A probable dual mode of action for both L- and D-lactate neuroprotection in cerebral ischemia

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Abstract

Lactate has been shown to offer neuroprotection in several pathological conditions. This beneficial effect has been attributed to its use as an alternative energy substrate. However, recent description of the expression of the HCA1 receptor for lactate in the central nervous system calls for reassessment of the mechanism by which lactate exerts its neuroprotective effects. Here we show that HCA1 receptor expression is enhanced twenty-four hours after reperfusion in an MCAO stroke model, in the ischemic cortex. Interestingly, intravenous injection of L-lactate at reperfusion led to further enhancement of HCA1 receptor expression in the cortex and striatum. Using an *in vitro* oxygen-glucose deprivation model we show that the HCA1 receptor agonist 3,5-dihydroxybenzoic acid reduces cell death. We also observed that D-lactate, a reputedly non-metabolizable substrate but partial HCA1 receptor agonist also provided neuroprotection in both *in vitro* and *in vivo* ischemia models. Quite unexpectedly, we show D-lactate to be partly extracted and oxidized by the rodent brain. Finally, pyruvate offered neuroprotection *in vitro* while acetate was ineffective. Our data suggest that L- and D-lactate offer neuroprotection in ischemia most likely by acting as both an HCA1 receptor agonist for non-astrocytic (most likely neuronal) cells as well as an energy substrate.

Keywords: Focal Ischemia, Lactate, Neuroprotection, Neuron-glia interactions, energy metabolism.

Introduction

Stroke is a frequent and often disabling disease whose early identification and treatment within a few hours from symptom onset are essential. In ischemic stroke, the most common stroke subtype resulting from an arterial occlusion, energy failure due to lack of oxygen and glucose supply to the brain is the central mechanism leading to neural damage. Brain metabolism therefore plays a critical role in the pathophysiological mechanisms underlying neuronal damage in stroke and for recovery. Even though glucose is known as the brain energy substrate *par excellence*, other metabolic intermediates such as monocarboxylates including lactate, pyruvate, acetate and ketone bodies have been shown to be oxidized for energy production (1-3) under certain conditions. Brain energy metabolism is a compartmentalized process involving neurons as well as astrocytes and oligodendrocytes, with metabolic interactions between different cell types. Monocarboxylates can be transported to and from different cell types by monocarboxylate transporters (MCTs) with different degrees of affinity. MCTs are a family of proton-dependent carriers where MCT1, MCT2 and MCT4 are the main MCTs in the central nervous system. Under physiological conditions, MCT1 is expressed in endothelial cells, astrocytes and oligodendrocytes, MCT4 in astrocytes and MCT2 is the main transporter in neurons. Under hypoxic conditions, MCT1 and MCT2 are also expressed in activated microglia (4-6).

Beside its role as an energy substrate, lactate also acts as a signaling molecule via the HCA1 receptor in adipose tissue and skeletal muscle (7). Interestingly, recent work revealed the presence of the HCA1 receptor in different brain structures, including cortex, hippocampus and cerebellum (8) and it has been shown that lactate administration to cortical neurons induced specific modulations in calcium transients frequencies in the same manner as a specific HCA1 receptor agonist (9). HCA1 receptor involvement has also been suggested in neurological disorders such as cerebral malaria (10).

We have previously shown that L-lactate administration is neuroprotective in models of cerebral ischemia both *in vitro* and *in vivo*: it attenuated neuronal death *in vitro* in organotypic hippocampal slice cultures subjected to oxygen and glucose deprivation (11). Both, intracerebroventricular and intravenous administration of L-lactate after transient middle cerebral artery occlusion (tMCAO) lead to a reduction in lesion size and improved neurological outcome (11, 12). While it was assumed so far that lactate exerts its neuroprotective effect by acting as a metabolic substrate for energy-deprived neurons, the mode of action is so far not characterized and recent data on the presence and role of the lactate receptor HCA1 in the CNS challenges this assumption. In the present study, we have explored both possible modes of action, as a metabolic substrate and signaling molecule. Understanding the mechanisms by which lactate exerts its neuroprotective effect is essential in order to adequately translate this promising preclinical neuroprotection strategy to a clinical setting.

Materials and methods

All experiments were conducted in accordance with ordinance 455.163 of the Swiss Federal Veterinary Office and approved by the Service Cantonal des Affaires Vétérinaires (license number VD2017.4b to LH) and the Kanton Zürich Gesundheitsdirektion Veterinäramt (license number ZH53/2007 to BW) (cantonal veterinary authority).

Transient middle cerebral artery occlusion in the mouse

A total of 55 male CD1 mice (body weight 26-35 g, Charles River, L'arbresle, France) were housed in the animal facility of the Department of Fundamental Neuroscience of the Université de Lausanne with 12:12 light/dark cycle with normal illumination, in groups of 5 per cage during one week for acclimatization prior to surgery. For experiments, mice were maintained anesthetized with isoflurane (1.5 – 2 % in nitrous oxide /oxygen 70 %/30 %) using a face mask. Body temperature was maintained at 37.0 ± 0.5°C throughout surgery (FHC Inc., Bowdoinham, ME, USA). Regional cerebral blood flow (rCBF) was measured and continuously recorded throughout the operation in all animals by laser-Doppler flowmetry (Perimed) with a flexible probe fixed on the skull (1 mm posterior and 6 mm lateral from bregma). Transient focal cerebral ischemia (30 min or 45 min depending on the set of experiments) was induced by occlusion of the left MCA with an intra-arterial suture as described elsewhere (11). Briefly, the left common carotid artery and the left external carotid artery were exposed and ligated following a ventral midline neck incision. Ischemia was induced by inserting a silicon-coated nylon monofilament (0.17 mm diameter) through the left common carotid artery into the internal carotid artery until mild resistance was felt and a drop to less than 20 % of initial rCBF was registered. rCBF was monitored and maintained below 20 % of the baseline level during ischemia. Reperfusion was considered successful if the rCBF rose above 50 % of baseline. Only mice that had a drop of 80% of rCBF and accomplished a reperfusion of above 50% of baseline in the rCBF were included in the study. They received five microliters per gram of body weight of either sodium D-lactate solution (200 mM), pyruvate solution (200 mM) or vehicle solution (PBS) injected randomly in the tail vein at reperfusion using a 1 ml syringe with a 25-gauge needle and a mouse restrainer (Braintree Scientific Inc., Braintree, Mass., USA). All surgeries were performed during day-light, between 9am and 5pm. The surgeon was not blinded. Each mouse was considered as an experimental unit.

At the beginning of the surgery, mice were administered 0.025 mg/kg of buprenorphine subcutaneously for post-surgery analgesia. Once the animals were awake, they were housed overnight in an incubator at 28°C.

Behavioral evaluation

The neurologic deficit was evaluated after reperfusion and before euthanasia, with a composite neuroscore graded for severity after surgery (0: no observable neurologic deficit; 1: failure to extend the right forepaw; 2: circling to the contralateral side, and 3: loss of walking or righting reflex) as previously described (11) and motor performance assessed on the Rotarod treadmill (UgoBasile, Milan, Italy). In this test, mice were placed on the rotating cylinder, set to accelerate

uniformly from 4 to 40 r.p.m., and their latency to fall was recorded before 600 seconds. The animals were trained on 2 different days before surgery, with two trials in each training session. The test was then performed 24 and 48 h after MCAO, with two consecutive trials for each animal. The better of the two trials was selected. Points were attributed on the basis of performances expressed as a percentage of the best performance before ischemia (0: 90% to 100% and then 0.5 point for each decrease of 15% until a maximum of 5 points for the highest neurological deficit). The behavior evaluation was assessed in a blinded manner.

Determination of ischemic lesion volumes

Animals were sacrificed 48 h after the onset of focal ischemia and 20-µm-thick, 720-µm-distant, coronal cryostat sections were stained with cresyl violet for histologic determination of lesion size. Digitalized images of the Nissl-stained tissue were acquired under a light stereomicroscope (Leica MZ16FA) and the lesion area was measured using ImageJ software. Infarct volume was calculated by multiplying the sum of the lesion areas on each section by the distance between sections (13).

Immunohistochemistry

Mice were injected intraperitoneally with a lethal dose of pentobarbital (10mL/kg, Sigma, Switzerland) and then perfused with 150mL of 4% paraformaldehyde (Sigma-Aldrich, USA) dissolved in 1 x PBS at pH 7.4. Brains were dissected, postfixed overnight at 4°C, cryoprotected 24h in 30% sucrose solution (Sigma-Aldrich, USA) and rapidly frozen. Twenty µm thick coronal microtome-cryostat (leica MC 3050S) sections were stored in cryoprotectant (30% ethylene glycol and 25% glycerin in 1 x PBS) at -20°C. For immunostaining, sections were washed three times in 1 x PBS and blocking of non-specific binding was achieved by incubating in 1 x PBS containing 1% bovine serum album, 0.1% Triton X-100 and 10% normal goat serum during 1h. Double labeling was carried out overnight at 4°C in the 1 x PBS solution without normal goat serum and different antibodies: monoclonal mouse anti-neuronal nuclear antigen (NeuN) antibody (1:500 dilution, Sigma) or monoclonal mouse anti-microtubule associated protein (MAP2) antibody (1:300 dilution, Sigma) and polyclonal rabbit anti-hydroxycarboxylic acid receptor (HCA1) (1:500 dilution; Sigma). After washing three times with 1 x PBS, brain sections were incubated for 2h at room temperature with the following fluorescent secondary antibodies: donkey anti-rabbit Alexa-594 (1:250, Invitrogen), donkey anti-mouse Alexa-488 (1:250, Invitrogen) for 2h at room temperature. After immunostaining brain sections were incubated for 10 min with 4,6 diamidino-2-phenylindole (DAPI) (Sigma, Buchs, Switzerland) dissolved in 1 x PBS (1/100,000) to reveal nuclei. Preparations were then maintained at 4°C until observation with a Zeiss LSM 710 Quasar Confocal Microscope (Zeiss, Feldbach, Switzerland).´

Western blotting

For protein expression experiments, young adult male mice were subjected to 30 minutes MCAO as described above and sacrificed either at 1h, 3h, 8h, 24 h or 48h after reperfusion. Striatum (caudate and putamen), primary motor and somatosensory cortex as well as hippocampus were collected using a rodent brain matrix (adult mouse, coronal sections, ASI Instruments, MI, USA).

Total proteins were extracted from brain tissues by cellular lysis in ice-cold RIPA buffer (#9806, Cell Signaling, Beverly, MA, USA) supplemented with a mixture of protease inhibitors (Complete 11257000; Roche, Basel, Switzerland). Protein quantification was performed with the Pierce BCA Protein Assay kit (#23227, Thermo Fisher Scientific, Pierce, Lausanne, Switzerland) and about 20 µg of proteins were denatured (95 °C) for 5 minutes in SDS-PAGE sample buffer (60 mM Tris-HCl pH 6.8, 5 % SDS, 6.6 % glycerol, 5 mM EDTA, 5 % β-mercaptoethanol and 0.1 % bromophenol blue). Samples were separated on a 10 % acrylamide gel with a 4 % stacking gel using an Electrophoresis Unit (Bio-Rad, Cressier, Switzerland). Proteins were then electroblotted onto nitrocellulose membranes (0.45 µm; #162-0115, Bio-Rad, Cressier, Switzerland) using the Electrophoresis Unit. Nonspecific binding sites were blocked for 1 hour at room temperature with a solution of Tris-Buffered-Saline (TBS-T; 50 mM Tris-HCl pH 7.5, 150mM NaCl) supplemented with 0.1 % Tween-20 and containing 10 % (wt/vol) skimmed milk. Blots were then incubated overnight at 4°C with specific primary antibodies in TBS-T 0.1 % containing 1 % skimmed milk: rabbit anti-mouse GPR81-S296 (HCA1) (1:500 dilution; #SAB1300790, Sigma, Buchs, Switzerland). Blots were washed three times in TBS-T 0.1% and subsequently incubated 2 h at room temperature with horseradish peroxidase-conjugated donkey anti-rabbit IgG (#NA9340V, 1:10.000 dilution; GE Healthcare, Glattbrugg, Switzerland). After being washed three times in TBS-T 0.1 %, blots were processed using Immun-StarTMWesternCTM Chemiluminescent Kit (#170-5070, Bio-Rad, Cressier, Switzerland). Chemiluminescence detection was performed with the ChemiDoc™ XRS System (#170-8070, Bio-Rad, Cressier, Switzerland). Total protein content assay was performed with the Pierce Reversible Protein Stain kit (#24580, Thermo Fisher Scientific, Pierce, Lausanne, Switzerland) and revealed with the ChemiDoc™ XRS System (#170-8070, Bio-Rad, Cressier, Switzerland). Both types of labeling were quantified with the ImageLab 3.0 software (Bio-Rad, Cressier, Switzerland) and the HCA1 (GPR81) protein expression was normalized to the total protein content.

Organotypic hippocampal slice cultures

Hippocampal slice cultures were prepared as described elsewhere (Berthet et al., 2009) from P8 to P10 rats (OFA Sprague Dawley, Janvier, France). Coronal hippocampal sections (350µm) were cultured on sterile porous membrane units (Millicell, Millipore, Billerica, MA, USA) in wells containing 1mL of culture medium with D-glucose 36 mM, 25% horse serum, 50% MEM (minimal essential medium supplemented with HEPES and sodium bicarbonate; Gibco), 25% HBSS (Hank's balanced salt solution; Gibco), and L-glutamine 2 mM (Sigma-Aldrich, St. Louis, MO, USA). Cultures were kept at 33°C, 5% CO_2, 100% humidity for 4 days. The medium was replaced by fresh identical medium at day 4, and then by culture medium with 15% horse serum, 60% MEM, 25% HBSS, and L-glutamine 2 mM at day 7 and 10. Experiments were conducted after 10 days of culture.

Oxygen and glucose deprivation on organotypic hippocampal slice cultures

Oxygen and glucose deprivation (OGD) experiments were performed in serum-free low glucose medium, DMEM (Dulbecco's modified Eagle's medium; D5030, Sigma-Aldrich, St. Louis, MO, USA) supplemented with D-glucose 1 mM and L-glutamine 2 mM and equilibrated for 1 h at 37 °C, in a humidified hypoxic chamber (COY,MI,USA) with an atmosphere of 5 % O_2, 5 % CO_2, completed by N_2. Hippocampal slices were transferred into this medium and placed into the hypoxic chamber for 1 h. Control cultures were kept in 60 % MEM, 15 % horse serum, 25 % HBSS, and L-glutamine 2 mM for 1 h at 37 °C in a humid normoxic atmosphere (11, 14). For recovery, cultures where then transferred into fresh culture medium at 33 °C for 48 h. Cultures were randomly treated with either D-lactate, pyruvate, acetate (Sigma-Aldrich, St. Louis, MO, USA, diluted in 1 x phosphate-buffered saline, PBS, pH 7), 3,5-dihydroxybenzoic acid (3,5-DHBA, Sigma-Aldrich, St. Louis, MO, USA, diluted in ethanol) or an equal volume of culture medium. Sodium D-lactate at a final concentration of 4 mM, pyruvate at 10 mM, sodium acetate at 0.13, 0.2, 4 or 8 mM or 3,5-DHBA at 4 mM was administered immediately after OGD.

Assessment of cell death in hippocampal slices

Cell death was determined in the CA1 region using the fluorescent viability indicator propidium iodide (PI). PI was applied in each dish (50 µg/mL) 1 h before measurement. PI fluorescence emission (excitation wavelength 536nm, emission wavelength 617nm) was measured 48 hours after hypoxia using an epifluorescence microscope with a 5x lens coupled to a camera (Leica). PI images were acquired with standardized camera settings and signal intensity was measured with ImageJ software (ImageJ 1.36b, National Institute of Health). After subtracting the background fluorescence on each slice, the results were expressed as a percentage of maximal cell death obtained by submerging slices in PBS for 24 h at 4°C. Cell death was averaged for the 4 slices of each culture well. The experimenter was not blinded.

Experiments using ^{11}C-D-lactate in Sprague Dawley rats

Radiotracer: The D- and L-enantiomers of 1-^{11}C-lactic acid were produced by a previously described radiosynthetic method, which includes separation of racemic 1-^{11}C-lactic acid by preparative chiral ligand exchange HPLC. Prior to use, the fractions corresponding to 1-^{11}C-D- or 1-^{11}C-L-lactic acid were further purified and finally formulated as sodium salts in physiologically acceptable sodium phosphate buffered saline (pH 7). The chemical and enantiomeric purity of each isolated radiolabeled enantiomer was routinely monitored by analytical chiral ligand exchange HPLC as part of the radiosynthesis quality control and typically revealed over 99% enantiomeric excess of the final product. The specific activity at the end of the synthesis was about 400 GBq/µmol. Both preparative and analytical chiral ligand exchange chromatography were performed on reversed-phase C_{18} columns (ODS), coated with a D- or L-penicillamine derived chiral selector. The preparation of the chiral selectors, the coating procedures for the HPLC columns and the HPLC separation conditions have been described in detail earlier (15).

Surgical preparation: Surgery was performed under isoflurane anesthesia (2 – 3 % in air/oxygen 70 %/30 %) and involved the placement of an arteriovenous shunt (av-shunt) from the right femoral artery to the right femoral vein, tracheotomy for mechanical ventilation and craniotomy for the placement of the beta scintillator. The actual experiments were performed under α-chloralose anesthesia (44 mg/kg s.c.). The av-shunt was run through a coincidence scintillator (GE Medical Systems). The online arterial sampling procedure is described in detail elsewhere (16). In short, total radioactivity in arterial blood was continuously recorded and was then corrected for i) a different tracer concentration in whole blood and plasma and ii) the build-up of labeled metabolites.

D-lactate kinetics in the somatosensory cortex: For the measurement of the time-course of 1-^{11}C-D-lactate in the brain, an intracortical beta probe was used (17, 18). Twenty minutes of data were acquired at baseline condition after injection of 200-300 MBq of radiotracer in 5 animals. In one of these animals baseline cerebral blood flow was determined prior to 1-^{11}C-D-lactate acquisition using ^{15}O-H_2O and the methodology described previously (17). The first pass extraction fraction of 1-^{11}C-D-lactate was then calculated using the relationship EF = K_1/CBF where K_1 is the transport parameter describing (1-^{11}C-D-lactate) transport from blood to tissue (figure 2a).

Kinetic modeling: Acquired radioactivity data was analyzed using the software package PMOD (PMOD Technologies GmbH, Zürich, Switzerland). The investigated methods consisted of standard compartmental modeling using the arterial input function and the one-tissue compartment model. The parameters are as follows: K_1 describes the transport of the tracer across the blood brain barrier and k_2 represents the back-diffusion of label from tissue to the blood system. Carbon is labeled as “C”. Label exchange between compartments is described by the following differential equation:

$$\frac{dC_{tiss}}{dt} = K_1 C_{plasma}(t) - k_2 C_{tiss}(t)$$

The basis of the calculation of the CBF measurement was also the one-tissue compartment model including a partition coefficient for ^{15}O-H_2O [for details see (17)].

Metabolite analysis in the blood: Samples (about 400 µL) were collected at different time points after tracer injection, with a maximum of 4-5 blood samples per animal, to determine the time-course of the ratio of the ^{11}C activity in plasma to whole blood and for analysis of authentic tracer and metabolites. These samples were first centrifuged for 3 minutes at 2000 rpm. Proteins were then precipitated with 75 µL acetonitrile in 50 µL plasma. After centrifugation for 3 minutes at 2000 rpm, the composition of the ^{11}C-derived radioactivity in the supernatant (80 µL) was analyzed by high-performance liquid chromatography (HPLC) on a polymeric column (PRP-1, 5-µm, 250 × 4.1 mm i.d., Hamilton, U.S.A.) with 3 mmol/L phosphoric acid in water (pH 2.67) as the mobile phase (1 mL/min). The retention times of ^{11}C-HCO_3^- (3.3 min) and lactic acid (5.1 min) were determined by using aqueous solution of $NaHCO_3$ and DL-lactic acid as reference compounds, detected by UV absorption at 220 nm. The amount of authentic tracer was expressed as a fraction of total plasma counts.

Metabolite analysis in brain: At the end of the experiment the rats were perfused with PBS, brains were removed and prepared for measurements with the HPLC system. Each brain was first homogenized before adding acetonitrile (150 %, i.e. 1.5x volume / weight of brain sample). The subsequent procedure was the same as with the blood samples described above, except that the amount of supernatant injected into the HPLC system was 200 µL.

Statistical analysis.

All data are presented as mean ± s.d. Statistical analyses were performed using non-parametric tests; one way ANOVA (Kruskal Wallis followed by Dunn's multiple comparison test) was used for analysis of *in vitro* experiments and the Mann-Whitney test was used for comparison between two groups in the *in vivo* experiments and for the behavioral experiments. Paired t-test was used to compare protein expression. $P < 0.05$ was considered statistically significant.

Results

HCA1 receptor expression and a possible neuroprotective role in ischemia

Recent studies revealed the presence and mechanism of action of the HCA1 receptor (formerly known as GPR81) in the central nervous system (8-10). Since L-lactate acts as an endogenous ligand for this receptor, it was of interest to describe the cellular expression of this receptor in several brain regions affected by ischemic damage in the mouse tMCAO model. We confirmed a strong neuronal expression of the HCA1 receptor in the hippocampus (CA1 and dentate gyrus), the cortex and the striatum as revealed by double labeling immunofluorescence for the HCA1 receptor and the neuronal markers NeuN or MAP2 (figure 1: NeuN [green], MAP2 [green], HCA1 [red], DAPI [blue]). The next step was to evaluate whether such HCA1 receptor expression was altered at different time points following 30 minutes tMCAO. For this purpose, the level of HCA1 receptor expression was evaluated on Western blots in protein extracts from hippocampus, cerebral cortex and striatum at 1, 3, 8, 24 and 48 hours following 30 minutes of tMCAO. Results at 1, 3 and 8 hours did not reveal significant differences between the three brain structures investigated (see supplemental table 1). However, a significant increase in HCA1 receptor expression was seen in the ischemic cortex after 24 hours (1.2 ± 0.2, $p < 0.05$ vs. contralateral cortex; figure 2a). Even though there is neuronal loss in the lesion core (ischemic striatum), we did not observe a significant decrease in HCA1 receptor expression in this structure (0.8 ± 0.3, n.s. vs. contralateral cortex) which might reflect a compensatory increase in HCA1 receptor expression in remaining neurons (figure 2a). At 48 hours post-ischemia, no difference could be observed between ischemic and contralateral tissues (see supplemental table 1). Based on this result and the possibility that lactate regulates HCA1 receptor expression we injected L-lactate intravenously at the beginning of the reperfusion period and monitored any changes in HCA1 receptor expression at 24 hours after the ischemic episode. We found an increase in HCA1 receptor expression in both the ischemic cortex (1.57 ± 0.34, $p < 0.04$ vs. contralateral cortex) and ischemic striatum (1.7 ± 0.4, $p < 0.05$ vs. contralateral striatum) while no significant difference was

observed for the hippocampus (figure 2b). These observations suggest that the expression of the HCA1 receptor may be modulated by ischemia and potentially by lactate injected at reperfusion.

We went on to further investigate the role of HCA1 receptors under ischemic conditions in an *in vitro* model. For this purpose, we used rat organotypic hippocampal slices exposed to an oxygen-glucose deprivation (OGD) protocol. We observed a significant increase in HCA1 receptor expression 48 hours after OGD (1.45 ± 0.16, $p < 0.01$ vs. control slices; figure 2c). In order to determine if the enhanced HCA1 receptor expression could play a role in the neuroprotective effect of L-lactate in this model, organotypic hippocampal slices were exposed to the HCA1 receptor agonist 3,5-dihydroxybenzoic acid (DHBA) after OGD. Results show that DHBA produced significant protection as it reduced neuronal cell death in the CA1 region of the hippocampus 48 hours after insult from 13.5 ± 7.2 % to 7.4 ± 5.2 % ($p < 0.05$; figure 2d).

D-Lactate neuroprotection in ischemia models

The D enantiomer of lactate has been described to act at least as a partial agonist of the HCA1 receptor (9, 19). For this reason, we tested the neuroprotective effect of D-lactate both *in vitro* and *in vivo*. The administration of 4 mM D-lactate to rat organotypic hippocampal slices directly after OGD also resulted in significantly reduced neuronal cell death in the CA1 region of the hippocampus 48 hours after OGD, from 29.2 ± 14.3 % to 11.3 ± 7.3 % ($p < 0.05$; figure 3a). It is noteworthy that the neuroprotective effect approaches that previously reported for L-lactate in this model (11). We then tested D-lactate *in vivo* by intravenous administration of 1 µmol/g D-Lactate after 45 minutes tMCAO. Mice with satisfactory ischemia (rCBF < 20% of baseline) and reperfusion (rCBF above 50% of baseline) received a single intravenous administration of D-lactate or vehicle, 10-15 minutes after the silicon-coated monofilament was removed allowing reperfusion. A total of 9 mice were not included in the study as they did not fulfill the rCBF inclusion criteria. Single administration of D-lactate significantly decreased the infarct volume from 81.8 ± 40.1 mm^3 (control group) to 45.5 ± 32 mm^3 (D-lactate group) measured 48 hours after ischemia ($p < 0.05$; figure 3b). Again, the magnitude of neuroprotection was comparable to that reported previously for L-lactate *in vivo (12)*. Behavioral performances on motor task assessment showed a clearly milder neurological deficit at 48 hours ($p < 0.05$) in the D-Lactate group, from a median of 2.5, with scores ranging from 1 to 5 (control group), to a median of 1, with scores ranging from 0 and 1 (D-lactate group, $p < 0.05$, figure 3c). One mouse from the control group and two mice from the D-lactate group died during the first night after surgery. Three mice per group were sacrificed within the first 6 hours after surgery due to the presence of repeated seizures as required by the veterinary authority.

Cerebral D-lactate metabolism

Different *in vivo* and *in vitro* studies have suggested that D-lactate is not metabolized in cerebral tissue (6, 20, 21) based on the apparent lack of expression of the specific enzyme D-lactate dehydrogenase in mammals. In order to clarify this issue in the rodent brain we set up a series of experiments using radiolabeled D-lactate to trace its metabolism and kinetics (figure 4a). To evaluate the brain uptake of D-lactate, we determined the first pass extraction fraction, which

was 16 %. The tissue kinetics of 1-^{11}C-D-lactate displayed an increase over 5 minutes and slow washout thereafter upon tracer injection (figure 4b). The average respective first-order kinetic rate constants obtained by kinetic modeling were 0.059 ± 0.01 ml/min/ml tissue (K_1) and 0.063 ± 0.01 min^{-1} (k_2). Average lactate levels in the blood were 2.2 ± 0.93 mmol/L.

^{11}C-CO_2 turned out to be the only metabolite in blood and brain. The time course of the fraction of authentic tracer in arterial plasma is shown in figure 4c. The pooled fraction data of all animals was approximated by a fit of a quadratic polynomial. This function was subsequently employed to convert the total plasma activity to the time-course of authentic ^{11}C-lactate (= input curve). At 40 minutes the fraction of true tracer in plasma dropped to about 30 percent. Measurements in brain tissue at the end of the experiment revealed about one third CO_2 and two thirds lactate (figure 4d).

Effect of pyruvate and acetate in ischemia models

Results obtained on D-lactate metabolism suggest that in addition to a possible activation of the HCA1 receptor, utilization of D-lactate as an energy substrate might also contribute to its beneficial effect in ischemia. Therefore, we explored the specificity of its metabolic mode of action by testing the neuroprotective effect of two other monocarboxylates. Pyruvate is the immediate downstream product of either L- or D-lactate and is the main substrate entering the Krebs cycle to provide energy. Other groups have previously described the neuroprotective effect of pyruvate in different models of cerebral ischemia (2, 22). In agreement with these observations, administration of 10 mM pyruvate directly after OGD significantly decreased cell death in the hippocampal CA1 region at 48 h from 25.4 ± 14.5% to 13.9 ± 10.3% ($p < 0.05$; figure 5a and 5b). In contrast, we did not observe a significant benefit *in vivo* after tMCAO. In our hands pyruvate administration at reperfusion after 30 minutes tMCAO induced only a slight reduction in lesion size ($p = 0.4$), from 91.9 ± 53.6 mm^3 (control group, n= 7) to 64.6 ± 53.4 mm^3 (pyruvate, n=5) 48 hours after ischemia. A total of 4 mice were not included because they did not fulfill rCBF inclusion criteria. There was no mortality in this experiment. Due to the inherent variability of the model our experiment was however not powered to detect a significant neuroprotective effect (the sample size calculation using an alpha error of 5% and a beta error of 50% resulted in a sample size of 21 per group, www.dssresearch.com).

As both L- and D-lactate could be taken up and metabolized in principle by either neurons or astrocytes, we wanted to determine whether the beneficial metabolic effect of lactate *in vitro* could be exerted via its use by astrocytes. For this purpose, we made use of acetate which is known to be specifically taken up and used as energy substrate by astrocytes (23). However, the addition of different concentrations of acetate (0.13, 0.2, 4 and 8 mM) to organotypic hippocampal slices 1 hour after OGD did not provide significant neuroprotection (figure 5a and 5c).

Discussion

After being considered for decades as a waste-product of metabolism lactate has been shown to be able to support synaptic activity (1, 24) and is nowadays recognized as a prominent energy substrate in the central nervous system (25). Lactate is produced under physiological conditions (26, 27) and it is suggested to play an important role in the metabolic support of long axons (6, 28) as well as in long-term memory formation (29). Lack of adequate lactate supply has also been implicated in some neurodegenerative diseases such as amyotrophic lateral sclerosis (30). Moreover, exogenous lactate supplementation seems beneficial after traumatic brain injury (31-33). In ischemic conditions, our group and others have demonstrated neuroprotection following L-lactate administration at reperfusion after tMCAO (11, 12, 34). Similarly, brain lactate was found to be essential to improve post ischemic outcome after cardiac arrest (35). Lactate can be transported from one cell type to another by monocarboxylate transporters that are selectively expressed in different cell types and which can transport monocarboxylates with different affinities [for a comprehensive review please refer to (36)]. More recently, lactate has been described to act as a signaling molecule in the central nervous system, either through the HCA1 receptor (8, 9) or through an unknown receptor yet to be specified (37).

Our initial characterization of the distribution of the HCA1 receptor in three brain regions affected following tMCAO confirmed a prominent neuronal expression in the central nervous system (8, 9). Interestingly, our data also suggested that the cerebral expression of HCA1 can be modulated under certain conditions. Lactate itself seems to be a signal for the induction of its own receptor. However, whether lactate accumulation following ischemia is responsible for the enhancement of the HCA1 receptor observed after 24 hours of reperfusion remains to be directly demonstrated. Nonetheless our results provide the first evidence that activation of HCA1 receptors alone is sufficient to provide neuroprotection, at least in an *in vitro* ischemic model. Our demonstration that neuroprotection was also achieved with D-Lactate confirms this observation in that D-lactate was shown to exhibit at least partial agonist activity on HCA1/GPR81 receptor (19, 38) and that D-lactate was considered as a non-metabolizable lactate enantiomer and often used as a negative control to demonstrate the necessity of L-lactate use as an alternative energy substrate. However unexpectedly, we found that D-lactate is extracted by the rodent brain and oxidized. In fact, one third of D-lactate was metabolized to CO_2 which is only marginally less than the previously reported fraction of CO_2 produced from L-lactate [42 ± 8 % CO_2 after 40 minutes; (1)]. This observation suggests that the rodent brain might possess a D-Lactate dehydrogenase activity. In accordance, the isolation of human and mouse transcripts encoding a homolog of the yeast D-lactate dehydrogenase was reported and its expression was found in various tissues, including the brain (39). However, it remains to be determined whether the presence of a cerebral D-Lactate dehydrogenase enzyme is sufficient to explain our results or if the activity is carried out by another *a priori* unrelated enzyme.

In the central nervous system, pyruvate is transported mainly by MCT1 and MCT2 in the direction of the concentration gradient and these MCTs are located in astrocytes and neurons, respectively (4, 40). Possible explanations for the neuroprotection against ischemic damage induced by either L- or D-lactate are the fact that lactate is converted to pyruvate by the

mitochondrial lactate dehydrogenase (mLDH) to fuel the Krebs cycle and therefore used as energetic substrate and that this conversion produces reduced nicotinamide adenine dinucleotide (NADH) that will in turn act as a reactive oxygen species (ROS) scavenger (41). Evidence pointing towards this metabolic explanation is the protection exerted by the administration of 10 mM pyruvate *in vitro*. Protection is not seen *in vivo* though. However, in support of a metabolism-mediated neuroprotective effect others (2, 22) have shown protection *in vivo* using an extended i.v. mode of administration of pyruvate (22). This difference in the mode of administration might explain the different *in vivo* results, although we cannot exclude that lactate might prove to be a more efficient neuroprotective agent, notably *in vivo*, in particular due to the ability of both L- and D-lactate to activate the HCA1 receptor.

It has been shown that acetate is preferentially transported by MCT1 and metabolized in astrocytes (18, 23). During ischemia it was observed that ^{14}C-acetate uptake is decreased as early as 3 minutes after the onset of ischemia. The degree of reduction in ^{14}C-acetate uptake correlates with the severity of ischemia which, according to Hosoi et al., relates to the depression of glial metabolism (42). Even though the reduction in ^{14}C-acetate uptake was reversible and reached full recovery 3 hours after 30 minutes MCAO reperfusion, Hosoi et al. did not evaluate the functional outcome of acetate administration. We showed here that the administration of acetate did not exert protection against neuronal death in hippocampal slices subjected to oxygen and glucose deprivation. Even though there is an increased energy demand in neurons and astrocytes subjected to ischemic insult, this result suggests that energy supply to astrocytes is not enough to exert neuroprotection. It should be noted that using an *in vitro* assay of ligand-induced ^{35}S-GTPγS binding with membranes from CHO cells expressing human HCA1 receptor, Cai et al., described that neither sodium pyruvate nor sodium acetate showed binding activity (19). As neither acetate nor pyruvate bind to the HCA-1 receptor, the results shown here cannot be related to HCA1 signaling pointing to the use of pyruvate and acetate as metabolic substrates.

Some limitations to consider in the present study are that (a) our in vivo stroke model involves a transient occlusion with removal of the silicon filament, therefore our results are limited to be translated to patients with either a spontaneous reperfusion or successful intravenous thrombolysis or intrarterial recanalisation. Evaluating the effect of lactate administration in permanent ischemia is an important step before aiming for translation in clinical practice. (b) Ischemia is induced under isoflurane anesthesia and this compound has been described as neuroprotective by itself. Therefore, we might not be observing the neurological damage to its fullness. In our in vitro model, oxygen and glucose deprivation on organotypic hippocampal slice cultures, the tissue architecture is only partially preserved and while some neuronal connections are maintained many are interrupted. Furthermore, there is no blood circulation. Despite these limitations, both models complement each other and strengthen our observations. (c) It should be considered as well that lactate was shown to increase regional CBF in physiologically activated but not resting human brain (43). Although we cannot rule out a contribution from CBF changes, the effect observed in the *in vitro* model, independently from blood flow, shows that lactate exerts a neuroprotective effect independently from possible effects on cerebral blood flow.

Taken together, these data point to the possibility that lactate, independently of the enantiomer type, may confer neuroprotection following ischemia by two distinct mechanisms: One, through the classical metabolic pathway providing an alternative energy supply to deprived neurons and the other through a receptor-mediated signal transduction mechanism in neurons. In both cases the exact targets within the cell still need to be identified.

Supplementary material is available at the JCBFM web site (www.nature.com/jcbfm).

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Disclosure of Interests/Conflicts statement: Authors have no conflict of interest

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Figures and tables

Figure 1. Confocal visualization of HCA1 receptor expression. Using two different antibodies against neuronal cells, we confirmed with immunohistochemistry the localization of the HCA1 receptor is in neurons. The brain structures analyzed were hippocampus (CA1 [1a-1f] and Dentate Gyrus (DG) [2a-2f] regions) primary cortex [3a-3f] and Striatum [4a-4f]. Color code: NeuN [green, a and c], MAP2 [green, d and f], HCA1 [red, b-c and e-f], DAPI [blue]. Magnification: 40µm.

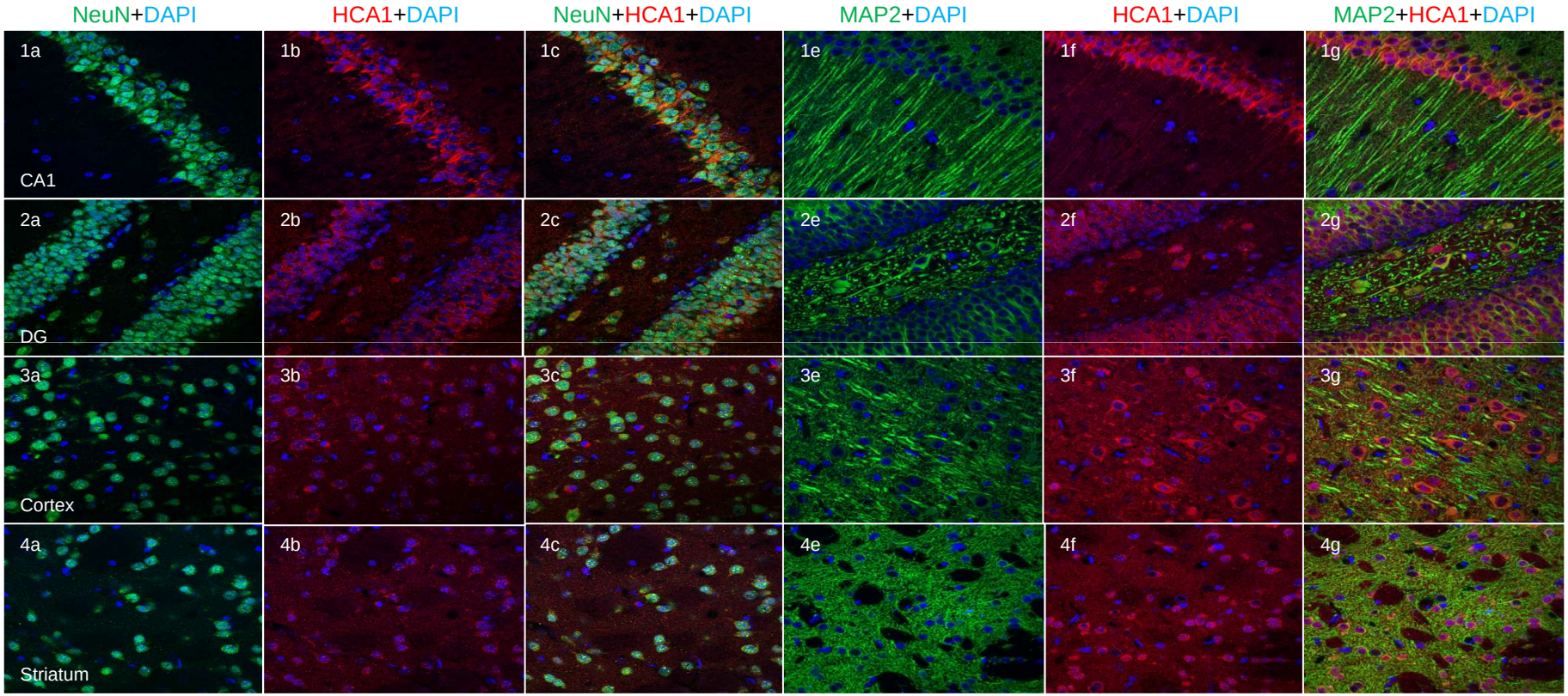
Figure 2. The HCA1 receptor expression is promoted by ischemia and plays a role in the neuroprotective effect. (a) Western blot analysis showed an increase in the HCA1 receptor expression at 24 h after tMCAO in the region surrounding the lesion site (primary motor and somatosensory cortex), in comparison to the contralateral hemisphere. (b) Intravenous L-lactate injection prompted an increase also in the lesion site (striatum) at the same time point (*$p < 0.05$; paired t-test ischemic vs contralateral hemisphere). (c) Western blot analysis showed an increase in the HCA1 receptor expression at 48 h after OGD (**$p < 0.01$ paired t-test). (d) Administration of 4 mM 3-5 DHBA, an HCA1 receptor agonist, attenuated cell death when administered directly to the medium after OGD (*$p < 0.05$, ***$p < 0.001$ One way ANOVA Kruskal-Wallis test followed by Dunn's multiple comparison test).

Figure 3. Neuroprotection by D-lactate. a) *In vitro*, administration of 4 mM D-lactate induced a significant reduction in cell death, assessed by PI staining, 48 h after treatment (*$p < 0.05$, One way ANOVA Kruskal-Wallis followed by Dunn's multiple comparison test). *In vivo,* intravenous injection of 1 µmol/g D-lactate after 45 min tMCAO decreased total infarct volumes measured 48 h after ischemia (b) and improved the neurological outcome (c), neurologic deficit scores from 0, no deficit – white, to 5 worst outcome – black; *$p < 0.05$ Mann-Whitney test, two-tailed p-value).

Figure 4. D-lactate metabolism. a) Schematic of biochemical pathways involved in the degradation of lactate and the proposed interpretation of the rate constants K_1 and k_2 which are mathematically defined by the one-tissue compartment model used for 1-^{11}C-D-lactate data analysis. b) Measured radioactivity concentration in the brain (open circles), model fit (black line) and arterial input curve (gray line). The inset displays the residuals of the fitting to the one-tissue compartment model. Equal distribution around the neutral line supports the adequacy of the applied model. c) Fraction of native radiolabeled lactate over 40 minutes after intravenous injection of 1-^{11}C-D-lactate in the blood. The filled circles represent data points from individual measurements and the solid line is the corresponding fit. d) Percentage of identified metabolites in brain tissue after 40 minutes.

Figure 5. Administration of pyruvate and acetate after OGD. (a) Administration of 10 mM pyruvate directly to the medium after 1 h OGD in rat organotypic hippocampal slices was able to protect against ischemic damage. (b) Administration of various doses of acetate did not protect against ischemic damage. Cell death was assessed by PI staining 48 h after treatment. (c) Representative images of PI staining among each group can be observed in panel (*$p < 0.05$; One way ANOVA Kruskal-Wallis test followed by Dunn's multiple comparison test).

Figure 1


NeuN+DAPI
HCA1+DAPI
NeuN+HCA1+DAPI
MAP2+DAPI
HCA1+DAPI
MAP2+HCA1+DAPI
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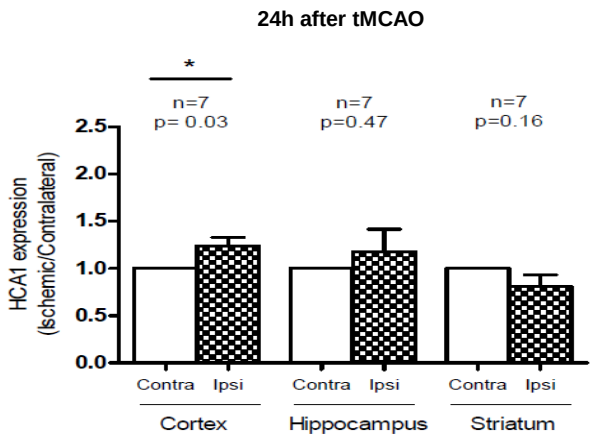
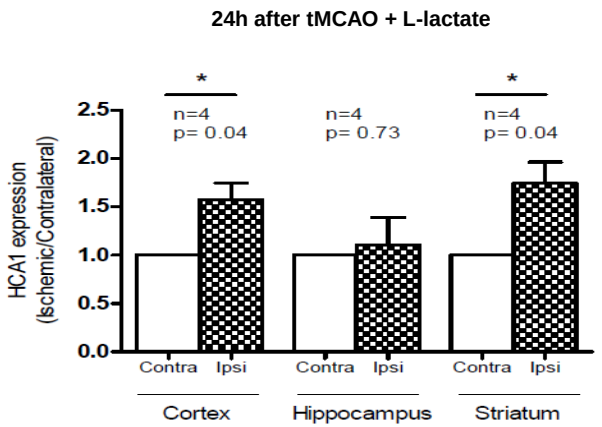
1b
1c
1e
1f
1g
CA1
2a
2b
2c
2e
2f
2g
DG
3a
3b
3c
3e
3f
3g
Cortex
4a
4b
4c
4e
4f
4g
Striatum

Figure 2

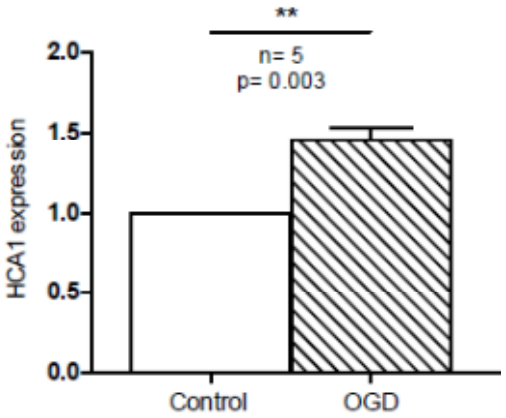
a



b



c



d

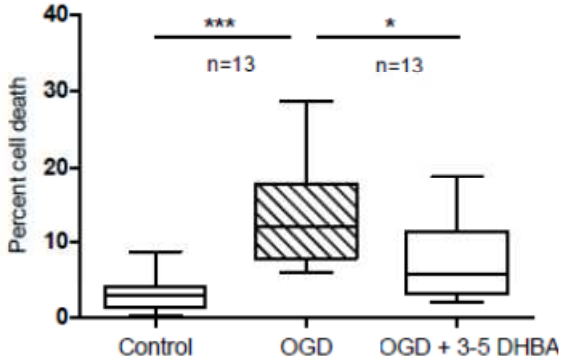
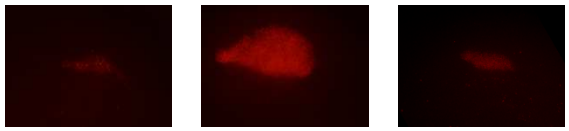
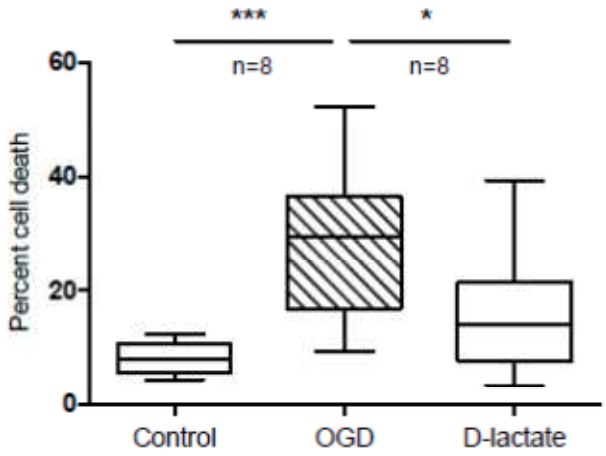


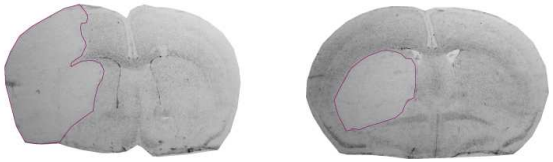
Figure 3

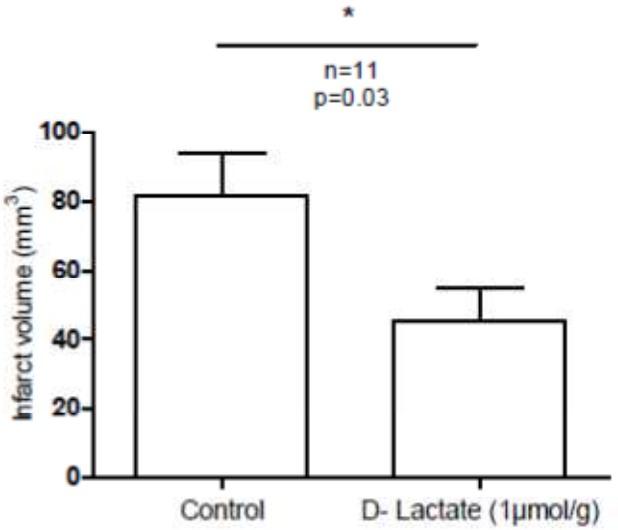
a




***
*
n=8
n=8
Percent cell death
60
40
20
0
Control
OGD
D-lactate

b




*
n=11
p=0.03
Infarct volume (mm3)
100
80
60
40
20
0
Control
D- Lactate (1µmol/g)

c

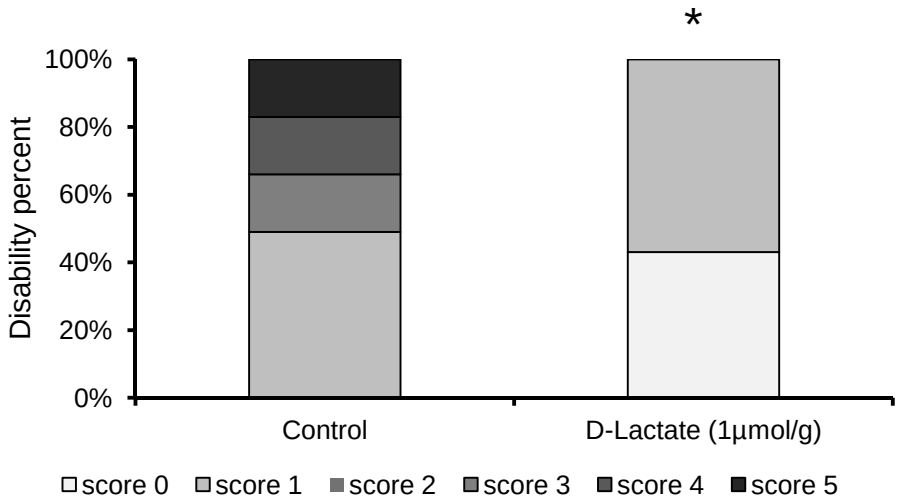
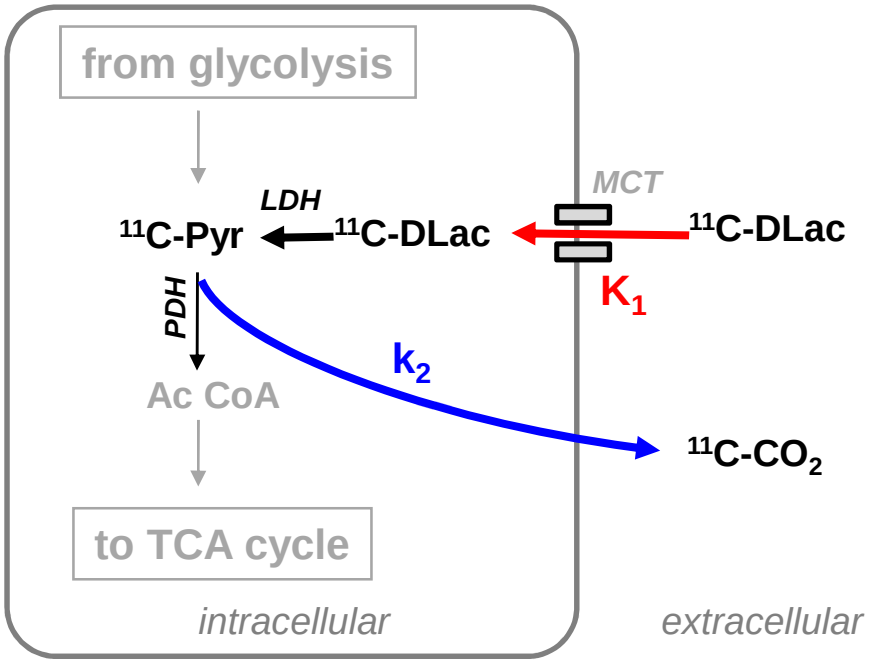
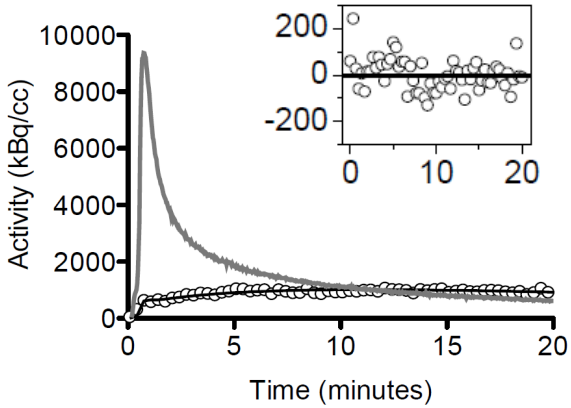

*
Disability percent
100%
80%
60%
40%
20%
0%
Control
D-Lactate (1µmol/g)
score 0
score 1
score 2
score 3
score 4
score 5

Figure 4

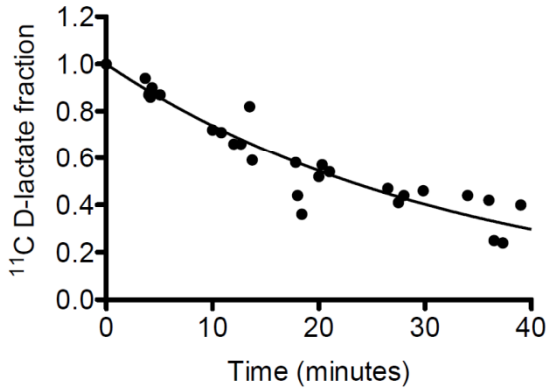
a



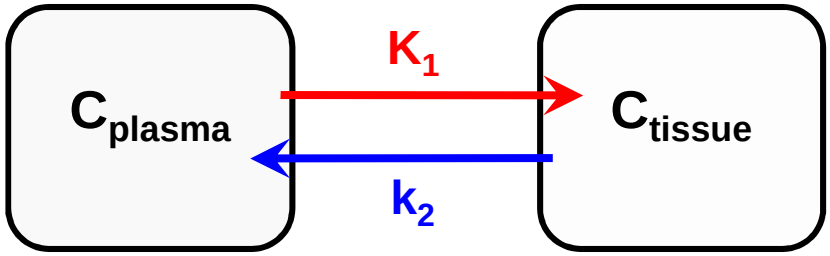
b



c



1-Tissue compartment model:



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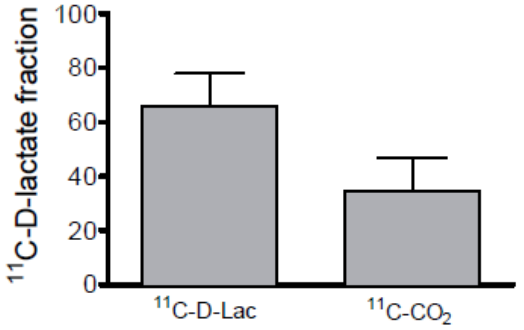
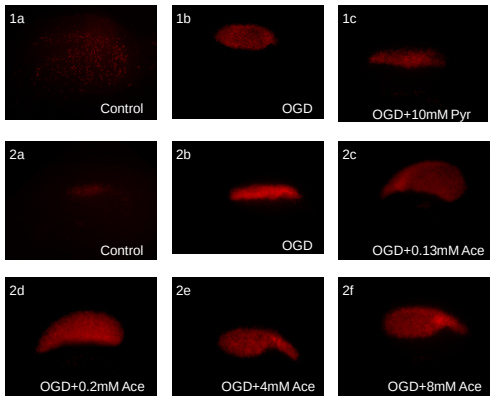
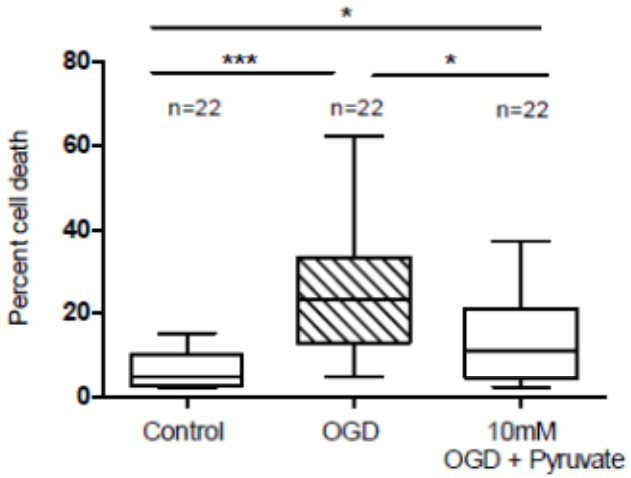


Figure 5

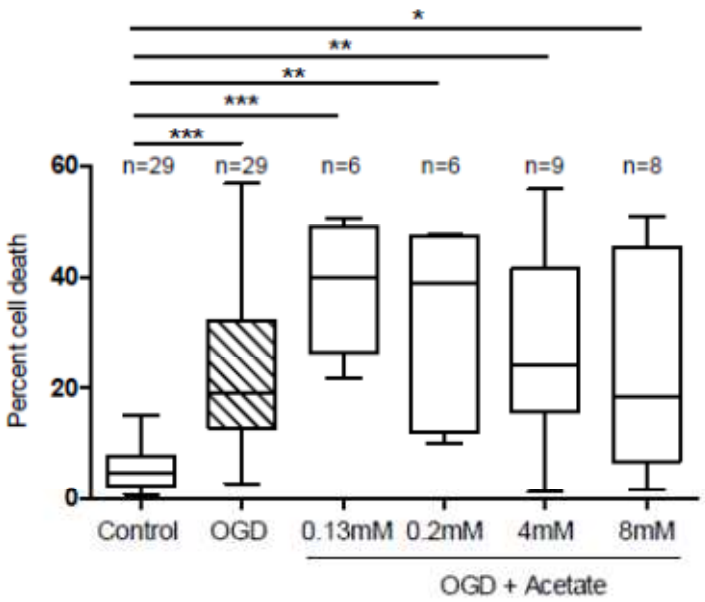
a


1a
Control
1b
OGD
1c
OGD+10mM Pyr
2a
Control
2b
OGD
2c
OGD+0.13mM Ace
2d
OGD+0.2mM Ace
2e
OGD+4mM Ace
2f
OGD+8mM Ace

b


*
***
*
n=22
n=22
n=22
Percent cell death
80
60
40
20
0
Control
OGD
10mM
OGD + Pyruvate

c


*
**
**
***
***
n=29
n=29
n=6
n=6
n=9
n=8
Percent cell death
60
40
20
0
Control
OGD
0.13mM
0.2mM
4mM
8mM
OGD + Acetate

Suppl. figure 1

Time after 30' tMCAO	Primary motor and somatosensory cortex	Hippocampus	Striatum
1h	1.34 ± 0.76	1.16 ± 0.41	0.74 ± 0.28
3h	0.93 ± 0.12	0.72 ± 0.23	1.07 ± 0.25
8h	1.10 ± 0.15	1.13 ± 0.40	0.84 ± 0.24
48h	1.08 ± 0.25	1.27 ± 0.25	0.82 ± 0.38