Drug-Induced Hypothermia as Beneficial Treatment before and after Cerebral Ischemia

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Key Words

Cell density · Endothelial cells · Growth factor · Hypoxia-ischemia · Necrosis · Pathogenesis · Pathology

Abstract

Objectives: Hypothermia is still unproven as beneficial treatment in human stroke, although in animal models, conditioning the brain with hypothermia has induced tolerance to insults. Here, we delineate the feasibility of drug-induced mild hypothermia in reducing ischemic brain damage when conditioning before (preconditioning) and after (postconditioning) experimental stroke. **Methods:** Hypothermia was induced in rats with a bolus of 6 mg/kg talipexole followed by 20 h continuous talipexole infusion of 6 mg/kg in total. Controls received similar treatment with saline. The core body temperature was continuously monitored. In preconditioning, hypothermia was terminated before either reversible occlusion of the middle cerebral artery (MCAO) for 60 min or global ischemia for 10 min with 2-vessel occlusion and hypotension. In postconditioning, rats experienced 60 min of MCAO before hypothermia was induced either immediately or with 3 h delay. Rats survived ischemia for 2, 7 or 90 days.

Infarct volumes were quantified by stereology. Additional experiments of methodological relevance were included in the study. **Results:** Talipexole induced mild hypothermia (35.1 ± 1.1 to $36.0 \pm 0.5^\circ\text{C}$) for <20 h. Hypothermic pre- and postconditioning reduced infarct sizes by more than 60% as monitored during the first 90 days after experimental stroke ($p < 0.05$). **Conclusion:** Talipexole is registered for use as a dopamine substitute in humans with Parkinson's disease. Although dosages cannot be directly translated to patients, our study exemplifies in an animal model that drug-induced hypothermia in a clinical setting might reduce cerebral ischemic damage before neuro- and cardiac surgical procedures and after stroke.

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Introduction

Only a small number of stroke patients benefit from thrombolysis due to a narrow therapeutic time window [1]. For the majority of patients, an alternative acute therapy is needed. Moreover, both neuro- and cardiac surgical procedures carry the risk of ischemic injury with sig-

nificant implications for mortality and morbidity [2]. Also, 42% of coronary artery bypass patients suffered from neurocognitive deficits 5 years after surgery [2]. From a social and an economical point of view, it is important to develop new therapeutic strategies that can be applied before (preischemic) and after cerebral ischemia (postischemic) in order to attenuate brain damage.

Ischemic tolerance can be induced if the brain is subjected to a weak ischemic stimulus prior to (preconditioning) or after (postconditioning) the primary ischemic insult [3]. Due to cross-tolerance, hypothermia as conditioning stimulus can also elicit such a transient adaptive cellular response, promoting tolerance towards ischemia [4]. Hypothermia is one of the most promising neuroprotective therapies, as assessed by the Stroke Therapy Academic Industry Roundtable criteria [5], affects a wide range of cell death mechanisms and has a broad safety margin [6–11]. Altogether, data suggest that mild to moderate hypothermia is a promising and safe conditioning stimulus in patients with cerebral ischemia.

So far, in most clinical studies, the target temperatures have been moderate hypothermia. Cooling to 32–34°C is related to serious adverse events and requires admission to an intensive care unit [9–11]. Evaluating animal models of acute ischemic stroke, a meta-analysis of hypothermia effects concluded that cooling to 35°C consistently reduced the infarct volume [12]. Thus, use of mild prolonged drug-induced hypothermia may help to overcome the above-mentioned obstacles. We have previously shown that the dopamine D₂ receptor agonist, talipexole, induced hypothermia and significantly reduced small infarcts in experimental stroke, as compared to controls [13]. Talipexole was chosen because it is registered for use as a dopamine substitute in humans with Parkinson's disease; therefore, results in our present study may more easily be extended to a clinical setting compared to using non-registered compounds.

This talipexole-induced hypothermic regime has been studied by us in moderately sized cortical infarcts in rats treated with hypothermia before and after cerebral ischemia.

In preconditioning experiments, we investigated the effects of drug-induced hypothermia given before cerebral ischemia in two well-known models of either 60-min middle cerebral artery occlusion (MCAO; infarct size <45 mm³) or 10 min of global cerebral ischemia (2-vessel occlusion model with hypotension; hippocampal CA1 infarction). This part of the study mirrors neuro- and cardiac surgical procedures in patients where drug-induced hypothermia as a preconditioning stimulus may be used prior to surgery to reduce cerebral ischemic damage.

In postconditioning experiments, we investigated if drug-induced hypothermia could also be used after MCAO and still permanently reduce brain damage, even if the infarct size was assessed as long as 90 days after the insult or if treatment was delayed for 3 h after the experimental stroke. This second part of the study exemplifies that drug-induced hypothermia as a postconditioning stimulus may be given to stroke patients immediately at pick-up for hospitalization and may thereby both shorten the time to treatment, and possibly, also expand the therapeutic window for other treatments at the hospital.

As proof of principle, and supplementary to methodological questions, we investigated if hypothermia could be considered the primary cause of infarct reduction when using talipexole. A group of rats were kept normothermic with a feedback heating lamp during drug treatment after MCAO, and then the infarct size was measured 7 days later. Further examinations of typical hypothermic conditioning effects were determined by measuring the presence of vascular endothelial growth factor (VEGF) in brains using Western blotting after animals without ischemia were treated with talipexole and allowed to survive for a further 24 h. Additional methodological studies encompassed investigations of the influence of talipexole on mean arterial blood pressure, as well as measurements of the influence of cranial window temperatures on brain infarct sizes.

Materials and Methods

This study was approved and conducted according to guidelines by the Animal Research Committee of Copenhagen University (No. 2006/561-1263). All procedures including steps to eliminate animal suffering were carried out in accordance with the guidelines of the Faculty of Health and Medical Sciences, University of Copenhagen, Copenhagen, Denmark.

Animals and Experiments

Male Wistar Hannover rats approximately 3 months old and weighing between 280 and 340 g were used in all experiments. Animals were housed 2 per cage under a 12-hour light/12-hour dark cycle with free access to water and food. Talipexole was kindly supplied by Boehringer Ingelheim (Ingelheim, Germany). A total of 119 out of 135 animals were included in the study (for those excluded, see Results). All animals were treated and handled by investigators blinded to treatments. The study contained groups for treatment with talipexole before cerebral ischemia (preconditioning) as illustrated in figure 1 and for treatment with talipexole after cerebral ischemia (postconditioning) as illustrated in figure 2.

Proof of Principle: Studies A and B

In study A, two groups of rats were kept normothermic with a feedback heating lamp during either drug or saline treatment after MCAO. After 60 min of MCAO, the rats received 20 h of con-

Fig. 1. An overview of the different groups included in the preconditioning study where talipexole was administered before cerebral ischemia.

tinuous infusion with talipexole ($n = 7$) or saline ($n = 5$) together with a temperature clamp (fan/heating lamp) between 36.5 and 37.5°C during the first 24 h after reflow, followed by 7 days survival.

In study B, to investigate if talipexole induced hypothermia in itself without ischemia-promoted VEGF formation in the brain as indicator of conditioning, animals were solely treated with either talipexole ($n = 4$) or saline ($n = 4$), as described below, and allowed to survive for 24 h before the brains were taken out and prepared for Western blotting.

Methodological Studies C and D

In study C, the influence of talipexole infusion on blood pressure was tested. Rats were either subjected to 60 min MCAO ($n = 7$) or sham operation ($n = 3$) followed by talipexole infusion for 20 h during which blood pressure was continuously measured in the first 24 h. Animals were euthanized under deep anesthesia at the end of the experiment.

In study D, the influence of the cranial window temperature on infarct size was also tested by subjecting rats ($n = 9$) to 60 min MCAO and, at the same time, both the cranial window temperature and the rectal temperature were monitored. After 7 days survival, the infarct volumes were estimated in each animal.

Anesthesia, Physiological Variables and Temperature

Anesthesia was induced by brief exposure to 3% halothane in a N_2O/O_2 (2:1) mixture. The rats were intubated and further mechanically ventilated (New England Medical Instruments Inc., South Natick, Mass., USA) during surgery with 0.5–1% halothane in N_2O/O_2 (2:1).

The femoral vein was cannulized for continuous talipexole infusion (equal doses in all experiments as indicated below) initiated 30 min after MCAO or as indicated in figure 1. The femoral artery was cannulized for monitoring of pCO_2 , pO_2 , blood gases, electrolytes and blood glucose (Radiometer ABL555; Radiometer Medical, Brønshøj, Denmark) before, during and immediately after surgery. The mean arterial blood pressure was measured during surgery in all rats and additionally during the first 24 h after reflow in experiment C. Long-term measurements of arterial blood pressure as well as intravenous drug infusion in freely moving rats were performed with catheters tunneled subcutaneously and exteriorized at the back of the neck in a lightweight tethering spring attached to a swivel device at the top of the cage (SAI Infusion Technologies, Libertyville, Ill., USA).

Rectal temperature was measured during surgery in all animals. A temperature sensor radio pill (TA-FA20; Data Sciences International, St. Paul, Minn., USA) was surgically placed in the

Fig. 2. An overview of the different groups included in the postconditioning study where talipexole was administered after cerebral ischemia.

peritoneal cavity of all rats for continuous recording of body temperature after surgery (Physio Tel Receiver RPC-1; Data Sciences International). In the additional 36.5–37.5°C temperature clamp in experimental group A, the recording device further regulates (on/off) a fan and heating lamp placed above the animal, such that a temperature <36.5°C turned on the heating lamp and a temperature >37.5°C turned on the fan. In experimental group D, an additional temperature probe was placed within the cranial window on the surface of the brain for simultaneous measurement of both the cranial window temperature and the rectal temperature.

Surgical Procedures and Drug Administration

For surgery, all rats subjected to MCAO were placed in a stereotaxic frame (David Kopf Instruments, Tujunga, Calif., USA) and then subtemporal craniotomy using sterile procedures for exposure of the left middle cerebral artery was performed [13, 14]. The rectal temperature was monitored and kept within 36.5–37.5°C during the operation with a thermostatic feedback heating lamp. The arcus zygomaticus was trephined with an air-driven drill, and the main trunk of the middle cerebral artery was exposed and clamped distal to the striatal trunk for 60 min with a microsurgical hook guided by a micromanipulator (David Kopf Instruments). The cranial window was constantly covered with saline heated to 37.0°C throughout the operation. Reperfusion was established by removal of the hook around the middle cerebral artery, and halothane anesthesia was then turned off. Animals were

removed from the stereotaxic frame after the end of MCAO and further ventilated with medical air until spontaneous respiration occurred within the first 10–15 min after disconnection of halothane. During this recovery period, incisions were quickly sutured after the skin was infiltrated with lidocaine (20 mg/ml).

Global ischemia was induced by 2-vessel occlusion combined with systemic hypotension [14]. Both common carotid arteries were exposed and isolated using loose ligatures. Normal blood pressure was recorded and followed by exsanguinations from the arterial catheter to lower the blood pressure. The ischemic insult was initiated by tightening the carotid ligatures bilaterally for 10 min. During the ischemic period, the blood pressure was maintained at 50 mm Hg by withdrawal or infusion of blood. Ischemia was terminated by loosening the carotid ligatures and by slow re-infusion of exsanguinated blood, if necessary, supplied with slightly heparinized saline, to restore normotension. Anesthesia was promptly turned off and the animals were further ventilated with medical air until spontaneous respiration occurred within 10–15 min. The skin was infiltrated with lidocaine (20 mg/ml) and the incisions were sutured.

Hypothermia was induced as previously described [13] in all experiments. According to body weight, talipexole rats received an intravenous bolus injection of 6 mg/kg talipexole [6-allyl-2-amino-5,6,7,8-tetrahydro-4H-thiazolo(4, 5-d)azepine dihydrochloride; Boehringer Ingelheim] dissolved in 0.5 ml saline followed by continuous infusion for 20 h of a total of 6 mg/kg talipexole (1 $\mu\text{l h}^{-1} \text{g}^{-1}$; concentration of 3 mg talipexole dissolved in 10 ml

saline). Control rats received similar volumes of saline as a bolus and continuous infusion for 20 h. In preconditioning experiments, talipexole or saline infusion was given after intravenous catheters were placed during anesthesia as described. In all other experiments, venous catheters were maintained for talipexole or saline infusion starting 30 min after reflow was established at the end of MCAO.

Tissue Sampling and Estimation of Ischemic Damage

All rats mentioned above were sacrificed under deep anesthesia (3% halothane in 2:1 N₂O/O₂) by transcardial perfusion with 4% phosphate-buffered formalin (pH 7.2). The brains were removed and further postfixed in formalin solution for 24 h at 4°C, then dehydrated and embedded in paraffin for coronal sectioning. At 100- μ m intervals, 10- μ m-thick coronal sections were collected and stained with cresyl violet. Infarct size was quantified directly by computerized stereological examination (CAST-Grid; Olympus, Albertslund, Denmark) according to the principles of Bonaventura Francesco Cavalieri adjusted to the guidelines of Swanson et al. [15]. Briefly, the area of surviving tissue in the infarcted hemisphere is subtracted from the area of the contralateral non-infarcted hemisphere to get the infarct area used for volume calculation – hereby the influence of both edema and shrinkage becomes negligible. In global ischemia, 10- μ m-thick coronal sections through the dorsal hippocampus were cut at 100- μ m intervals and stained with hematoxylin and eosin. CA1 pyramidal cells could be divided into neurons with unchanged morphology showing a distinct nucleus and nucleolus, and ischemic neurons with both shrunken cell body and fragmentized nucleus [14]. Based on the overall rostrocaudal extension of the ischemic CA1 pyramidal cell damage and number of cell loss, the degree of hippocampal CA1 injury could be scored as 0 = no ischemia, 1 = mild ischemia, 2 = moderate ischemia, and 3 = severe ischemia. This scoring system has previously been evaluated in detail in our model [14]. Briefly, we compared whether counting surviving hippocampal CA1 neurons, or counting neurons with ischemia, or estimating the volume of ischemic CA1 damage, or scoring the overall rostrocaudal extension of the ischemic CA1 infarct resolved to the best CA1 damage, and came to the conclusion that both volume estimation and scoring of overall neuropathology best reflected the ischemic damage.

Determination of VEGF Expression Using Western Blotting

Following transcardial perfusion with 250 ml saline, the brains were isolated after decapitation under deep anesthesia and snap-frozen at -80°C until homogenization in PBS (pH 7.2) containing protease inhibitors (Complete, Mini; Roche, Penzberg, Germany). The tissue was disrupted using the Ultra-Turrax® T25 basic tissue homogenizer (Rose Scientific Ltd., Edmonton, Alta., Canada). The homogenates were pelleted by centrifugation at 13,000 g at 4°C for 20 min, and the supernatants were removed for further analysis including total protein determination, using the Bradford protein assay kit (Thermo Scientific, Mass., USA), according to the manufacturer's instructions.

Homogenates (30 μ g protein/lane) and the protein ladder (Pageruler; Fermentas, St. Leon-Rot, Germany) were loaded onto 12% Tris-glycine gels and transferred onto Hybond-P membranes (GE Healthcare, Little Chalfont, UK) using the mini-Protein electrophoresis system (Biorad, Hercules, Calif., USA). Following blocking (5% skim milk powder; Sigma-Aldrich, St. Lou-

is, Mo., USA), the membranes were incubated in primary antibody (mouse anti-rat VEGF antibody 1:100; Novus Biologicals, Littleton, Colo., USA) overnight at 4°C, washed thoroughly and incubated with horseradish peroxidase-conjugated goat anti-mouse antibody (1:20,000; Jackson, Newmarket, UK) for 1 h followed by thorough washing. Chemiluminescence (Supersignal West Pico Chemiluminescent Substrate; Thermo Scientific, Rockford, Ill., USA) was detected on Hyperfilm ECL (GE Healthcare). The intensities of the bands on the films were measured using the software ImageJ (US National Institutes of Health, Bethesda, Md., USA). Ratios between VEGF and α -tubulin were calculated from these intensities, and the results are expressed relative to values from the control group. Western blotting was performed twice.

Statistical Analysis

Results are described as the mean $\pm$ SD, unless otherwise stated. Differences in physiological data were analyzed using Student's t test. Correlation analyses between rectal and cranial window temperatures and the difference between rectal and cranial temperature and infarct size were performed using Spearman's rank correlation. Differences in infarct volumes between groups were analyzed using Student's t test as saline and talipexole were alternately administered to the animals in all experimental groups. Changes in CA1 pyramidal cell damage were compared using the χ^2 test. Differences in VEGF expression were analyzed using Student's t test. p values <0.05 were considered significant.

Estimation of sample sizes in the MCAO model experiments was predicted from our previous results [13]. If the α (confidence level) and β error levels (statistical power) were 5 and 50%, respectively, groups required a sample size of 4, and if α and β were 5 and 20%, respectively, groups required a sample size of 9.

Results

Preconditioning Experiments

All animals subjected to 60 min MCAO and 2 days survival (fig. 1) had cortical infarcts in the left hemispheres which in the talipexole group measured 7.0 ± 3.5 mm³ and in the saline group measured 26.8 ± 9.3 mm³ (fig. 3). This difference between the infarct sizes in the two groups corresponded to a 74% reduction in infarct volume after regulated hypothermic preconditioning ($p < 0.05$). During the 20 h of infusion, the average body core temperature was $35.5 \pm 0.4^\circ\text{C}$ in the talipexole group and $36.9 \pm 0.3^\circ\text{C}$ in the saline group ($p < 0.05$).

When global ischemia was induced 2 and 3 days after preconditioning (fig. 1), there was no statistical significance in the degree of hippocampal damage between the talipexole and the saline group (fig. 4). However, at 4 days after preconditioning, global ischemia induced significantly less damage to CA1 pyramidal cells ($p < 0.05$) in those rats that received talipexole preconditioning (fig. 4). The difference in core body temperatures between tali-

Fig. 3. Effect of treatment on infarct size following focal ischemia. Volumes of cortical infarctions following 60 min MCAO in various experimental groups, as indicated. Black bars represent the talipexole-induced hypothermic groups while white bars represent saline-treated normothermic rats. Details on groups and treatment are given in figures 1 and 2. The mean $\pm$ SD is shown for each experimental group. * $p < 0.05$, talipexole versus saline group. Pre = Preconditioning; post = postconditioning.

Fig. 4. Delayed effect of treatment on hippocampal CA1 damage following global ischemia. Animals had 20 h with talipexole-induced hypothermia or saline followed by 2 days ($n = 4$ and 6 , respectively), 3 days ($n = 4$ and 3) or 4 days ($n = 4$ and 4) rest before they were subjected to 10 min of global ischemia and yet another 7 days of survival. The overall ischemic CA1 pyramidal cell damage was in agreement with previous verification [14] and scored as follows: 0 = no ischemia, 1 = mild ischemia, 2 = moderate ischemia, and 3 = severe ischemia. Four days after talipexole-induced hypothermic preconditioning, CA1 damage was significantly reduced compared to saline ($p < 0.05$), as demonstrated by the brighter shades.

talipexole and saline animals corresponded to a $1.2\text{--}1.3^\circ\text{C}$ reduction during regulated hypothermic preconditioning (saline vs. talipexole; 2 days: $37.1 \pm 0.3^\circ\text{C}$ vs. $35.9 \pm 0.6^\circ\text{C}$; 3 days: $37.2 \pm 0.3^\circ\text{C}$ vs. $36.0 \pm 0.5^\circ\text{C}$; 4 days: $37.2 \pm 0.6^\circ\text{C}$ vs. $35.9 \pm 0.4^\circ\text{C}$; all $p < 0.05$).

Postconditioning Experiments

Infarct size in all animals subjected to 60 min MCAO measured $8.1 \pm 3.9 \text{ mm}^3$ in the talipexole group and $22.9 \pm 5.4 \text{ mm}^3$ in the saline group after 2 days survival (fig. 2, 3) and 15.8 ± 7.2 and $39.4 \pm 6.7 \text{ mm}^3$ after 90 days survival (fig. 2, 3), corresponding to 65 and 60% reductions in infarct volumes comparing talipexole and saline groups, respectively ($p < 0.05$). For animals with 2 days survival, during the 20 h of either talipexole postconditioning or saline infusion, the average core body temperatures were $35.1 \pm 1.1^\circ\text{C}$ in the talipexole rats and $37.4 \pm 0.6^\circ\text{C}$ in the saline rats ($p < 0.05$; fig. 5a). For animals with 90 days survival, during the 20 h of infusion, the average core body temperature was $35.5 \pm 0.7^\circ\text{C}$ in the talipexole rats and $36.9 \pm 0.5^\circ\text{C}$ in the saline rats ($p < 0.05$).

A therapeutic window of postconditioning with 3 h delay in drug infusion following reflow (fig. 2) showed a significant 78% decrease in infarct size ($5.9 \pm 2.1 \text{ mm}^3$ in the talipexole group vs. $26.1 \pm 11.0 \text{ mm}^3$ in the saline group; $p < 0.05$, after 7 days survival; fig. 3).

Proof of Principle of Experiment A: Clamping of Body Temperature

During the 20 h of talipexole or saline administration together with temperature clamping (fig. 5b), average body core temperatures were not significantly different in the two groups (37.0 ± 0.5 vs. $37.1 \pm 0.3^\circ\text{C}$ for the talipexole and saline groups, respectively). Keeping the animals normothermic resulted in infarct sizes that did not differ between the two groups (42.8 ± 12.2 vs. $43.1 \pm 7.8 \text{ mm}^3$ for the talipexole and saline groups, respectively) after 7 days survival.

Proof of Principle of Experiment B: VEGF as a Marker for Conditioning

Since VEGF has been identified as one of the major beneficial players in conditioning [16, 17], we treated 4 rats with talipexole and 4 rats with saline followed by 24 h of survival. Without further treatment, brain homogenates were then investigated with Western blotting for the expression of VEGF (fig. 6). More than a doubling of VEGF expression levels was seen following talipexole administration compared to the saline group ($p < 0.05$).

Methodological Investigations of Experiment C: Blood Pressure

Cumulated data (<24 h after surgery) from the mean arterial blood pressure after talipexole treatment in rats subjected to 60 min MCAO was $133.4 \pm 15.0 \text{ mm Hg}$, and

Fig. 5. Core body temperature during treatment. **a** Core body temperature as a function of time in saline (upper curve, $n = 8$) and talipexole (lower curve, $n = 8$) treated rats recorded 30 min after the end of 60 min MCAO and during the next 22 h. This is consistent with previously published work [13] and, as previously noted, the core body temperature returns to normal levels after drug withdrawal [13]. **b** Core body temperature as function of time in saline (upper

curve, $n = 5$) and talipexole (lower curve, $n = 7$) treated rats recorded 30 min after the end of 60 min MCAO and during the next 22 h when animals are kept normothermic by maintaining the temperature between 37.0 and 38.0°C with a thermoregulated heating lamp and fan. Note that the initial drop in temperature seen in rats without temperature regulation (**a**, upper curve) after MCAO is abolished in **b** (upper curve). The mean $\pm$ SD is shown for each experimental group.

Fig. 6. Semiquantitative determination of VEGF expression. **a** VEGF expression in brain homogenates from rats without ischemia subjected to talipexole-induced hypothermia ($n = 4$, arrows) or saline infusion ($n = 4$). **b** VEGF expression (approximately 25 kDa) relative to loading control (α -tubulin, approximately 50 kDa) is quantified. Data are given as the mean $\pm$ SEM from two separate Western blot experiments. * $p < 0.05$, talipexole versus saline group.

in the sham-operated group, it was 123.2 ± 13.0 mm Hg; these figures were not statistically different ($p > 0.05$). Measurements were performed in freely moving rats, and figures are not different from measurements in rats subjected to moderate stress reported by others [18].

Methodological Investigations of Experiment D: Rectal and Cranial Window Temperatures

Rectal and cranial window temperatures were simultaneously registered during the 60 min MCAO, with average temperatures as shown in figure 7a. Results showed that both temperature curves followed the same pattern. Figure 7b depicts the average cranial window temperature versus the infarct size in each animal. There was no statistically significant correlation between cranial window temperature and infarct size. Finally, figure 7c demonstrates the difference between the average rectal and cranial window temperatures versus the infarct size in each animal; results showed that there was no statistically significant correlation between temperature differences and infarct size.

Animal Exclusions and Mortality

In the preconditioning studies, 1 saline rat died during surgery, and 1 saline and 2 talipexole rats were excluded because of subcortical infarcts. In the postconditioning studies, 4 saline and 2 talipexole rats died during surgery, and 3 talipexole rats were excluded because of subcortical infarcts. In additional experiment A, 2 saline rats died during surgery, and in experiment C, 1 saline rat died during surgery.

Fig. 7. Influence of the open cranial window on brain temperature and infarct size. **a** Rectal and cranial window temperatures (Tcw) were simultaneously registered during 60 min MCAO (n = 9, mean temperatures). The two temperature curves nicely reflect one another. **b** Cranial window temperature versus infarct size in each animal is plotted. No statistically significant correlation ($p > 0.05$) between cranial window temperature and infarct size was found. **c** The difference between the rectal and cranial window temperatures versus the infarct size in each animal is plotted. There was no statistically significant correlation between temperature differences and infarct size.

Physiological Data

Physiological data from arterial blood was collected just before, during and immediately after global and focal cerebral ischemia. Physiological data from both the global [14] and the focal ischemia model [13, 14] have been reported and discussed in detail previously, and results in this study are not different from these reports. Within each experimental group, data from talipexole- and saline-treated groups were compared: variations between these data corresponding to pCO_2 , pO_2 , sodium, potassium, pH, and glucose as well as interischemic body temperature and mean arterial blood pressure during the operation were not statistically significant ($p < 0.05$).

Discussion

Therapeutic Drug-Induced Hypothermia Applied before and after Cerebral Ischemia

Our major finding was a long-term infarct reduction following talipexole-induced hypothermia, both when mild hypothermia was initiated before and up to 3 h after

reperfusion in rodent models of focal and global ischemia. Hypothermia has been shown to mediate cross-tolerance towards ischemia when used as a preconditioning stimulus [4]. In our model of talipexole-induced hypothermia, we also demonstrated neuroprotection when talipexole-induced hypothermia was completed hours before focal as well as global ischemia. Conditioning was further supported in our study by the finding of VEGF induction in the brain after talipexole-induced hypothermia [16, 17]. In line with showing (pre)conditioning effects of talipexole-induced hypothermia, the more broad-term postconditioning [19] can be used for poststroke talipexole-induced hypothermia. We anticipate an effect from the hypothermia stretching beyond the time of treatment, which is supported by the fact that postischemic hypothermia has been shown to alter gene expressions [20, 21]. Again, when talipexole was administered after reperfusion, results showed a 65–60% reduction in infarct size after 2 and 90 days survival, respectively.

As anticipated, we observed a slight progression in infarct size up to 90 days after the insult, but still, the infarct

reduction was significant and substantial. Pioneer studies showed that physiologically forced cooling after ischemia only reduced infarct size temporarily for the first 7 days but not after 60 days survival. However, when interischemic hypothermia was applied, the neuroprotection remained 60 days after the insult [22]. Thus, long-term survival studies must ensure that significant pathological mechanisms are irreversibly terminated and not just delayed, a test that our study passed, possibly because of both a prolonged period of 20 h of hypothermia and a less stressful regulated hypothermia without counter-regulation.

Investigating the therapeutic window, a 3-hour delay in treatment after stroke also significantly reduced infarct size. However, when temperature reduction was eliminated during talipexole treatment by keeping the rats normothermic with a heating lamp, the reduction in infarct size did not materialize. It is therefore most likely the hypothermia in itself and not other effects from the talipexole that are primarily responsible for the reduced brain damage. This is further strengthened by our measures of an unchanged blood pressure in talipexole-treated rats as compared to controls.

Experimental Models for Studying Regulated Hypothermia in Cerebral Ischemia

In experimental stroke, one can question the relevance of the model. We have previously and now chosen a reversible focal stroke model that reproducibly results in cortical infarcts of small to medium size ($<45 \text{ mm}^3$) leaving the thermoregulatory hypothalamic preoptic nucleus undamaged [23]. In figures, 83 rats entered our 60-min MCAO model out of which 8 died during surgery and 6 were discarded because in addition to cortical infarcts, they had subcortical infarcts. Therefore, we find our model suited for studies of regulated drug-induced hypothermia. In contrast, models of permanent MCAO and intraluminal filament MCAO also damage subcortical thermoregulatory structures [24, 25] with induction of hyperthermia [26] as an inappropriate result. When we performed coordinated measurements of both rectal and cranial window temperature during surgery in relation to infarct development in our model, it clearly showed that variations in cranial window temperature had no influence on the infarct size.

Nishio et al. [4, 27] demonstrated a significant reduction in infarct size by forced hypothermic preconditioning initiated 24 h before MCAO. Therefore, we also started induction of regulated hypothermic preconditioning at 26 h before MCAO and we obtained similar results. In

our global ischemia model, a typical late-phase neuroprotection [3] was seen on day 4 after hypothermic preconditioning. This time frame is in agreement with the original reports by Kato et al. [28], Kirino et al. [29] and Kitagawa et al. [30]. Interestingly, it has been shown that infarct protection afforded by postconditioning is as effective as that afforded by preconditioning [31]. This was confirmed by our results when comparing for example pre- and postconditioning in MCAO with 2 days survival.

Dopamine-Induced Hypothermia in Stroke

Hypothermia is a common side effect of numerous drugs [32]. Dopamine plays a key role in temperature regulation in the preoptic medial hypothalamic nucleus [23, 33], and dopamine is in general either directly or indirectly involved when drug-induced hypothermia occurs [33]. As an example, we have previously demonstrated neuroprotection following cannabinoid-induced hypothermia [14] which is related to endogenously released dopamine [34]. In fact, core temperature measurement in rats, reflecting intrinsic efficacy at dopamine receptors [35, 36], is used for drug screening in the industry. Various dosages were initially tested to find a treatment that in our model induced $1.2\text{--}2.3^\circ\text{C}$ temperature reduction during the first 20 h of administration. Remarkably, continuation of talipexole administration beyond 20 h could not prevent the core body temperature from returning to normothermia [13]. The very same was seen after continuous slow cannabinoid injection [14] pointing towards a common dopaminergic thermoregulation as mentioned [34]. The dopaminergic D_2 - D_3 agonist talipexole may also in part gain its hypothermic effect from additional 2-adrenoreceptor agonist effects [37, 38].

Indeed, translating the dose from experimental rat studies directly to humans simply based on body weight revealed that prolonged hypothermia in patients would require doses more than a hundredfold of current recommendations (maximum 3.5 mg/day). The only observation on hypothermia in humans following talipexole treatment was reversible hypothermia of 35.4°C in a 55-year-old male with Parkinson's disease who took a daily dose of 2 mg talipexole [39]. This may indicate that sufficient mild temperature reductions occur in humans using much lower talipexole doses than in rats [40, 41]. Dopamine receptor agonists for Parkinson's disease frequently (approximately 30%) cause nausea and vomiting triggered by dopamine receptors [42]. More serious adverse events are the risk of causing psychosis [43–45]. In that context, the use of dopamine agonists in rats may result in stereotypic behaviors [46, 47], but we did not

observe behavioral stereotypes in the talipexole-treated rats.

Finally, it remains possible that talipexole additionally conveys a neuroprotective effect independent of hypothermia [48]: attenuation of glutamate neurotoxicity [49], inhibition of apoptosis [50] and increased superoxide dismutase antioxidant activity [51] are among the mechanisms suggested.

Implications of Drug-Induced Hypothermia in Stroke

The overall purpose of our studies was to investigate in animal models if mild drug-induced hypothermia in a clinical setting might be useful before neuro- and cardiac surgical procedures and, after stroke, to ameliorate ischemic brain damage. We exemplified this perspective by choosing the clinically approved drug talipexole and speculate that drug-induced regulated mild hypothermia may cause less severe, and easier-to-treat, adverse events than forced mechanical cooling. Furthermore, drug-induced cooling can be initiated instantly and anywhere – also outside of hospitals. Since hypothermia is a common side effect to many registered drugs, we suggest that more attention is directed towards this perspective. Briefly,

these drugs are serotonergic 5HT_{1A} agonists, dopaminergic D₂ agonists and possibly also α_2 -receptor agonists with hypothermic regulatory actions in the brain, as well as drugs like 2-deoxy-glucose and iodine solution with direct and indirect effects on metabolic heat production, and finally, maybe also α_1 -receptor antagonists with selective peripheral vasodilator effects increasing heat loss.

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Disclosure Statement

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