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LACTATE NEUROPROTECTION IN CEREBRAL ISCHEMIA : A PROBABLE DUAL MECHANISM OF ACTION

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LACTATE NEUROPROTECTION IN CEREBRAL ISCHEMIA : A PROBABLE DUAL MECHANISM OF ACTION

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ABSTRACT

In cerebral ischemia, a central mechanism leading to neural damage is energy failure due to the lack of oxygen and glucose supply but it has been shown that besides glucose, other metabolic intermediates such as lactate, pyruvate or acetate can be oxidized for energy production. L-lactate is neuroprotective when administered either after oxygen and glucose deprivation (OGD) or during reperfusion after transient middle cerebral artery occlusion (tMCAO). New evidence has risen concerning the presence and possible involvement of the Hydroxy-Carboxylic Acid Receptor-1 (HCA1) in nervous system effects of lactate. Therefore, the objective of the present work was to elucidate whether the neuroprotective effects of lactate are exerted as an energy substrate or acting through the HCA1 receptor.

In this work, we show that the HCA1 receptor expression is enhanced 24 hours after reperfusion in a 30min MCAO stroke model, in the lesion core. Interestingly, intravenous injection of L-lactate at reperfusion led to further enhancement of HCA1 receptor expression in the ischemic cortex and striatum (lesion site). To test the role of the HCA1 receptor we evaluated the effect of a receptor agonist, 3,5-dihydroxybenzoic acid (3-5 DHBA), in the *in vitro* OGD. The administration of 3-5 DHBA to the organotypic hippocampal cultures after 1h OGD reduced cell death. Protein expression analysis revealed that the hippocampal cultures receiving 3-5 DHBA showed an increase protein expression of HCA1 in comparison to the control hippocampal slices. We also report that D-lactate, a reputedly non-metabolizable substrate but a partial HCA1 receptor agonist also provided neuroprotection in ischemia models both *in vitro* and *in vivo*. It decreased the percentage of cell death in hippocampal cultures subjected to OGD as well as the lesion size and the neurological deficit in the mice receiving intravenous administration of D-lactate at reperfusion, after 45min MCAO. Quite unexpectedly, and thanks to collaboration with the group of Prof. Bruno Weber from the University Hospital of Zürich, we show D-lactate to be partly extracted and oxidized by the rodent brain. Finally, to explore a bit further the metabolic pathway we tested other monocarboxylates, pyruvate and acetate, in the *in vitro* model. Pyruvate offered neuroprotection by decreasing the percentage of cell death while acetate was ineffective.

Altogether, 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.

RÉSUMÉ

Dans l'ischémie cérébrale, le manque d'énergie relatif à un manque d'apport d'oxygène et de glucose est le mécanisme central menant à l'atteinte neural. Il a été montré que, mis à part le glucose, d'autres intermédiaires métaboliques comme le lactate, le pyruvate ou l'acétate peuvent être oxydés pour produire de l'énergie. Le L-lactate est neuroprotecteur lorsqu'il est administré soit après la privation d'oxygène et de glucose (OGD) ou durant la reperfusion après occlusion transitoire de l'artère cérébrale moyenne (MCAO). Il a été montré récemment que le récepteur 1 de l'acide hydroxy-carboxylique (HCA1) était impliqué dans certains effets du lactate dans le système nerveux. Ce travail a donc pour objectif de déterminer si le lactate exerce ses effets neuroprotecteurs en tant que substrat énergétique ou à travers les récepteurs HCA1.

Dans cette étude, nous avons montré que l'expression des récepteurs HCA1 est augmentée 24 heures après la reperfusion, dans le centre de la lésion, dans le modèle d'accident vasculaire cérébrale chez la souris avec MCAO de 30 minutes. Fait intéressant, l'injection intra-veineuse de L-lactate au moment de la reperfusion conduit à une augmentation de l'expression des récepteurs HCA1 au niveau du cortex et du striatum ischémiques (sites de lésion). Afin de tester le rôle des récepteurs HCA1, nous avons étudié l'effet d'un de ses agonistes, l'acide 3,5-dihydroxybenzoique (3-5 DHBA), dans un modèle *in vitro* d'OGD. L'application de 3-5 DHBA sur des cultures organotypiques de l'hippocampe après 1 heure d'OGD a réduit la mort cellulaire. L'analyse de l'expression des protéines a révélé une augmentation de l'expression des récepteurs HCA1 dans des cultures d'hippocampe ayant reçu du 3-5 DHBA comparé à des cultures contrôles de l'hippocampe. Nous avons aussi montré que le D-Lactate, substrat réputé non métabolisable mais aussi un agoniste partiel des récepteurs HCA1 a aussi un rôle neuroprotecteur dans les modèles d'ischémie non seulement *in vitro* mais aussi *in vivo*. En effet, il diminue le taux de mort cellulaire dans les cultures d'hippocampe soumises à l'OGD, tout comme la taille de la lésion et les déficits neurologiques chez des souris ayant reçues une injection intra-veineuse de D-Lactate au moment de la reperfusion, après 45 minutes de MCAO. Nous avons découvert, en collaboration avec le groupe du Prof. Bruno Weber le résultat assez inattendu que le D-Lactate pouvait être partiellement extrait et oxydé par le cerveau de rongeur. Finalement, afin d'explorer un peu plus en détail la voie de signalisation métabolique, nous avons testé d'autres monocarboxylates, le pyruvate et le lactate dans le modèle *in vitro* : le pyruvate apporte une neuroprotection en diminuant le pourcentage de mort cellulaire alors que l'acétate est inefficace.

Toutes nos données combinées montrent que le L- et le D- lactate offrent une protection dans l'ischémie en agissant le plus probablement à la fois comme agonistes des récepteurs HCA1 pour les cellules non astrocytaires (probablement neuronales) et comme substrat énergétique.

LIST OF ABBREVIATIONS

2DG	2-deoxy-D-glucose
3,5-DHBA	3,5-Dihydroxybenzoic acid
4-CIN	alpha-cyano-4-hydroxycinnamate
AHA	American Heart Association
ALS	Amyotrophic Lateral Sclerosis
ANLS	Astrocyte-Neuron Lactate Shuttle
ANOVA	Analysis of Variance
AT	Anaerobic Threshold
ATP	Adenosine Tri-Phosphate
cAMP	cyclic Adenosine Monophosphate
CA1	*Cornus Ammonis*-1
CA3	*Cornus Ammonis*-3
CBF	Cerebral Blood Flow
CCA	Common Carotid Artery
CO_2	Carbon dioxide
CoA	acetyl-coenzyme-A
CT	Computer Tomography
DAPI	4',6-Diamidino-2-Phenylindole
DG	Dentate Gyrus
DMEM	Dulbecco's Modified Eagle Medium
DWI	Diffusion-Weighted Imaging
EP	Ethyl pyruvate
ERK	Extracellular signal-Regulated Kinase
GFAP	Glial Fibrilar Acidic Protein
Glu	Glutamate
GLUT	Glucose Transporter
Gln	Glutamine
HBSS	Hank's Buffered Salt Solution
HCA1	Hydroxy-carboxylic Acid Receptor 1
HEPES	Hydroxyethyl-Piperazineethane Sulfonic acid
HLa	Lactic acid
H_2O_2	Hydrogen peroxide
ICA	Internal Carotid Artery
ICV	Intracerebroventricular
IgG	Immunoglobulin G
IP	Intraperitoneal
IV	Intravenous
LC	Locus Coeruleus
LDH	Lactate Dehydrogenase
MAP2	Microtubule Associated Protein
MCAO	Middle Cerebral Artery Occlusion
MCT	Monocarboxylate transporter
MEM	Minimum Essential Medium
MRI	Magnetic Resonance Imaging
MRS	Magnetic Resonance Spectroscopy

NAA	N-acetylaspartate
NADH	Nicotinamide adenine dinucleotide
NeuN	Neuronal Nuclei
NO	Nitric Oxide
OGD	Oxygen and Glucose Deprivation
PBS	Phosphate Buffer Solution
PET	Positron Emission Tomography
Pi	inorganic Phosphate
PI	Propidium Iodide
PIDs	Peri-infarct depolarizations
PO_2	Oxygen partial pressure
rCBF	regional Cerebral Blood Flow
RIPA	Radioimmunoprecipitation Assay
rtPA	recombinant tissue Plasminogen Activator
s.d.	Standard Deviation
SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
si-RNA	small interfering- RNA
SLC16	Solute Carrier 16
TAT1	T-type amino-acid transporter 1
Tau	Taurine
TBI	Traumatic Brain Injury
TBS-T	Tris-Buffered Saline-Tween
TGI	Transient Global Ischemia
TOAST	Trial of Org 10172 in Acute Stroke Treatment
X-ALD	X-linked adrenoleukodystrophy

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LIST OF PUBLICATIONS

Modulation of monocarboxylate transporters expression contributes to the metabolic reprogramming during cerebral ischemia.
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Berthet C, **Castillo X**, Magistretti PJ, Hirt L (2012) New evidence of neuroprotection by lactate after transient focal cerebral ischaemia: extended benefit after intracerebroventricular injection and efficacy of intravenous administration. *Cerebrovasc Dis* 34(5-6): 329-335.

I. INTRODUCTION

1. CLINICAL AND MOLECULAR CONSIDERATIONS OF STROKE

a) Epidemiological Burden

In 1971 the World Health Organization started the first attempt to collect data on stroke in communities in a uniform manner and from countries with different social, cultural, and environmental backgrounds. For this purpose stroke was defined as "rapidly developing clinical signs of focal (at times global) disturbance of cerebral function lasting more than 24 h or leading to death with no apparent cause other than that of vascular origin" (Hatano, 1976).

Since then, it has been described that stroke is a frequent and potentially disabling disease, present worldwide, and for which early identification and treatment is essential. Currently there is an estimate of around 800,000 new or recurrent strokes (either ischemic or hemorrhagic) every year (Go et al., 2014). Incidence of stroke varies greatly between high income and low to middle income countries, the latter ones showing an increase over the last three decades in the incidence of stroke subtypes (Feigin et al., 2009). According to the American Heart Association in high-income countries the incidence of stroke has decreased in the last decades thanks to increased awareness and control of risk factors as well as early identification of the disease. The different incidence among high, middle and low income countries is possibly due to the lack of adequate technology for proper and fast diagnosis in low and middle income countries. On the other hand, the reported increase in stroke incidence in the these countries might reflect the slow shift from a traditional to a Westernized diet and lifestyle increasing the cardiovascular and cerebrovascular risk factors for stroke(Reddy, 2004).Population risk factors are: hypertension, diabetes mellitus, hyperlipidemia, cigarette smoking, cardiac disease (atrial fibrillation, rheumatic heart disease, mitral valve disease, infective endocarditis, atrial myxoma and mural thrombi complicating myocardial infarction) and a family history of stroke (Go et al., 2014).

b) Pathological subtypes

Stroke has been subdivided pathologically into hemorrhagic and ischemic types (thrombotic or embolic).According to the American Heart Association, approximately 87% of strokes are due to ischemic and the remaining 13% are due to hemorrhages. Clinicians must rapidly identify between these two types because they have distinct etiologies, prognosis and treatments. Some classical stroke clinical features may help to differentiate these subtypes: headache, neck stiffness,

vomiting and coma are more common in hemorrhagic stroke whereas previous transient ischemic attacks, atrial fibrillations and atherosclerosis risk factors are more common in ischemic stroke. Unfortunately these features are not sufficiently reliable and brain imaging is required in the acute phase to distinguish between ischemic and hemorrhagic stroke.

Table 1. Major ischemic stroke subtypes. TOAST classification, adapted from Adams et al., 1993.

Stroke type and subtype	Clinical features	Diagnosis
Ischemic stroke		
Large-artery atherosclerosis	Cerebral cortical impairment (aphasia, neglect, restricted motor involvement) or brain stem or cerebellar dysfunction.	Imaging findings on CT/MRI: significant stenosis (>50%) or occlusion of a major brain artery. Cortical or cerebellar lesions and brain stem or subcortical hemispheric infarcts >1.5cm. Duplex imaging or arteriography of a stenosis of greater than 50% of an appropriate intracranial or extracranial artery is needed.
Cardioembolism	Findings similar to those of large-artery atherosclerosis (cortical or cerebellar dysfunction).	Cortical, cerebellar, brain stem, or subcortical infarct >1.5cm. A cardiac source of emboli should be found.
Small-artery occlusion (lacune)	One of the traditional lacunar syndromes without evidence of cerebral cortical dysfunction. History of diabetes mellitus or hypertension.	Normal CT/MRI or brain stem or subcortical hemispheric lesion smaller than <1.5cm. Cardiac sources for embolism and stenosis greater than >50% of large extracranial arteries should be excluded.
Acute stroke of other determined etiology	Rare causes such as nonatherosclerotic vasculopathies, hypercoagulable states, or hematologic diseases.	CT/MRI findings of an acute ischemic stroke, regardless size or localization. Blood tests or arteriography should reveal the unusual cause of stroke. Cardiac sources for embolism and stenosis greater than >50% of large extracranial arteries should be excluded.
Stroke of undetermined etiology	Instances when cause of stroke cannot be determined despite an extensive evaluation or with at least two potential causes of stroke so that a final diagnosis cannot be determined.	

Table 2. Major hemorrhagic stroke subtypes and treatment. Adapted from Aminoff and Kerchner, 2013.

Stroke type and subtype	Clinical features	Diagnosis	Treatment
Hemorragic stroke			
Spontaneous intracerebral hemorrhage	Commonly associated with hypertension, occurs most frequently in the basal	Noncontrast CT; laboratory tests to identify bleeding disorders.	Generally conservative, but decompression is helpful when a hematoma

	ganglia. Cortical localizations may results from cerebral amyloid angiopathy	Lumbar puncture is contraindicated.	exerts a mass effect and with cerebellar hemorrhages.
Subarachnoid hemorrhage	Severe sudden headache that may be followed by nausea and vomiting and loss or impairment of consciousness; signs of meningeal irritation.	CT to confirm diagnosis. Lumbar puncture in case of negative CT to look for red blood cells or xanthochromia; angiography to determine source of bleeding.	General measurements to prevent further hemorrhage. Definitive treatment requires surgical clipping of the aneurysm base or endovascular treatment by interventional radiologist.
Intracranial aneurysm	Focal neurologic deficit by compressing adjacent structures.	Angiography indicates size and site of the lesion.	If symptomatic, surgical or endovascular techniques.
Arteriovenous malformations.	Supratentorial lesions: hemorrhage, recurrent seizures and headaches. Infratentorial lesions: Often silent.	Arteriography	If unrupted, pharmacological therapy for existent medical disorders or vascular risk factors (Moore et al., 2014

1.1 ISCHEMIC STROKE

Brain ischemia is a diminution of cerebral blood flow to below a critical threshold that induces brain damage. It can be divided into global or focal ischemia; global cerebral ischemia entails diminution over the entire brain and happens after different clinical conditions including transient circulatory/cardiac arrest, occlusion of cerebral or extracerebral vessels supplying nervous tissue, or periods of prolonged systemic hypotension. Focal ischemia follows a transient or permanent blood flow reduction in the territory of a cerebral artery typically due to embolic or thrombotic vessel occlusion.

1.1.1 Global cerebral ischemia

Global ischemia has been studied in many animal models including mice, gerbils, rabbits and dogs (Owens et al., 2014; Liu et al., 2014; Ishiyama et al., 2010; Sieber et al., 1994)where it has been described that specific neuronal populations in the brain, including CA1 pyramidal neurons of the hippocampus, medium-sized neurons of the striatum and the Purkinje cells of the cerebellum are the most susceptible to injury(Pulsinelli et al. 1982; Smith et al. 1984;Sheng et al. 1999; Wellons et al. 2000; Onken et al. 2012). The sensitivity of these neuronal populations was studied with a model of global cerebral ischemia with analysis of mitochondrial fusion and fission after ischemia (Owens et al., 2014). They showed that mitochondrial fission is activated in all hippocampal areas

with neurons in the CA1 zone are the most sensitive to ischemic insult whereas neurons in the CA3 and DG are able to re-fuse after 24h of recirculation, making them more resistant to ischemic insult (Owens et al., 2014).

Regarding the therapeutic approach in humans, hypothermia has been shown to be a safe treatment for global ischemia for adults and newborns. The Hypothermia After Cardiac Arrest Study group (THACAS, 2002) reported that55 percent of patients who had been resuscitated after cardiac arrest due to ventricular fibrillation and that underwent therapeutic hypothermia (32°C – 34°C) for 24 hours had a more favorable neurologic outcome and reduced mortality compared to patients that received standard treatment with normothermia. In newborns having suffered neonatal hypoxic-ischemic encephalopathy, hypothermia has been shown to be a safe therapy resulting in decreased neurological deficits and death (Shankaran et al., 2005). The exact mechanism for this protective effect has not been fully elucidated but it has been proposed that hypothermia may be beneficial by promoting a decrease in cerebral blood flow that in turn leads to a reduction in cerebral oxygen consumption (Mezrow et al., 1992), by limiting cerebral tissue acidosis during ischemia and recirculation (Chopp M et al., 1989), or by decreasing leukotriene B4 production 10 minutes after reperfusion and brain edema 2h after reperfusion (Dempsey et al., 1987).

1.1.2 Focal cerebral ischemia

A characteristic of focal ischemia occurring after arterial occlusion is a central core of cell death surrounded by a region that remains metabolically active and known as the “penumbra”. Depending on the rate of residual blood flow and the duration of ischemia the penumbra maybe “rescued” when cerebral blood flow is restored early enough otherwise cell death mechanisms continue and the penumbra becomes incorporated into the central core if reperfusion is not effective. Therefore, the ischemic penumbra has been defined as cerebral tissue with“…potential for the post-ischemic recovery of functionally impaired cells, determined not only by the level of residual flow in the ischemic phase but also by the duration of the flow disturbance” (Heiss WD., 2012).

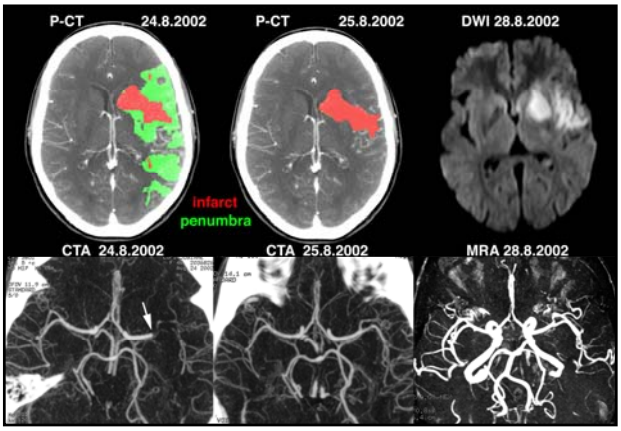

Figure 1.Ischemic stroke in the left middle cerebral artery.
Left and middle upper panels show a cerebral perfusion computerized tomography (P-CT) with the ischemic core (red) surrounded by penumbra (green). The ischemic core corresponds to the tissue with irreversible damage due to ischemia; whereas the penumbra corresponds to the brain parenchyma with reduced blood flow but which may be rescued by a prompt restoration of blood flow. The upper right panel shows a diffusion weighted image from MRI with hyperintensity in the core of the ischemic lesion in the territory of the left middle cerebral artery. Lower panels show angiographic sequences of cerebral CT (left and middle panels) with occlusion of the left middle cerebral artery (arrow). After intravenous administration of rtPA, angiographic sequence of brain MRI shows complete repermeabilisation of the left middle cerebral artery (right lower panel). (With courtesy of the Division of Neuroradiology, University Hospital, Lausanne, Switzerland).

What does the ischemic core and penumbra represent?

Almost 40 years ago, Astrup et al., tested the association between electrical function (synaptic transmition) and extracellular activities of K^+ and H^+ at the ischemic threshold of electrical failure by lowering regional cerebral blood flood (rCBF) of four adult baboons by middle cerebral artery occlusion to below 20 ml / 100 g per minute, described previously as the ischemic threshold for the somatosensory evoked response (Branston et al., 1974). They found that the evoked cortical potential was completely abolished at a rCBF of 15 ml / 100 g per minute and that K^+ release occurred when systemic blood pressure and rCBF was decreased to about 8 ml / 100 g per minute, demonstrating that the ischemic threshold for K^+ release is below that for complete electrical failure. Following these results, two critical levels of decreased perfusion were described: a functional threshold, representing the flow threshold for reversible functional failure (ischemic penumbra); and a lower threshold below which irreversible membrane failure and morphological damage occur (ischemic core) (Astrup et al., 1977).Following this studies with baboons, in patients with acute stroke the thresholds of ischemic penumbra have been studied with two common imaging techniques: diffusion-weighted MRI (DWI), used to detect cellular

edema which reflects irreversible ischemic damage and positron emission tomography (PET) of a central benzodiazepine receptor ligand, used as an indicator of neuronal viability. Both methods are good for predicting the final lesion in the cortex (Heiss et al., 2000; 2004).

Considering then that neuronal damage could be analyzed depending on the duration of ischemia, cerebral damage can be differentiated into different phases: an acute and a delayed phase of neuronal damage.

1.1.2.1 Biochemistry of ischemic stroke

Ischemic brain injury results from a sequential series of events from energy depletion to cell death. Intermediate factors include an excess of extracellular excitatory amino acids, increase of intracellular Ca^{2+}, and inflammation.

a) Acute neuronal damage

Shortly after the onset of ischemia, and because neurons and glia do not store alternative energy sources, the lack of oxygen and glucose supply leads to a reduction in metabolites such as ATP(energy depletion) that will in turn promote membrane potential loss and depolarization of neurons and glia (Katsura et al., 1994). Following this depolarization, voltage-dependent calcium channels become activated and excitatory amino acids are released into the extracellular space. As the re-uptake system of excitatory amino acids is energy-dependent, this process is impaired and promotes further accumulation of glutamate into the extracellular space which leads to excitotoxicity: the presence and continuation of peri-infact depolarizations (PIDs) in the penumbra (see below). Disturbance of the energy-dependent ionic pumps leads to an increase in intracellular sodium and extracellular potassium concentrations as well as calcium influx into cells. This increase in intracellular calcium concentration promotes activation of proteolytic enzymes such as gelsolin that degrade structural constituents of the cell and promotes generation of free-radical species that surpass scavenging mechanisms enhancing structural damage (Furukawa et al., 1997; Chen et al., 1997). Anaerobic metabolism causes increases in brain tissue osmolality and osmotic cell swelling (cytotoxic edema). The final result is terminal depolarization of cell membranes in the core of the lesion.

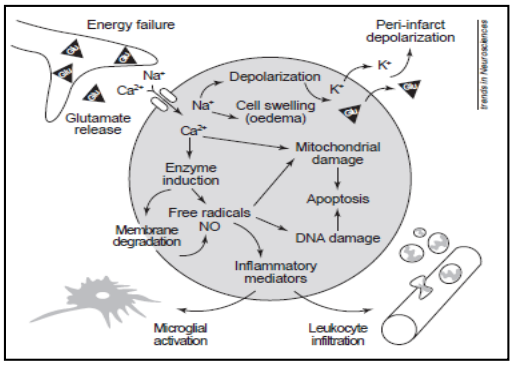

Figure 2. Major pathways involved in ischemic cell damage. Shortly after the onset of ischemia, excitotoxic mechanisms are activated in neurons from an increase in the extracellular concentration of glutamate and intracellular concentration of Ca^{2+} (calcium). The increase in this intracellular messenger over-activates different enzymes that will lead in turn to membrane degradation, generation of free radicals such as nitric oxide (NO) and mitochondrial damage (Dirnagl et al., 1999).

b) Progression of ischemic damage

Importantly, if perfusion of the ischemic tissue is restored within minutes, cytotoxic edema (hyperosmotic) might be reversible. Nevertheless if the ischemic episode persists different proteases are induced which leads to disruption of tight junctions, degradation of the basal lamina, and breakdown of the blood-brain barrier (Battey et al., 2014; Yang and Rosenberg, 2011). The opening of the blood-brain barrier allows influx of neutrophils and formation of cytokines and chemokines; activated microglia and macrophages are found especially in peripheral regions of the ischemic territory since they play a role in the clearance of damaged tissue and have been found in patients with ischemic stroke in the territory of the middle cerebral artery(Gerhard et al., 2005; Cagnin et al., 2007; Benakis et al., 2010). In the penumbra, where some energy supply is preserved, cells can re-polarize and depolarize again in response to increased glutamate levels. Repetitive depolarizations or PIDs can last for hours after ischemia, promoting a misrelation between the increased metabolic workload and the low oxygen supply leading to further transient episodes of hypoxia. Therefore, the higher the rate of PIDs the faster the tissue evolves into terminal depolarizations (Back et al., 1996) Further cell death in the ischemic penumbra is mainly driven by apoptosis promoted in turn by the release of cytochrome c from the outer mitochondrial membrane (Doyle et al., 2008).

In summary, each of the above pathophysiological processes has a distinct time frame, some occurring within minutes, others over hours and days after the ischemic injury. These processes share overlapping and redundant features and cause injury to neurons, glia and endothelial cells. Within the core of the ischemic territory, where blood flow is most severely restricted, excitotoxic and necrotic cell death occurs within minutes. In the periphery of the ischemic area, where

collateral blood flow can counteract the most detrimental effects of the stroke, the degree of ischemia and the timing of reperfusion determine the fate of individual cells. In this ischemic penumbra cell death occurs less rapidly mainly via active cell death mechanisms such as apoptosis but also as a result of the inflammatory reaction (astroglial scar formation) and the presence of peri-infarct depolarizations.

1.1.3 Models of cerebral ischemia in rodents

To study the molecular mechanisms involved in the neurological damage after cerebral ischemia with the goal of finding therapeutic targets suitable for translation to the clinical practice, many different models of *in vivo* and *in vitro* cerebral ischemia have been developed in rodents. To try to replicate the diverse manifestations *in vivo*, causes and anatomic sites of stroke in humans rodent models of ischemia that involve either the whole brain (global cerebral ischemia), or just the region irrigated by a specific artery (focal cerebral ischemia) have been developed. The *in vitro* approach has been used mainly to profit the fact that being an environment without blood flow, is easier to control what the cells are receiving and to analyze the outcome, but always considering that any given effect cannot be immediately extrapolate to a stroke situation since interaction of ischemic tissue with blood borne constituents is missing in these models. In general, with these models researchers can target cellular pathways that control neuronal fait or administer different drugs with the purpose of preserve brain function, following the neuronal damage after different time points and or under different degrees of severity (permanent vs transient ischemia). The end point is to achieve neuroprotection and improved outcome in an experimental setting, defining appropriate doses, modes and time of administration that may then be tested in clinical trials for an eventual translation “from the bench to bed-side”. A brief introduction to most common cerebral ischemia models used are described next, focusing on the models used to perform the experiments described in this work.

In vivo models of global cerebral ischemia

- Two-vessel occlusion model of forebrain ischemia: produced by bilateral common carotid artery (CCA) occlusions combined with a controlled arterial hemorrhage to elicit systemic hypotension sufficient to reduce forebrain blood flow markedly (Smith et al., 1984). Adequate hypotension is crucial for successful ischemia.

- Four-vessel occlusion model of forebrain ischemia: elicit high-grade forebrain ischemia in either anesthetized or awake rats. This technique is divided into two stages: the first takes place under anesthesia and consists of an “atraumatic arterial clasp” placement around each common carotid artery and the electrocoagulation of vertebral arteries through the alar foramina. After 24 hours, carotid clasps are tightened but they could be removed to allow reperfusion (Pulsinelli et al., 1979).
- Levine preparation of hypoxia-ischemia: Originally described as unilateral CCA ligation in rats followed by a gradual exposure to an anoxic environment for 45 minutes, 24h after occlusion. Even though this model was originally described for use in adult animals it is now used more commonly to assess the effects of neonatal hypoxia-ischemia in an adaptation proposed by Rice et al. in 1981. This adaptation consists of unilateral CCA ligation follow by 3.5 hours hypoxia P7 rats four to eight hours after CCA ligation (Rice et al., 1981).

In vivo models of focal ischemia

- Photochemically induced focal cerebral thrombosis: Starting from the principle that platelet aggregation can be induced in an organ following the administration of a photosensitizing dye by irradiating it with light of a specific wavelength, a model involving IV administration of the rose Bengal dye has been developed. This technique involves the retraction of the scalp of an anesthetized rat and stimulation of the rose Bengal with light at 560nm to generate singlet oxygen, which peroxidizes lipid molecules at the vascular endothelium and promotes microvascular platelet aggregation (Watson et al., 1985).
- Suture Middle Cerebral Artery Occlusion (MCAO): This method can be used either for permanent or transient ischemia generation and is widely accepted as a reliable model for studying focal cerebral ischemia. The MCAO method used for the experiments described in this thesis is a variation of the model proposed by Longa et al. (Longa et al., 1989), consisting of a transient regional cerebral ischemia elicited by occlusion of the CCA by advancing an intraluminal suture from the CCA to the internal carotid artery (ICA) until occlusion of the MCA is reached (Huang et al., 1994). A small difference to the method described by Longa et al., is that there is no occlusion of additional extra-cranial vessels (i.e. vertebral arteries) as reperfusion to at least 50% of the initial CBF from the collaterals was desired to control the variability resulting from different degrees of reperfusion. The MCA artery arises from the internal carotid artery and along its path it branches and projects to the frontal, parietal and temporal lobe and so with this model, ischemia is

induced predominantly in the middle and posterior portions of the caudoputamen region, including the internal capsule and the anterior thalamus, as well as frontoparietal cortex. An advantage of this model is that middle cerebral artery occlusion is responsible for many of the ischemic stroke episodes in humans making the model particularly relevant for clinical translation. The limitation of the model is the high variability innate to the individual anatomical characteristics of the different mice strains and requires extensive training in small animal surgery.

In vitro model of cerebral ischemia

- *In vitro* transient oxygen and glucose deprivation of organotypic hippocampal slice cultures is a widely used model of ischemia since it has been shown to promote necrotic and apoptotic cell death phenotypes depending on the magnitude of the environmental ischemic insult (Gwag et al., 1995; Kalda et al., 1998). As the environment can be more easily controlled, this technique is useful not only to elucidate molecular mechanisms behind neuronal cell death / protection, but also to assess pharmacological influences over the same detrimental / beneficial mechanisms. The limitation of course is that interactions between different brain structures are not considered in this approach, which definitely has an important weight in the final outcome of ischemic stroke. Confirmation of *in vitro* observations in an *in vivo* model of ischemic stroke is necessary.

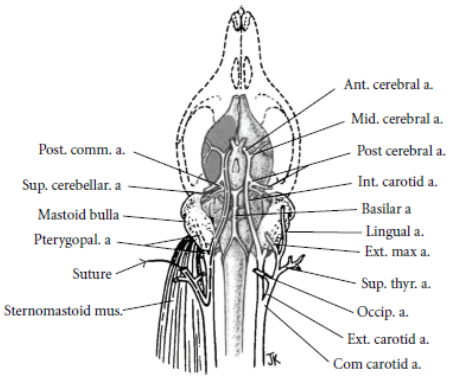

Figure 3. Diagram of cerebrovascular anatomy of a rat. The middle cerebral artery occlusion technique consists of the insertion of a filament through the common carotid artery into the internal carotid until reaching the beginning of the middle cerebral artery where the filament is left in place for a set time to exert occlusion and the subsequent ischemia (Longa et al., 1989)

1.1.4 Current acute therapy: thrombolysis, revascularization

Because ischemic stroke is most frequently due to a thromboembolic occlusion of an intracranial artery, the American Heart Association (AHA) states that the first line of treatment in the acute phase of ischemic stroke should be the restoration of blood flow to the ischemic area as an attempt to salvage the ischemic penumbra. Because ischemic stroke triggers a series of molecular mechanisms that leads to neuronal death the interval from the onset of symptoms and the time of reperfusion is critical and it is of extreme importance that reperfusion is achieved as quickly as possible. Currently, only the intravenous administration of recombinant tissue plasminogen activator (rtPA), a thrombolytic agent, is approved to be administered up to 4.5h after the beginning of symptoms in selected patients. The earlier the drug can be supplied the better the outcome expected. Nevertheless, treatment with rtPA is associated with symptomatic intracranial hemorrhage that is complicated to manage and can be fatal. Therefore, a careful selection of patients and scrupulous monitoring and secondary care is mandatory (Adams et al., 2003).

It was recently described that intraarterial thrombolysis of the blood clot causing ischemia administered within 6 hours after stroke onset in patients with a proximal intracranial occlusion of the anterior circulation is safe and beneficial. Mechanical clot removal increased the rate of functional independence of the patients that received the intraarterial treatment, in comparison with patients that did not (Berkhemeret al., 2015). It should be noted that this treatment is not approved yet by the FDA and that the availability of intra-arterial thrombolysis should not preclude the administration of intravenous rtPA in otherwise elegible patients.

Based on different clinical trials, the AHA and the European Stroke Organization has emitted some recommendations for treatment of patients with acute ischemic stroke (Adams et al., 2003; Hacke et al., 2008):

1. Urgent routine anticoagulation is not recommended because of the high-risk of serious intracranial bleeding complications in patients with moderate to severe stroke, nor within 24h of treatment with rtPA.
2. Unless contraindicated, aspirin should be given within 24 to 48h of stroke onset but not as a substitute for intravenous administration of rtPA.
3. Regarding surgical interventions, carotid endarterectomy is not recommended for asymptomatic individuals with significant carotid stenosis, except for those at high risk of stroke.

4. Early mobilization and measures to prevent subacute complications of stroke are highly recommended. Subcutaneous administration of anticoagulants or the use of external compression stockings are recommended to prevent deep vein thrombosis in immobilized patients.

1.1.5 Letters from basic research: is translation of neuroprotection from bench to bed possible?

Despite the tremendous amount of efforts worldwide and the incredible progress in the understanding of fundamental mechanisms of neuronal cell death it is unfortunately not yet possible to translate these molecular and cellular principles to clinically effective neuroprotective therapies for stroke. In 2001 there was a report of the history of acute ischemic stroke trials of the 20th century. The analysis was shocking: out of a total of 178 controlled trials analyzed, more than 50 neuroprotective agents were studied in about 114 trials and only few trials showed positive results: the Neurological Institute of Neurological disorders and Stroke (NINDS) rtPA trial, Prolyse in Acute Cerebral Thromboembolism (PROACT II), a low-molecular-weight heparin trial (Kidwell et al., 2001). Another analysis that included not only the drugs tested in clinical trials but also the experimental ischemia results was released in 2006. Systematic research of controlled *in vivo* and *in vitro* experiments using functional or histological end points were selected for analysis: a total of 8516 experimental results were extracted from approximately 3500 papers. Final analysis included7,554 experimental results from models of focal ischemia (3,867 results), global ischemia (1,546 results), and *in vitro* models (1,341 results) (O'Collins et al., 2006).And even though out of the 1026 candidate stroke drugs identified, neuroprotective efficacy was shown in 62% of the focal ischemia experimental results, in 70% of global ischemia models and in 74% of the *in vitro* models, only few studies show efficacy when administered to humans (studies mentioned above).

Importantly, the consensus is that neuroprotection could be achieved but by studying the molecular mechanisms triggered not only in neurons after stroke but also in other cell types that likely participate in neuronal survival or play a role in repair mechanisms. For example, the supply of energetic substrates or the secretion of trophic factors from reactive glia. Efforts should be made also to target not only the acute phase of ischemic stroke, but to support remodeling / late repair phases of stroke, having as the main goal a decrease in the disability outcome.

2. BRAIN ENERGY METABOLISM

In an adult human, the brain represents about 2% of the total body weight. Regardless its small size, impressively the brain uses about 20% of the total oxygen and caloric intake (Raichle and Gusnard, 2002). Brain energy metabolism is a compartmentalized process involving different cells such as neurons, astrocytes and oligodendrocytes, with interactions between the different cell types.

Glucose is known as the brain energy substrate par excellence and generation of metabolic energy from glucose starts with glycolysis, which is a sequence of reactions that converts glucose into pyruvate with concomitant production of ATP (substrate phosphorylation). In normal resting brain cells, pyruvate then enters mitochondria, where it is converted to acetyl-CoA and is completely oxidized to carbon dioxide through the citric acid (Krebs) cycle. Most of the glucose-derived ATP is generated in mitochondria as the result of the transfer of electrons from $FADH_2$ and NADH, formed in the citric acid cycle, to oxygen (oxidative phosphorylation). Before ATP and pyruvate are generated in the cytosol, a hydrogen ion and two electrons must be transferred from glyceraldehyde 3-phosphate oxidizing NAD^+ to NADH. To sustain the continued operation of glycolysis, the regeneration of NAD^+ is absolutely necessary. There are several ways to regenerate cytosolic NAD^+ and one of the primary ways is a transfer of electrons from NADH to mitochondria by glycerol-phosphate or malate-aspartate shuttles. These shuttles not only allow re-oxidation of cytosolic NAD^+ but also provide more ATP (1.5 mol by means of the glyceron-phosphate shuttle or 2.5 mol by means of the malate-aspartate shuttle) by oxidative phosphorylation (Brand and Chappell, 1974; Minn and Gayet, 1977; Dennis and Clark, 1978). Reduction of pyruvate to lactate is another important way to regenerate NAD^+, because it is extremely rapid and coupled to an equimolar re-oxidation of NADH generated by glyceraldehyde 3-phosphate to NAD^+ generated by lactate dehydrogenase(Mintun et al., 2004). It has been reported that during physiological activation glycolysisis elevated to a substantially greater degree than oxygen utilization and reduction of pyruvate to lactate results in a rise in tissue lactate levels (Fox and Raichle, 1986; Fox et al., 1988).

This increase in lactate levels is of special interest since it was clear as early as 1953 that other metabolic intermediates such as monocarboxylates including lactate, pyruvate, acetate and ketone bodies could also be oxidized for energy production. In his pioneering experiments, McIlwain showed that lactate and pyruvate were able to maintain the respiration rate of cells in

the human cerebral cortex, as efficiently as glucose and enable the tissue to fully respond to stimulation (McIlwain H, 1953). Since then, interest in brain metabolism has grown exponentially; especially trying to understand the role that each of (or most of) the different brain cells play in homeostatic maintenance. Much more recently, it was shown that lactate for example, is capable of sustaining neuronal activity for periods of up to hours in the almost complete absence of glucose in the living organism (hypoglycemia induced by insulin injection) (Wyss et al., 2011). Pyruvate and acetate have also been subjects of intense research in the metabolic field and will looked at more in detail in the following section.

BRAIN ENERGY METABOLISM AND ISCHEMIA

As mention before, the time window for administration of the only currently approved treatment for the acute phase of ischemic stroke is short and in an important number of patients is not possible to determine the onset of the ischemia, therefore leaving them without treatment option in the acute phase. Therefore, Dr. Berthet and collaborators studied the changes in biochemical patterns following ischemia *in vivo* by using a magnetic resonance spectroscopy (^{1}H-MRS) in mice subjected to tMCAO (Berthet et al., 2011).They were able to estimate at a very early time point the severity of cerebral ischemia induced by MCAO and distinguish transient ischemic attacks from minor strokes or moderate strokes based on metabolite concentration in the ipsilateral striatum, where the lesion core is located. Specifically, a scatter plot of a combination score of N-acetylaspartate (NAA), glutamate (Glu) and taurine (Tau) against glutamine (Gln) measured in the infarcted region 3 hours after ischemia onset, allowed the distinction between moderate strokes , minor insult, transient ischemic attacks and sham mice. (Figure 4).

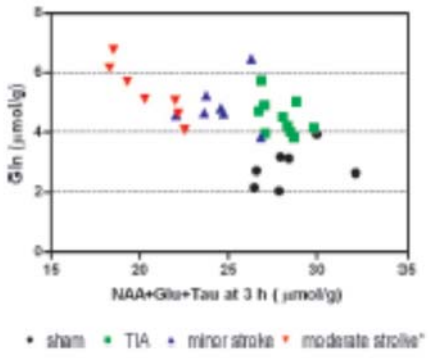

Figure 4. Scatter plot showing a combination of 4 metabolites. Combination of the 4 metabolites at 3 hours after reperfusion allows a good separation among the 4 groups. Glutamine (Gln) is increased in all ischemic conditions with reperfusion independently of the outcome (Berthet et al., 2011).

Because in the set of experiments described before only mice with good reperfusion after removal of the silicon filament were considered for the biochemical analysis and considering that permanent ischemia is a condition observed in stroke patients in need of revascularization, further investigation was performed with the same non-invasive approach to permanent ischemia (MCAO without reperfusion) in mice (Berthet et al., 2014). They demonstrated that the metabolite spectra changes can be identified as early as 1h after ischemia (Figure 5) with an increase in lactate concentration observed as early as 1h that can be traced up to 24 hours after the onset of the ischemia, while levels of glucose decreased. This metabolite spectra changes are important to provide a good estimate of the ischemia onset time within 6 hours after the onset of the ischemic insult, an extremely relevant finding if this can be extended to stroke patients and used to determine whether they qualify for intravenous thrombolytic treatment or other therapies that have a strict therapeutic window.

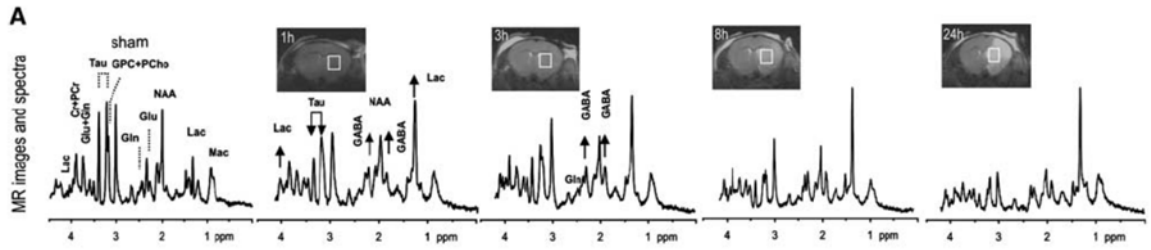

Figure 5. Representative T_2-weighted magnetic resonance images and corresponding spectra from control mice and mice subjected to permanent ischemia at selected time points. White boxes indicate measured regions of interest (Berthet et al., 2014)

2.1 MONOCARBOXYLATES INVOLVED IN THE METABOLIC PATHWAY: LACTATE, PYRUVATE AND ACETATE.

2.1.1 LACTATE

2.1.1.1 Lactate and exercise

Lactate was considered for a long time as a dead end metabolite of glycolysis resulting from muscle hypoxia and a major cause of muscle fatigue. In the early 80's muscular lactate production was used to study the anaerobic threshold (AT) at which metabolic acidosis (during exercise) accelerates the stimulation to breathing, reducing exercise endurance (Wasserman, 1984;

Ingemann-Hansen et al., 1981). The AT theory is that elevated lactic acid (HLa) production and concentration during muscular contraction or exercise are the result of O_2-limited oxidative phosphorylation. Nevertheless, over the last 30 years, there has been evidence showing that anoxia and hypoxia are not necessarily the cause of increased HLa production (Banqsbo et al., 1994; Richardson et al., 1998; Gladden, 2000). Therefore, we can consider that O_2 is one of the several factors that promote an increase in muscle and blood HLa at submaximal exercise intensity. Because lactic acid is almost completely dissociated into lactate anions and protons (H^+) at physiological pH and the reported increase in lactate and H^+ levels in muscle and blood during exercise, it was considered for a long time that any detrimental effect of HLa on muscle and exercise performance is due a promoted acidosis rather than the increase in lactate. This concept has been challenged over the last 10 years by studies that point to inorganic phosphate (Pi) as a major cause of fatigue (Westerblad et al., 2002).

In the field of sports medicine, muscle fatigue and the effect of lactate release either during rest, moderate or intense exercise has been widely studied. It is now known that at rest, lactate is released into the blood stream. During exercise, muscles produce lactate rapidly while its clearance is slowed. This promotes an intramuscular accumulation of lactate and an increased release of lactate into the blood. The lactate that remains in the muscle can then be taken up by neighboring oxidative muscles and used as fuel, in a cell to cell lactate shuttle manner (Stanley et al., 1986). Most of the lactate taken up by muscles undergoes oxidation, mainly by skeletal muscles contracting in a submaximal steady state. The lactate released into the blood stream will reach the cardiac muscle, which is an active lactate consumer or will be taken up by the brain (Stanley, 1991).

2.1.1.2 Lactate and brain metabolism

Considering their electrical activity, neurons have higher energy requirements than astrocytes but they are slower at metabolizing glucose probably because of a weaker expression of glycolytic enzymes in comparison to astrocytes (for a comprehensive review, please refer to Barros, 2013) Briefly, differences between neurons and astrocytes energetic metabolism are more important at the post-translational level. For example, neurons have a functional block at phosphofructokinase that secures a sustained flux through the pentose phosphate pathway, the problem being that neurons cannot activate glycolysis efficiently on ATP demand. By contrast, astrocytes maintain high rates of glycolysis and respond to ATP demand with robust glycolytic stimulation. Glycogen is

a polymer of glucose that in the brain is worth 10-15 minute of the resting metabolic rate and even though astrocytes do not make glucose from glycogen, they can produce lactate. Importantly, neurons have glycogen synthase but keep it inactive. Mutations of the inactivation mechanism lead to accumulation of aberrant glycogen and the development of Lafora disease, a fatal genetic condition characterized by progressive ataxia and dementia (Dulac et al., 2014).

Astrocyte-neuron lactate shuttle

The astrocyte-neuron lactate shuttle (ANLS) proposed in 1994 by Pellerin and Magistretti, postulates that activated neurons use lactate provided by astrocytes as energetic fuel (Pellerin and Magistretti, 1994). According to this hypothesis at glutamatergic synapses the effect of glutamate is terminated by an uptake system located in the astrocytes that surround the synaptic complex. The glutamate uptake is coupled with Na^+ uptake and the increase in intracellular Na^+ concentration in the astrocyte stimulates the Na/K ATPase pump, which in turn will activate astrocyte metabolism to supply ATP to restore the Na^+/K^+ balance and to supply energy for glutamine synthesis from the glutamate that has been taken up. Therefore glucose will enter astrocytes from the blood stream and the lactate produced as the result of metabolism will be exported to the extracellular space from where neurons can take it up, convert lactate to pyruvate and fuel the Krebs cycle, ultimately using lactate as a supplementary energetic substrate especially during periods of high neuronal activity. To support this hypothesis, they showed that in cultured astrocytes uptake of exogenous glutamate is strongly associated with increased lactate production (Pellerin and Magistretti, 1994).

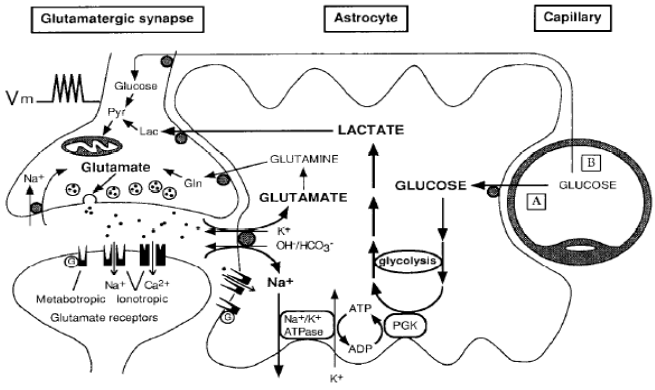

Figure 6. Schematic representation of the neurovascular unit showing hypothetical interactions in the astrocyte-neuron lactate shuttle. Glutamate release at the glutamatergic synapse is re-uptaken by the astrocyte together with Na^+. This will activate astrocyte metabolism leading to the production of lactate as a result of glucose glycolysis. The lactate produced will be exported to the extracellular space from where it can enter neurons thanks to the monocarboxylate transporters located in its membrane (Pellerin and Magistretti, 1994).

A number of studies have given evidence supporting the ANLS hypothesis. For example, it has been reported that lactate and pyruvate serve as fuel supporting axon function in the rat optic nerve (Brown et al., 2001) and that lactate could substitute (or be preferred to) for glucose in cultured cortical neurons (Pellerin et al., 1998; Bouzier-Sore et al., 2003). The ANLS has also been proposed in other cell-neuron energetic support interactions such as those between Schwann cells and peripheral neurons (Véga et al., 1998), Müller glial cells and photoreceptors (Poitry-Yamate et al., 1995). *In vivo* in anesthetized rats using a paradigm involving radiotracer kinetics and brain activation it was shown that lactate, as the primary energy source was able to maintain neuronal activity during severe insulin-induced hypoglycemia (Wyss et al., 2011). Supporting information also comes from humans since it has been demonstrated by positron emission tomography using ^{18}fluorodeoxyglucose that infusion of sodium lactate decreases the whole-brain rate of glucose uptake in healthy men at rest (Smith et al., 2003).

ANLS has also been implied in physiological processes as memory formation where a specific role for glycogen-derived lactate has been suggested from the observation that pharmacological inhibition of glycogen phosphorylase in rat hippocampus impaired the formation of long-term memory, an effect that could be rescued by exogenous lactate (Suzuki et al., 2011; Newman et al., 2011). Genetic knockdown of the lactate transporters of astrocytes (MCT4), oligodendrocytes (MCT1), and neurons (MCT2) induced amnesia. Interestingly, exogenous lactate rescued memory formation in MCT4 and MCT1 knockdowns but not in MCT2 knockdown, emphasizing the relevance of lactate transfer between glial cells and neurons. Disruption of the astrocyte-neuron lactate supply has also been involved in the development of neurodegenerative diseases such as amyotrophic lateral sclerosis (Lee et al., 2012).

Even considering the compelling evidence supporting the ANLS hypothesis, this theory has been subject to different challenges and criticisms (Fillenz, 2005). Assuming energy supply would necessarily need to match energy consumption to sustain neuronal activity (propagation of action potentials), different groups have used a battery of techniques to measure the time course of changes in lactate resulting from neuronal activation, ranging from nicotinamide adenine dinucleotide (NADH) flurorescence in rat hippocampus to magnetic resonance spectroscopy in humans. They have reported that brief stimuli (either tail pinch in the rat, electrical stimulation of Schaffer collaterals or a 1-s visual stimulus in healthy volunteers) promote an initial oxidative phosphorylation localized in neurons, involving mainly glucose oxidation (Hu and Wilson, 1997). Furthermore, using a lactate sensor that allows direct and continuous measurements of lactic acid

concentration with high temporal resolution in the hippocampus of rat brain, Hu and Wilson showed an increase in extracellular lactate concentrations in response to acute neuronal activation that will supply local energy to keep homeostasis and to counteract transport limitation of glucose from the blood stream through the blood brain barrier. By comparing the reduction of extracellular glucose (21%) versus the reduction in the extracellular pool of lactate (7%) after electrical stimulation of the Schaffer collaterals they conclude that glucose is the main substrate used by activated neurons and that lactate acts as a complementary energetic supply (Hu and Wilson, 1997a; 1997b).

Another point that ANLS has failed to explain is the effect exerted by the glutamate released from astrocytes. It is important because it has been described that proper stimulation will provoke the release of glutamate as a consequence of the rise in intracellular astrocytic Ca^{2+}. According to Bernardinelli et al., this increase in intracellular Ca^{2+} promotes a further increase in glucose uptake by astrocytes leading to the same increase in lactate release as the one promoted by the uptake of synaptically released glutamate. The main difference being that by the first mechanism lactate release will take longer and be more prolonged in comparison to the lactate release triggered by the uptake of synaptically released glutamate a characteristic that could in turn explain the pattern of lactate release described in the studies mentioned in humans (abrupt increase seconds after acute neuronal activation and maintenance of high levels of lactate afterwards) (Bernardinelly et al., 2004; Kasischke et al., 2004; Mangia et al., 2003).

This information allows us to conclude that local lactate production is indeed taking place in the brain under specific circumstances and lactate is acting as a key player in brain energy metabolism. Nevertheless, this information does not allow us to state that lactate is more important than glucose (as an energetic substrate for neuronal activation).

2.1.1.3 Lactate neuroprotection

Following the early evidence given by McIlwain in 1953, , that different monocarboxylates such as lactate and pyruvate could maintain electrical activity in human brain slices, Schurr et al., demonstrated the ability of brain tissue to maintain normal neuronal activity in rat hippocampal slices using lactate as the sole energy substrate (Schurr et al., 1988). This early observation was followed by several *in vitro* studies where was shown using hippocampal slices that lactate accumulated during a period of oxygen deprivation is an obligatory aerobic energy substrate for

recovery of neuronal function upon re-oxygenation, thus demonstrating that lactate could be used as an effective energy substrate (Schurr et al., 1997b; Cater et al., 2003). *In vivo* confirmation of this neuroprotective effect was obtained with a model of transient global cerebral ischemia (TGI) in rats where the monocarboxylate transporter inhibitor alpha-cyano-4-hydroxycinnamate (4-CIN) was administered by intraperitoneal injection (IP). After 5 min of TGI, rats pretreated with 4-CIN showed increased neuronal damage compared to control rats prompting the authors to conclude that lactate is a critical oxidative energy substrate immediately post-ischemia in the rat brain (Schurr et al., 2001).

The protective effect of lactate has been also shown against glutamate excitotoxicity *in vitro* by exposing hippocampal slices to two different concentrations of glutamate with or without previous treatment with 4-CIN. They showed that hippocampal slices treated with glucose could tolerate 15 min activation induced by glutamate administration except if the monocarboxylate transport was blocked. If cells are unable to transport lactate, administration of glutamate yields permanent loss of neuronal function. Interestingly, when glycolysis was inhibited by the glucose analog 2-deoxy-D-glucose (2DG) administration of glutamate reduced the number of slices capable of normal neuronal function after the stimulus, but when lactate was administered concomitantly with 2DG the majority of the hippocampal slices showed normal function after the glutamate challenge (Schurr et al., 1999). By using an *in vivo* excitotoxicity model Ros et al., tested lactate ability to protect against glutamate neurotoxicity by infusing medium containing glutamate with or without lactate into the cortex of non-anesthetized rats. When glutamate was infused with lactate a reduction in the lesion size was observed in comparison to the lesion elicited by perfusion of glutamate alone (Ros et al., 2001).

Taking this information into account, in this lab Dr. Berthet performed a series of experiments in vitro to test the effect of lactate administration after reoxygenation, demonstrating that administration of L-lactate to rat organotypic hipoccampal slices directly after oxygen and glucose deprivation (OGD) was protective in that it resulted in decreased cell death in the CA1 region of the hippocampus at 48h.Berthetcould also show that L-lactate administration in vivo either by intracerebroventricular (ICV) or intravenous (IV)injection was protective when administered after either 15, 30 or 60 min middle cerebral artery occlusion (MCAO) in young adult mice, decreasing the lesion size and improving the neurological outcome measured 48h or 14 days after ischemic injury (Berthet et al., 2009; 2012).

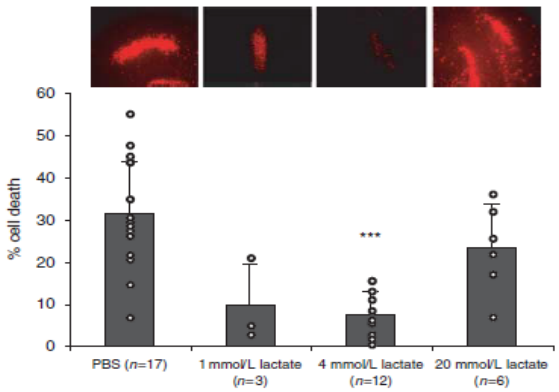

Figure 7. Lactate neuroprotection. Lactate exerts protection to organotypic hippocampal slice cultures subjected to 30 minutes oxygen and glucose deprivation by decreasing the percent of cell death (Berthet et al., 2009).

Lactate neuroprotection has also being studied in traumatic brain injury (TBI) in humans. It was recently shown that in patients with TBI, cerebral extracellular lactate was above normal ranges and this elevation did not match with brain hypoxia but was mainly associated to normal pyruvate and oxygen levels, meaning that an increase in lactate was rather due to activated glycolysis than to an ischemic episode providing important data regarding endogenous effects of lactate after increased energy demands (Sala et al., 2013).To further elucidate if exogenous lactate administration improves cerebral energy metabolism, Bouzat et al., provided an intravenous infusion of hypertonic sodium lactate to patients with severe TBI and monitored the PO_2 in brain tissue using cerebral microdialys is as well as intracranial pressure. They reported an increase in the amount of extracellular pyruvate and glucose as well as a decrease in extracellular glutamate levels. This would mean that supplemental lactate from the systemic circulation may be converted by the brain into pyruvate to be utilized aerobically, helping the injured human brain by not only providing an energetic substrate but also by sparing cerebral glucose (Bouzat et a., 2014). This study demonstrates that lactate is used as an alternative energy source, while glucose is “saved” for important rescue processes such as the regulation of reactive oxygen species, neurotransmission and biosynthesis.

2.1.2 Other monocarboxylates of interest in brain energy metabolism: pyruvate, acetate.

Glycolysis described by Krebs around 1937 is a sequence of reactions that convert glucose into pyruvate with a small production of ATP. Further oxidation of pyruvate in the mitochondria produces most of the energy contained in glucose. This dogma has been challenged in more recent times by many studies showing lactate as an integral part of the oxidative energy

metabolic pathway, beginning with glucose and ending with the production of carbon dioxide(CO_2), water involving the mitochondrial electron transport chain, thanks to the enzyme lactate dehydrogenase (LDH) that converts lactate to pyruvate (Schurr, 2014). Therefore, other monocarboxylates located downstream of lactate oxidation such as pyruvate and acetate have been studied as alternative energy substrates in conditions of increased energy demand. Indeed, many *in vitro* studies have shown neuroprotection using pyruvate acting as a glutamate and hydrogen peroxide (H_2O_2) scavenger. For example, it has been described that pyruvate administration improves survival of cultured mouse striatal neurons from mouse embryos exposed to H_2O_2 (Desagher et al., 1997; Boyko et al., 2011), and decreases neurotoxicity in cultured mouse cortical neurons induced by either chronic or acute exposure to zinc (Sheline et al., 2000). It has also been shown to protect rat astrocytes subjected to oxygen and glucose deprivation (Sharma et al., 2003). Its beneficial effect has also been shown *in vivo* in a model of global ischemia in the mouse when administered by IP injection after 12 minutes of forebrain ischemia where it decreases neuronal death and improves overall survival evaluated 30 days after the insult (Lee et al., 2001), and also by decreases infarct size with less neurological deficit in rats subjected to either transient or permanent middle cerebral artery occlusion (MCAO) analyzed at 24h or 14 days after the ischemic insult (Yi et al., 2007). It was necessary however to use an incredible number of animals to prove the beneficial effect of pyruvate administration (402 rats), perhaps due to pyruvate instability in aqueous solutions. To overcome this problem some groups have used the ethyl ester of pyruvic acid, i.e. ethyl pyruvate (EP), showing a beneficial effect in rats that received an IP injection of EP following transient focal ischemia. This rats developed smaller lesions and showed less neurological deficit when EP was administered as late as 24h after tMCAO (Yu et al., 2005; Kim et al., 2005). Nevertheless, the question regarding if the modification in the compound might be the responsible for the protection observed by EP administration and not by the pyruvate itself has been raised.

Another monocarboxylate of interest in brain energy metabolism is acetate. It enters the Krebs cycle by getting converted to acetyl-CoA by acetyl-CoA synthetase and subsequently enters the Krebs cycle by condensing with oxaloacetate to form citrate. It is important to mention that in the brain, acetate is considered as a specific energy substrate for astrocytes probably due to the rapid uptake of acetate into astrocytes by the monocarboxylate transporter (MCT) 1, expressed in astrocytes and absent in the synaptosomes (Waniewski and Martin, 1998), rather than a higher enzymatic activity (Waniewski and Martin, 2004). Considering the tight relationship existing between neurons and astrocytes in the metabolic unit, acetate as an astrocytic metabolic marker

has arose in recent years as a molecule of interest in the brain energy metabolism field(Wyss et al., 2009; Hosoi et al., 2009).*In vivo* a marked reduction in acetate uptake has been demonstrated after short-term MCAO, mainly in the ischemic core (striatum) (Hosoi et al., 2007) and interestingly it has been shown the important contribution of glial cells to the survival of neuronal cells after ischemic injury using infusion of fluorocitrate, a selective glial toxin directly into the striatum of rats 4h before 10 min MCAO, causing an increase in cell damage in comparison to MCAO only or the infusion of fluorocitrate alone (Hosoi et al., 2006).

2.2 Monocarboxylate transporters

Monocarboxylates such as lactate, pyruvate, acetate and ketone bodies cannot cross membranes by diffusion but use a specific transport system to cross the blood-brain barrier into each cell type, or to transfer from one cell type to the other when monocarboxylates are produced locally. Monocarboxylates can be transported to and from different cell types with different degrees of affinity by monocarboxylate transporters (MCTs), a family of proton-dependent carriers. Also known as the solute carrier 16 (SLC16) gene family, the MCT family is composed of14 members based on sequence homology, grouped as MCT1-9, MCT11-14 and T-Type amino-acid transporter-1 (TAT1). Nevertheless, MCT8 has been described to be a thyroid hormone transporter instead of a monocarboxylate transporter (Friesema et al., 2003) and only the first four (MCT1-4) were shown to cotransport monocarboxylates and protons (Halestrap and Price, 1999). Studies performed mainly on MCT1 using xenopus laevis oocyte mutagenesis on and molecular modeling, rotating the C-termini domain have demonstrated that MCTs have 12 transmembrane domains with the N- and C- termini located intracellularly and the transport action itself taking place as follows: binding of the proton followed by binding of the anion, domain rearrangement which allows the passage from the extracellular to the intracellular space of the anion and the proton, release to the cytosol of the anion followed by the proton and return of the empty substrate binding site to the external surface(Figure 8) (Wilson et al., 2009).

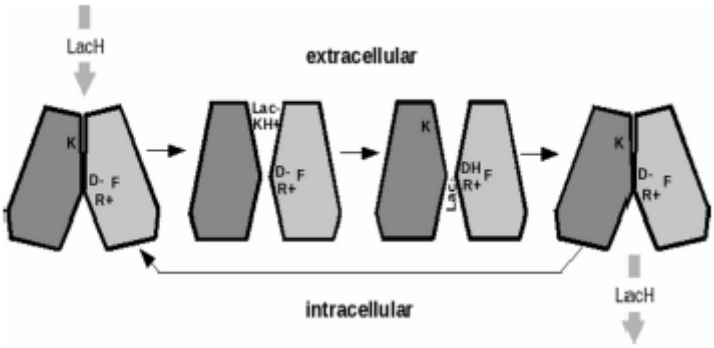

Figure 8. Cartoon illustrating the proposed mechanism of lactic acid transport by MCT1. Lactic acid protonates Lys^{38} (*K*) causing the channel to open. Lactate then moves into the open extracellular side of the pore and forms an ion pair with Lys^{38}. In the next step the proton on Lys^{38} is transferred to aspartate 302 (D-) neutralizing the aspartate side chain (DH). This is followed by migration of lactate through the pore where it forms an ion pair with R306 (R+). The size of the adjacent side chain of residue 360 (F) governs the ability of this site to bind the α-hydroxy acid. Once $Lys^{38}$is deprotonated, and lactate is occupying the specificity filter, the transporter relaxes back towards the closed state and releases lactic acid into the intracellular space (Wilson et al., 2009)

They serve to equilibrate substrate concentrations across cell membranes using the concentration gradients as the driving force allowing the migration of the monocarboxylates from the site of production to the site of consumption. As such, MCTs have an important role in metabolism, since an important part of metabolic communication between different cell types depends on their proper function. Of the four monocarboxylate transporters known to cotransport monocarboxylates and protons, MCT1, MCT2 and MCT4 are the main MCTs in the central nervous system and their distribution can be distinguished as follows:

- MCT1 was found to be present in almost all tissues including muscle, kidney, liver and heart; in the central nervous system, MCT1 is expressed throughout the whole brain of rodents mostly abundant in the astrocytes of the cortex, hippocampus and cerebellum. It is also abundantly expressed in endothelial cells forming the blood brain barrier and was recently described in oligodendrocytes (Pellerin et al., 2005; Rinholm et al., 2011)
- MCT2 is expressed in the liver, kidney and testis and it has been described to be the main MCT expressed in neurons (Pierre et al., 2002).
- MCT4 is strongly expressed in skeletal muscle and astrocytes (Pierre and Pellerin, 2005).

Regarding affinity, MCT1 transports many short-chain monocarboxylates with Km values decreasing as the chain length increases. Km values of around 3.5mM for lactate and around 1.0 mM for pyruvate were reported. MCT2 displays the lowest km values of around 0.7mM and 0.08 mM for lactate and pyruvate, respectively. This observation is in line with a role for MCT2 in tissue (liver or kidney) or cells (neurons) exhibiting important monocarboxylate uptake and / or consumption. MCT4 has been identified as a major lactate transporter of muscle cells and astrocytes with a Km of around 30mM. Moreover, the very low affinity of MCT4 for pyruvate (Km 150 mM) prevents the loss of pyruvate from the cell and thus permits high rates of glycolysis and cytosolic ATP production (Bergersen, 2014).

2.3 Hydroxy-carboxylic Acid Receptor 1 (HCA1)

Formerly known as GPR81, HCA1 used to be part of a big family of GPR's orphan receptors until 2008 when lactate was identified as the endogenous ligand by using a ^{35}S-GTPγS binding assay and the involvement of the GPR81 receptor in the previously described lactate-mediated suppression of adipose lipolysis (Cai et al., 2008; Boyd et al., 1974).

The HCA receptor family has three members encoded by three genes located near one another on human chromosome 12: HCA1, HCA2 and HCA3 and whereas HCA1 is activated by lactic acid, HCA2 and HCA3 (formerly known as GPR109A and GPR109B) are activated by the ketone body 3-hydroxy butyric acid and the β-oxidation intermediate 3-hydroxy-octanoic acid, respectively (Offermans, 2014). Of the three HCA receptors, HCA1 appears to be the phylogenetically oldest receptor and is found in most mammals as well as in fish (Kuei et al., 2011).Functional HCA2 ketone body receptors have been described only in mammals, whereas the gene encoding HCA3 is present only in the genome of higher primates (Zellner et al., 2005). In humans, the three receptors have been involved in the inhibition of lipolysis under different physiological conditions. As will be described below, HCA1 is involved in the insulin-induced inhibition of lipolysis, while HCA2 and HCA3 reduce lipolysis during starvation and increased fatty acid oxidation (Figure 9)

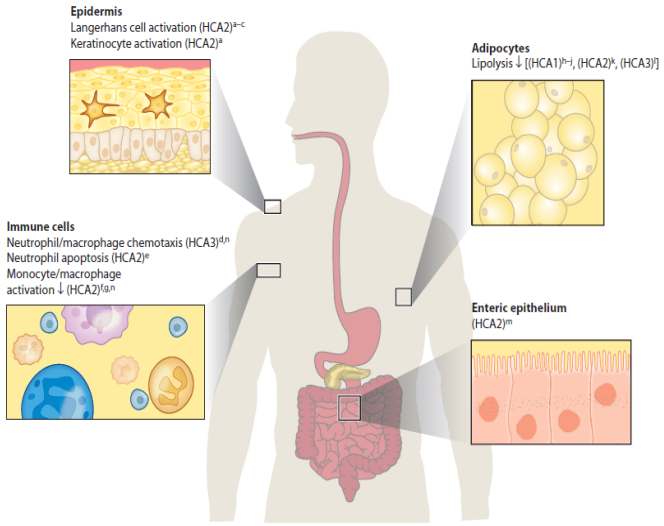

Figure 9.Distribution of different HCA receptors expression in the human body including the cellular functions regulated via hydroxyl carboxylic acid receptors (Offermanns, 2014).

Originally described as being expressed in a "highly restricted" manner in adipose tissues and upregulated during adipocyte differentiation (Wise et al., 2003; Ge et al., 2008) for many years the focus of HCA1 receptor research was on adipose tissue and its possible involvement in the insulin-induced inhibition of lipolysis. In fact, it was described that activation of the HCA1 receptor by lactate was able to inhibit cAMP synthesis inhibiting lipolysis (Liu et al., 2009) and this result was confirmed and replicated using transgenic mice lacking the HCA1 receptor (Ahmed et al., 2010). Since it was reported that mice lacking HCA1 showed reduced weight gain when they were fed a high fat diet, it has been suggested that the chronic inhibition of the anabolic effects of insulin in adipocytes in the absence of HCA1 results in reduced body weight under a hypercaloric diet. More recently, it was shown that the signaling pathway downstream of inhibition of cAMP leads to the dissociation a G_i subunit ($G_{\beta\gamma}$), subsequently inducing activation of ERK1/2 (Li et al., 2014).

Interestingly it is now known that the HCA1 receptor is also expressed throughout the brain with prevalence in the cerebellar Purkinje neurons and their dendrites, pyramidal cells in the hippocampus, neurons in the dentate hilus and in the cerebral neocortex (Figure 10) (Lauritzen et al., 2014) which has prompted the idea that lactate may act not only as an energetic substrate but also as an endogenous agonist of the HCA1 receptor in the brain, therefore acting as a "volume transmitter" (Bergersen and Gjedde, 2012). Supporting this hypothesis, it has been described that

lactate administration to cortical neurons induced specific modulations in calcium transient frequencies in the same manner as a specific HCA1 receptor agonist (Bozzo et al., 2013).

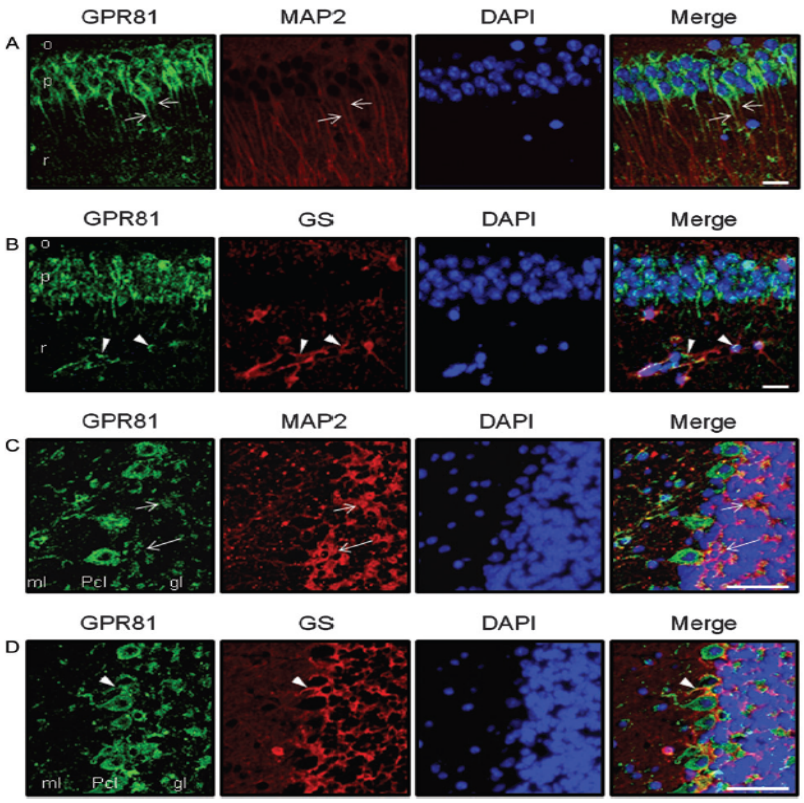

Figure 10. HCA1 is present in neurons (A and C) and in astrocytes (B and D).Immunolabeling of coronal paraffin-embedded sections demonstrates the presence of HCA1 in cellular elements in hippocampus CA1 (A and B) and cerebellum (C and D). In the hippocampus, HCA1 is concentrated in perikarya and proximal parts of dendrites of pyramidal cells. In the cerebellum, HCA1 is concentrated in Purkinje cell perikarya or synaptic glomeruli, in the granule cell layer. Sections were counterstained with DAPI to show cell nuclei (Lauritzen et al., 2014)

It is important to mention that a recent study on locus coeruleus (LC) has described lactate's stimulation of norepinephrine release from LC neurons through a receptor mechanism although a –yet- unknown receptor has been implied in this action (Tang et al., 2014).The HCA1 receptor may also be involved in the mechanisms of damage promoted by lactate accumulation in neurological disorders such as cerebral malaria (Mariga et al., 2014), and a rather controversial recent work implies the HCA1 receptor in neuronal damage after ischemic stroke (Shen et al., 2014).

AIMS OF THE PRESENT WORK

Previous observations on lactate administration at reperfusion after a transient ischemic episode have shown the neuroprotective effect exerted by lactate by decreasing the percent of cell death after oxygen and glucose deprivation and the lesion size and improving the neurological outcome in mice subjected to tMCAO. It was assumed that this neuroprotective effect is related to lactate being carried by the monocarboxylate transporters across the cell membrane and being used as an energetic substrate by conversion to pyruvate and further oxidation in the mitochondria. Therefore, the recent finding of HCA1 as lactate receptor and its expression in the central nervous system is of extreme interest for elucidating the mechanism taking place behind lactate neuroprotection and the main focus of this project.

GENERAL:

To study the neuroprotective effect of lactate: is it related to metabolization of lactate as an energetic substrate or by lactate acting as a signaling molecule through the HCA1 receptor?

SPECIFIC:

- To corroborate HCA1 receptor expression in central nervous system and analyze possible changes in the expression resulting from ischemia, with and without administration of L-lactate.

- To corroborate HCA1 expression in the central nervous system, different brain regions will be analyzed with confocal microscopy by using an immunohistochemistry approach in samples of sham mice. Double labeling will enable us to know with which cellular or subcellular marker HCA1 receptor co-localizes.

- Protein expression will be analyzed by Western blot in samples obtained from different brain regions of mice subjected to 30 minutes tMCAO and comparison of the total protein content will be performed between the two hemispheres.

- To analyze if the administration of L-lactate after tTMCAO promotes changes in HCA1 expression, L-lactate will be administered by intravenous injection at reperfusion in mice subjected to 30 minutes tMCAO and protein expression will be analyzed by Western blot.

- To analyze if the sole activation of HCA1 receptor could exert protection after an ischemic insult, 3,5-DHBA will be administered to organotypic hippocampal slice cultures after being subjected to oxygen and glucose deprivation and comparison of cell death percent will be performed between control slices, slices subjected to OGD that did not received 3,5-DHBA and treated slices.

- Glucose, pyruvate and acetate administration, metabolites known to be oxidized in the mammalian brain, as well as D-lactate, will be analyzed using the *in vitro* ischemic model to evaluate the participation of the metabolic pathway in the protection exerted by lactate administration. The different substrates will be administered after OGD to organotypic hippocampal slice cultures and cell death will be analyzed at 48 hours. Comparison will be performed between control slices and slices subjected to OGD with or without treatment. Those substrates that show a beneficial effect will then be tested in the in vivo ischemic model to analyze the lesion size and neurological outcome.

II.METHODS

All experiments were conducted in accordance with Swiss guidelines for animal experimentation and approved by the veterinary authority.

1. Organotypic hippocampal slice cultures

Hippocampal slice cultures were prepared 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.

2. 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. 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 4mM, pyruvate at 10mM, sodium acetate at 0.13, 0.2, 4 or 8mMor 3,5-DHBA at 4mM was administered immediately after OGD.

3. 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.

4. Transient middle cerebral artery occlusion in the mouse

Male CD1 mice (body weight 26-35 g, Charles River, L'arbresle, France) were 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) was induced by occlusion of the left MCA with an intra-arterial suture (Longa et al., 1989). 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. Five microliters per gram of body weight of either sodium D-lactate solution (200 mM), pyruvate solution (200 mM) or vehicle solution (PBS) were 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). The surgeon was not blinded.

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.

5. 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 elsewhere (Longa et al., 1989) 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.

6. 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 (Swanson et al., 1990).

7. 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.

8. *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).

9. 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 behavior. Paired t-test was used to compare protein expression. $P < 0.05$ was considered statistically significant.

III. RESULTS

1. New evidence of neuroprotection by lactate after transient focal cerebral ischaemia: extended benefit after intracerebroventricular injection and efficacy of intravenous administration.

Carole Berthet, **Ximena Castillo**, Pierre J. Magistretti, Lorenz Hirt.

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This work was carried out mainly by Dr. Carole Berthet and is a continuation of the work published by our group in 2009 describing the neuroprotective effect of intracerebroventricular (ICV) injection of lactate after tMCAO (Berthet et al., 2009). In this study, we went on to show a long term neuroprotective effect after ICV administration of lactate that yielded smaller hemispheric atrophy 14 days after tMCAO and better neurological outcome in the mice that received lactate compared to control mice. Considering the aim of translation of lactate as a neuroprotectant in clinics we decided to test a more suitable route of lactate administration for clinical practice and tested intravenous (IV) lactate administration. We showed that IV injection of lactate was also able to exert protection by significantly decreasing the lesion size at48h after tMCAO (13.7 ± 12.2 mm^3, n=12 vs. 29.6 ± 25.4 mm^3, n=12, $p<0.05$) and attenuating the neurological deficit in the mice that received the IV treatment (median=1, min=0, max=2.5, n=12) compared to control mice (median=1.5, min=1, max=8, n=12, $p<0.05$).

My personal contribution to this work involved showing by immunohistochemistry analysis that there was a decrease in neuronal death in the lesion site (striatum) in mice treated with lactate and by measuring the acid-base status that IV injection of lactate did not promote an increase in the blood pH (7.34 ± 0.09 lactate vs. 7.29 ± 0.07 control, n=4),pCO_2 (40.75 ± 4.81 lactate vs. 38.97 ± 2.03 control, n=4) and pO_2 (106.35 ± 10.02 lactate vs. 118 ± 14.11 control, n=4) when lactate injected mice were compared to those receiving IV injection of the vehicle control.

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

2.1 Possible involvement of the HCA1 receptor in ischemia

2.1.1 HCA1 receptor localization in brain tissue.

As described above, recent studies revealed the presence and mechanism of action of the HCA1 receptor in the central nervous system (Bozzo et al., 2013; Lauritzen et al., 2013; Mariga et al., 2014). 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. Using an immunohistochemistry approach, we were able to confirm strong neuronal expression of the HCA1 receptor in the hippocampus (CA1 and dentate gyrus, row 1 (1a-f and 2 (2a-f) respectively), the cortex (row 3, 3a-f) and the striatum (row 4, 4a-f) revealed by double immunofluorescence labeling for the HCA1 receptor and the neuronal markers NeuN (panels a to c) or MAP2 (panels d to f). Figure 11: NeuN [green], MAP2 [green], HCA1 [red], DAPI [blue].

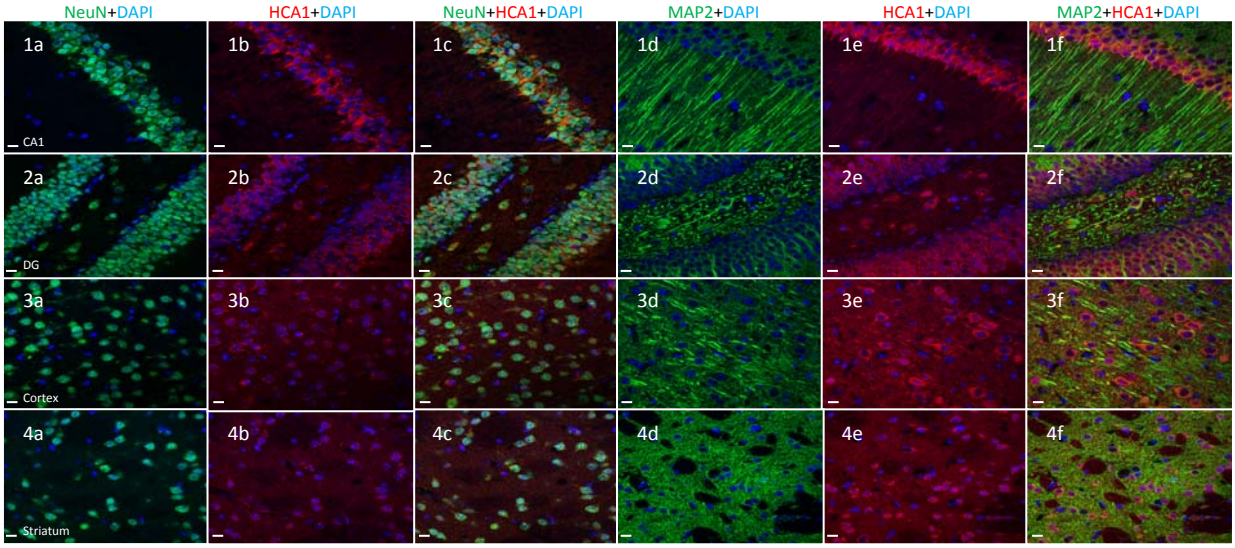


Figure 11. Confocal visualization of HCA1 receptor expression, c**olocalization with neuronal markers.** Using an antibody against neuronal cells, we confirmed with immunohistochemistry the localization of the HCA1 receptor is mainly in neurons. The brain structures analyzed were primary cortex, hippocampus (CA1 and Dentate Gyrus (DG) regions) and Striatum. Color code: NeuN [green], MAP2 [green], HCA1 [red], DAPI [blue]. Magnification: 40x. Scale bar: 20µm

The same approach was used to study possible HCA1 receptor expression in astrocytes by using double immunofluorescence with the HCA1 receptor and the astrocytic marker against glial fibrilar acidic protein (GFAP), in the same three brain regions analyzed before. We did not observe

Obvious co-localization of the HCA1 receptor in reactive astrocytes (Figure 12: GFAP [green], HCA1 [red], DAPI [blue]).

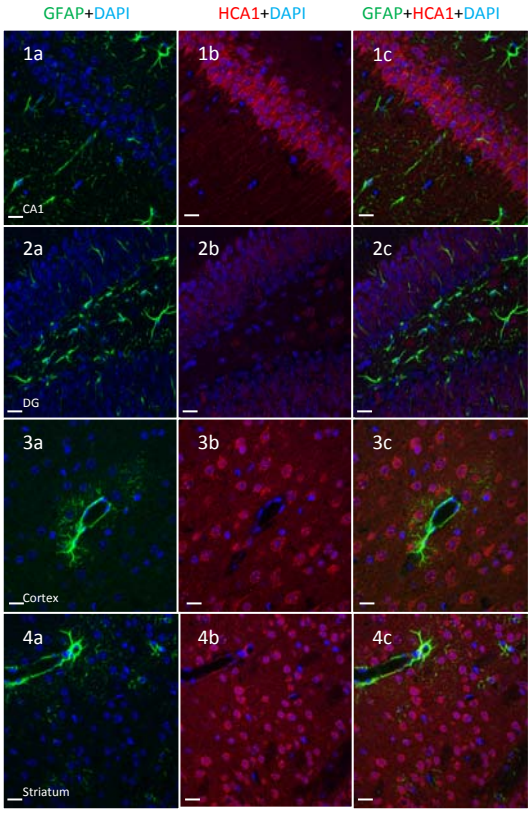


Figure 12. Confocal visualization of HCA1 receptor expression. HCA1 receptor is not expressed in astrocytes. Using an astrocyte marker we did not observe by immunohistochemistry co-localization of the HCA1 receptor with reactive astrocytes. The brain structures analyzed were primary cortex, hippocampus (CA1 and Dentate Gyrus (DG) regions) and Striatum. Color code: GFAP [green], HCA1 [red], DAPI [blue]. Magnification: 40x. Scale bar: 20µm

2.1.2 HCA1 receptor expression in ischemic conditions

To evaluate whetherHCA1 receptor expression was altered at different time points after30 minutes tMCAO we evaluated the HCA1 total protein expression on Western blots in protein extracts from hippocampus, cerebral cortex and striatum at 1, 3, 8, 24 and 48 hours after tMCAO in collaboration with the group of Prof. Pellerin from the Physiology department of the University of Lausanne. Normalization of ischemic hemisphere was performed against the contralateral hemisphere. Results at 1, 3, 8 and 48 hours did not reveal significant differences between the three brain structures investigated (Table 3).

Table 3.Expression of HCA1 receptor at different time points after 30 minutes tMCAO.

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

However, a significant difference in the ratio of HCA1 receptor expression was seen in the ischemic cortex after 24 hours (1.2 ± 0.2, $p < 0.05$ vs. contralateral cortex; figure 8) and 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 13). An immunohistochemistry approach showed the apparent upregulation of the HCA1 receptor in the ischemic hemisphere, in comparison to the contralateral hemisphere (Figure 14).

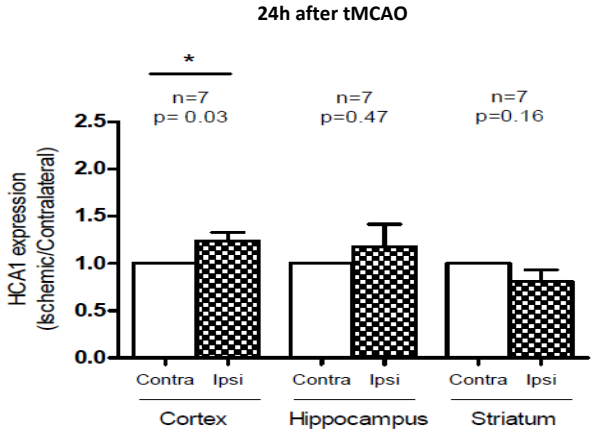


Figure 13. HCA1 receptor expression is increased by ischemia. Western blot analysis showed an increase in HCA1 receptor expression 24 h after tMCAO in the region surrounding the lesion site (primary motor and somatosensory cortex), compared to the contralateral hemisphere. (*p < 0.05; paired t-test ischemic vs contralateral hemisphere).

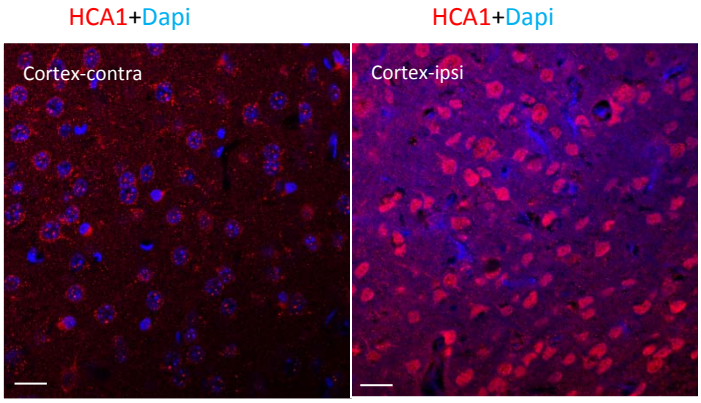


Figure 14. HCA1 receptor expression is increased in the ipsilateral (ischemic) cortex. Detailed section of the ipsilateral and contralateral cortex, were an increased in the HCA1 staining can be appreciated. Sections were counterstained with DAPI to show cell nuclei. Color code: HCA1 [red], DAPI [blue]. Magnification: 63x. Scale bar: 20µm

Diaschisis, as describe in 1914 by von Monakow, established that functional changes in brain structures remote from the site of a focal brain damage were processes underlying functional recovery. Modern neuroimaging studies have shown the existence of diaschisis by reveling that focal brain lesions are accompanied by widespread metabolic changes involving the affected cerebral hemisphere but extending into brain areas supplied by contralateral and cerebellar arteries (Feeney and Baron, 1986). In order to control that the differences observed between the ipsilateral vs contralateral hemispheres were present regardless the possible changes that happened in the contralateral hemisphere, we decided to compared the HCA1 receptor expression between both hemispheres and a sham cortex. We observed that even though there are changes in the expression taking place in the contralateral hemisphere, the difference is maintained between the ischemic and the contralateral sides (Figure 15).

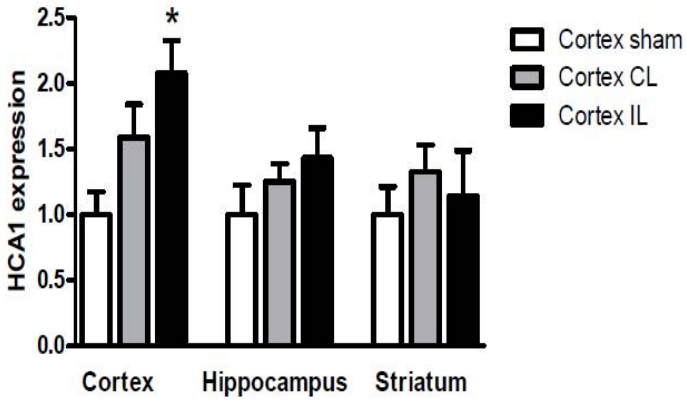


Figure 15. Western blot analysis of HCA1 expressed in cortex (primary motor and somatosensory cortex), hippocampus and striatum from 30 minutes MCAO and sham mice. The HCA1 values were normalized to the total protein content values. (*p<0.05, ANOVA followed by Dunnett's multiple comparison test. Sham samples n=4, MCAO samples n=7).

As mentioned above the upregulation appears to be transitory since at 48 hours post-ischemia there was no longer a difference between ischemic and contralateral tissues (Table 3 and figure 16). It is possible that a temporary upregulation reflects a specific time window when the ischemic cortex needs to effectively use the substrates available for survival. At 48h the lesion is considered mature and the region that could not be saved is already incorporated into the irreversibly damaged tissue.

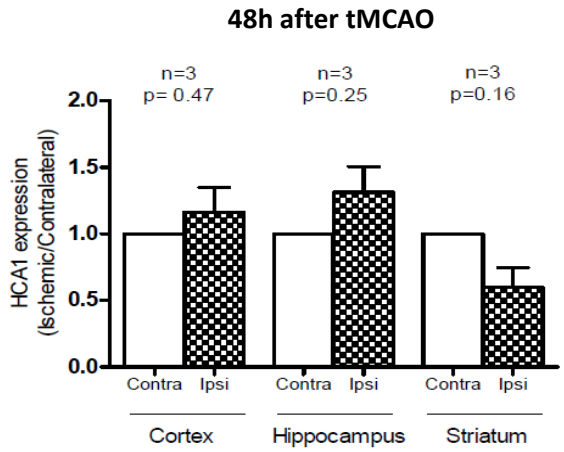


Figure 16. HCA1 receptor expression 48h after ischemia. Western blot analysis showed no significant difference in HCA1 receptor expression 48 h after tMCAO in any of the brain areas analyzed, compared to the contralateral hemisphere.

2.1.3 Regulation of HCA1 receptor expression by L-lactate under ischemic conditions.

Based on this result and considering the neuroprotective effect of lactate administered at reperfusion, we decided to test if lactate administration regulates HCA1 receptor expression by injecting L-lactate intravenously at the beginning of the reperfusion period and monitoring 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 in the hippocampus. This difference was again evident when an immunohistochemistry approach was used to illustrate the quantitative data (figure 17 and 18). These observations suggest that the expression of the HCA1 receptor may be modulated by ischemia and potentially by lactate injected at reperfusion.

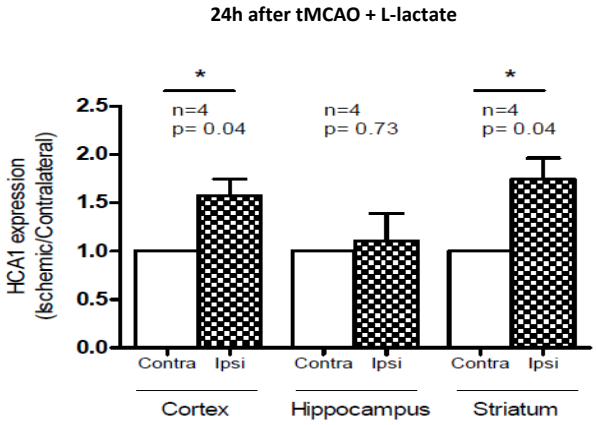


Figure 17.The HCA1 receptor expression is promoted by ischemia and enhanced by L-lactate administration. Intravenous L-lactate injection lead to an increase in HCA1expression in the lesion site (striatum) at 24h (*$p < 0.05$; paired t-test ischemic vs contralateral hemisphere).

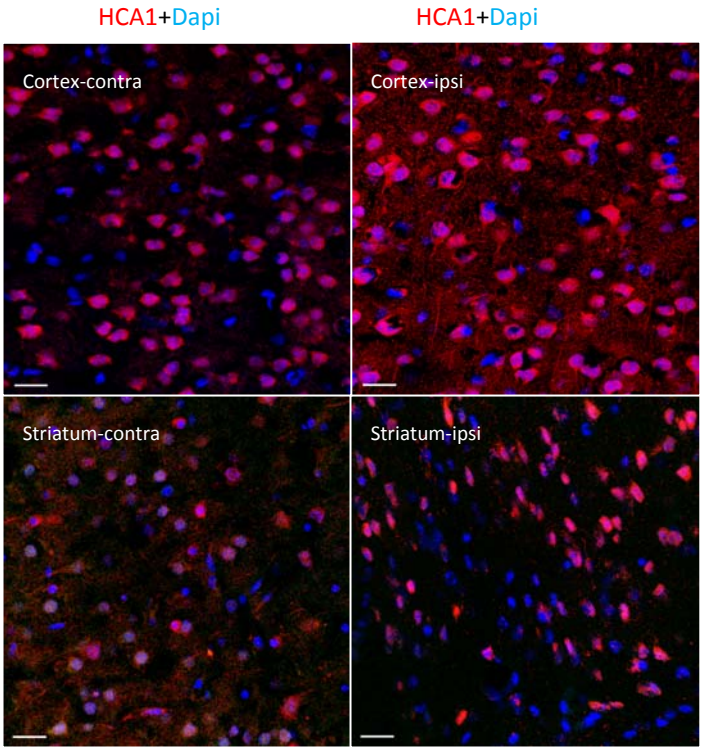


Figure 18. Administration of L-lactate promoted an increase in the HCA1 receptor expression in the ipsilateral (ischemic) cortex and striatum. Detailed section of the ipsilateral and contralateral cortex and striatum, were an increased in the HCA1 staining can be appreciated. Sections were counterstained with DAPI to show cell nuclei. Color code: HCA1 [red], DAPI [blue]. Magnification: 63x. Scale bar: 20µm

2.1.4 Neuroprotective role of the HCA1 receptor

We then investigated the role of HCA1 receptors in ischemic conditions in an *in vitro* model. The *in vitro* model has the advantage that we could further investigate the physiological role of the HCA1 receptor upregulation observed *in vivo*, by applying a HCA1 receptor agonist after OGD. 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 19).

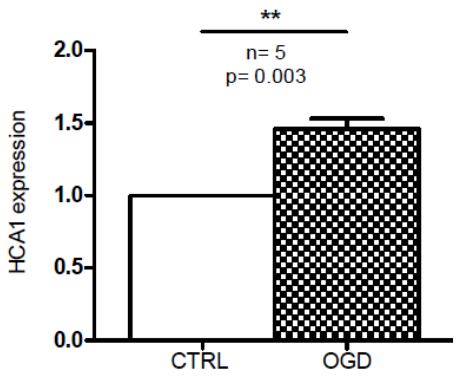


Figure 19. HCA1 receptor expression is increased after an *in vitro* ischemic insult. Western blot analysis showed an increase in HCA1 receptor expression 48 h after OGD (**p < 0.01; One way ANOVA Kruskal-Wallis test followed by Dunn's multiple comparison test and paired t-test).

As mention above, in order to determine if enhanced HCA1 receptor expression could play a role in the neuroprotective effect of L-lactate after OGD, organotypic hippocampal slices were exposed to the HCA1 receptor agonist 3,5-dihydroxybenzoic acid (DHBA) 1 hour 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 OGD from 13.5 ± 7.2 % to 7.4 ± 5.2 % ($p < 0.05$; figure 20).

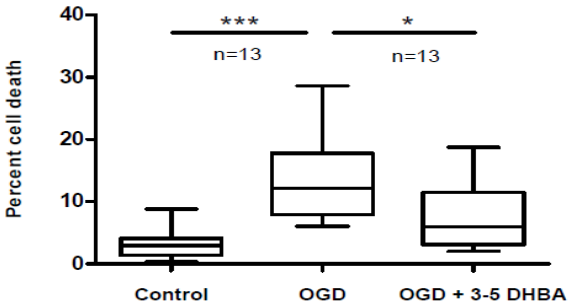


Figure 20.Activation of the HCA1 receptor is neuroprotective *in vitro*. Administration of 4 mM 3-5 DHBA, an HCA1 receptor agonist, attenuated cell death when administered directly to the medium after OGD (*$p < 0.05$, ***$p < 0.001$).

2.2 D-Lactate neuroprotection in ischemia models

2.2.1 D-lactate neuroprotection *in vitro* after oxygen and glucose deprivation

The D enantiomer of lactate was considered a non-metabolized isoform of lactate, and was described to act at least as a partial agonist of the HCA1 receptor (Cai et al., 2008; Bozzo et al., 2013). We were interested to determine if D-lactate could exert a neuroprotective effect both *in vitro* and *in vivo*. To our surprise, 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 21). It is noteworthy that the neuroprotective effect approaches that previously reported for L-lactate in this model (Berthet et al, 2009).

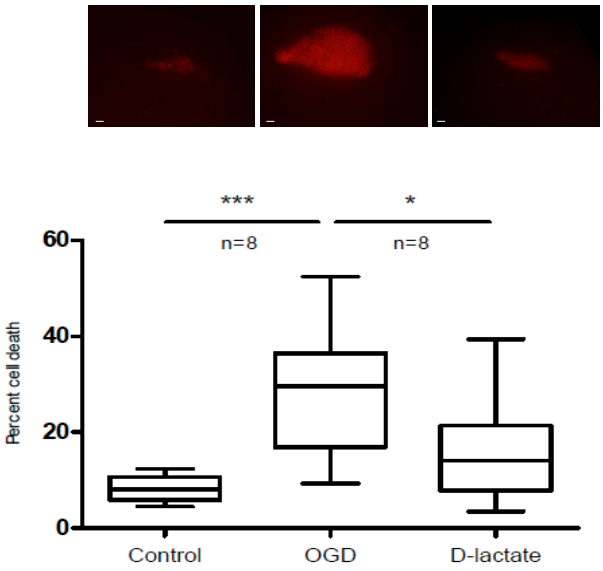


Figure 21.*In vitro* neuroprotection by D-lactate. Administration of 4 mM D-lactate induced a significant reduction in cell death, assessed by PI staining, 48 h after treatment (*p < 0.05, One way ANOVA Kruskal-Wallis followed by Dunn's multiple comparison test). Magnification: 5x. Scale bar: 20µm

2.2.2 D-lactate neuroprotection is observed *in vivo* when administered after 45 minutes tMCAO

We then tested D-lactate *in vivo* by intravenous administration of 1 µmol/g D-Lactate after 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 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 22). Again, the magnitude of neuroprotection was comparable to that reported previously for L-lactate *in vivo* (Berthet et al, 2009)

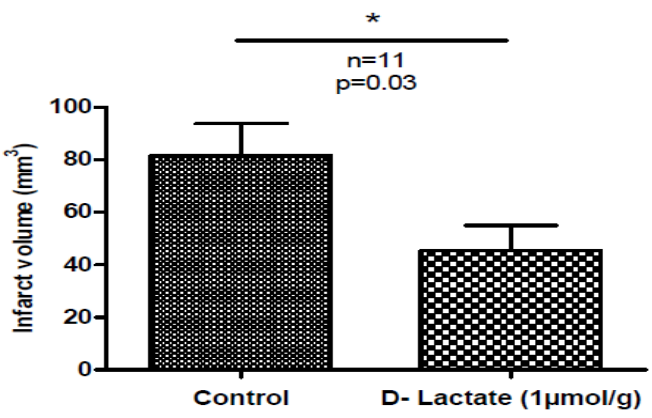


Figure 22. *In vivo* neuroprotection by D-lactate. Intravenous injection of 1 µmol/g D-lactate after 45 min tMCAO decreased total infarct volumes measured 48 h after ischemia (*p < 0.05 Mann-Whitney test, two-tailed p-value).

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 23).

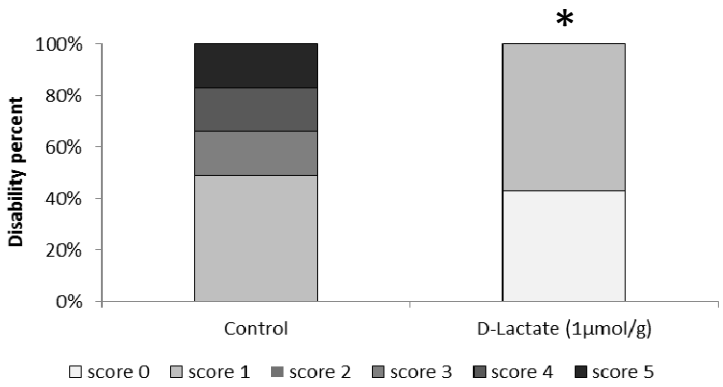


Figure 23.D-lactate neuroprotection is reflected also in the neurological outcome. Mice receiving intravenous administration of D-lactate show less neurological deficit than the mice that received the control solution Neurologic deficit scores from 0, no deficit – white, to 5 worst outcome – black; *$p < 0.05$ Mann-Whitney test, two-tailed p-value).

As stated above, different *in vivo* and *in vitro* studies suggested that D-lactate is not metabolized in cerebral tissue (Borg et al., 2003; Tekkök et al., 2005; Rinholm et al., 2011) thought to be due to the apparent lack of expression of the specific enzyme D-lactate dehydrogenase in mammals. The observation of neuroprotection exerted by D-lactate in both our in vivo and in vitro ischemic models, prompted us to study a bit further this issue in the rodent brain in collaboration with the group of Prof. Bruno Weber at the Neuroscience centre of the University Hospital of Zürich to.

They previously described L-lactate metabolism using radiolabeled L-lactate to trace its metabolism and kinetics in a one-tissue compartment model (Wyss et al., 2011). Using this technique and D-Lactate they reported that $^{11}C\text{-}CO_2$ was the only metabolite in blood and brain (Appendix 1), concluding that D-lactate could be effectively metabolized in the rodent brain.

2.3. Effect of glucose, pyruvate and acetate in ischemia models

2.3.1 Detrimental effect of glucose application *in vitro*

It has been previously described that glucose administered before ischemic stroke exerts a detrimental effect resulting in increased lesion size in a rat model of global cerebral ischemia (Schurr et al., 2001). As glucose is the main energetic substrate of the brain, we decided to test the effect of glucose administration after OGD in vitro. Administration of glucose to organotypic hippocampal cultures after OGD was detrimental increasing the percentage of cell death compared to control slices that received PBS after OGD (Fig. 24).

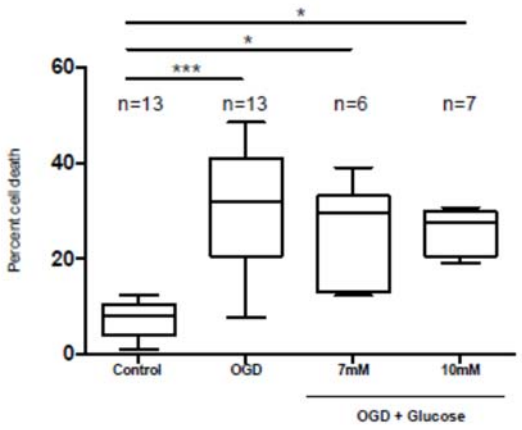


Figure 24. Administration of glucose after OGD. Administration of two doses of glucose did not protect against ischemic damage and was indeed detrimental. Cell death was assessed by PI staining 48 h after treatment. (One way ANOVA Kruskal-Wallis test followed by Dunn's multiple comparison test).

2.3.2 *In vitro* neuroprotection exerted by pyruvate

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 (Lee et al., 2001; Ryou et al., 2012). 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 25).

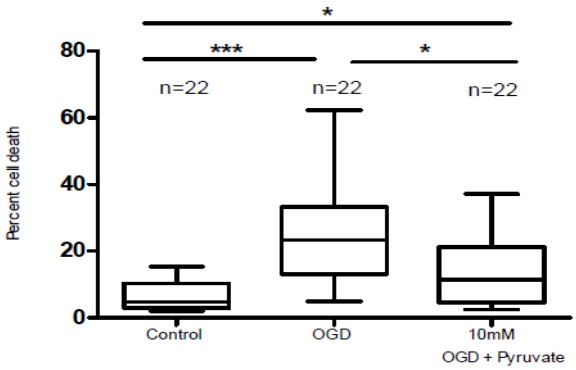


Figure 25.Administration of pyruvate after OGD. Administration of 10 mM pyruvate directly to the medium after 1 h OGD in rat organotypic hippocampal slices was able to protect against ischemic damage. Cell death was assessed by PI staining 48 h after treatment. (One way ANOVA Kruskal-Wallis test followed by Dunn's multiple comparison test).

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. Due to the inherent variability of the model our experiment was however underpowered 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) (Figure 26).

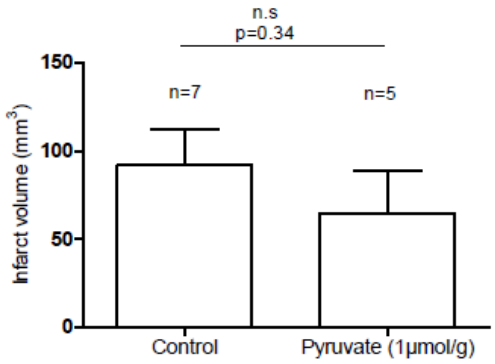


Figure 26. Administration of pyruvate after 30 minutes tMCAO. Administration of 1µmol/g pyruvate intravenously at reperfusion after 30 minutes tMCAO in adult mice was not able to induce a significant protection against ischemic damage although a trend was observed (p= 0.4).

2.3.3 Acetate administration to hippocampal slices subjected to OGD

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 an energy substrate by astrocytes (Waniewski and Martin, 1998). 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 27).

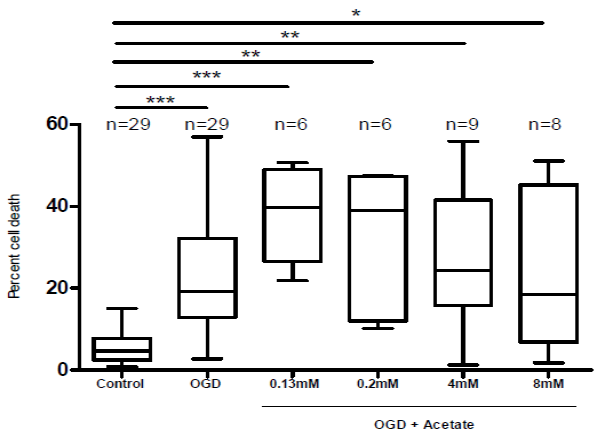


Figure 27. Administration of acetate after OGD. Administration of various doses of acetate did not protect against ischemic damage. Cell death was assessed by PI staining 48 h after treatment. (One way ANOVA Kruskal-Wallis test followed by Dunn's multiple comparison test).

2.4. Effect of 4-CIN application to organotypic hippocampal slice cultures.

To further investigate lactate being used as a metabolic substrate in the post-ischemic neuroprotection, we decided to test the administration of the monocarboxylate transporter inhibitor 4-CIN to organotypic hippocampal slices cultures prior to oxygen and glucose deprivation, and posterior application of 4mM lactate, the concentration that has shown to exert protection in our previous experiments. If lactate is not able to enter the cells to be metabolized, the resultant effect should reflect HCA1 receptor activation. Toxicity experiments were carried out to elucidate a concentration that did not promote cell death by itself and could be used in the subsequent experiments. Results showed that several doses of 4-CIN exerted a cytotoxic effect. (figure 28).

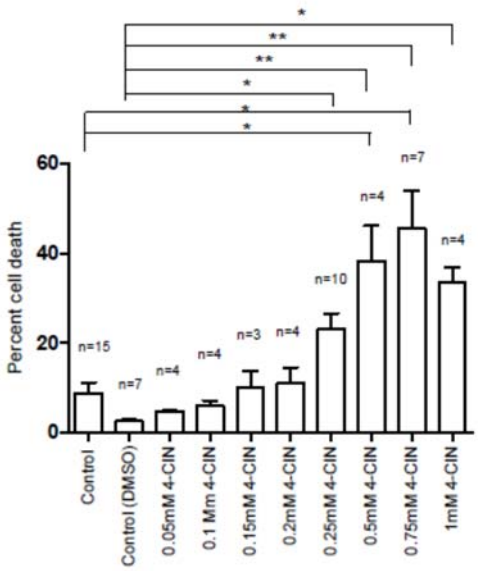


Figure 28. Administration of 4-CIN to organotypic hippocampal slice cultures. Administration of various doses of 4-CIN showed a cytotoxic effect by increasing the percentage of cell death without a noxious stimuli, in comparison with the control slices. Cell death was assessed by PI staining 48 h after treatment. (One way ANOVA Kruskal-Wallis test followed by Dunn's multiple comparison test).

(Part of these results have been submitted for publication in *Journal of Cerebral Blood Flow and Metabolism* as: A probable dual mode of action for both L- and D- lactate neuroprotection in cerebral ischemia. Castillo X, Rosafio K, Wyss M, Buck F, Pellerin L, Weber B, Hirt L)

IV. DISCUSSION

Glucose is the main energy substrate of the brain but under special circumstances lactate plays an important role as an alternative energy substrate and has been shown to support synaptic activity in the central nervous system (Pellerin 2003; Wyss et al., 2011; Schurr 2014; Bergersen 2014). The movement to consider lactate as a useful molecule for brain function instead of a waste product of metabolism has been long and still is not unanimously accepted.

The ANLS hypothesis has been severely criticized and one of the main criticisms is provided by Simpson et al., who proposed an alternative, mathematical model whereby taking into consideration concentration and kinetic characteristics of the blood brain barrier and neuronal and glial transporter proteins (GLUT1 – 3 and MCT1 – 2), they concluded that is in the neuron – and not in the astrocyte- that glucose gets metabolized and the astrocyte is the chief exporter of lactate (Neuron-Astrocyte lactate shuttle, NALS) (Simpson et al., 2007). This report somehow revived the long fight between scientists that support or discard the ANLS hypothesis, claiming besides the direction of lactate flux, the overall contribution of lactate to cerebral metabolism is small (Fillenz M, 2005; Mangia et al., 2009; Dinuzzo et al., 2010; Jolivet et al., 2010; Mangia et al., 2011). In another set of experiments described in the introduction and realized by Hu and Wilson(1997b) it was described that in the active brain after stimulation there was a dip in lactate concentration smaller than the one observed in glucose, which has been interpreted by skeptics of the ANLS as a proof of the preferential utilization of glucose as energetic substrate. It should be mentioned though, that a closer analysis performed by Schurr and Gozal (2012) estimated that since the lactate basal level before stimulation was 1.19 mM and that of glucose was 2.60 mM, and that stimulation prompted a fall of lactate levels of 0.08 mM and on glucose levels of 0.36mM. At first sight it would appear that a significant portion of the energy required for this activation is supplied via glycolysis as 0.36mM glucose were consumed, while a smaller portion of that energy is supplied via aerobic consumption of lactate, since only 0.08mM of the monocarboxylate were consumed. These quantities, however, could be misleading since 0.36mM glucose produces a net amount of 0.72 mM ATP (1 mol of glucose produces 2 mol of ATP glycolytically), while 0.08 mM lactate produces 2.72 mM ATP (1 mol of lactate produced 34 mol of ATP via the mitochondrial tricarboxylic acid (TCA) cycle). Consequently, the “small” amount of lactate consumed upon the first stimulation actually produced 3.8 times more ATP than the amount produced by the consumption of glucose upon the first stimulation.

According to Avital Schurr, the main point of the long discussion appears to be the misunderstanding about lactate being claimed as the most important brain energy substrate,

therefore prompting several groups to execute research and invest effort into proving that concept wrong, trying to keep the “most important brain energy substrate” crown for glucose (Schurr, 2014). Glucose is the main energy substrate for brain activity and it is not the focus of this thesis to argue against this concept. The focus instead is to show that lactate is an important molecule not only for a series of physiological processes but that it can exerts neuroprotectant effects, and to investigate the possible mechanism of action.

Since McIlwain’s description in 1953 that lactate can act as an energy substrate to sustain the metabolic rate of the brain (McIlwain H, 1953) many efforts have been made and different techniques have been developed in an attempt to obtain more accurate information. For example, it has been shown *in vivo* that different stimuli such as mild stress and stimulation of the perforant pathway in the dentate gyrus are enough to promote elevation of the local extracellular lactate concentration, measured in real time by microdialysis in freely moving rats (Hu and Wilson 1997; Fellows et al. 2003). Lactate has also been involved in important physiological processes such as memory consolidation as well as long-term memory formation (Suzuki et al. 2011) and it

was shown using a transient global cerebral ischemia model in rats and a monocarboxylate transporter inhibitor that brain lactate is essential to improve post ischemic outcome(Schurr et al. 2001).

Lactate has been shown as in important molecule involved in the physiological functions of the central nervous system not only in lactate-neuron interactions, but has also been described to be important for the metabolic homeostasis of other cell types such as oligodendrocytes. Oligodendrocytes consume more lactate than neurons in culture, possibly due to use as a metabolic substrate and as a precursor to build carbon skeletons (lipid synthesis) (Sánchez-Abarca et al., 2001). Furthermore, Rinholm et al., showedthat lactate can be taken up by MCT1 and support oligodendrocyte development and myelination when glucose levels are low, such as during the early postnatal period (Rinholm et al., 2011). Funfschilling et al., used a transgenic mice approach to show that enhanced glycolysis increases production and release of lactate, pyruvate and acetyl-coenzyme-A (CoA) from oligodendrocytes that can supply glycolysis products to support axonal energy needs via MCT1 and MCT2 (Funfschilling et al., 2012). Finally, a proper oligodendrocyte metabolism has proven to be of extreme importance since missregulation is now linked to the presence of neurodegenerative diseases such as amyotrophic lateral sclerosis (ALS) and X-linked adrenoleukodystrophy (X-ALD) (Lee et al., 2012; Kassmann et al., 2007).

So far I have mainly pointed out the importance of endogenous lactate production in physiological conditions as well as the importance of a working transport system. Nevertheless, in some situations where oxygen and glucose are restricted, lactate has been shown to have a beneficial effect when administered exogenously such as after traumatic brain injury and ischemia, regardless of the pathway of administration (directly to the lesion site, in the parenchyma or by intracerebroventricular or intravenous injections) (Bouzat et al., 2014; Berthet et al., 2009; 2012; Horn and Klein, 2013).

As mentioned above it is well known that the monocarboxylate transporter family is responsible not only for lactate's fluxes from different cell types but also those of other monocarboxylates such as pyruvate, acetate and ketone bodies. The monocarboxylate transporters show selective expression in different cell types as well as transporting the monocarboxylates from one cell to another with different affinities (Pierre and Pellerin, 2005). Considering this information, the recent work describing lactate acting as a signaling molecule in the central nervous system, either through the HCA1 receptor (Lauritzen et al., 2013; Bozzo et al., 2013) or through an unknown receptor yet to be specified (Tang et al., 2014) was of extreme interest for our work and lead to the question of whether the neuroprotective effect of lactate is exerted by it's being metabolized as an energetic substrate or by lactate acting as a signaling molecule.

In an attempt to elucidate if lactate is acting as a signaling molecule the first part of this study was focused on the presence of the HCA1 receptor in the central nervous system and the changes observed in its expression when mice were subjected to ischemia. We also used the lactate enantiomer D-lactate, since it has been described to be transported by the MCTs and also acts as a weak agonist of the HCA1 receptor. In the second part of this work, we used pyruvate and acetate to assess neuroprotection after *in vitro* ischemia since these monocarboxylates are located downstream in the metabolic pathway and would therefore help us to elucidate if the protection is taking place due to the use of lactate as a metabolic substrate.

HCA1 receptor expression and cerebral ischemia

To address this we decided to first confirm the presence and distribution of the HCA1 receptor in three brain regions (cortex, hippocampus and striatum) and our results show that the HCA1 receptor is expressed throughout the brain regions analyzed, mainly in the cytoplasm and processes of neurons. Interestingly, we also showed that the cerebral expression of HCA1 can be

modulated by ischemia and that L-lactate seems to be a signal for the induction of its own receptor.

One interesting feature of the modulation of the HCA1 receptor 24h after ischemia is that without exogenous lactate stimulation there appears to be an increase in the cortex that surrounds the lesion site, probably in a region where the detrimental mechanisms of neuronal death can still be reversed. Another interesting observation is that in the ischemic striatum, where the lesion core is located, there is no difference in the HCA1 receptor expression compared to the contralateral hemisphere. As mentioned, the HCA1 receptor appears to be expressed mainly in neurons and the fact that there is no detectable change in its expression ratio in the lesion core might be an indirect indication that HCA1 receptor is over-expressed in the few remaining neuronal cells located in the ischemic core. However, with the information we have we cannot affirm that lactate accumulation following ischemia is in fact the responsible for the enhancement of the HCA1 receptor observed after 24 hours of reperfusion.

When L-lactate was administered exogenously by intravenous injection we observed a further increase in the ratio of the HCA1 receptor expression not only in the cortical region surrounding the lesion core but also in the ischemic striatum, supporting the idea that HCA1 signaling might be promoting protection and survival of energy deprived neurons. In line with this observation is the fact that the analysis of the HCA1 protein expression in organotypic hippocampal slices subjected to OGD also showed a difference in the ratio of the HCA1 protein expression compared to control slices. To further investigate if upregulation of the HCA1 receptor under ischemic conditions both *in vivo* and *in vitro* has a functional effect we used 3,5-dihydroxybenzoic acid, a specific agonist (Liu et al., 2012) of the HCA1 receptor, in our *in vitro* experiments and report that activation of the HCA1 receptor is beneficial by decreasing the percentage of cell death.

Even though in the peripheral tissue it has been demonstrated that lactate acts as an agonist on the HCA1 receptor, to the best of our knowledge the results shown in this thesis are the first evidence that the HCA1 receptor is involved in the neuroprotective effects of lactate in ischemic stroke. This is an important observation since it highlights the necessity to consider that the effects of lactate in the central nervous system might not be completely abolished when monocarboxylate transport is disrupted, a widely used approach to block the effects of lactate. Nevertheless, it remains to be elucidated to which extent the effects of lactate are due to its use as an energetic substrate or as a signaling molecule and importantly, if these two pathways are used under different physiological or pathological circumstances.

Here it is important to mention the recent work of Shen et al., who showed that a vector containing a green fluorescent protein coupled with the GPR81 (HCA1) receptor (GFP-GPR81) transfected into mouse neuroblastoma neuro-2a (N2A) cells aggravated the OGD-induced cell death compared to control cells. In this work they used 3-hydroxy-butyrate (3-OBA) as an antagonist of the HCA1 receptor (GPR81) and observed protection (decreased the cell death) when this ketone body was applied to cells subjected to OGD. Similar results were obtained *in vivo* when 3-OBA was injected intracerebroventricularly at the onset of permanent MCAO and a reduction in the lesion size was observed (Shen et al., 2014). The work is intriguing in that they used a ketone body (3-OBA) as antagonist of the HCA1 receptor, knowing that ketone bodies are agonists of another members of the hydroxycarboxylic acid receptor family, includingHCA2, and that it has been recently described that activation of the HCA2 receptor by ketone bodies and nicotinic acid exerts protection decreasing infarct volume and activating monocytes and / or macrophages (Rahman et al., 2014). Therefore they cannot rule out the possibility that their results are due to activation of the HCA2 receptor instead of antagonism of the HCA1 receptor. Nonetheless, the model used in the work performed by Shen et al., is not the same as in our experiments (30 minutes ischemia vs permanent ischemia; organotypic hippocampal slice cultures vs neuronal cultures transfected with HCA1 receptor) and 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.

D-lactate neuroprotection

In the literature, D-lactate, the enantiomer form of lactate, has been described on several occasions to be a non-metabolize (inactive) enantiomer. Nevertheless, if we look more carefully it has been described that D-lactate can also be transported by the monocarboxylate transporter family (Kang KP et al., 2006) although with less affinity than L-lactate and also isolation of human and mouse transcripts encoding a homolog of the yeast D-lactate dehydrogenase was reported with expression in various tissues, including the brain (Flick and Konieczni, 2002). It is known that accumulation of D-lactate in humans and ruminants results in acidosis (Uribarri et al., 1998; Vella and Farrugia, 1998; Kang KP et al., 2006) but contrary to the initial assumption that D-lactic acid is not metabolized in humans and therefore accumulation causes D-lactic acidosis, it is now known that D-lactic acidosis risk increases for several reasons such as the presence of a malabsorption disease (e.g. short bowel disease), diabetes mellitus, an increase in the consumption of colonic

bacterial flora capable of producing D-lactic acid, large amount of carbohydrates or a diminished colonic motility that would allow nutrients into the colon to undergo bacterial fermentation. In fact, it has been reported that healthy infants and adults are able to metabolize D-lactate (Haschke-Becher E et al., 2000; Uribarri et al., 1998; Talasniemi JP et al., 2008) and that impaired metabolism of D-lactate is almost a prerequisite for the development of D-lactic acidosis. Furthermore, it has been shown that daily consumption of 6.4 mmol/kg body weight DL-lactic acid for 5 weeks, did not result in accumulation of plasmatic D-lactate in healthy volunteers, nor in an increased urinary excretion since less than 2% of the administered dose of D-lactate was excreted in urine when measured 24h after intake (de Vrese et al., 1990) which might imply a high rate of metabolism of D-lactate.

In our experiments, we found that neuroprotection was also achieved when D-lactate was administered either *in vitro*, after 1h OGD or *in vivo*, at reperfusion after 45 minutes MCAO. Thanks to a collaboration we established with the group of Bruno Weber from the Neuroscience Center of the University Hospital in Zürich we know that, despict previous works in basic research describing D-lactate as the non-metabolize enantiomer, this metabolite is effectively extracted by the rodent brain and metabolized to CO_2 only marginally less than the previously reported fraction of CO_2 produced by L-lactate (Wyss et al., 2011; Appendix, figure 21). So its metabolism might explain the neuroprotection we observed however, other observations by several groups show that D-lactate exhibits at least a partial agonist activity on the HCA1 receptor (Cai et al., 2008; Bozzo et al., 2013; Liu et al., 2009; Ahmed et al., 2010). Therefore, we cannot conclude using D-lactate protection if neuroprotection takes place as a result of lactate being used as an energy substrate or acting through the HCA1 receptor.

Considering that lactate is transported by MCTs, we decided to try another approach by blocking the MCTs with α-cyano-4-hydroxy-cinnamate (4-CIN) a compound widely used to inhibit MCT function *in vitro* and *in vivo* (Erlichman et al., 2008; Tang et al., 2014; Schurr et al., 2001). The first step of this experiment was to establish a safe concentration of 4-CIN that elicited blockage of MCTs but did not result in toxicity of the cultures. Unfortunately, these toxicity assays took longer than expected since the doses previously reported as safe in the literature promoted increased cell death in controls without OGD. These experiments are being continued.

Effect of glucose, pyruvate and acetate in ischemia models

The detrimental effects observed with glucose administration after OGD to organotypic hippocampal slice cultures are in line with previous observations reported in the literature. In 1977 Myers and Yamaguchi found, by serendipity that administration of glucose (instead of saline) to monkeys before a 14 min cerebral ischemia resulted in a worse neurological outcome (Myers and Yamaguchi, 1977). This finding, confirmed by many groups in basic science prompted establishing clinical recommendations in the management of stroke patients and today, minimizing the risk of hyperglycemic conditions with insulin administration is routine. This effect of hyperglycemia-aggravated ischemic damage has been called the "Glucose paradox" and it was assumed for years that the elevated pre-ischemic glucose levels were creating an elevated intra-ischemic lactic acid levels. Regardless this long standing dogma, lactate might have a safe "way out" as the cause of the observed detrimental effects. Schurr et al., described that glucose administered 2h before inducing ischemia had a beneficial effect lowering the degree of neuronal damage, and that glucose administered from 15 to 60 minutes before ischemia was detrimental exacerbating neuronal damage in comparison to the control rats. When lactate transport was blocked with the MCT inhibitor 4-CIN administered concomitantly with glucose 2h before ischemia, they observed that the beneficial effect of glucose was abolished and there was a significant increase in the degree of delayed neuronal damage 7 days postischemia. Interestingly, they also reported a significant increase in blood corticosterone after glucose administration and when an inhibitor of corticosterone synthesis was used, a lower degree of neuronal damage was observed (Schurr et al., 2001). From this work, corticosterone is exposed as the real culprit behind the glucose paradox and lactate's beneficial role in postischemic outcome is again apparent.

In the central nervous system, pyruvate is transported mainly by MCT1in astrocytes and blood vessels and MCT2 in neurons in the direction of the concentration gradient (Pierre et al., 2002; Pellerin et al., 2005). As mentioned above, lactate can be converted to pyruvate by lactate-dehydrogenase to fuel the Krebs cycle and therefore be used as an energetic substrate. Trying to elucidate the impact that the metabolic pathway might have on the neuroprotective effects observed with the administration of lactate, we administered pyruvate *in vitro* after 1h OGD and *in vivo* at reperfusion after 30 minutes MCAO. Evidence supporting ametabolic effect is the protection we obtain with the administration of 10 mM pyruvate *in vitro*, although we fail to obtain protection *in vivo* when pyruvate was administered at reperfusion after 30 minutes MCAO. It is important to mention that even though *in vivo* we observed a minor decrease in the lesion size in mice that received intravenous injection of pyruvate in comparison with the control mice,

this difference did not reach a statistical significance probably due to pyruvate instability in aqueous solutions (Fink MP, 2007) which could increment the variability of the results therefore increasing the number of animals needed to find a statistically significant effect. In work published in 2007, authors showed that pyruvate was able to reduce the infarct volume as well as the motor deficits when administered in both transient and permanent MCAO compared to the control mice not receiving pyruvate. In this work, they used a total of 402 rats (Yi et al., 2007).

Nevertheless, in agreement with our observations other groups have described the *in vitro* neuroprotective effect of pyruvate (Ryou et al., 2012; 2013) and to overcome the compound instability problem some experiments have been carried out by continuous infusions of pyruvate from the beginning of ischemia elicited by MCAO until 30 minutes after reperfusion (Ryou et al., 2012) or by using a compound called ethyl-pyruvate, supposed to increase pyruvate's stability in aqueous solutions (Kim et al., 2005; Yu et al., 2005; Shen et al., 2010). The problem with ethyl-pyruvate is that it is not completely clear if the protective effects observed are due only to an increase in the stability of pyruvate or perhaps due to the conformational change itself. However, regarding the results reported by Ryou et al., using normal pyruvate, differences 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.

Regarding acetate, it has been shown that this monocarboxylate is preferentially transported by MCT1 and metabolized in astrocytes (Waniewski and Martin, 1998; Wyss et al., 2009). 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 (Hosoi et al., 2007). 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 analyzed functional outcome of acetate supplementation in our *in vitro* ischemic model and found that administration of acetate did not exert protection against neuronal death in hippocampal slices subjected to oxygen and glucose deprivation, in any of the doses that we applied. 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 after an ischemic insult. 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 (Cai et al., 2008). As neither acetate nor pyruvate binds 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.

V. CONCLUSION AND PERSPECTIVES

The data obtained so far point to the possibility that lactate, independently of the enantiomer type, confers neuroprotection after ischemia perhaps by two mechanisms: One is through classical metabolic pathways providing an alternative energy supply to deprived neurons and the other is through a receptor-mediated signal transduction mechanism in neurons. In both cases the exact targets within the cell still need to be identified.

Future directions:

HCA1 Receptor expression and cerebral ischemia

In order to get a clearer picture of the role that the HCA1 receptor might be playing in the neuroprotective effects of lactate, I envisage a knock down approach of the protein either *in vitro* using si-RNA or *in vivo* using the HCA1 -/- mice produced by the group of Stefan Offermanns in Germany. Unfortunately I did not have the time to perform these experiments but would expect that HCA1-/- mice subjected to tMCAO would still show some protection after lactate administration since lactate should still be able to be used as an energy substrate. In these transgenic mice it would also be interesting to analyze the expression of the different MCTs since preliminary analysis of HCA1 and MCTs expression in wild type CD1 mice subjected to 30 min MCAO and supplemented with either L-lactate or PBS at reperfusion have shown the existence of some sort of counter regulation between MCT2, the monocarboxylate transporter expressed mainly in neurons and reputedly in charge of lactate's import into neurons, and HCa1 expression in the different brain structures analyzed (Appendix, figure 22 and 23). When analyzed at the same time points and tissue samples used to analyze HCA1 receptor expression, MCT2 showed no difference in the ratio of MCT2 expression in the ischemic hemisphere compared to the contralateral hemisphere in mice subjected to 30 minutes MCAO. Interestingly, L-lactate administration promoted a decrease in the ratio expression of the MCT2 in the cortex and striatum of ischemic hemispheric compared to contralateral hemisphere. This on-going work has also been performed in collaboration with the group of Prof. Pellerin.

Dissection of the metabolic pathway in lactate neuroprotection.

In order to elucidate the importance of lactate as metabolic substrate in the post-ischemic outcome, lactate transport could be blocked *in vitro* with 4-CIN supplemented in the medium before subjecting the organotypic hippocampal cultures to OGD. Supplementation of 3-5 DHBA after OGD in cultures where lactate transport has been blocked would be a feasible approach to try to determine if sole activation of the HCA1 receptor is able to exert protection after oxygen and glucose deprivation.

APPENDIX

Figure 29. D-lactate metabolism. a) Schematic of the 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 (Matthias Wyss, personal communication).

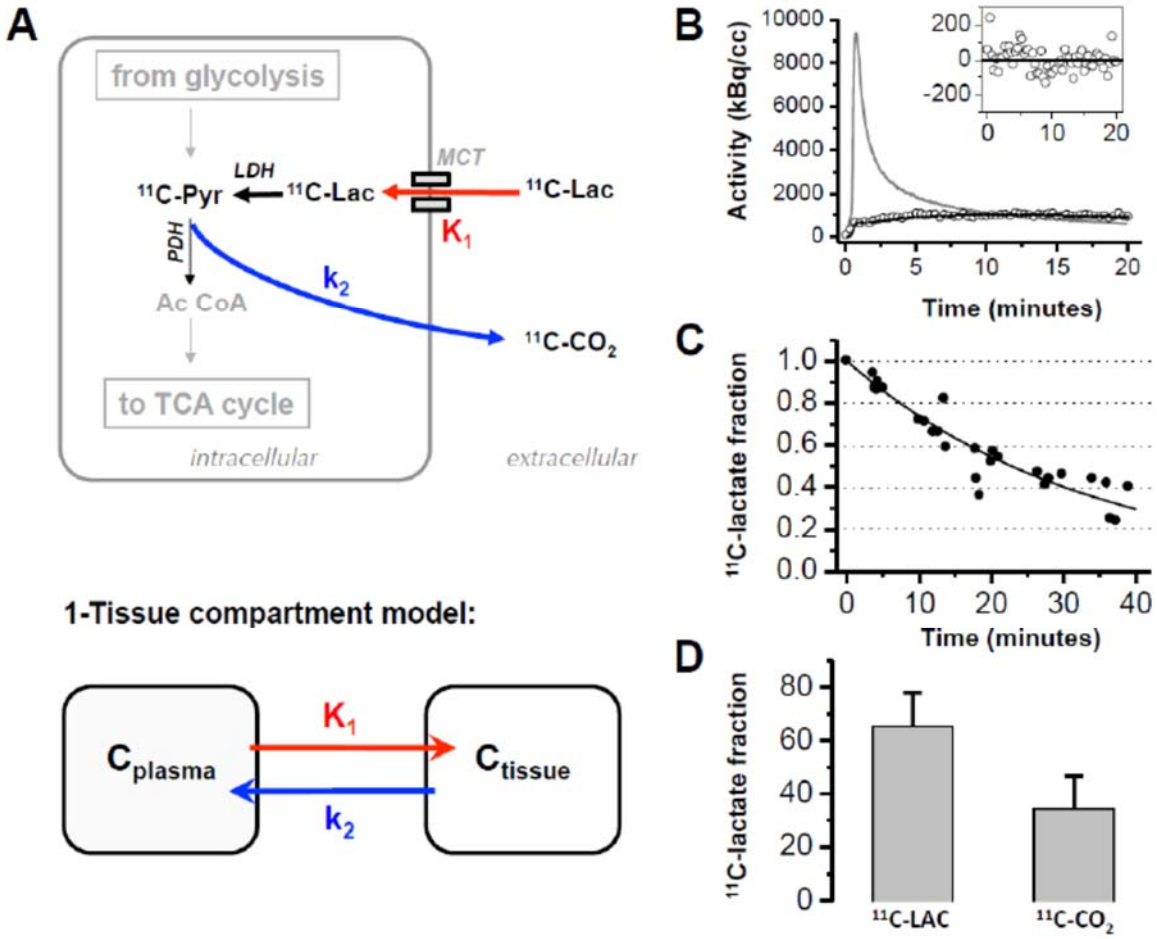


Figure 30. MCT2 expression 24h after ischemia. Western blot analysis showed no difference in the ratio of the HCA1 receptor expression at 24 h after tMCAO in any of the brain areas analyzed, compared to the contralateral hemisphere.

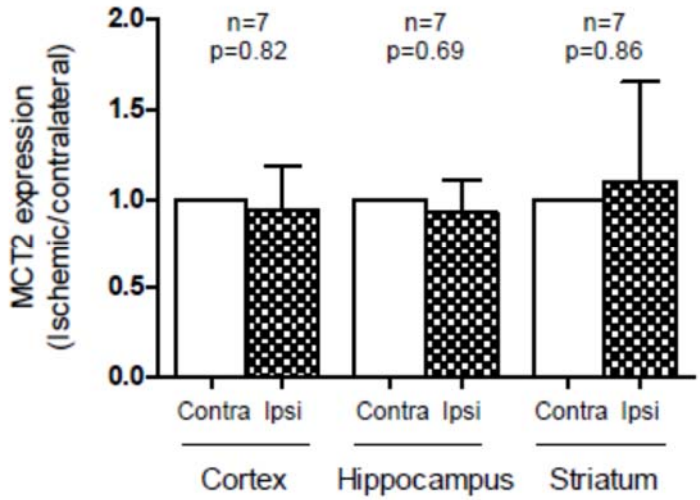
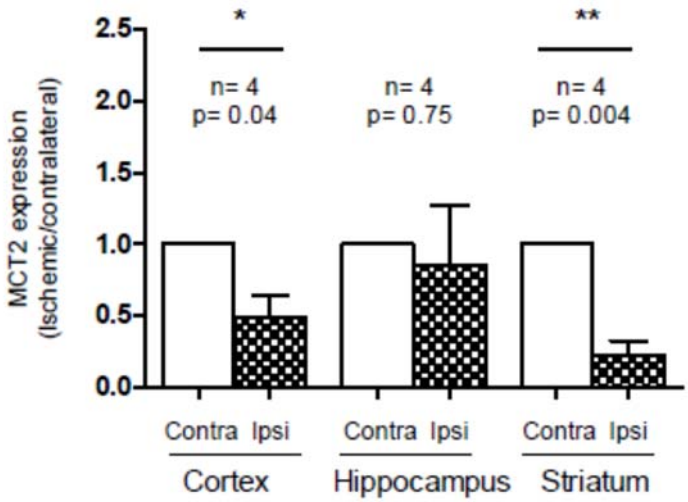


Figure 31. MCT2 expression is inhibited by ischemia and L-lactate administration. Intravenous L-lactate injection prompted a decrease in the MCT2 expression ratio in the region surrounding the lesion core (ischemic cortex) and also in the lesion site (striatum) 24h after tMCAO (*p < 0.05; paired t-test ischemic vs contralateral hemisphere).



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New Evidence of Neuroprotection by Lactate after Transient Focal Cerebral Ischaemia: Extended Benefit after Intracerebroventricular Injection and Efficacy of Intravenous Administration

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Key Words

Lactate · Cerebral ischaemia · Stroke · Neuroprotection · Translational research · Middle cerebral artery occlusion

Abstract

Background: Lactate protects mice against the ischaemic damage resulting from transient middle cerebral artery occlusion (MCAO) when administered intracerebroventricularly at reperfusion, yielding smaller lesion sizes and a better neurological outcome 48 h after ischaemia. We have now tested whether the beneficial effect of lactate is long-lasting and if lactate can be administered intravenously. ***Methods:*** Male ICR-CD1 mice were subjected to 15-min suture MCAO under xylazine + ketamine anaesthesia. Na L-lactate (2 μl of 100 mmol/l) or vehicle was administered intracerebroventricularly at reperfusion. The neurological deficit was evaluated using a composite deficit score based on the neurological score, the rotarod test and the beam walking test. Mice were sacrificed at 14 days. In a second set of experiments, Na L-lactate (1 μmol/g body weight) was administered intravenously into the tail vein at reperfusion. The neurological deficit and the lesion volume were measured at 48 h. ***Results:*** Intracerebroventricularly injected lactate induced sustained neuroprotection shown by smaller neurological deficits at 7 days (median = 0, min = 0, max = 3, n = 7 vs. median = 2, min = 1, max = 4.5, n = 5, $p < 0.05$) and 14 days after ischaemia (median = 0, min = 0, max = 3, n = 7 vs. median = 3, min = 0.5, max = 3, n = 7, p = 0.05). Reduced tissue damage was demonstrated by attenuated hemispheric atrophy at 14 days (1.3 ± 4.0 mm^3, n = 7 vs. 12.1 ± 3.8 mm^3, n = 5, $p < 0.05$) in lactate-treated animals. Systemic intravenous lactate administration was also neuroprotective and attenuated the deficit (median = 1, min = 0, max = 2.5, n = 12) compared to vehicle treatment (median = 1.5, min = 1, max = 8, n = 12, $p < 0.05$) as well as the lesion volume at 48 h (13.7 ± 12.2 mm^3, n = 12 vs. 29.6 ± 25.4 mm^3, n = 12, $p < 0.05$). ***Conclusions:*** The beneficial effect of lactate is long-lasting: lactate protects the mouse brain against ischaemic damage when supplied intracerebroventricularly during reperfusion with behavioural and histological benefits persisting 2 weeks after ischaemia. Importantly, lactate also protects after systemic intravenous administration, a more suitable route of administration in a clinical emergency setting. These findings provide further steps to bring this physiological, commonly available and inexpensive neuroprotectant closer to clinical translation for stroke.

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Introduction

In the neurovascular unit, astrocytes line cerebral capillaries with their end feet and interact closely with neurons at the synapses. Brain activation is coupled to glucose uptake from the blood stream, which is likely to involve astrocytes due to the spatial organization of the neurovascular unit. Lactate, a product of anaerobic glycolysis, can be shuttled from astrocytes to neurons and used as an efficient energy substrate [1]. Lactate transport from astrocytes to neurons plays a physiological role as it was recently shown that this transport is critical for long-term memory [2]. Lactate shuttling also has an important role in physiopathological events [3, 4]. Lactate protects neural tissue against excitotoxicity as it attenuates neuronal death induced by intracortical glutamate injection [3, 5]. Based on the knowledge of this beneficial role of lactate, we recently demonstrated that lactate could also prevent ischaemic neuronal death in vitro in hippocampal slice cultures subjected to oxygen and glucose deprivation [6] and in vivo, intracerebroventricular lactate injection after transient middle cerebral artery occlusion (MCAO) in mice attenuates both the lesion volume and the neurological deficit 2 days after ischaemia. We now show the effect of lactate neuroprotection on outcome at later time points as well as demonstrate a beneficial response at 48 h with intravenous administration. Lactate is a natural and easily available substance and our recent results are critical steps for its translation to clinical stroke patients.

Materials and Methods

Transient MCAO in the Mouse

All animal experiments were conducted according to Swiss guidelines for animal experimentation and approved by the veterinary authority. Male ICR-CD1 mice (body weight 26–35 g, Charles River, L'Arbresle, France) were anaesthetized by intraperitoneal injection of 8 mg/kg xylazine (Rompun 2%, Bayer, Zurich, Switzerland) and 100 mg/kg ketamine (Ketanarkon 100, Streuli Pharma, Uznach, Switzerland). At 0 h, ischaemia was induced by inserting a silicone-coated 8-0 filament (Doccol Corp., Redlands, Calif., USA) through the left common carotid artery into the internal carotid artery [6, 7]. The filament was withdrawn after 15 min for reperfusion. Regional cerebral blood flow (rCBF) was measured by laser Doppler flowmetry (Periflux 5000, Perimed, Stockholm, Sweden) with a flexible probe fixed on the skull, 1 mm posteriorly and 6 mm laterally from the bregma and monitored throughout surgery. Rectal temperature was maintained at 37 ± 0.5 °C throughout surgery, using a temperature control unit (FHC, Bowdoinham, Me., USA). Mice were administered 0.025 mg/kg of buprenorphine subcutaneously for analgesia after surgery. They were then housed overnight in an incubator at 31 °C. Mice were sacrificed after 48 h or 14 days. Among intracerebroventricularly injected mice, 5/18 died before the 14-day time point (2 died during surgery before treatment; 2 vehicle-treated mice died on day 4; 1 lactate-treated animal died on day 6). Among intravenously injected mice, 4/52 died before 48 h (1 died during surgery before treatment; 3 vehicle-treated animals died on day 2).

Lactate Treatment

Two microlitres of either the 100 mmol/l Na L-lactate (Fluka, Sigma-Aldrich, Buchs, Switzerland) solution corresponding to 200 mmol/l or vehicle (1× PBS, pH 7.4) were randomly injected by a surgeon blinded to the treatment solution intracerebroventricularly at reperfusion into the left lateral ventricle (0.9 mm laterally, 0.1 mm posteriorly, 3.1 mm deep from the bregma [6, 8]) using a Hamilton syringe (Hamilton Company, Bonaduz, Switzerland).

Five microlitres per gram of body weight of Na L-lactate solution (200 mmol/l) or vehicle solution were injected by a surgeon blinded to the treatment solution intravenously (tail vein) administered at reperfusion using a 1-ml syringe with a 25-gauge needle and a mouse restrainer (Braintree Scientific Inc., Braintree, Mass., USA).

Lesion Volume Measurement

The lesion volume was calculated by multiplying the distance between sections by the lesion area, measured on 20-μm-thick cryostat sections stained with cresyl violet and by an examiner blinded to the treatment group. The hemispheric atrophy was calculated by subtracting the volume of the hemisphere which suffered ischaemic injury from the unaffected hemisphere.

Immunohistochemistry

Frozen cryostat brain sections were fixed with 4% *p*-formaldehyde for 1 h at 4 °C, washed in 1× PBS and incubated overnight at 4 °C in 1× PBS, 1% bovine serum albumin, and 0.1% Triton with neuronal nuclear antigen (NeuN) (Sigma) antibody (1:300 dilution). The sections were incubated for 1 h at room temperature in the same buffer with anti-mouse FITC (Jackson ImmunoResearch Laboratories, Baltimore, Md., USA) antibody. Sections were mounted in Vectashield (Vector Labs, Burlingame, Calif., USA). Images were acquired with the Axiovision v4.8 software using a Zeiss Axiovision microscope (488 nm absorbance).

Behavioural Evaluation

A composite neuroscore [6, 9] was used to assess the neurological deficit, as previously described [6]. The composite score is based on three tests, each with a maximum of 3 points: the neuroscore (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); secondly, the beam walking test [10] (0 = the mouse walks directly to the end of the beam; 1 = the mouse walks along the beam with a few slips; 2 = the mouse cannot walk more than a few steps, and 3 = the mouse does not move); finally, the rotarod treadmill (UgoBasile, Comerio, Italy) test [10], i.e. the mice are placed on a rotating drum, set to accelerate uniformly from 4 to 40 rpm, and their latency to fall from it is recorded. Animals were trained 3 times over the 2 days before sur-

gery, with 2 trials for each session. The test was then performed at different time points after MCAO, and the better of the 2 consecutive trials was selected. Points were attributed on the basis of performance expressed as a percentage of the best performance before ischaemia (0 point: 90–100% and then 0.5 point for each 15% decrease) [6].

Exclusion Criteria

The surgeon applied strict exclusion criteria before statistical analysis: mice with temperature outside the 36.5–37.5°C limit, an rCBF above 20% of baseline during ischaemia or below 50%, 10 min after reperfusion. In intravenously injected mice, those mice for which the surgeon doubted the quality of injection were excluded. In total, 1 mouse out of 13 was excluded from the intracerebroventricular group and 24/48 from the intravenous group.

Brain Lactate Measurements

Mice underwent cerebral ischaemia followed by lactate or vehicle injection (intravenous or intracerebroventricular). Thirty minutes after injection, animals were sacrificed by intracardiac PBS perfusion for 2 min to remove blood. Brains were isolated and frozen in liquid nitrogen vapour.

Brain lactate was measured in each hemisphere by an enzymatic-spectrometric protocol in which lactate contained in the sample is incubated with lactate dehydrogenase (14 U/ml) and 3 mmol/l NAD in glycine-semicarbazide 0.2 mol/l buffer, pH 10. NADH resulting from the reaction is measured by its absorbance at 340 nm [11, 12].

Blood Gas Analysis

Mice were injected intravenously with either lactate or PBS. Arterial blood (125 μl) was withdrawn from the carotid artery 15–26 min after injection and collected in Clinitubes (Radiometer, Denmark). Samples were analysed for blood gases and lactate in a Radiometer ABL800 Flex machine.

Statistics

Parametric data were presented as mean ± standard deviation and scores as median, minimum, maximum. Statistical analyses were carried out using the non-parametric two-tailed Mann-Whitney test (Instat, Graphpad, La Jolla, Calif., USA). A probability of 0.05 or less was considered significant.

Results

Intracerebroventricular Injection of Lactate Attenuates Ischaemia-Induced Hemispheric Atrophy and Improves the Functional Outcome 14 Days after Transient MCAO in Mice

To extend our previous observation of neuroprotection with intracerebroventricular injection of lactate after mouse MCAO, we subjected mice to 15 min transient MCAO under xylazine + ketamine anaesthesia. In this model, the striatal and small cortical lesions seen at early time points were even smaller at 14 days in both groups. Therefore, lactate treatment did not appear to induce a significant reduction in lesion size at this late time point (5 ± 3 mm^3, n = 7 vs. 8 ± 3 mm^3 in vehicle treatment, n = 5, p = 0.17) (fig. 1a). Although the small size of lesions may have a confounding effect, reduced tissue damage in lactate-treated mice was demonstrated indirectly by attenuated hemispheric atrophy (1 ± 4 mm^3, n = 7, vs. 12 ± 4 mm^3, n = 5, p = 0.048) (fig. 1b). Very convincing results were obtained by looking at the neurological impairment detected by an increase in the composite behavioural outcome score, peaking at 2 days and with partial recovery at 7 and 14 days after MCAO. Lactate treatment induced a sustained neuroprotection attested by smaller neurological deficits at 2 days (median neuroscore = 1.5, min = 1, max = 3.5, n = 6 vs. median = 4.75, min = 1.5, max = 7.0, n = 6 in vehicle-treated animals, p = 0.036), 7 days (median = 0, min = 0, max = 3, n = 7 vs. median = 2, min = 1, max = 4.5, n = 5, p = 0.028) and 14 days after ischaemia (median = 0, min = 0, max = 3, n = 7 vs. median = 3, min = 0.5, max = 3, n = 7, p = 0.05) (fig. 1c). To evaluate delayed neuronal loss, we performed immunohistochemistry with an antibody against NeuN in brain slices from mice 14 days after 15 min MCAO and injected with either PBS (fig. 1d) or lactate (fig. 1e). The ischaemic lesion corresponds to a region of reduced intensity of NeuN staining compared to the contralateral hemisphere in both treatment groups, demonstrating neuronal loss at this delayed time point.

There was no difference in temperature, body weight and rCBF during surgery and reperfusion between the groups.

Lactate Injected Intravenously at Reperfusion Attenuates both the Lesion Volume and Neurological Deficit 48 h after Transient MCAO in Mice

Early treatment to halt the progression of ischaemic damage is very important in acute stroke, hence the importance of a practical and quick route of administration for a medication. We therefore tested intravenous injection of lactate into the tail vein at reperfusion. In healthy human brains, lactate injected intravenously (0.75–3 μmol/g) was shown to be metabolized. Lactate administered intravenously was also shown to protect the human brain against intracranial hypertensive episodes in severe traumatic brain injury [13–15]. We tested doses of 1 and 3 μmol/g in a preliminary experiment (injection of 3 μmol/g was toxic and led to the death of 2 mice tested). We compared the concentration of lactate in the target organ, the brain, with intracerebroventricular and intravenous (1 μmol/g) injections and showed that tail vein lactate injection after reperfusion was able to increase

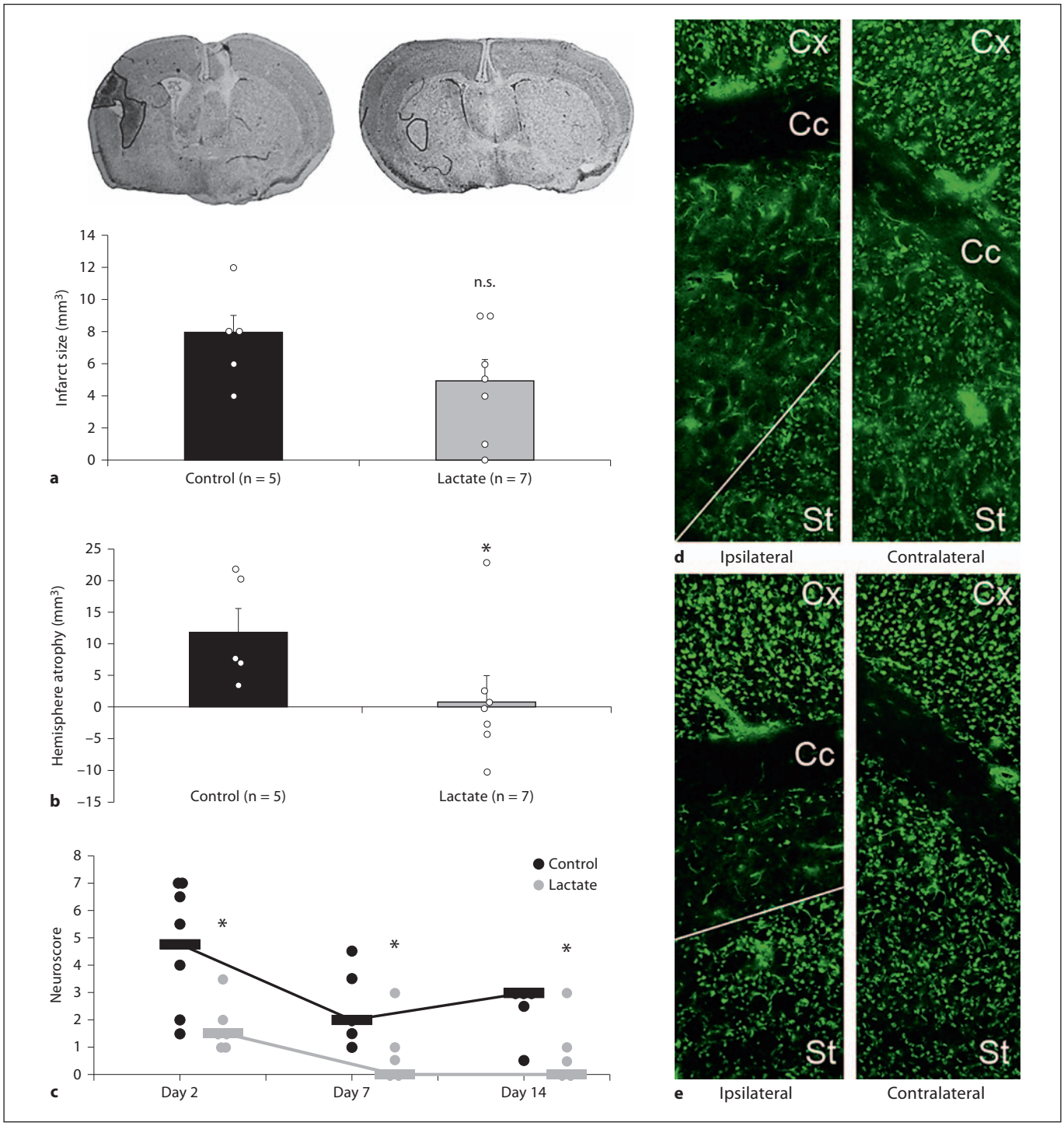

Fig. 1. Intracerebroventricular injection of 200 mmol/l lactate induces sustained neuroprotection after 15-min MCAO. **a** Infarct size measured at 14 days after MCAO represented as mean ± SD. **b** Atrophy of the injured hemisphere measured at 14 days after MCAO represented as mean ± SD. **c** Evolution of neurologic deficit scores (from 0 = no deficit, to 9 = most severe deficit) up to 14 days after MCAO, represented as medians. **d**, **e** Immunohistochemistry with an antibody against NeuN was performed on brain slices from mice 14 days after 15-min MCAO and injected with either PBS (**d**) or lactate (**e**). The ischaemic lesion, outlined in white, corresponds to a region of reduced intensity of NeuN staining compared to the contralateral hemisphere in both treatment groups. * $p \leq 0.05$ for the Mann-Whitney test, two-tailed p value. Circles represent individual animals. Cx = Cortex; Cc = corpus callosum; St = striatum.

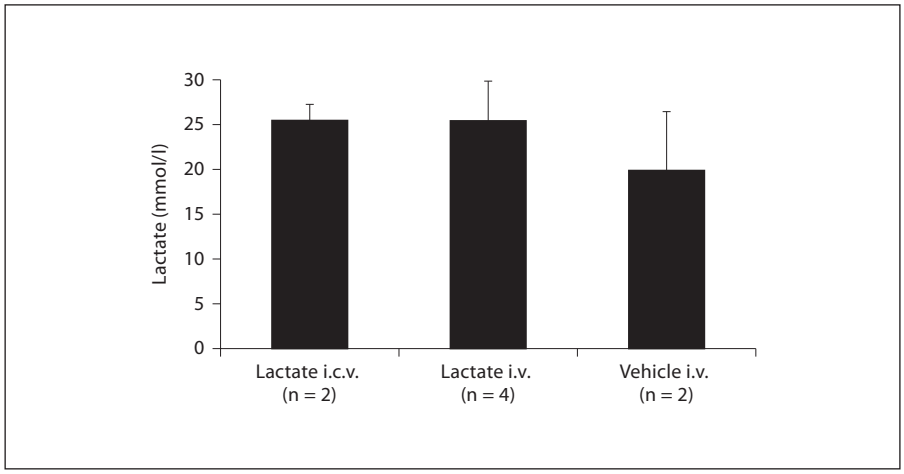

Fig. 2. Brain lactate concentration is similar after intravenous (i.v.) injection of 1 μmol/g lactate or intracerebroventricular (i.c.v.) injection of 2 μl of 100 mmol/l lactate. Lactate concentration was measured in the ischaemic hemisphere 30 min after lactate injection.

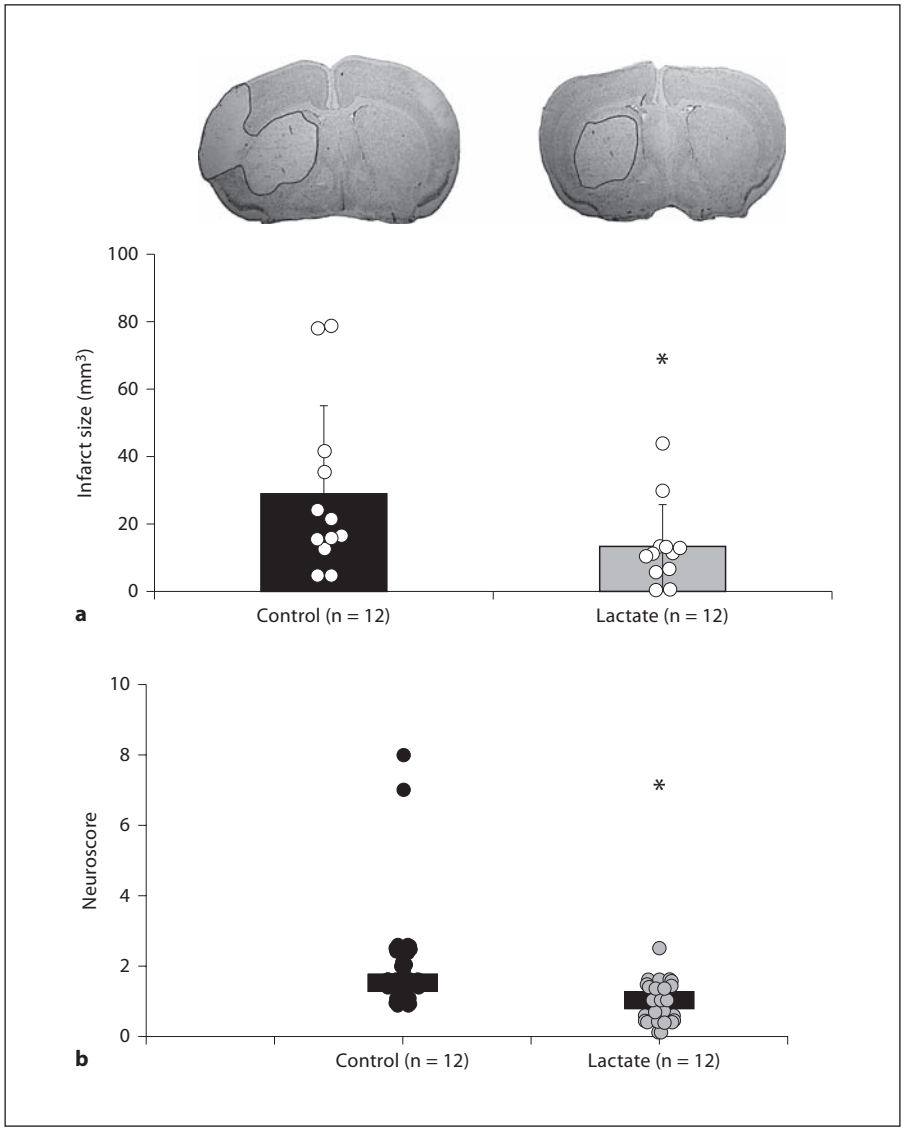

Fig. 3. Intravenous injection of 1 μmol/l lactate/g body weight at reperfusion after 15-min MCAO is neuroprotective. **a** Infarct size measured at 48 h after MCAO represented as mean ± SD. **b** Neurologic deficit scores (from 0 = no deficit, to 9 = most severe deficit) at 48 h after MCAO, represented as medians. * $p \leq 0.05$ for the Mann-Whitney test, two-tailed p value. Circles represent individual animals.

the brain lactate concentration to a comparable level (after 30 min) as an intracerebroventricular injection of 2 μl 100 mM lactate, recently shown to be neuroprotective [6] (fig. 2). Experiments were done with a dose of 5 μl of 200 mmol/l L-lactate solution per gram of body weight, corresponding to 1 μmol/g of body weight. Controls received an equal volume of vehicle solution. Lactate administered intravenously attenuated the lesion volume (13.7 ± 12.2 mm^3, n = 12 vs. 29.6 ± 25.4 mm^3, n = 12, p = 0.046) as well as, importantly, the neurological deficit (median = 1, min = 0, max = 2.5, n = 12) compared to vehicle treatment (median = 1.5, min = 1, max = 8, n = 12, p = 0.024) at 48 h (fig. 3). These results show that a very practical intravenous administration of lactate induces neuroprotection.

There was no difference in the temperature, body weight and rCBF during surgery and reperfusion between the groups. Similarly, the pH, pCO_2, pO_2 and lactate measured in the arterial blood after intravenous injection were not different between the groups (data not shown).

Discussion

In this paper, we show that the neuroprotective effect of lactate, now tested in a 15-min MCAO under xylazine-ketamine anaesthesia, extends beyond 48 h, with improvement in the behavioural outcome and attenuated tissue destruction with significantly less hemisphere atrophy at 14 days. It is important to determine if neuroprotection is long-lasting as long-term neurological outcome is clinically more relevant than short-term outcome, attested by the NINDS recombinant tissue plasminogen activator study [16] and the more recent ECASS 3 [17] trial, both of which used the behavioural outcome at 3 months as a primary outcome measure.

Cerebral ischaemia causes rapidly progressing and devastating tissue damage in the ischaemia territory. An early intervention is of critical importance to impede the lesion progression. This is why we needed to establish a clinically more feasible route of administration than injection into the brain. We chose intravenous administration as it is reliable and practical in the case of stroke patients. The results presented here support the ability of intravenously injected lactate to cross the blood-brain barrier [13, 14] and efficiently protect the brain as was also seen in traumatic brain injury patients, a very different injury model [15]. However, its mode of action remains unclear. Indeed, in the case of ischaemia, lactate is known to increase in the brain in two waves: a first increase during ischaemia due to anaerobic glycolysis and a second increase beginning 1 h after reperfusion and continuing up to 72 h [18–20]. Lactate levels in the brain are thus very high during ischaemia and after reperfusion and consequently the addition of a small amount of lactate may appear pointless. However, a careful look at the lactate kinetics shows that between 15 min and 1 h after reperfusion, lactate concentrations are back to normal and it is exactly at this time point that we inject lactate. At reperfusion, there is a huge energy demand, which cannot be met by glucose as ATP stores are depleted and glucose cannot be converted to glucose-6P for glycolysis. In these conditions, lactate accumulated during ischaemia by anaerobic glycolysis represents an ideal source of energy, which can explain the rapid decrease of the accumulated lactate and return to baseline in the first hour of reperfusion and the beneficial effect of additional lactate supply at this time point.

The second increase in lactate is not due to anaerobic glycolysis as it occurs after reperfusion with restored oxygen supply and may be explained by the astrocyte-neuron shuttle model [1]. Astrocytes are more resistant to ischaemia than neurons. When neurons are dying, the lactate produced by surviving astrocytes cannot be consumed by neurons, hence an increase in lactate. Lactate production could also reflect an attempt of the ischaemic brain to rescue its suffering neurons. In less severe ischaemia, the second lactate increase occurs later, coinciding with a more delayed neuronal demise [21]. Similarly, locating where the lactate increase occurs in the brain using spatial mapping by magnetic resonance spectroscopy 24 h after ischaemia shows a gradient with highest lactate concentrations in the centre of the ischaemic lesion correlating with the severity of tissue damage [19].

Another mode of action of lactate shown in traumatic brain injury is an attenuation of cellular swelling [15]. The proposed explanation for this observation is that the injected lactate solution contains both metabolizable (lactate) and non-metabolizable (Na) ions. The use of lactate by brain cells induces an imbalance between positive and negative charges which needs to be compensated by an efflux of anions from cells, accompanied by water, thus counteracting the detrimental cellular swelling [15].

Recent exciting results on the role of oligodendrocytes in enhancing axon function and neuronal survival in models of amyotrophic lateral sclerosis also point towards a role for lactate. There is a metabolic interaction

between oligodendroglia and neurons with lactate transport from oligodendroglia to neurons [22].

In conclusion, although some aspects of its role as a neuroprotectant remain intriguing, lactate is an exciting candidate for neuroprotection in ischaemic stroke. The results presented here bring this natural and inexpensive agent closer to a translation to stroke patients.

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Disclosure Statement

The authors report no conflict of interest with this work.

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ORIGINAL ARTICLE

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

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Lactate has been shown to offer neuroprotection in several pathologic 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 24 hours after reperfusion in an middle cerebral artery occlusion 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* whereas 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.

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Keywords: energy metabolism; focal ischemia; lactate; neuron-glia interactions; neuroprotection

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 because of lack of oxygen and glucose supply to the brain is the central mechanism leading to neural damage. Brain metabolism therefore has a critical role in the pathophysiologic 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. Monocarboxylate transporters are a family of proton-dependent carriers where MCT1, MCT2, and MCT4 are the main MCTs in the central nervous system (CNS). Under physiologic 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 neurologic 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 neurologic outcome.[11,12] Although 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

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neuroprotective effect is essential 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). The animal studies are reported according to the ARRIVE guidelines.

Transient Middle Cerebral Artery Occlusion in the Mouse

A total of 55 male CD1 mice (body weight 26 to 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 1 week for acclimatization before surgery. For experiments, mice were maintained anesthetized with isoflurane (1.5% to 2% in nitrous oxide/oxygen 70%/30%) using a face mask. Body temperature was maintained at 37.0 ± 0.5 °C throughout surgery (FHC, Bowdoinham, ME, USA). Regional cerebral blood flow (rCBF) was measured and continuously recorded throughout the operation in all animals by laser-Doppler flowmetry (Perimed AB, Stockholm, Sweden) with a flexible probe fixed on the skull (1 mm posterior and 6 mm lateral from bregma). Transient focal cerebral ischemia (30 or 45 minutes depending on the set of experiments) was induced by occlusion of the left middle cerebral artery (MCA) with an intraarterial suture as described elsewhere.[11] Briefly, the left common carotid artery and the left external carotid artery were exposed and ligated after 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. Regional cerebral blood flow 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 5 μL per gram of body weight of either sodium D-lactate solution (200 mmol/L), pyruvate solution (200 mmol/L), or vehicle solution (phosphate-buffered saline, 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, Braintree, MA, USA). All surgeries were performed during day-light, between 9 am and 5 pm. The surgeon was not masked. 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 rpm, and their latency to fall was recorded before 600 seconds. The animals were trained on two different days before surgery, with two trials in each training session. The test was then performed 24 and 48 hours 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 neurologic deficit). The behavior evaluation was assessed in a masked manner.

Determination of Ischemic Lesion Volumes

Animals were killed 48 hours 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, Heerbrugg, Switzerland) and the lesion area was measured using ImageJ software (ImageJ 1.36b, National Institute of Health). 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 (10 mL/kg, Sigma, Buchs, Switzerland) and then perfused with 150 mL of 4% paraformaldehyde (Sigma-Aldrich, St Louis, MO, USA) dissolved in $1 \times$PBS at pH 7.4. Brains were dissected, postfixed overnight at 4 °C, cryoprotected 24 hours in 30% sucrose solution (Sigma-Aldrich), and rapidly frozen. Twenty micrometer thick coronal microtome-cryostat (Leica MC 3050S) sections were stored in cryoprotectant (30% ethylene glycol and 25% glycerin in $1 \times$PBS) at − 20 °C. For immunostaining, sections were washed three times in $1 \times$PBS and blocking of non-specific binding was achieved by incubating in $1 \times$PBS containing 1% bovine serum albumin, 0.1% Triton X-100, and 10% normal goat serum during 1 hour. Double labeling was performed overnight at 4 °C in the $1 \times$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 $1 \times$PBS, brain sections were incubated for 2 hours at room temperature with the following fluorescent secondary antibodies: donkey anti-rabbit Alexa-594 (1:250, Invitrogen, Eugene, OR, USA), donkey anti-mouse Alexa-488 (1:250, Invitrogen). After immunostaining, brain sections were incubated for 10 minutes with 4,6 diamidino-2-phenylindole (Sigma) dissolved in $1 \times$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 killed either at 1, 3, 8, 24, or 48 hours 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 Warren, 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 approximately 20 μg of proteins were denatured (95 °C) for 5 minutes in sodium dodecyl sufate-polyacrylamide gel electrophoresis sample buffer (60 mmol/L Tris-HCl pH 6.8, 5% SDS, 6.6% glycerol, 5 mmol/L 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) using the Electrophoresis Unit. Nonspecific binding sites were blocked for 1 hour at room temperature with a solution of Tris-Buffered-Saline (50 mmol/L Tris-HCl pH 7.5, 150 mmol/L 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 Tris-Buffered-Saline 0.1% containing 1% skimmed milk: rabbit anti-mouse GPR81-S296 (HCA1) (1:500 dilution; #SAB1300790, Sigma). Blots were washed three times in Tris-Buffered-Saline 0.1% and subsequently incubated 2 hours 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 Tris-Buffered-Saline 0.1%, blots were processed using Immun-StarTMWesternCTM Chemiluminescent Kit (#170-5070, Bio-Rad). Chemiluminescence detection was performed with the ChemiDoc XRS System (#170-8070, Bio-Rad). Total protein content assay was performed with the Pierce Reversible Protein Stain kit (#24580, Thermo Fisher Scientific, Pierce) and revealed with the ChemiDoc XRS System (#170-8070, Bio-Rad). Both types of labeling were quantified with the ImageLab 3.0 software (Bio-Rad) 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[11] from P8 to P10 rats (OFA Sprague Dawley, Janvier, France). Coronal hippocampal

sections (350 μm) were cultured on sterile porous membrane units (Millicell, Millipore, Billerica, MA, USA) in wells containing 1 mL of culture medium with D-glucose 36 mmol/L, 25% horse serum, 50% minimal essential medium (supplemented with HEPES and sodium bicarbonate; Gibco, Paisley, UK), 25% HBSS (Hank's balanced salt solution; Gibco), and L-glutamine 2 mmol/L (Sigma-Aldrich). 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% minimal essential medium, 25% HBSS, and L-glutamine 2 mmol/L 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, Dulbecco's modified Eagle's medium (D5030, Sigma-Aldrich) supplemented with D-glucose 1 mmol/L and L-glutamine 2 mmol/L and equilibrated for 1 hour at 37 °C, in a humidified hypoxic chamber (COY, Grass Lake, 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 hour. Control cultures were kept in 60% minimal essential medium, 15% horse serum, 25% HBSS, and L-glutamine 2 mmol/L for 1 hour 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 hours. Cultures were randomly treated with either D-lactate, pyruvate, acetate (Sigma-Aldrich; diluted in 1 × PBS, pH 7), 3,5-dihydroxybenzoic acid (Sigma-Aldrich; diluted in ethanol) or an equal volume of culture medium. Sodium D-lactate at a final concentration of 4 mmol/L, pyruvate at 10 mmol/L, sodium acetate at 0.13, 0.2, 4, or 8 mmol/L, or 3,5-dihydroxybenzoic acid at 4 mmol/L 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. Propidium iodide was applied in each dish (50 μg/mL) 1 hour before measurement. Propidium iodide fluorescence emission (excitation wavelength 536 nm, emission wavelength 617 nm) was measured 48 hours after hypoxia using an epifluorescence microscope with a × 5 lens coupled to a camera (Leica). Propidium iodide images were acquired with standardized camera settings and signal intensity was measured with ImageJ software. 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 hours at 4 °C. Cell death was averaged for the four slices of each culture well. The experimenter was not masked.

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 high-performance liquid chromatography (HPLC). Before 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 more than 99% enantiomeric excess of the final product. The specific activity at the end of the synthesis was approximately 400 GBq/μmol. Both preparative and analytical chiral ligand exchange chromatography were performed on reversed-phase C_{18} columns (octadecyl-silica), 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% to 3% in air/oxygen 70%/30%) and involved the placement of an arteriovenous 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 subcutaneously). The arteriovenous shunt was run through a coincidence scintillator (GE Medical Systems, Waukesha, WI, USA). The online arterial sampling procedure is described in detail elsewhere.[15] In short, total radioactivity in arterial blood was continuously recorded and was then corrected for (1) a different tracer concentration in whole blood and plasma and (2) 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.[16,17] Twenty minutes of data were acquired at baseline condition after injection of 200 to 300 MBq of radiotracer in 5 animals. In one of these animals, baseline cerebral blood flow was determined before 1-^{11}C-D-lactate acquisition using ^{15}O-H_2O and the methodology described previously.[16] 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 4A).

Kinetic modeling. Acquired radioactivity data were 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:

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

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.[16]

Metabolite analysis in the blood. Samples (approximately 400 μL) were collected at different time points after tracer injection, with a maximum of 4 to 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 270 g. Proteins were then precipitated with 75 μL acetonitrile in 50 μL plasma. After centrifugation for 3 minutes at 270 g, the composition of the ^{11}C-derived radioactivity in the supernatant (80 μL) was analyzed by HPLC on a polymeric column (PRP-1, 5-µm, 250 × 4.1 mmol/L intradermally, Hamilton, Franklin, MA, USA) with 3 mmol/L phosphoric acid in water (pH 2.67) as the mobile phase (1 mL/minute). The retention times of ^{11}C-HCO_3^- (3.3 minutes) and lactic acid (5.1 minutes) were determined by using aqueous solution of $NaHCO_3$ and DL-lactic acid as reference compounds, detected by ultraviolet 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., 1.5 × 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 analysis of variance (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 CNS.[8–10] Because 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

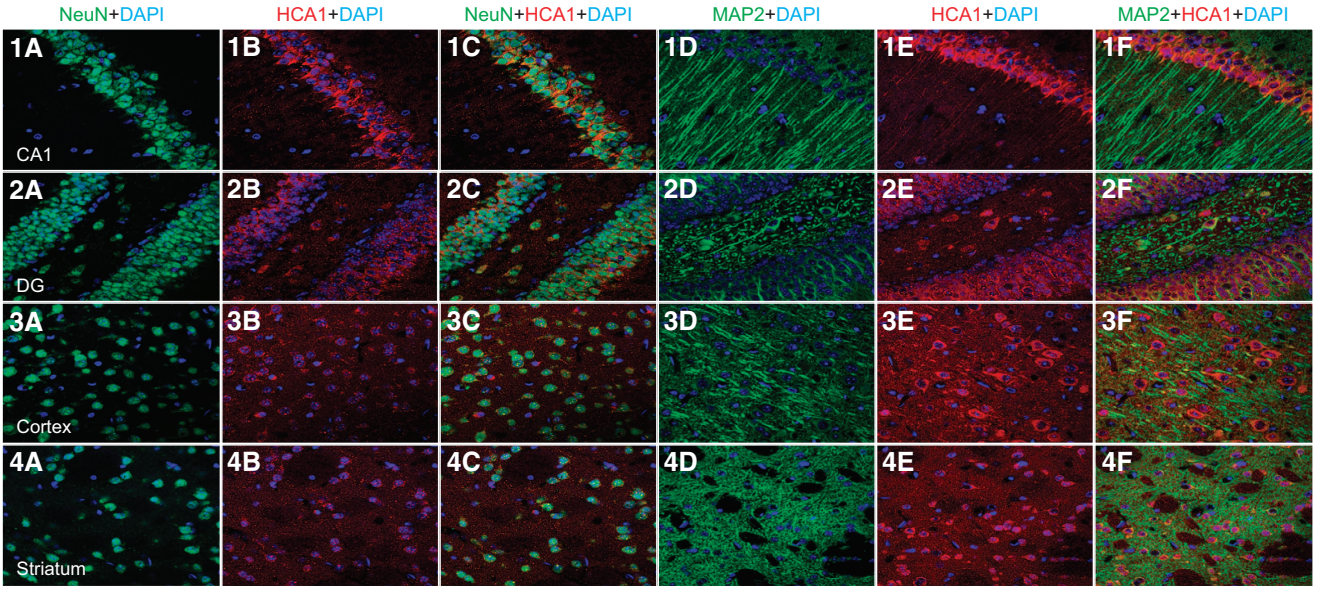

Figure 1. Confocal visualization of HCA1 receptor expression. Using two different antibodies against neuronal cells, we confirmed with immunohistochemistry the localization of the HCA1 receptor is in neurons. The brain structures analyzed were hippocampus (CA1 (1a–1f) and Dentate Gyrus (DG) (2a–2f) regions) primary cortex (3a–3f) and Striatum (4a–4f). Color code: NeuN (green, a and c), MAP2 (green, d and f), HCA1 (red, b and c and e and f), 4,6 diamidino-2-phenylindole (blue). Magnification: 40 μm.

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), 4,6 diamidino-2-phenylindole (blue)). The next step was to evaluate whether such HCA1 receptor expression was altered at different time points after 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 after 30 minutes of tMCAO. Results at 1, 3, and 8 hours did not reveal significant differences between the three brain structures investigated (see Supplementary 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 may reflect a compensatory increase in HCA1 receptor expression in remaining neurons (Figure 2A). At 48 hours after ischemia, no difference could be observed between ischemic and contralateral tissues (see Supplementary Table 1). On the basis of 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 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). To determine whether the enhanced HCA1 receptor expression could have 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 after OGD. Results show that 3,5-dihydroxybenzoic acid produced significant protection as it reduced neuronal cell death in the CA1 region of the hippocampus 48 hours after insult from $13.5 \pm 7.2\%$ to $7.4 \pm 5.2\%$ ($P < 0.05$; 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.[9,18] For this reason, we tested the neuroprotective effect of D-lactate both *in vitro* and *in vivo*. The administration of 4 mmol/L 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 $29.2 \pm 14.3\%$ to $11.3 \pm 7.3\%$ ($P < 0.05$; 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 to 15 minutes after the silicon-coated monofilament was removed allowing reperfusion. A total of nine 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 with 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 to 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

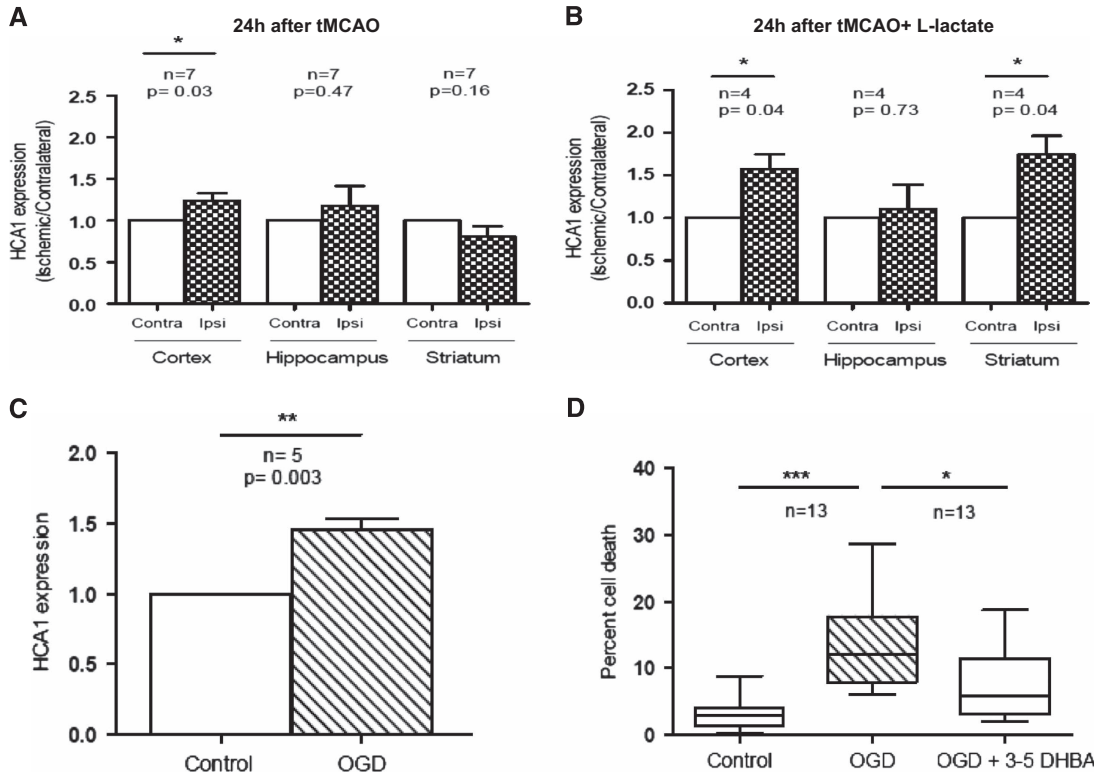

Figure 2. The HCA1 receptor expression is promoted by ischemia and has a role in the neuroprotective effect. (**A**) Western blot analysis showed an increase in the HCA1 receptor expression at 24 hours after transient middle cerebral artery occlusion (tMCAO) in the region surrounding the lesion site (primary motor and somatosensory cortex), in comparison with 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 hours after oxygen and glucose deprivation (OGD) (**$P < 0.01$ paired *t*-test). (**D**) Administration of 4 mmol/L 3,5-dihydroxybenzoic acid (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 analysis of variance (ANOVA) Kruskal–Wallis test followed by Dunn's multiple comparison test).

mice per group were killed within the first 6 hours after surgery because of 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[6,19,20] based on the apparent lack of expression of the specific enzyme D-lactate dehydrogenase in mammals. 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 of more than 5 minutes and slow washout thereafter on tracer injection (Figure 4B). The average respective first-order kinetic rate constants obtained by kinetic modeling were 0.059 ± 0.01 mL/minute/mL tissue (K_1) and 0.063 ± 0.01 per minute (k_2). Average lactate levels in the blood were 2.2 ± 0.93 mmol/L.

$^{11}C\text{-}CO_2$ 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 were approximated by a fit of a quadratic polynomial. This function was subsequently used 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 approximately 30%. Measurements in brain tissue at the end of the experiment revealed approximately 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, the use of D-lactate as an energy substrate may 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.[2,21] In agreement with these observations, administration of 10 mmol/L pyruvate directly after OGD significantly decreased cell death in the hippocampal CA1 region at 48 hours from $25.4 \pm 14.5\%$ to $13.9 \pm 10.3\%$ ($P < 0.05$; Figures 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 four mice were not included because they did not fulfill rCBF inclusion criteria. There was no mortality in this experiment. Because of 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

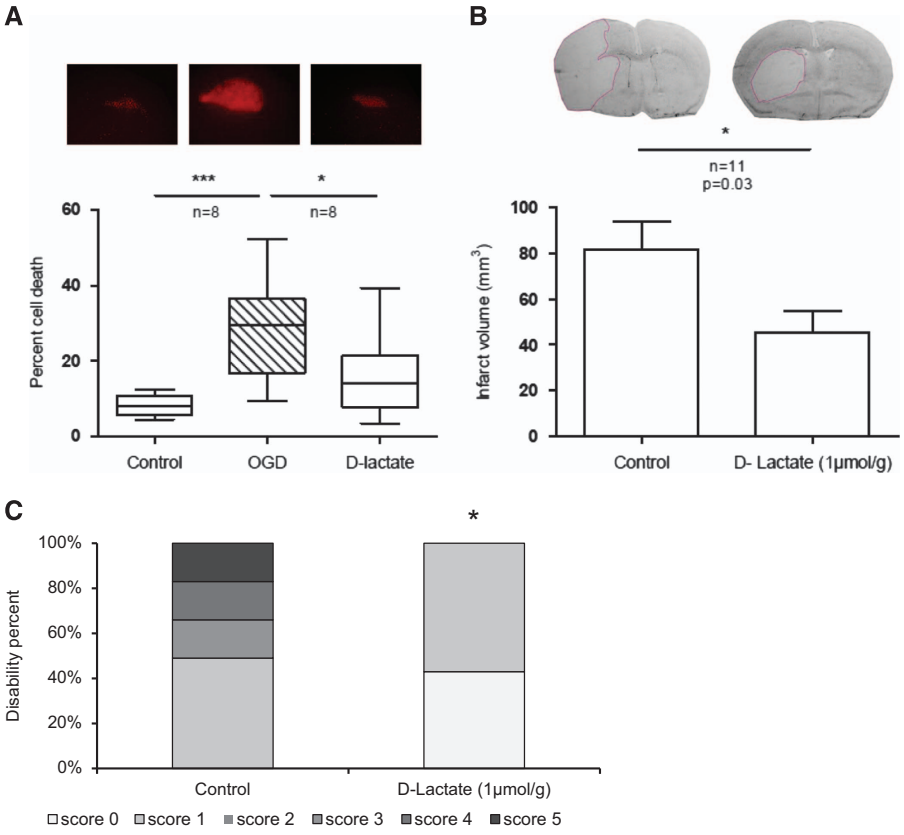

Figure 3. Neuroprotection by D-lactate. (**A**) *In vitro,* administration of 4 mmol/L D-lactate induced a significant reduction in cell death, assessed by propidium iodide (PI) staining, 48 hours after treatment (*$P < 0.05$, one-way analysis of variance (ANOVA) Kruskal–Wallis followed by Dunn's multiple comparison test). *In vivo,* intravenous injection of 1 µmol/g D-lactate after 45 minutes transient middle cerebral artery occlusion (tMCAO) decreased total infarct volumes measured 48 hours after ischemia (**B**) and improved the neurologic 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.

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.[22] However, the addition of different concentrations of acetate (0.13, 0.2, 4, and 8 mmol/L) to organotypic hippocampal slices 1 hour after OGD did not provide significant neuroprotection (Figures 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[1,23] and is nowadays recognized as a prominent energy substrate in the CNS.[24] Lactate is produced under physiologic conditions,[25,26] and it is suggested to have an important role in the metabolic support of long axons[6,27] as well as in long-term memory formation.[28] Lack of adequate lactate supply has also been implicated in some neurodegenerative diseases such as amyotrophic lateral sclerosis.[29] Moreover, exogenous lactate supplementation seems beneficial after traumatic brain injury.[30–32] In ischemic conditions, our group and others have shown neuroprotection after L-lactate administration at reperfusion after tMCAO.[11,12,33] Similarly, brain lactate was found to be essential to improve post-ischemic outcome after cardiac arrest.[34] Lactate can be transported from one cell type to another by MCTs that are selectively expressed in different cell types and which can transport monocarboxylates with different affinities.[35] More recently, lactate has been described to act as a signaling molecule in the CNS, either through the HCA1 receptor[8,9] or through an unknown receptor yet to be specified.[36]

Our initial characterization of the distribution of the HCA1 receptor in three brain regions affected after tMCAO confirmed a prominent neuronal expression in the CNS.[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 after ischemia is responsible for the enhancement of the HCA1 receptor observed after 24 hours of reperfusion remains to be directly shown. 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[18,37] and that D-lactate was considered as a non-metabolizable lactate enantiomer and often used as a negative control to show 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 ($42 \pm 8\%$ CO_2 after 40 minutes[1]). This observation suggests that the rodent brain may 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

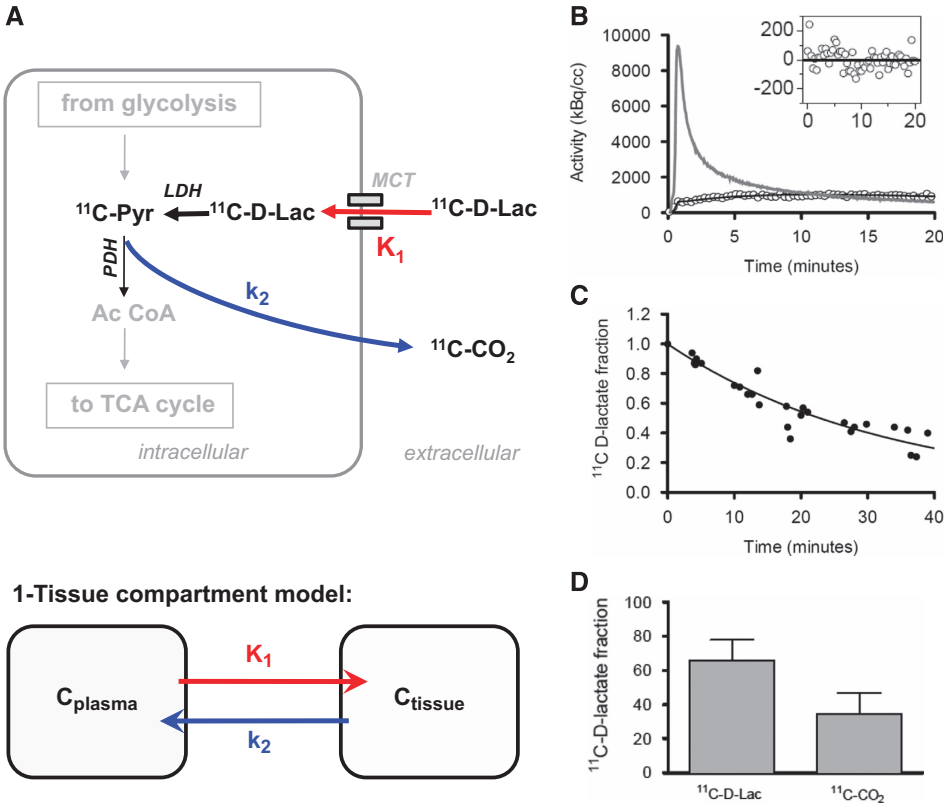

Figure 4. D-lactate metabolism. (**A**) Schematic of biochemical pathways involved in the degradation of lactate and the proposed interpretation of the rate constants K_1 and k_2 which are mathematically defined by the one-tissue compartment model used for 1-^{11}C-D-lactate data analysis. (**B**) Measured 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 more than 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.

tissues, including the brain.[38] However, it remains to be determined whether the presence of a cerebral D-Lactate dehydrogenase enzyme is sufficient to explain our results or whether the activity is performed by another *a priori* unrelated enzyme.

In the CNS, 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.[4,39] 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 to fuel the Krebs cycle and therefore used as energetic substrate and that this conversion produces reduced nicotinamide adenine dinucleotide that will in turn act as a reactive oxygen species scavenger.[40] Evidence pointing towards this metabolic explanation is the protection exerted by the administration of 10 mmol/L pyruvate *in vitro*. Protection is not seen *in vivo* though. However, in support of a metabolism-mediated neuroprotective effect, others[2,21] have shown protection *in vivo* using an extended intravenous mode of administration of pyruvate.[21] This difference in the mode of administration may explain the different *in vivo* results, although we cannot exclude that lactate may prove to be a more efficient neuroprotective agent, notably *in vivo*, in particular because of 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.[17,22] 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*,[41] relates to the depression of glial metabolism. Even though the reduction in ^{14}C-acetate uptake was reversible and reached full recovery 3 hours after 30 minutes MCAO, 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*[18] described that neither sodium pyruvate nor sodium acetate showed binding activity. As neither acetate nor pyruvate binds 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 (1) 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. (2) Ischemia is induced under isoflurane anesthesia and this compound has been described as

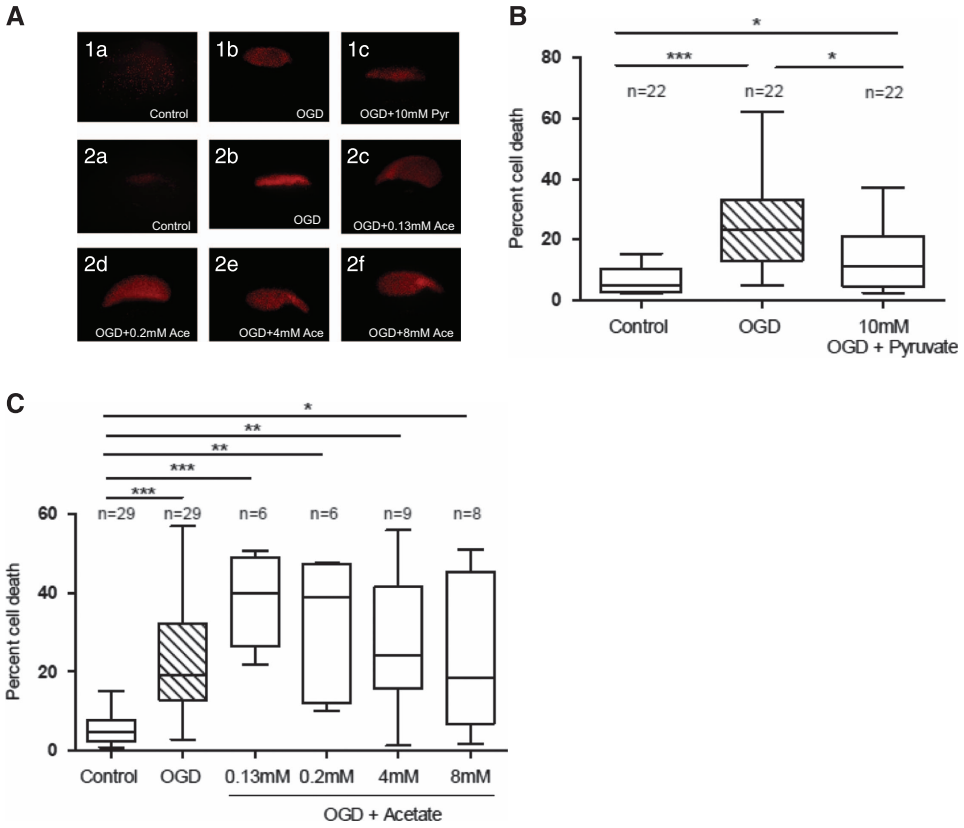

Figure 5. Administration of pyruvate and acetate after OGD. (**A**) Administration of 10 mmol/L pyruvate directly to the medium after 1 hour oxygen-glucose deprivation (OGD) in rat organotypic hippocampal slices was able to protect against ischemic damage. (**B**) Administration of various doses of acetate did not protect against ischemic damage. Cell death was assessed by propidium iodide (PI) staining 48 hours after treatment. (**C**) Representative images of PI staining among each group can be observed in panel (*$P < 0.05$; one-way analysis of variance (ANOVA) Kruskal–Wallis test followed by Dunn's multiple comparison test).

neuroprotective by itself. Therefore, we may not be observing the neurologic damage to its fullness. In 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. (3) It should be considered as well that lactate was shown to increase regional CBF in physiologically activated but not resting human brain.[42] 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 after ischemia by two distinct mechanisms: One, through the classic 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.

AUTHOR CONTRIBUTIONS

XC, KR, MW, KD, and AB designed and performed experiments; LP, BW, and LH designed experiments. XC, MW, LP, and LH wrote the paper.

DISCLOSURE/CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Supplementary Information accompanies the paper on the Journal of Cerebral Blood Flow & Metabolism website (http://www.nature.com/jcbfm)