Journal of Neuroscience Methods 204 (2012) 366–373

Contents lists available at SciVerse ScienceDirect

Journal of Neuroscience Methods

journal homepage: www.elsevier.com/locate/jneumeth

Basic Neuroscience

Impaired hippocampal glucoregulation in the cannabinoid CB_1 receptor knockout mice as revealed by an optimized *in vitro* experimental approach

Cristina Lemos[a,1], Ângela Valério-Fernandes[a,1], Gabriele C. Ghisleni[a,1,2], Samira G. Ferreira[a], Catherine Ledent[b], María L. de Ceballos[c,d], Attila Köfalvi[a,*]

[a] *Center for Neuroscience and Cell Biology of Coimbra, Faculty of Medicine, University of Coimbra, 3004-517 Coimbra, Portugal*
[b] *IRIBHN, Université libre de Bruxelles, Brussels B-1070, Belgium*
[c] *Neurodenegeration Group, Department of Cellular, Molecular and Developmental Neurobiology, Instituto Cajal, CSIC, Doctor Arce, 37, 28002 Madrid, Spain*
[d] *Centre for Biomedical Research on Neurodegenerative Diseases (CIBERNED), Spain*

ARTICLE INFO

Article history:
Received 17 September 2011
Received in revised form
28 November 2011
Accepted 28 November 2011

Keywords:
Hippocampus
Cannabinoid
Glucose
Uptake
Metabolism
2-Deoxyglucose
3-O-Methylglucose

ABSTRACT

Several techniques exist to study the rate of glucose uptake and metabolism in the brain but most of them are not sufficiently robust to permit extensive pharmacological analysis. Here we optimized an *in vitro* measurement of the simultaneous accumulation of the metabolizable and non-metabolizable ^{3}H and ^{14}C D-glucose analogues; permitting convenient large-scale studies on glucose uptake and metabolism in brain slices. Next, we performed an extensive pharmacological characterization on the putative glucoregulator role of the endocannabinoid system in the hippocampal slices of the rat, and the wild-type and the CB_1 cannabinoid receptor (CB_1R) knockout mice.

We observed that ^{3}H-3-O-methylglucose is a poor substrate to measure glucose uptake in the hippocampus. ^{3}H-2-deoxyglucose is a better substrate but its uptake is still lower than that of ^{14}C-U-D-glucose, from which the slices constantly metabolize and dissipate ^{14}C atoms. Thus, uptake and the metabolism values are not to be used as standalones but as differences between a control and a treatment.

The CB_1R knockout mice exhibited ~10% less glucose uptake and glucose carbon atom dissipation in comparison with the wild-type mice. This may represent congenital defects as acute treatments of the rat and mouse slices with cannabinoid agonists, antagonists and inhibitors of endocannabinoid uptake/metabolism failed to induce robust changes in either the uptake or the metabolism of glucose.

In summary, we report here an optimized technique ideal to complement other metabolic approaches of high spatiotemporal resolution. This technique allowed us concluding that CB_1Rs are at least indirectly involved in hippocampal glucoregulation.

1. Introduction

Systemic deregulation of glucose metabolism harms the brain (Bauduceau et al., 2010), while brain disorders involve deregulation of local glucose metabolism (Stone and Seidman, 2008; Mosconi and McHugh, 2011). To achieve new modes of detection and treatment of brain disorders, there is still much to understand about the modes the brain utilizes glucose.

Several techniques have been established to assess glucose uptake and/or metabolism in the brain. *In vivo*, local rates of glucose uptake can be measured by positron emission tomography with ^{11}C-3-O-methylglucose (Bingham et al., 2005) or with ^{18}F-2-fluoro-2-deoxyglucose (Herholz, 2010); or can be *ex vivo* analyzed by autoradiography after the *in vivo* injection of radioactive 2-deoxyglucose (Freedland et al., 2002). *In vitro*, one can measure the uptake of the fluorescent glucose analogues, for instance, of 6-NBDG (Barros et al., 2009), or of the radioactive (^{14}C or ^{3}H) glucose or its analogues, 3-O-methylglucose or deoxyglucose in cell culture (Mannerström and Tähti, 2004; Uemura and Greenlee, 2006). However, non-metabolizable glucose probes should be used with care as their affinity to the glucose transporters and hexokinases is different from that of glucose itself (Dick et al., 1984; Carruthers,

Abbreviations: 2/6-NBDG, 2/6-(N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)-6-deoxyglucose; 2-AG, 2-arachidonoyl glycerol; ABHD6, α/β-hydrolase domain 6; BCA, bicinchoninic acid assay; CB_1R and CB_2R, cannabinoid type-1 and type-2 receptors; dpm, disintegration per minute; FAAH, fatty acid aminohydrolase; GPR18 and GPR55, G protein-coupled orphan receptors 18 and 55; KO, knockout; MAGL, monoacylglycerol lipase; $TRPV_1$R, transient receptor potential vanilloid type-1 receptor; WT, wild-type.

* Corresponding author. Tel.: +351 96 8802309; fax: +351 239 822776.
E-mail address: akofalvi@yahoo.com (A. Köfalvi).
[1] Equivalent first authors.
[2] Current address: Universidade Católica de Pelotas, Centro de Ciências da Vida e da Saúde, Rua Almirante Barroso 1202 sala G109, Centro, Pelotas, RS CEP 96010-280, Brazil.

0165-0270/$ – see front matter © 2011 Elsevier B.V. All rights reserved.
doi:10.1016/j.jneumeth.2011.11.028

1990; Barros et al., 2007, 2009). Hexokinases – the rate-limiting enzymes of glycolysis – may differently handle deoxyglucose and its derivatives than glucose itself, and deoxyglucose may even be toxic (Bachelard et al., 1971; Kurtoglu et al., 2007). Furthermore, the *in vivo* approaches are expensive, slow, and provide minimal spatial, cellular and temporal resolution. In contrast, cell culture experiments have fewer of these limitations but omit the important neuron-glia coupling (Magistretti, 2006), rendering the system artificial.

Among the remaining approaches, the elegant FRET glucose nanosensor technique reported by Bittner et al. (2010) gives second and micrometer scale resolutions but does not serve information about the rate of glucose uptake. ^{13}C NMR spectroscopy provides cellular resolution of ^{13}C-U-glucose metabolism in the brain. It can trace simultaneously the fate of the ^{13}C label through glycolysis and the TCA cycle; and can distinguish the neuronal and glial compartments (García-Espinosa et al., 2004), even *in vivo* (Duarte et al., 2011b). Yet, NMR spectroscopy lacks information on the uptake and temporal resolution (Badar-Goffer et al., 1990).

Here we optimized an *in vitro* approach offering rapid mapping of glucose uptake and metabolism in brain slices and cell cultures. We took advantage of the dual ^{3}H and ^{14}C β scintillation protocols allowing measuring the simultaneous accumulation of ^{3}H and ^{14}C labels derived from ^{3}H-2-deoxyglucose or ^{3}H-3-O-methylglucose and ^{14}C-U-glucose (James et al., 1985; Nakai et al., 1988; Ito et al., 1990).

The endocannabinoid system is an important systemic/peripheral glucoregulator (Pagano et al., 2007; Matias et al., 2008; Quarta et al., 2010; Song et al., 2011). In the brain, we observed that the hippocampal CB_1 cannabinoid receptor (CB_1Rs) density is increased in the streptozotocin model of type-1 diabetes (Duarte et al., 2007), which suggests its involvement in diabetic encephalopathy and glucoregulation. Thus, here we aimed at optimizing the glucose uptake and metabolism assay and at running an extensive pharmacological analysis of the association of the endocannabinoid system with local hippocampal glucoregulation.

2. Materials and methods

2.1. Animals

All studies were conducted in accordance with the principles and procedures outlined in the EU guidelines (86/609/EEC) and by FELASA, and in accordance with the recommendations of the NC3Rs Reporting Guidelines Working Group (2010), and were approved by the local Animal Care Committee of the institute. Animals were housed in an SPF facility, with 12 h light on/off cycles and *ad libitum* access to food and water. Male Wistar rats (180–200 g, 8- to 10-week old) were purchased from Charles-River (Barcelona, Spain). CB_1R null-mutant (knockout) male mice (Ledent et al., 1999) and their wild-type littermates on CD-1 background were genotyped from the tail, housed as detailed above and sacrificed daily in pair (one WT and one KO), until 16 weeks of age. CB_1R knockout mice (39.5 ± 0.82 g) were 11% lighter than their wild-type littermates (44.3 ± 0.77 g; $n = 47$, $p < 0.01$) at the moment of euthanasia, indicating the anabolic role of CB_1Rs in systemic metabolism.

2.2. Brain slice experiments

Animals were anesthetized with halothane before decapitation (around 14:00 o'clock each experimental day to reduce putative circadian hormonal effects). Brains were immediately collected in ice-cold assay solution (see below). After cooling down, hippocampi and neocortices were removed on ice within 4 min after decapitation, and sliced into 450 μm-thick transversal slices with the help of a McIlwain tissue chopper. We previously characterized with 50 μm steps from 300 μm to 1 mm thickness that 450 μm is optimal for oxygenation, drug penetration in the centre of the slice vs. physical resistance (data not shown). Slices were gently separated in ice-cold carboxigenated (95% O_2 and 5% CO_2) assay solution of the following composition (in mM): NaCl 113, KCl 3, KH_2PO_4 1.2, $MgSO_4$ 1.2, $CaCl_2$ 2.5, $NaHCO_3$ 25, glucose 5.5, HEPES 10. Slices were then transferred into a multichamber slice incubator (40 ml of assay solution at 37 °C), and carboxigenated (5% CO_2 and 95% O_2) until the end of the experiment.

2.3. Metabolic recovery experiments

After preparation, the rat hippocampal slices were subjected to pre-incubation for 0, 15, 30, 45, 60, 75, 90 and 105 min at 37 °C, respectively. Once this pre-incubation period has been over we bath applied the following two radioactive glucose analogues combined for 15 min: ^{14}C-U-D-glucose (^{14}C-U-glucose; 50 nM; specific activity: 360 mCi/mmol; American Radiolabeled Chemicals, Inc. [ARC]; Saint Louis, MO 63146, USA) and 2-^{3}H(N)-deoxy-D-glucose (^{3}H-2-deoxyglucose; 2.5 nM; 60 Ci/mmol; ARC). After the assay, the slices were washed twice in ice-cold uptake solution for 5 min, and collected in 1 ml NaOH (0.5 M).

2.4. Testing the suitability of glucose analogues

Once we established that at least 60 min preincubation is necessary for the metabolic recovery (see Section 3), we bath applied radioactive glucose analogues after 90 min recovery preincubation for 30 and 45 min. Four hippocampal and 2 cortical slices of the rat per condition in duplicate were caused to undergo the following treatments: (A) ^{14}C-glucose (50 nM) alone; (B) ^{3}H-2-deoxyglucose (2.5 nM) together with ^{14}C-glucose (50 nM); and (C) 3-O-[methyl-^{3}H]-D-glucose (2.5 nM; 60 Ci/mmol; ARC) together with ^{14}C-glucose (50 nM). Upon completing the incubation, the slices were washed twice in ice-cold uptake solution for 5 min, and collected in 1 ml NaOH (0.5 M).

2.5. Pharmacological assays

From each animal for each of the experimental conditions (chambers), four slices were used from the pair of hippocampi (max. four conditions in duplicate). Each of the chambers held 40 ml assay solution and represented a solvent control or a treatment. Even if slices are kept at 4 °C, we found that they lose their physiological quality after 3 h (~30–40% less glucose uptake, lack of response to treatment), and thus, duplicate experiments can only be run simultaneously.

Drugs or vehicle DMSO (≤0.1%) were added from min 80 of the preincubation period, except for the treatment with KCl. When treatment with KCl occurred, at min 89 of the preincubation period, control and treated slices were moved to the appropriate solutions (the same as before for the control, but isomolar substitution of NaCl with KCl for the stimulated slices). Radioactive glucose analogues, ^{14}C-U-glucose (50 nM) and ^{3}H-2-deoxyglucose (2.5 nM) were bath applied from min 90 for 30 min. Then, the slices were washed twice in ice-cold uptake solution for 5 min, and collected in 1 ml NaOH (0.5 M).

2.6. Calculations for glucose uptake and metabolism

After dissolving the slices in 1 ml NaOH, 800 μl of the samples was assayed for ^{3}H (X disintegration/minute [dpm]) and ^{14}C (Y dpm) counts with the help of a Tricarb β-counter (PerkinElmer, USA), and the rest for protein concentration (P mg) with the bicinchoninic acid assay (Merck Biosciences, Germany). The incubation