidal layer. Scale bar: 10 μ m.

We extended our histological analysis to peripheral tissues, focusing specifically on renal morphology. Kidney sections from the cocktail-treated group exhibited marked histological alterations (see Fig 14) compared to vehicle-treated controls (mean histology scores: 10.5 ± 0.96 vs. 1.7 ± 0.36 , respectively; $p < 0.001$, Mann-Whitney U-test). For the scoring and categorizations of specific types of renal alterations, see Table 4.

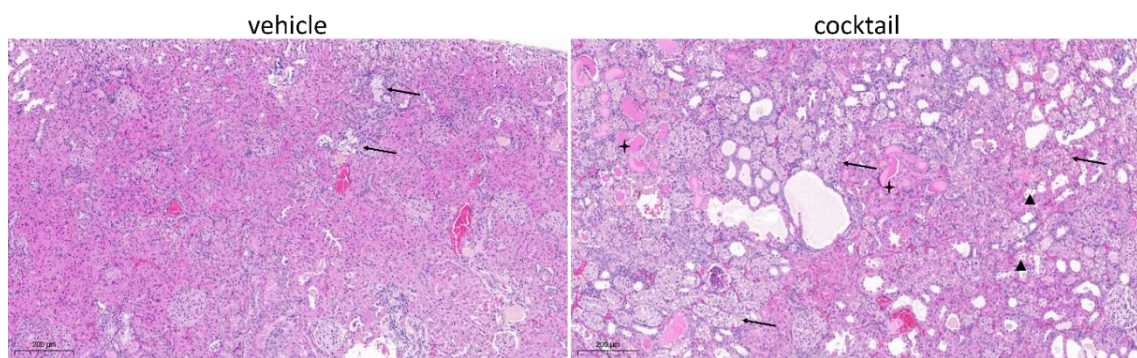


Figure 14. Kidney histology: severe nephrotoxicity in cocktail-treated animals. Example sections of kidney histology (haematoxylin - eosin staining). 'Vehicle': section from a vehicle-treated rat (#A). Focal vacuolation of tubular epithelium (arrow). 'Cocktail': section from a cocktail-treated rat (#13). Multifocal vacuolation of tubular epithelium (arrow). Multifocal tubular dilation and eosinophilic casts in the tubular lumen (asterisk). Apoptotic cells in the tubules (arrowhead). Scale bar: 200 μ m.

Table 4. Scoring of alterations caused by treatment in kidney tissue sections. Assigned score for each category correspond to the following: 0 = No pathological changes, 1 = Minimal changes (affecting ≤ 10 % of the section), 2 = Mild changes (affecting 11–25 % of the section), 3 = Moderate changes (affecting 26–50 % of the section), 4 = Severe changes (affecting > 50 % of the section).

vehicle-treated	animal ID											
Alteration	1	1C	C	11	20	E	2	4	5	6	A	15
Vacuolization of tubular epithelium (focal)	0	1	0	1	0	0	1	1	1	0	1	1
Vacuolization of tubular epithelium (multifocal or diffuse)	0	0	0	0	0	2	0	0	0	0	0	0
Tubular dilatation	0	0	0	0	0	0	0	0	0	1	1	0
Eosinophil casts in the tubular lumen	0	0	0	0	0	0	0	0	0	0	0	0
Brown pigments in the tubular epithelium	0	0	0	0	0	1	2	2	1	1	1	1
Apoptosis in the tubular epithelium	0	0	0	0	0	0	0	0	0	0	0	0
Apoptosis in the medulla	0	0	0	0	0	0	0	0	0	0	0	0
Total score	0	1	0	1	0	3	3	3	2	2	3	2
cocktail-treated	animal ID											
Alteration	0	8	12	13	14	1A	21	3	19	1F	1E	
Vacuolization of tubular epithelium (focal)	0	0	0	0	0	0	0	0	0	0	0	
Vacuolization of tubular epithelium (multifocal or diffuse)	3	3	3	2	2	2	0	3	2	2	2	
Tubular dilatation	2	2	1	4	3	2	2	3	3	2	2	
Eosinophil casts in the tubular lumen	2	2	1	3	2	2	1	1	1	2	2	
Brown pigments in the tubular epithelium	3	3	3	3	2	2	0	0	1	0	0	
Apoptosis in the tubular epithelium	1	3	1	1	1	1	0	0	1	1	1	
Apoptosis in the medulla	0	2	3	2	1	3	1	1	1	2	2	
Total score	11	15	12	15	11	12	4	8	9	9	9	

4.4. Exp 4: *Isolation of MS-PPOH's effects in young, naïve Long-Evans rats*

To determine whether MS-PPOH contributed to the observed mild efficacy, we tested it alone at 10 mg/kg/day in young rats. We measured CBF, Y-maze performance, blood pressure, 11,12-EET levels, and kidney histology. The results confirmed that MS-PPOH alone is ineffective at engaging its target in the brain.

The cocktail-treated group exhibited a greater mean change from baseline in CBF compared to controls due to two outlier values, however, this difference was not statistically significant (Fig 15).

No significant effects were observed for either systolic blood pressure or heart rate across measurements. However, a significant time effect was revealed on diastolic blood pressure (time: $F(3,39)=2.941$, $p < 0.05$).

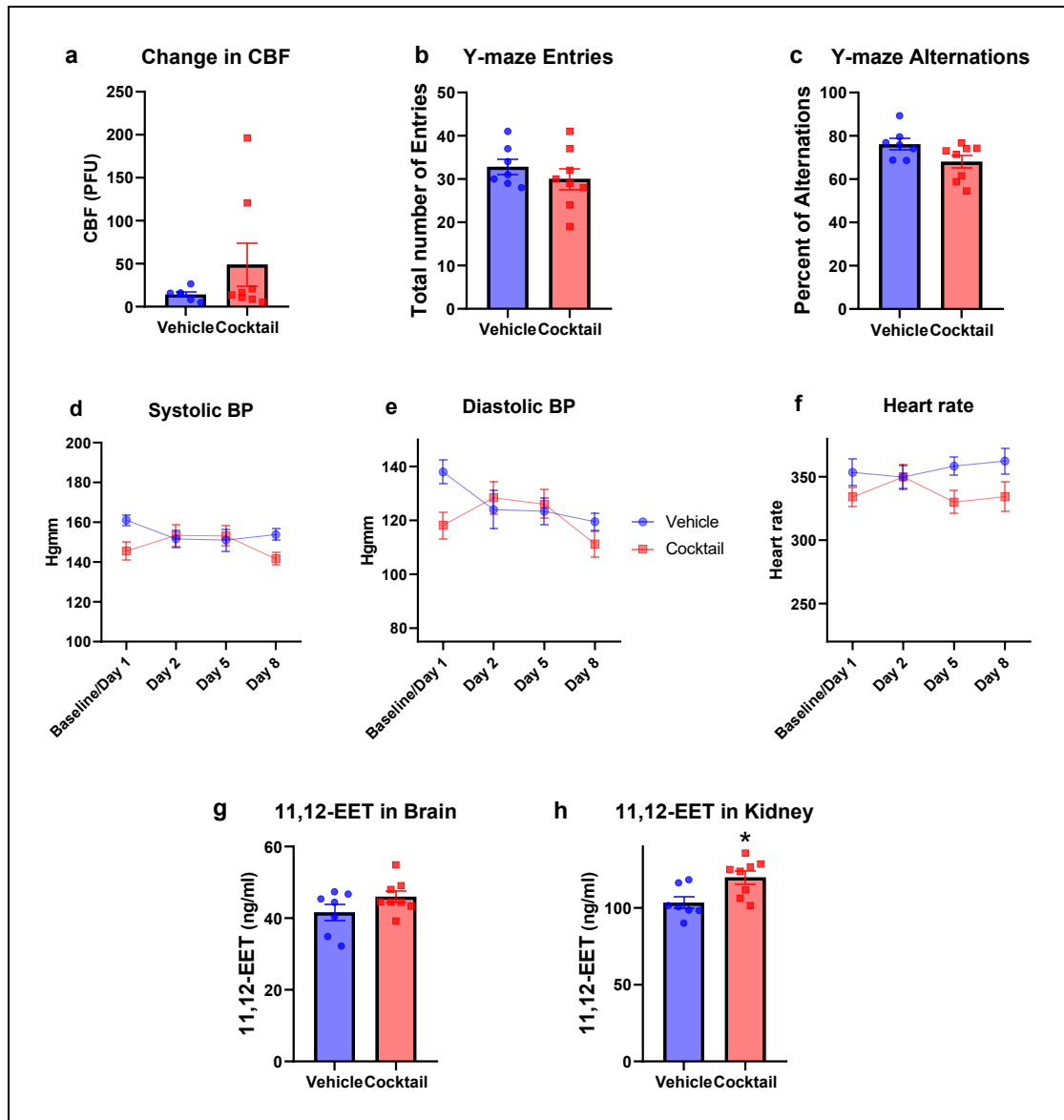


Figure 15. MS-PPOH alone: no effect on CBF, cognition, blood pressure, or brain 11,12-EET levels in Exp 3 – **a)** increase in CBF in the barrel cortex in response to whisker stimulation (mean $\pm$ SEM). **b) and c)** Number of entries and percent of alternations in the Y-maze, respectively (mean $\pm$ SEM). **d), e) and f)** systolic, diastolic blood pressure and heart rate, respectively, over the course of vehicle or cocktail treatment (mean $\pm$ SEM). **g) and h)** 11,12-EET levels in the brain and kidney, respectively (mean $\pm$ SEM). *: $p < 0.05$, t-test.

Y-maze performance analysis revealed no significant between-group difference in spontaneous alternation behaviour.

EET levels in renal tissue showed a statistically significant difference between groups ($t(13)=2.849$, $p < 0.05$). No other measured analytes demonstrated significant variations. We did not observe notable histological alterations in kidney sections (mean histology scores of the vehicle-treated and cocktail-treated groups were 1.2 ± 0.48 and 0.33 ± 0.21 , respectively; $p > 0.05$, Mann-Whitney U-test).

4.5. Exp 5: *Conditioned taste aversion (CTA) testing in aged rats to investigate possible gastrointestinal malaise effects*

To explain the decreased food intake observed in aged animals, we performed a conditioned taste aversion test with the cocktail to check whether it causes gastrointestinal malaise. We paired saccharin consumption with injection of the drug cocktail and measured subsequent saccharin preference. The results confirmed significant aversion, indicating the cocktail induces gastrointestinal distress in aged rats. We validated the assay with LiCl as the standard positive control.

Conditioned taste aversion test with LiCl

After conditioning, during experimental Days 4 and 5 when animals had simultaneous access to water and saccharin solution, animals treated with LiCl showed significantly reduced saccharin preference compared to controls (Day 4: $t(10) = 19.905$, $p < 0.001$; Day 5: $t(10) = 29.651$, $p < 0.001$, Fig 16).

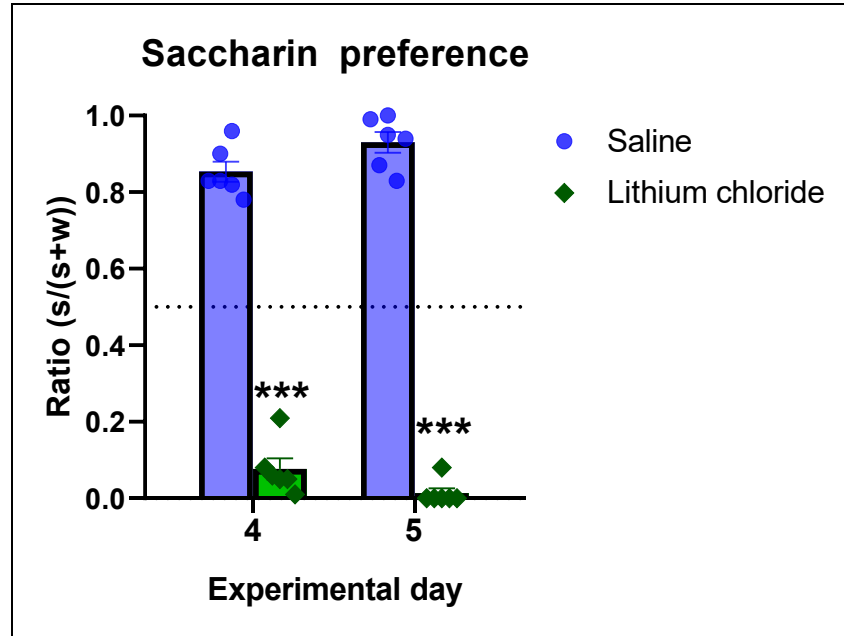


Figure 16. CTA evoked by LiCl - Proportion of saccharin solution consumption in a two-bottle choice situation (saccharin solution and water) after pairing saccharin consumption with LiCl or saline treatment (mean $\pm$ SEM). The dotted line marks the 'no-preference' value. ***: $p < 0.001$, vs 'Saline' t -test.

CTA measurement with the cocktail

Following conditioning, during Days 5-6 when animals had simultaneous access to water and saccharin solution, treated animals demonstrated significantly reduced saccharin preference compared to controls (Day 5: $t(9) = 4.219$, $p < 0.01$; Day 6: $t(9) = 3.813$, $p < 0.01$; Fig 17a).

Additionally, the 'Cocktail' treatment group showed significantly greater food pellet leftovers versus controls across multiple days (Day 4: $t(9) = -2.842$, $p < 0.05$; Day 5: $t(9) = -2.306$, $p < 0.05$; Day 6: $t(9) = -3.406$, $p < 0.01$; Fig 17b).

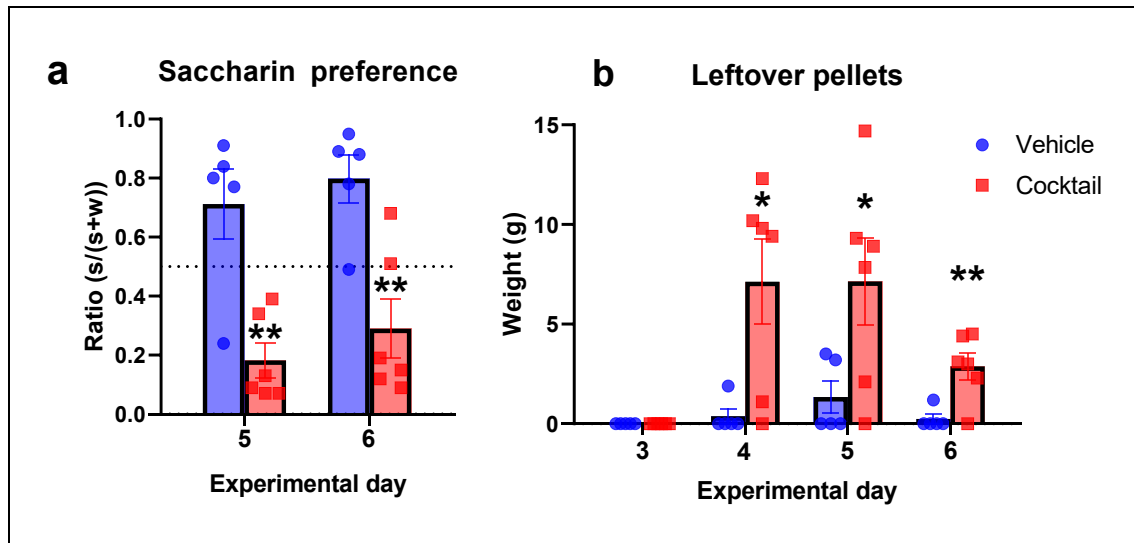


Figure 17. Saccharin consumption and leftover pellets in the CTA test with the cocktail. **a)** Proportion of saccharin solution consumption in a two-bottle choice situation (saccharin solution and water) after pairing saccharin consumption with cocktail or vehicle treatment (mean $\pm$ SEM). The dotted line marks the 'no-preference' value. **b)** weight of leftover food pellets from Day 3 to 6 of their treatment (mean $\pm$ SEM). * and **: $p < 0.05$ and $p < 0.01$, respectively, vs 'Vehicle' t-test.

5. Discussion

The primary objective of this doctoral work was to develop a pharmacologically induced NVU model in rats that could serve as a valid translational platform for modelling human cognitive decline. Rats were selected as the preferred species due to their superior learning capabilities compared to mice, making them more suitable for investigating complex cognitive functions across multiple domains. While previous work in mice demonstrated promising results for pharmacological NVU induction no such approach had been reported in the literature in rats.

A summary of the key points of the performed experiments and their respective designs are shown in Table 5.

*Table 5. Summary of the performed experiments. Each experiment is indicated with the number) of rats (N) involved. Apart from Exp 5, all treatment consisted of two injections per treatment day (b.i.d.). In the case of Exp 5, only 2 days involved ‘cocktail-treatment’. In Exp 3, because of toxic side-effects, starting dosages had to be adjusted. Final amounts are indicated with bolded numbers. Performed cognitive task abbreviations: MWM: Morris water maze, 5C: 5CSRTT, PJ: pot jumping test, TS: touchscreen pairwise visual discrimination, Coop: cooperation task, YM: Y-maze alternation test, FC: fear conditioning, LP: lever-press learning, NOR: novel object recognition. *: single dose*

Study	Subjects	Duration (day)	Dosage (mg/kg, b.i.d.)			Performed cognitive tasks
			MS-PPOH	L-NAME	Indomethacin	
Exp 1 (N=28)	Naïve, young – Hannover Wistar	8	5	10	1	FC, MWM
Exp 2 (N=17)	Naïve, young – Hannover Wistar	8	2.5	5	.5	YM, LP, NOR
Exp 3 (N=25)	Experienced, aged – Long-Evans	14	5 2.5	7 5	.7 .5	MWM, 5C PJ, TS, COOP, YM
Exp 4 (N=15)	Naïve, young – Long-Evans	8	5	-	-	YM
Exp 5 (N=11)	Naïve, aged – Hannover Wistar	6 (2)	5*	10*	1*	-

It was evident from the literature on the applied pharmacons that significant dose adjustments would be required for successful translation from mice to rats. Thus, in our initial experiment (Exp 1) we applied substantially reduced daily doses: 2x5 mg/kg MS-PPOH instead of 20 mg/kg, 2x10 mg/kg L-NAME instead of 100 mg/kg and 2x1 mg/kg indomethacin instead of 7.5 mg/kg, administered given. With these doses we successfully induced NVU but still observed substantial adverse effects including elevated blood pressure and intestinal pathology. These findings necessitated further refinement of the model, most of all, further dose reduction.

In Exp 2, we implemented a halving dose reduction of all cocktail components (MS-PPOH, L-NAME, and indomethacin) which successfully eliminated the previously observed toxic effects. However, this modification also abolished the intended NVU effects, as evidenced by the absence of significant changes in neurovascular coupling responses, working memory performance, and brain EET levels. This outcome highlighted the narrow therapeutic range achievable with this pharmacological approach.

A fundamental aspect of our conceptual framework was the use of aged, cognitively experienced animals, which more accurately reflects the human clinical population targeted by potential cognitive enhancers. For pharmacologically induced NVU, such subjects offer a specific advantage: although a short-term treatment may be insufficient to teach complex tasks, it can reveal the disruption of knowledge that has already been acquired.

In Exp 3, we employed an aged, experienced Long-Evans cohort with a modified dosing regimen intermediate between those of Exp 1 and 2. Unexpectedly, this protocol induced severe toxicity manifested as dramatically reduced food intake and significant weight loss, effects not previously observed in younger animals. These adverse reactions necessitated continuous dose adjustments to rescue the experiment, with the primary goal of determining whether any NVU or cognitive deterioration could be elicited.

Cognitive assessment in Exp 3 revealed limited but noteworthy effects. The most prominent finding was impaired performance in the 5CSRTT, characterized by decreased correct responses and increased omissions. While the elevated omission rate might reflect reduced motivation due to treatment-induced malaise, the diminished accuracy – observed specifically on Day 8 – suggests a genuine, though transient, attentional deficit.

The Morris water maze showed a trend toward poorer performance in treated animals, but this did not reach statistical significance and appeared more as a lack of improvement rather than an overt deficit.

Consistent with these subtle cognitive changes, we observed only a mild disruption in neurovascular coupling. It is noteworthy that the hyperaemic response to whisker stimulation was already quite low in the aged control group, with some animals producing undetectable signals. No adverse blood pressure effects were recorded. ELISA data indicated a moderate reduction in brain PGE₂, confirming partial cyclooxygenase inhibition, but EET levels were again unchanged. While the poor brain penetration of MS-PPOH could be speculated upon, 11,12-EET levels were also not decreased in the kidney.

Histological analysis revealed no brain alterations, but moderate toxicity was evident in kidney samples. Nephrotoxicity has been reported for all three compounds, though typically at higher doses than those used here (116–121). In a subsequent experiment (Exp 4), MS-PPOH alone did not cause notable kidney histopathology. However, these data were generated in young rats, and aged subjects may exhibit heightened sensitivity.

Given that Exp 3 failed to show a decrease in brain 11,12-EET, potentially due to the interim dose reduction of MS-PPOH, we sought to determine if the initial 2x5 mg/kg daily dose could effectively lower EET levels. Therefore, Exp 4 assessed the effects of MS-PPOH alone in young, naïve rats, again measuring blood pressure and Y-maze performance as cognitive outcome. The results demonstrated that even a 10 mg/kg/day dose of MS-PPOH did not reduce 11,12-EET levels in either the brain or the kidney of young rats, nor had any influence on other measured variables.

The pronounced reduction in food intake observed in Exp 3 prompted us to investigate potential gastrointestinal malaise using a conditioned taste aversion paradigm in Exp 5. Since the effect was age-dependent, we used aged Wistar rats for this purpose. The results clearly demonstrated that the drug cocktail induces significant aversion, providing a plausible explanation for the feeding disruptions seen in aged animals of Exp 3. This finding represents a critical limitation of the current pharmacological approach, particularly in aged populations where such side effects may substantially deteriorate behavioural and physiological assessments.

6. Conclusions

The creation of a dependable, pharmacologically induced model of NVU offers considerable promise. Such a model would not only deepen our knowledge of how cerebrovascular dysfunction leads to cognitive decline but could also serve as a translationally relevant platform for evaluating potential cognitive-enhancing drugs in discovery research. However, our investigations indicate that the present strategy (simultaneously inhibiting three key pathways of neurovascular coupling, namely EET synthesis, NO production, and prostaglandin synthesis) requires refinement before it can serve as a reliable NVU model. The following observations inform potential amendments to the approach:

1. Establishing individual target engagement

The first step would be to establish individual dose-response relationships for each compound by quantifying changes in EET, PGE₂, and NO levels in the brain. This step, which was also missing in the original mouse study (85), is essential to confirm target engagement at specific doses. Without it, the contribution of each component to the overall NVU effect remains unknown. For instance, the EET data from Exp 3 and 4 indicate that MS-PPOH may have been ineffective at the doses used. An added complication for this compound is its unknown blood-brain penetration efficacy.

2. Balancing efficacy and toxicity

A more significant challenge is identifying doses that produce a sufficient cerebral efficacy without causing pronounced peripheral effects. A key lesson from our work is that simple dose adjustment proved insufficient to circumvent the trade-off between central efficacy and peripheral safety; modified regimens either failed to produce NVU (Exp 2) or led to severe adverse events (Exp 3). Pronounced systemic toxicity can overshadow the intended cerebrovascular disruption and confound the interpretation of cognitive and physiological data.

3. Accounting for drug synergy and age sensitivity

Even with established individual effective doses, we must anticipate potential synergistic interactions between the compounds, especially with regard to their peripheral effects (see for example (116)). (Such synergism could also explain why even lowered doses provoked severe side effects in aged rats, whereas younger animals tolerated higher doses with fewer complications.) This possibility necessitates complementary measurements of EET, PGE₂, and NO levels following their combined administration.

4. Intracerebroventricular administration

In principle, the issue of peripheral toxicity could be circumvented by delivering the compounds directly into the brain, ideally via osmotic minipumps connected to an intracerebroventricular (icv.) cannula. This method would also resolve the uncertainty regarding MS-PPOH's brain penetration. Practically, however, this approach introduces major formulation challenges, as MS-PPOH and indomethacin require specific solvents (cyclodextrin and NaHCO₃, respectively). While we managed this for systemic administration, it is uncertain whether these formulations are suitable for the much higher concentrations needed in a one- to two-week minipump delivery.

5. Icv. dose-response curves

If the minipump formulation challenge can be overcome, the fundamental dose-finding studies mentioned in point 1 should be conducted using the icv. route of administration to establish effective concentrations directly within the central nervous system.

6. Methodological challenges in measuring NVC

Assuming all dosing obstacles are surmounted, a subsequent challenge involves the measurement of neurovascular coupling itself. Our experience showed that the measurable CBF signals in the barrel cortex in response to whisker stimulation exhibited significant inter-subject variability; a weak signal does not necessarily indicate impaired coupling. Due to this variability, demonstrating the uncoupling efficacy of an intervention

requires either very large sample sizes or, optimally, a self-controlled "before-after" experimental design. The latter would necessitate a chronic survival model using a less invasive measurement technique like fMRI or fUS, which would also permit longitudinal tracking of the induced NVU.

7. Targeting cognitively relevant brain regions

Although measuring whisker-stimulated hyperaemia in the barrel cortex is technically convenient, this region is not the most functionally relevant for studying cognitive functions. Assessing NVC in areas more directly linked to memory, such as the hippocampus, demands more sophisticated and methodologically challenging approaches. Furthermore, standardizing the conditions for CBF measurements (e.g., location, timing, stimulus parameters) across studies remains an ongoing difficulty.

8. Subject resilience and compensatory mechanisms

Finally, our prior work has indicated that cognitively experienced animals can exhibit resilience to impairment (97,109,110). The transient nature of the attention deficit observed in Exp 2 suggests a similar resilience may exist against NVU. Additionally, long-term compensatory mechanisms (e.g., upregulated enzyme synthesis) may develop to counteract the pharmacological inhibition over time.

Overall feasibility and translational value

Model development is a vital component of pharmaceutical research and development to ensure a smooth transition from preclinical results to clinical success. While the published mouse study (85) suggested that transferring this pharmacological NVU model to rats would yield a straightforward and attractive method for target validation or drug screening, our findings illustrate that achieving this goal is far more complex. It demands significant technological resources, time, and its overall feasibility remains uncertain. However, securing funds for developing such models should be considered as outmost importance, because decisions based on inadequately validated models are a primary cause of late-stage clinical failures.

To advance NVU modelling, we recommend prioritizing several key research directions. First, comprehensive dose-response studies should be conducted for each individual compound to establish clear relationships between dosage and target engagement, with particular focus on measuring EET, PGE₂ and NO levels in both brain and peripheral tissues. This foundational work must precede any combination therapy approaches. Second, alternative administration routes such as icv. delivery should be explored alongside innovative formulations that can enhance central nervous system efficacy while minimizing peripheral toxicity. Third, there is a pressing need to develop chronic, non-invasive NVU assessment methodologies, with fMRI representing a particularly promising approach for enabling longitudinal studies without requiring terminal procedures. Finally, dedicated investigation of potential compensatory mechanisms is warranted, as these may significantly obscure NVU effects in behaviourally experienced subjects and contribute to the transient nature of observed cognitive impairments. While our work has identified substantial barriers to reliable pharmacological NVU induction in rats, it simultaneously outlines a clear roadmap for model refinement. Successfully addressing these challenges through the recommended approaches will be crucial for creating robust preclinical models that can effectively bridge the gap to clinical applications in cerebrovascular cognitive impairment research.

7. Summary

The neurovascular unit regulates cerebral blood flow in response to neuronal activity, a process impaired in disorders like Alzheimer's disease but also in normal aging. Despite its clinical relevance, no reliable pharmacological models of NVU exist for preclinical drug development. This doctoral work aimed to establish such a model in rats by simultaneously blocking three vasodilatory pathways with a single "cocktail" of inhibitors: MS-PPOH for EETs, L-NAME for nitric oxide, and indomethacin for prostaglandins, a strategy based on prior mouse studies.

Initial experiments in young rats confirmed NVU induction (54 % reduced cerebral blood flow response) but revealed dose-limiting toxicity (hypertension, intestinal damage). Dose reduction improved tolerability but abolished NVU effects, highlighting a narrow therapeutic window. A key innovation was testing aged, cognitively experienced rats, a translationally more relevant population. Here, intermediate dosing caused transient attentional deficits and mild cerebral blood flow disruption (28 %), but severe weight loss due to decreased food intake. Conditioned taste aversion assay revealed gastrointestinal toxicity in aged animals. Stand-alone MS-PPOH treatment failed to alter brain EET levels, suggesting poor target engagement.

While partial NVU induction was achieved, the model's utility is limited by toxicity, particularly in aged rats. Future work should explore alternative dosing strategies, icv. delivery, and non-invasive cerebral blood flow monitoring (e.g., fMRI or fUS) to improve translational validity. This research provides a foundation for refining NVU models, essential for developing therapies targeting cerebrovascular dysfunction in cognitive decline.

8. References

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9. Bibliography of the candidate's publications

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