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Hierarchical glucocorticoid-endocannabinoid interplay regulates the activation of the nucleus accumbens by insulin

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ABSTRACT

Here we asked if insulin activation of the nucleus accumbens *in vitro* is reflected by an increase in ^{3}H-deoxyglucose ([^{3}H]DG) uptake, thus subserving a new model to study molecular mechanisms of central insulin actions. Additionally, we investigated the dependence of this insulin effect on endocannabinoids and corticosteroids, two major culprits in insulin resistance. We found that in acute accumbal slices, insulin (3 and 300 nM but not at 0.3 nM) produced an increase in [^{3}H]DG uptake. The synthetic cannabinoid agonist, WIN55212-2 (500 nM) and the glucocorticoid dexamethasone (10 μM), impaired insulin (300 nM) action on [^{3}H]DG uptake. The glucocorticoid receptor (GcR) antagonist, mifepristone (10 μM) prevented dexamethasone from inhibiting insulin's action. Strikingly, this anti-insulin action of dexamethasone was also blocked by two CB_1 cannabinoid receptor (CB_1R) antagonists, O-2050 (500 nM) and SR141716A (500 nM), as well as by tetrahydrolipstatin (10 μM), an inhibitor of diacylglycerol lipases—the enzymes responsible for the synthesis of the endocannabinoid, 2-arachidonoyl-glycerol (2-AG). On the other hand, the blockade of the post-synaptic 2-AG metabolizing enzymes, α,β-serine hydrolase domain 6/12 by WWL70 (1 μM) also prevented the action of insulin, probably via increasing endogenous 2-AG tone. Additionally, an anti-insulin receptor (InsR) antibody immunoprecipitated CB_1Rs from accumbal homogenates, indicating a physical complexing of CB_1Rs with InsRs that supports their functional interaction. Altogether, insulin stimulates glucose uptake in the nucleus accumbens. Accumbal GcR activation triggers the synthesis of 2-AG that in turn binds to the known CB_1R-InsR heteromer, thus impeding insulin signaling.

Abbreviations: [^{3}H]DG, [^{3}H]-2-deoxy-D-glucose; [^{18}F]FDG-PET, [^{18}F]-fluorodeoxyglucose-positron emission tomography; [^{3}H]GABA, tritiated γ-aminobutyric acid; 2-AG, 2-arachidonoyl-glycerol; 3Rs, replacement refinement and reduction of animals in research; α,βHD6/12, α,β hydrolase domain-containing protein 6/12; ARRIVE, animals in research reporting *in vivo* experiments; CB_1R(s), cannabinoid CB_1 receptor(s); DAGLα, sn-1-diacylglycerol lipase α; dexa, dexamethasone; DMAQB1, demethylasterriquinone B1, DMSO, dimethylsulfoxide; DPM, disintegration per minute; DTT, dithiothreitol; FELASA, federation for laboratory animal science associations; FR%, fractional release expressed as percentage; GcR(s), glucocorticoid receptor(s); HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; IGF-1R(s), insulin-like growth factor-1 receptor(s); IgG_{2A}, immunoglobulin G_{2A}; InsR(s), insulin receptor(s); IOMeTAG, I-OMe-tyrphostin AG 538; MAGL, monoacylglycerol lipase; McR(s), mineralocorticoid receptor(s); MOPS, 3-(N-morpholino)propanesulfonic acid; SDS-PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis; THL, tetrahydrolipstatin; Tris, tris(hydroxymethyl)aminomethane; Triton-X 100, polyethylene glycol p-(1,1,3,3-tetramethylbutyl)-phenyl ether; WIN-2, WIN55212-2.

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1. Introduction

Insulin signaling in the brain is crucial for both central and systemic metabolic homeostasis (Kleinridders et al., 2014; Kullmann et al., 2015). Research in humans and animals concluded that insulin influences glucose regulation in the forebrain (Duarte et al., 2012). For instance, [^{18}F]FDG-PET studies documented that global cerebral glucose uptake rates can be stimulated by acute hyperinsulinemia only in patients with impaired glucose tolerance (Hirvonen et al., 2011), unless endogenous insulin production is suppressed in the healthy control group (Bingham et al., 2002). These findings suggest that even under normoinsulinemia, brain insulin levels are high enough to mask certain central effects of exogenous insulin. Notably though, mapping local metabolic rates in different brain areas, both increased and decreased glucose uptake have been reported in response to an acute bolus of insulin (Anthony et al., 2006). Local metabolic rates do not necessarily match changes in global metabolic rates: insulin for instance stimulates glucose uptake on average by 27% in the ventral striatum of healthy humans, and only by 13% in insulin resistant patients (Anthony et al., 2006).

The ventral striatum is called the nucleus accumbens in non-primate mammals, and is a major relay nucleus of the reward circuitry (Kenny, 2011). Insulin signals converge here that underlie palatable food preference and food reward (Volkow et al., 2008; Kenny, 2011; Kullmann et al., 2015). The modulation of mesoaccumbal transmitter release and synaptic plasticity is also crucial in drug-induced reward and reinforcement of drug abuse – including that of cannabis—which are highly dependent on frontal cortical-mesoaccumbal endocannabinoid signaling (Robbe et al., 2003; Maldonado et al., 2013; Covey et al., 2014). Consequently, food and drug addiction share common mechanisms (Volkow et al., 2008; Kenny, 2011), and insulin resistance is associated with the physical and functional deterioration of the nucleus accumbens, which exacerbates addictive behaviour including the increased intake of caloric and palatable food (Isganaitis and Lustig, 2005; Chen et al., 2013; O'Dell et al., 2014).

Various molecular mechanisms can hinder insulin signaling at insulin receptors (InsRs). For example, glucocorticoids are well known to cause insulin resistance and obesity (Andrews and Walker, 1999). Strikingly, a recent study revealed that glucocorticoids can trigger the metabolic syndrome by engaging endocannabinoid signaling in the periphery (Bowles et al., 2015). Glucocorticoid receptor (GcR) activation has also been reported to stimulate endocannabinoid synthesis at central synapses (Di et al., 2003; Hill and McEwen, 2009; Hill et al., 2011). The metabotropic CB_1 cannabinoid receptor (CB_1R) is the principal cognate receptor in the brain of the two most-studied endocannabinoids, anandamide and 2-AG (Katona and Freund, 2012; Murataeva et al., 2014). The CB_1R is present in the nucleus accumbens (Mátyás et al., 2007; Pickel et al., 2006; Winters et al., 2012). Remarkably, CB_1Rs form functional and physical heteromers with the β-chain of the InsRs in both pancreatic islets and neuronal cell lines (Dalton and Howlett, 2012; Kim et al., 2012), and cannabimimetics prevent insulin-induced autophosphorylation of the InsR β-chain and the consequent activation of the PI_3K-Akt pathway.

Here we sought answers to two main questions, namely whether insulin also stimulates glucose uptake in the accumbal slice as it does *in vivo* (Anthony et al., 2006), and if the effect of insulin is influenced by glucocorticoid and endocannabinoid signaling. This would also support the use of *in vitro* [^{3}H]DG assays to study basic mechanisms of food intake regulation, providing a viable alternative to *in vivo* [^{18}F]FDG-PET studies.

2. Materials and methods

2.1. Ethics statement and animals

All studies were conducted in accordance with the principles and procedures outlined as "3Rs" in the guidelines of EU (86/609/EEC), FELASA, and the National Centre for the 3Rs (the ARRIVE; Kilkenny et al., 2010). Studies were approved by the Animal Care Committee of the Center for Neuroscience and Cell Biology of the University of Coimbra, Portugal. We also applied the ARRIVE guideline for the design and execution of *in vitro* pharmacological experiments (see below), as well as for data management and interpretation (McGrath et al., 2010; Curtis et al., 2015).

Sixty-seven male Wistar rats purchased from Charles-River (Barcelona, Spain) at 6 weeks of age. Animals were housed on 12 h light on/off cycles under controlled temperature (23 ± 2 °C), and *ad libitum* access to food and water. All efforts were made to minimize the number of animals used and to minimize their stress and discomfort, and different rat tissues were shared for various ongoing studies.

2.2. In vitro *measurement of glucose uptake in accumbal slices*

Experiments were carried out as before, with some modifications (Lemos et al., 2012, 2015). Before decapitation with a guillotine, rats were deeply anesthetized with halothane vapor in air, using 1 mL liquid halothane in a 9000 cm^3-volume box. Rats were killed around 2:00 PM each experimental day to reduce potential circadian hormonal effects, and their brain was immediately placed in ice-cold Krebs-HEPES assay solution of the following composition: (in mM: NaCl 133, KCl 3, KH_2PO_4 1.2, $MgSO_4$ 1.2, $CaCl_2$ 2.5, $NaHCO_3$ 25, glucose 5.5, HEPES 1.5, pH 7.4). The pairs of nuclei accumbens were rapidly dissected and 400 μm-thick transverse slices were cut parallel to the coronal plane with the help of a McIlwain tissue chopper, and the slices were gently separated in ice-cold assay solution (carboxygenated with 95% O_2 and 5% CO_2), then transferred and maintained at 37 °C in a multichamber slice incubator with 50 mL of carboxygenated assay solution until the end of the experiment. Each container had separated nylon-mesh bottom wells to keep 4 accumbal slices from each of the three animals per experiment, *i.e.* each container had approximately 3.5-4.0 mg protein in 50 mL assay medium. Chemicals were added to the assay at different time-points as explained in Fig. 1A: the DAGL inhibitor, tetrahydrolipstatin (10 μM), the GcR antagonist, mifepristone (10 μM), and the mineralocorticoid receptor (McR) antagonist, spironolactone (10 μM) or their vehicle, DMSO (0.1%) were added at the beginning of the 60 min recovery incubation period. The MAGL inhibitor, JZL184 (1 μM), the α,βHD6/12 inhibitor, WWL70 (1 μM), and the CB_1R-selective antagonists SR141716A (500 nM) and O-2050 (500 nM), and the IGF-1R antagonist, I-OMe-tyrphostin AG 538 (5 μM) or their vehicle, DMSO (0.1%) were added after 50 min recovery incubation. The synthetic cannabinoid agonist, WIN55212-2 (500 nM) and the glucocorticoid dexamethasone (1 and 10 μM) or their vehicle, DMSO (0.1%) were added after 55 min recovery time, and demethylasterriquinone B1 (10 μM) or its vehicle, DMSO (0.1%), as well as insulin (3 and 300 nM) were added after 60 min recovery time, *i.e.* 1 min before [^{3}H]-2-deoxyglucose ([^{3}H]DG; final concentration of 1 nM) administration. The slices were then incubated for more 30 min, which is an optimal assay length to obtain good specific *vs.* total uptake with linear kinetics (Schmidt et al., 1989). Two-three randomly selected slices were incubated with 1 nM [^{3}H]DG on ice along with the main experiment, to determine non-specific extracellular label.

Upon completion of the uptake period, the slices were washed four times in ice-cold Krebs-HEPES solution for 5 min and trans-