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

Running title: Insulin action on accumbal glucose uptake

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Abbreviations

[³H]DG, [³H]-2-deoxy-D-glucose; [¹⁸F]FDG-PET, [¹⁸F]-fluorodeoxyglucose-positron emission tomography; [³H]GABA, tritiated γ -aminobutyric acid; 2-AG, 2-arachidonoyl-glycerol; 3Rs, Replacement, Refinement and Reduction of Animals in Research; α , β HD6/12, α , β hydrolase domain-containing protein 6/12; ARRIVE, Animals in Research: Reporting *In Vivo* Experiments; CB₁R(s), cannabinoid CB₁ receptor(s); DAGL α , *sn*-1-diacylglycerol lipase α ; dexam, 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

Abstract (244/250)

Here we asked if insulin activation of the nucleus accumbens *in vitro* is reflected by an increase in ³H-deoxyglucose ([³H]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 [³H]DG uptake. The synthetic cannabinoid agonist, WIN55212-2 (500 nM) and the glucocorticoid dexamethasone (10 μ M), impaired insulin (300 nM) action on [³H]DG uptake. The glucocorticoid receptor (GcR) antagonist, mifepristone (10 μ M) prevented dexamethasone from inhibiting insulin's action. Strikingly, this anti-insulin action of dexamethasone was also blocked by two CB₁ cannabinoid receptor (CB₁R) antagonists, O-2050 (500 nM) and SR141716A (500 nM), as well as by tetrahydrolipstatin (10 μ M), 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 μ M) also prevented the action of insulin, probably via increasing endogenous 2-AG tone.

Additionally, an anti-insulin receptor (InsR) antibody immunoprecipitated CB₁Rs from accumbal homogenates, indicating a physical complexing of CB₁Rs 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 CB₁R-InsR heterodimer, thus impeding insulin signaling.

Keywords

2-arachidonoylglycerol; CB₁R cannabinoid receptor; glucocorticoid receptor; glucose uptake; insulin; nucleus accumbens.

1. Introduction

Insulin signaling in the brain is crucial for both central and systemic metabolic homeostasis (Kleinridders et al., 2014; Kullmann et al., 2014). Research in humans and animals concluded that insulin influences glucose regulation in the forebrain (Duarte et al., 2012a). For instance, [¹⁸F]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 CB₁ cannabinoid receptor (CB₁R) 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 CB₁R is present in the nucleus accumbens (Mátyás et al., 2006; Pickel et al., 2006; Winters et al., 2012). Remarkably, CB₁Rs form functional and physical heterodimers 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 PI₃K-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* [³H]DG assays to study basic mechanisms of food intake regulation, providing a viable alternative to *in vivo* [¹⁸F]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 cm³-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, KH₂PO₄ 1.2, MgSO₄ 1.2, CaCl₂ 2.5, NaHCO₃ 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% O₂ and 5% CO₂), 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,β HD6/12 inhibitor, WWL70 (1 µM), and the CB₁R-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 [³H]-2-deoxyglucose ([³H]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 [^3H]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 transferred to 1 mL NaOH (0.5 M) to dissolve them. An aliquot of 800 μL was then assayed for ^3H in a Tricarb 2900TR β -counter (PerkinElmer Portugal [ILC, Lisbon, Portugal]), while the remaining solution was used for protein quantification by the bicinchoninic acid assay (Merck Biosciences, Germany). Specific tritium uptake was multiplied by a factor of 5.5×10^6 to estimate the total glucose uptake (corresponding to the concentration difference between D-glucose and [^3H]DG in the uptake medium).

2.3. [^3H]GABA and [^{14}C]glutamate release assays from accumbal slices

Experiments were carried out as described earlier for striatal and hippocampal slices (Köfalvi et al., 2005; Bitencourt et al., 2015), with slight modifications. Accumbal slices were prepared as above, incubated at room temperature at carboxygenation for 60 min to allow their metabolic recovery (Lemos et al., 2012), and were then incubated (loaded) either with [^3H]GABA (specific activity, 60 Ci/mmol; final concentration, 300 nM) or [^{14}C]glutamate (200 mCi/mmol; 20 μM) for 10 min, in Krebs-HEPES solution that also contained aminooxyacetic acid (100 μM) - a dual inhibitor of glutamate decarboxylase and GABA transaminase - to prevent the metabolism of [^3H]GABA or [^{14}C]glutamate, respectively. The slices were trapped by layers of Whatman GF/B filters, and superfused continuously at a rate of 0.8 mL/min with Krebs-HEPES solution (37°C) until the end of the experiments. Upon termination of a 10-min washout period, three 2-min samples were collected serving as baseline, and then insulin (300 nM) was superfused for 12 min in half of the chambers (the other half served as control) (Fig. 2). All treatments and controls were run in duplicate and averaged as $n=1$. The [^{14}C] and [^3H] content of each superfusate sample and slice was counted by a dual-label protocol using a Tricarb β -counter (Perkin-Elmer). DPM values were normalized to the slice content as fractional release (FR%), *i.e.* the percent of actual content in the effluent as a function of the total content. The calculation for drug (insulin) effect on the baseline can be found in Köles et al. (2013).

2.4. Immunoprecipitation assays

Immunoprecipitation assays of the CB₁R were carried out essentially as previously described (Marques et al., 2013; Ferreira et al., 2015). After 55 min recovery, WIN55212-2 (500 nM), and 5 min later, insulin (300 nM) were added to the bath of the accumbal slices, which were left for more 15 min to stimulate the formation of receptor heterodimers. The slices were then collected and proteins were extracted as before, and subsequently incubated with 50% slurry protein A-Sepharose (GE Healthcare Europe GmbH, Carnaxide, Portugal) for 3 h at 4°C to eliminate non-specific binding. After incubation, the pre-cleared supernatants containing 700 µg of protein were incubated overnight with rotation at 4°C with 8 µg rabbit polyclonal anti-InsR C-terminal antibody (Sigma, SAB4501558) previously linked to protein-A sepharose (GE Healthcare), in the presence of 1% bovine serum albumin (Sigma-Aldrich Portugal, Sintra, Portugal) and protease inhibitors (Roche Diagnostics, Basel, Switzerland). The beads were washed three times with isolation buffer containing 150 mM KCl, 20 mM MOPS, 1% Triton X-100 (pH 7.4), and resuspended in 6× diluted SDS-PAGE sample buffer (0.35 M Tris, 30% glycerol, 10% SDS, 0.6 M DTT, 0.012% bromophenol blue, pH 6.8). Immune complexes were dissociated from the beads and denaturated by heating to 95 °C for 5 min and then separated by 10% SDS-polyacrilamide gel electrophoresis. Proteins were then transferred into nitrocellulose membranes (Panreac Química, Barcelona, España) and probed with goat polyclonal anti-CB₁R C-terminal antibody (Rozenfeld and Levi, 2008; Bitencourt et al., 2015) and affinity purified rabbit polyclonal anti-InsR (Santa Cruz Biotechnology, sc-711), diluted in blocking solution (Tris-buffered saline supplemented with 0.1% Tween-20 and 5% milk). Membranes were incubated with a horseradish peroxidase (HRP)-conjugated goat anti-rabbit or rabbit anti-goat secondary antibody (Pierce, Thermo Fisher Scientific Inc., Darmstadt, Germany), followed by incubation with SuperSignal West Pico Chemiluminescent Substrate (Pierce, Thermo Fisher Scientific Inc., Darmstadt, Germany), visualized using a VersaDoc3000 apparatus (BioRad, Amadora, Portugal) and analyzed with ImageLab software (BioRad, Amadora, Portugal). A negative control containing the same amount of rabbit polyclonal IgG instead of the rabbit anti-InsR antibody was performed in parallel.

2.5. Data presentation

All data represent means ± S.E.M. of "n" observations (animals) in duplicates (release experiments) or uniplicates (uptake experiments). For data presentation and manipulation, we followed the rules of Curtis et al. (2015). Raw data were normalized to the control value

measured from the same animal. Normalized data were tested for normal distribution by the Kolmogorov-Smirnov test. Statistical significance was determined by one-sample *t*-test against the hypothetical value of 100 (%), and $P < 0.05$ was accepted for significant difference, according to Curtis et al. (2015). Note that one-sample *t*-test does not take into account when more than one treatment is normalized to the same everyday control. Tests were performed using the GraphPad Prism 5.0 software package.

2.6. Materials and chemicals

[2,3-³H(N)]GABA ([³H]GABA; specific activity: 60 Ci/mmol) and ³H-2-deoxy-D-glucose ([³H]DG; 60 Ci/mmol) and rimonabant (SR141716A) were bought from American Radiolabeled Chemicals, Inc, (Saint Louis, MO, USA). [¹⁴C]-U-glutamate was from Perkin-Elmer Portugal (ILC Lisbon, Portugal). Dexamethasone human recombinant insulin, mifepristone, O-2050, spironolactone and WIN55212-2 mesylate were purchased from Tocris Biosciences, Bristol, UK. Aminoxyacetic acid, bovine serum albumin, DMSO, halothane, HEPES, I-OMe-tyrphostin AG 538, and 3-(N-morpholino)propanesulfonic acid (MOPS) were bought from Sigma-Aldrich, Sintra, Portugal. Inorganic reagents were obtained either from Merck Biosciences, Darmstadt, Germany or Sigma-Aldrich. Non-water soluble substances were diluted in DMSO and stored in aliquots at -20°C until use at a 1:1000 dilution. Insulin was reconstituted with HCl (final concentration in the assay medium: 7 nanomoles of HCl per each nanomol of insulin), aliquoted and stored at -20°C.

3. Results

3.1. Insulin receptor (*InsR*) activation stimulates glucose uptake in the accumbal slices

The first 30 min of deoxyglucose uptake in the brain displays linear kinetics (Schmidt et al., 1989; Lemos et al., 2012). The mean glucose uptake value, as calculated from specific [³H]DG uptake, amounted to 68.4 ± 1.9 nmol/mg protein in the 30 min uptake period in the accumbal slices ($n = 51$ rats, *i.e.* pooled from all control animals throughout the study). One-min pretreatment with insulin at 3 nM ($n = 7$) and at 300 nM ($n = 20$), but not at 0.3 nM ($n = 10$) stimulated [³H]DG uptake in the range of 20-24% ($P < 0.05$) (Fig. 1B). Since we got more consistent results with 300 nM insulin and we did not want to mitigate the possible

involvement of the insulin-like growth factor receptor-1 (IGF-1R) in the following assays, we opted for using the concentration of 300 nM for insulin throughout the study.

To test if insulin stimulated [^3H]DG uptake via the activation of IGF-1Rs, we pretreated the slices with the IGF-1R antagonist, I-OMe-Tyrphostin AG 538 (IOMeTAG) (5 μM) for 10 min, which neither affected [^3H]DG uptake alone ($P > 0.05$ vs. DMSO; Table 1), nor prevented insulin from stimulating [^3H]DG uptake ($n = 8$; $P < 0.05$) (Fig. 1B). Demethylasterriquinone B1 (DMAQB1) (10 μM), a selective activator of the Akt-pathway, specific to the InsR rather than other tyrosine kinases, also stimulated glucose uptake ($n = 25$, $P < 0.05$) (Fig. 1B), albeit lesser than insulin.

3.2. Insulin does not stimulate glucose uptake via the modulation of synaptic activity

An increase in either GABAergic or glutamatergic signaling (or both) is associated with greater glucose consumption in neurons and astrocytes (Ackerman et al., 1984; Pellerin and Magistretti, 1994). Thus, we next asked if insulin affects the release of fast neurotransmitters in the accumbal slices. In fact, insulin receptors and insulin receptor substrate p58/53 are present in CNS synapses (Abbot et al., 1999), and insulin is known to affect synaptic communication in the brain (McGregor et al., 2015). We therefore loaded accumbal slices with [^3H]GABA and [^{14}C]glutamate, and subsequently, measured the outflow of these two radiolabeled neurotransmitters under insulin superfusion. As Fig. 2 demonstrates, insulin (300 nM) did not affect the resting release of the two neurotransmitters (net changes for GABA, 0.020 ± 0.022 FR%, $n = 4$, $P > 0.05$ vs. 0 FR% change; and net changes for glutamate, 0.18 ± 0.55 FR%, $n = 4$, $P > 0.05$ vs. 0 FR% change). Hence, it is unlikely that insulin stimulates glucose uptake via the stimulation of fast synaptic transmission in the accumbal slice. Instead, insulin perhaps directly stimulates cellular [^3H]DG uptake in the nucleus accumbens.

3.3. Glucocorticoid receptor (GcR) activation prevents insulin from stimulating glucose uptake

Glucocorticoids are well known to impair insulin signaling (Andrews and Walker, 1999). We next asked if the effect of insulin in accumbal slices is also hindered by a glucocorticoid. Five min pretreatment of the slices with dexamethasone (1 and 10 μM) did not affect resting [^3H]DG uptake (Table 1) but prevented insulin (300 nM) from stimulating [^3H]DG uptake ($n = 6$; $P > 0.05$ for both concentrations of dexamethasone, vs DMSO CTRL) (Fig. 3). The GcR antagonist, mifepristone (10 μM) also had no effect on [^3H]DG uptake *per se* (Table 1), but

antagonized the action of dexamethasone and restored insulin-stimulated [3 H]DG uptake ($n = 11$; $P < 0.05$ vs DMSO CTRL) (Fig. 3). Interestingly, the mineralocorticoid receptor (McR) antagonist, spironolactone (10 μ M) (Fig. 3) stimulated [3 H]DG uptake by 21% ($P < 0.05$) on its own (Table 1), but it failed to antagonize the anti-insulin action of dexamethasone, as concluded from the normalization of the effect of the simultaneous treatment of insulin + dexamethasone + spironolactone to spironolactone alone ($n = 12$; $P > 0.05$) (Fig. 3). Thus, we conclude that it is the GcR, rather than the McR that impairs the stimulation of accumbal [3 H]DG uptake by insulin.

3.4. *CB₁ cannabinoid receptors mediate the anti-insulin action of GcRs*

The CB₁R has been shown to inhibit InsR signaling via heterodimer formation between the receptors both in a neuronal and a pancreatic β -cell line (Dalton and Howlett, 2012; Kim et al., 2012). Thus, we asked if CB₁R ligands affect resting and insulin-stimulated glucose uptake. Neither the potent synthetic cannabinoid agonist, WIN55212-2 (500 nM) nor the two CB₁R-selective antagonists, O-2050 (500 nM) and SR141716A (500) affected accumbal glucose uptake *per se* (Table 1). However, a 5-min-preincubation with WIN55212-2 ($n = 11$; $P > 0.05$ vs DMSO CTRL) prevented insulin from stimulating glucose uptake in the accumbal slices (Fig. 4). It has been previously proposed that endocannabinoids can mediate some synaptic actions of glucocorticoids (Di et al., 2003, 2005; Hill et al., 2011), and we recently found in the cerebral cortex and the hippocampus that post-synaptic GcR immunoreactivity co-localizes with DAGL α (Bitencourt et al., 2015), the major cerebral isoform of diacylglycerol lipase, which synthesizes the endocannabinoid, 2-AG (Murataeva et al., 2014). Furthermore, these co-localizing protein complexes are apposed to presynaptic CB₁R-positive elements, thus bolstering the idea that GcR activation can stimulate CB₁Rs via trans-synaptic 2-AG signaling (Di et al., 2003, 2005; Hill and McEwen, 2009). To test if a similar mechanism is accountable for the anti-insulin effect of dexamethasone, we first pretreated the slices with the CB₁R-selective antagonists, O-2050 ($n = 6$) or SR141716A ($n = 8$), and 5 min later added dexamethasone (10 μ M). Indeed, both CB₁R antagonists successfully restored the stimulatory action of insulin (Fig. 4). Of note, SR141716A pretreatment almost doubled the effect of insulin ($35.6 \pm 7.8\%$ stimulation over DMSO control; $P < 0.01$), which is probably due to the inverse agonist properties of this antagonist.

To prove that the identity of the endocannabinoid underlying dexamethasone's action was indeed 2-AG, we preincubated the slices with tetrahydrolipstatin (10 μ M), which inhibits

DAGL isoforms responsible for CB₁R activation in the brain (Palomäki et al., 2007). While tetrahydrolipstatin had no effect alone (Table 1), it restored the ~20% stimulation of glucose uptake by insulin in the presence of dexamethasone (n = 8; $P < 0.05$ vs. DMSO CTRL) (Fig. 4). It is believed that synaptic 2-AG is constantly generated at a basal rate, contributing to tonic CB₁R signaling (Lee et al., 2015), and 2-AG is removed by major metabolizing enzymes, MAGL and α,β HD6/12 (Murataeva et al., 2014). Hence, we pretreated the slices either with the MAGL inhibitor, JZL184 (1 μ M) or with the α,β HD6/12 inhibitor, WWL70 (1 μ M). These inhibitors did not significantly affect glucose uptake on their own (Table 1), and MAGL inhibition still permitted insulin to stimulate glucose uptake in the slices (by 22%; n = 5; $P < 0.01$). However, the blockade of α,β HD6/12 abolished the action of insulin (n = 5; $P > 0.05$ vs DMSO alone) (Fig. 4).

These pharmacological data together with the previously reported ability of the CB₁R to heterodimerize with the InsR (Dalton and Howlett, 2012; Kim et al., 2012) strongly suggest a physical association between InsRs and CB₁Rs here in the nucleus accumbens. To prove this, we co-incubated accumbal slices with insulin (300 nM) and WIN55212-2 (500 nM) for 15 min with the aim of stimulating heterodimerization (Kim et al., 2012), then extracted the membrane proteins, and made an attempt to pull the CB₁R down with an anti-InsR β -chain antibody. Indeed, as Fig. 5 illustrates, the CB₁R was immunoprecipitated with an anti-InsR antibody, thus providing evidence for the coexistence of a both the CB₁R and the InsR in a common protein complex in the rat nucleus accumbens.]

4. Discussion

The novel findings in this study indicate that insulin stimulates glucose uptake in the rat accumbal slice *in vitro* via insulin receptor (InsR) activation. Certainly, this is consistent with an earlier *in vivo* [¹⁸F]FDG-PET study in humans (Anthony et al., 2006), thus documenting that studying insulin action in rat accumbal slices is a viable alternative approach that may be useful for dissecting mechanisms. Furthermore, glucocorticoid receptor (GcR) activation triggers the release of the endocannabinoid 2-AG, which in turn binds to CB₁ cannabinoid receptors (CB₁Rs) that form heterodimers with InsRs. While CB₁R activation *per se* does not affect accumbal glucose uptake, it prevents insulin from stimulating glucose uptake.

Insulin and its receptors have been found throughout the brain, with their highest density in brain areas involved in the control of food intake (Havrankova et al., 1978; Pacold and Blackard, 1979). Cerebral insulin levels are dozens of times higher than those in the blood,

which cannot be explained solely by a putative concentrating insulin transport from the circulation into the brain, and indeed, local cerebral insulin synthesis has been identified, at least in neurons (Havrankova et al., 1978; Clarke et al., 1986; Devaskar et al., 2004; Ghasemi et al., 2013; Molnár et al., 2014). We conclude that insulin stimulated glucose uptake in the accumbal slices via the activation of InsRs rather than IGF-1Rs, based on the following observations: Insulin binds to the InsR with a K_d of 0.4 nM and is 13-32-fold less potent at IGF-1Rs in the rat brain (Werther et al., 1987, 1989). In fact, insulin already reached maximal effect at 3 nM in our assay, but even the effect of 300 nM insulin was insensitive to IGF-1R blockade. Finally, the selective insulin receptor activator, DMAQB1 also stimulated glucose uptake, although much less efficaciously. This latter discrepancy can be due to the fact that we used this insulinomimetic at a concentration close to its EC_{50} , or perhaps to that this ligand only activates the PI_3K -Akt pathway amongst the various second messenger systems associated with the InsR (Webster et al., 2003) which might not be enough for maximal effect.

The InsR is widely distributed in the mammalian brain (Pacold and Blackard, 1979; Folli et al., 1994; Schulingkamp et al., 2000), including the nucleus accumbens (Kleinridders et al., 2014), with highest levels in the shell of the accumbens among the striatal regions (Werther et al., 1987). Cerebral InsRs play a major role in the regulation of the energy metabolism of the whole body (Brüning et al., 2000; Varela and Horvath, 2012). InsR activation can also stimulate local and global rates of cerebral glucose metabolism, but only a few studies have documented these findings (Bingham et al., 2002; McNay et al., 2010).

The exact cellular distribution of InsRs has not yet been investigated in the nucleus accumbens. However, studies in brain cell cultures provided (often circumstantial) evidence that insulin may directly stimulate glucose uptake in both neurons (Benomar et al., 2006; Uemura and Greenlee 2006) and astrocytes (Clarke et al., 1984; Werner et al., 1989; Kum et al., 1992).

Insulin may also modulate cerebral energy metabolism via the regulation of the activity of the circuitry. The stimulation of either GABAergic or glutamatergic signalling is indeed associated with increased glucose uptake in neurons and astrocytes (Ackerman et al., 1984; Pellerin and Magistretti, 1994), supporting the notion of activity-dependent metabolic coupling in the brain (Patel et al., 2014; Lundgaard et al., 2015). There is evidence for modulation of excitatory synaptic transmission by insulin (500 nM) in the ventral tegmental area, which occurs trans-synaptically, because it involves insulin-mediated endocannabinoid release onto presynaptic glutamatergic terminals (Labouèbe et al., 2013; Liu et al., 2013).

Insulin can also affect synaptic transmission by modulating AMPA- and NMDA-dependent synaptic plasticity, which is manifested post-synaptically (Huang et al., 2004; Costello et al., 2012). In our hands, insulin did not affect the resting extracellular GABA and glutamate levels in the accumbal slices. Hence, it is unlikely that insulin stimulated glucose uptake in the accumbal slice via the modulation of the fast synaptic transmission.

Alternatively, insulin could act through neuromodulation, and dopamine is the major neuromodulator of the nucleus accumbens. Accumbal dopamine release accompanies the hedonic rather than the nutritional value of sugar intake (Tellez et al., 2016). Importantly, both systemic insulin injection (Potter et al., 1999) and *in vitro* insulin application on acute brain slices (Stouffer et al., 2015) increased accumbal dopamine release, which is consistent with the hypothesis that insulin evokes reward and satiety responses. In line with this evidence, a fresh publication suggests that *in vivo* CB₁R antagonism by AM6527 produces behaviors more consistent with satiety than reduced reward value (Thompson et al., 2016). Indeed, this is what we would expect to see if CB₁R antagonism in the nucleus accumbens facilitates insulin signaling *in vivo*.

Another study, however, reported in the ventral tegmentum that insulin decreased the excitatory drive via endocannabinoid release onto the dopaminergic efferents (Labouèbe et al., 2013), which is expected to *decrease* dopaminergic activity in the target nucleus accumbens. This contradiction may be resolved by assuming a putative local accumbal mechanism that increases extracellular dopamine levels even if the firing of the input terminals is decreased. One possible scenario is that insulin in the presence of low ambient GABA levels inhibits the major neuronal GABA_A receptor subtype (Williams, 2008). In the rat nucleus accumbens, GABA_A receptor activation indeed reduces dopamine release (Sperlágh et al., 2009), thus insulin may presynaptically disinhibit accumbal dopamine release. Accumbal CB₁Rs are predominantly confined to GABAergic nerve terminals (Mátyás et al., 2006). Strikingly, the activation of these presynaptic CB₁Rs also can disinhibit accumbal dopamine release via the reduction of GABA release (Sperlágh et al., 2009). At the moment, we can not exclude the possibility that insulin stimulated glucose uptake via dopaminergic mechanisms in the accumbal slices, although the chance for that is remote: CB₁Rs do not modulate accumbal dopamine release directly (Sperlágh et al., 2009), and CB₁R activation acted inversely to insulin on glucose uptake, while both insulin and endocannabinoids are expected to increase accumbal dopamine efflux. Nevertheless, there is a lack of information on how dopamine might modulate glucose metabolism in the brain, and earlier studies using L-DOPA also reported inconclusive data (Barash et al., 1985). This

means that even if insulin acts through dopamine to influence glucose uptake, a serious additional effort is required to sufficiently settle the intricate interaction among neuromodulators and accumbal energy metabolism.

We next observed that acute GcR activation prevented insulin from stimulating glucose uptake, *i.e.* caused insulin resistance. While in the periphery, it is well-known that glucocorticoids impair insulin sensitivity (Andrews and Walker, 1999; Barbera et al., 2001), the present study provides evidence that this phenomenon holds true for the brain, too. If so, it may partly explain the detrimental effects of long-term unpredictable stress, chronic glucocorticoid treatment and Cushing-syndrome on brain structure and function (Belanoff et al., 2001; Sotiropoulos et al., 2008; Cattaneo and Riva, 2015). Importantly, these outcomes of chronic overactivation of GcRs (*i.e.* obesity/diabetes, mood disorders and dementias including Alzheimer's disease) fully coincide with the neuropsychiatric profile of cerebral insulin resistance (Duarte et al., 2012a, Ghasemi et al., 2013). Curiously, it has been known for decades that adrenalectomy rapidly increases glucose uptake in the brain in a matter of hours (Kadekaro et al., 1988). It is tempting to find parallelism with our data that spironolactone stimulated glucose uptake in the accumbal slice, because the McRs are "always occupied" by corticosteroids while GcRs are subject to phasic activation (Funder, 1996). But taken that animal manipulation and anesthesia always trigger phasic GcR activation in the brain (Gil et al., 2007), the rescue of insulin-stimulated glucose uptake is equivalently possible as an underlying mechanism for the adrenalectomy-induced increased cerebral glucose uptake observed by Kadekarko et al. (1988).

Last but not least, we documented that the DAGL/2-AG/CB₁R axis mediated the anti-insulin effect of dexamethasone. In the periphery, it has been shown that chronic treatment with a high dose of corticosterone triggers metabolic syndrome in mice within weeks (Bowles et al., 2015). This is accompanied with an increase in circulating anandamide levels, and can be prevented by genetic or chemical ablation of the CB₁R (Bowles et al., 2015). Interestingly, in the hypothalamus of these animals, a significant decrease in 2-AG levels was observed, which may reflect desensitization as a result of chronic DAGL stimulation. Nevertheless, glucocorticoids do increase endocannabinoid release acutely in the hypothalamus to inhibit the glutamatergic input to the parvocellular neurons of the paraventricular nucleus (Di et al., 2003), and so they do in the medial prefrontal cortex (Hill et al., 2011). This can be achieved by post-synaptic DAGL α activation adjacent to GcR in the frontal cortex (Bitencourt et al., 2015), apposing to CB₁R-positive nerve terminals. Whether in the nucleus accumbens, the CB₁R-InsR heterodimers are located to cells different from those which bear GcR and

DAGL α cannot be determined by our study. It has been previously established that the CB₁R is predominantly neuronal (axonal and dendritic) in the nucleus accumbens (Pickel et al., 2006). It is quite likely that the MSN dendrites are also endowed with GcR and DAGL α , as their antisera give predominant post-synaptic labeling in the cerebral cortex and the hippocampus (Bitencourt et al., 2015). Assuming that these neuronal CB₁Rs impair the action of insulin, it is tempting to conclude that insulin stimulates glucose uptake in the MSN. Still, conclusive evidence is required to disprove the involvement of glial cells.

As of interest, two functional types of CB₁R antagonists can be distinguished. One is a neutral antagonist such as O-2050, and it is believed that it only prevents phasic CB₁R activation upon stimulated endocannabinoid release, while SR141716A (rimonabant, Acomplia) also prevents the constitutive activity of the CB₁R (Pertwee, 2005; Meye et al., 2013). This scenario is consistent with what we found in our experimental model with the overt facilitation of insulin action. Notably, the action of SR141716A (Acomplia) on human food intake is initially dependent on the blockade of central CB₁Rs (Horvath, 2006; Matias et al., 2008). However, neutral CB₁R antagonists are just as good for reducing food intake as the inverse agonist, SR141716A, but without affecting constitutive CB₁R signaling (Meye et al., 2013). Whether this beneficial central CB₁R antagonist action on calorie intake involves the nucleus accumbens and insulin signaling has not been fully elucidated, but is highly likely. This is because endocannabinoids and insulin in the mesoaccumbal reward system play a crucial role in palatable and caloric food intake (Potter et al., 1999; Volkow et al., 2008; Daws et al., 2011; Kenny et al., 2011; Meye and Adam, 2014; Thompson et al., 2016).

Taken together, our report here is the first to find a link among glucocorticoids, endocannabinoids and insulin signaling in the brain, specifically in the nucleus accumbens. The model is testable *in vivo* too, and may help us to better understand the intricate interaction among these signaling systems in the control of calorie intake as well as in the pathogenesis of obesity, and other neuropsychiatric disorders.

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Conflicts of interest

None.

List of Author Contribution

Study design: AK. Neurochemical assays: BSP, CL, FNK, CSS, AK, EC. Immunoprecipitation: JMM, RJR, KM, BSP, CSS. Financing: AK, RAC. First draft: AK. Final form of the manuscript: all authors.

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Legends to figures

Fig. 1 Insulin receptor activation stimulates glucose uptake in acute accumbal slices from the rat. (A) Flow-chart depicting the experimental steps in the principal assay and their timing. Abbreviations: dexamethasone; DMAQB1, demethylasterriquinone B1 THL, tetrahydrolipstatin; WIN, WIN55212-2. DMSO (0.1%) was given as vehicle control in place of the lipophilic compounds in the same time. (B) Insulin, rather than IGF-1 receptors mediate the action of insulin on glucose uptake. Bars represent the mean $\pm$ S.E.M. of $n \geq 7$ independent observations (rats). The control values from each experiment are averaged and taken as 100%, while effect amplitudes are normalized to the appropriate controls. Due to the relative comparison, the ordinate is preferably labeled as [3 H]DG ([3 H]deoxyglucose) uptake which is more exact than glucose uptake, although the two are used interchangeably in this study. * $P < 0.05$, *** $P < 0.001$, n.s., not significant.

Fig. 2 Release diagrams and relative values of the release of [3 H]GABA and [14 C]glutamate in rat accumbal slices. Insulin was administered in the superfusion medium according to the horizontal bars. FR%, fractional release %, *i.e.* the release value relative to the total radioactivity content in the slice at the given time-point. All data are mean $\pm$ S.E.M. of $n = 4$, in duplicate.

Fig. 3 Glucocorticoid receptor activation prevents insulin from stimulating glucose uptake. Bars represent the mean $\pm$ S.E.M. of $n \geq 6$ independent observations (rats). Abbreviations; dexamethasone; spirono, spironolactone (10 μ M). * $P < 0.05$, *** $P < 0.001$, n.s., not significant.

Fig. 4 CB₁ cannabinoid receptors mediate the anti-insulin action of dexamethasone. While the synthetic cannabinoid agonist, WIN55212-2 (500 nM) and the CB₁R antagonists, O-2050 (500 nM) and SR141716A (500 nM) had no effect on glucose uptake *per se* (Table 1), WIN55212-2 prevented insulin from stimulating glucose uptake. Both CB₁R antagonists

opposed the anti-insulin action of dexamethasone (10 μ M). The diacylglycerol lipase inhibitor, tetrahydrolipstatin (THL, 10 μ M) mimicked the effect of the CB₁R antagonists. Finally, the α,β HSD6/12 inhibitor, WWL70 (1 μ M) but not that the monoacylglycerol lipase inhibitor, JZL184 (1 μ M) mimicked the anti-insulin action of dexamethasone, thus supporting the involvement of 2-AG in the glucocorticoid-induced insulin resistance. Bars represent the mean $\pm$ S.E.M. of $n \geq 6$ independent observations (rats). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, n.s., not significant.

Fig. 5 The CB₁R was readily detected at around 53 kDa molecular weight, and enriched in complexes immunoprecipitated with the anti-insulin receptor β -chain antibody (anti-InsR β ; Sigma-Aldrich), but not with rabbit polyclonal IgG (44 kDa). InsR β was detected at 95 kDa (with a different anti-InsR β antibody from Santa-Cruz Biotechnology).

Table 1 Effect of treatments on [³H]DG uptake in acute slices

treatment	mean ± S.E.M.	n	<i>P</i> vs. 100
dexamethasone (1 µM)	94.9 ± 11.3	6	0.669
dexamethasone (10 µM)	108.6 ± 6.5 ₅	8	0.228
I-OMe-tyrphostin AG 538 (5 µM)	104.1 ± 6.2	8	0.523
JZL184 (1 µM)	88.9 ± 8.8	6	0.261
mifepristone (10 µM)	101.3 ± 16.5	7	0.940
O-2050 (500 nM)	106.9 ± 7.3	22	0.357
spironolactone (10 µM)	120.9 ± 7.8	7	0.036 *
SR141716A (500 nM)	87.0 ± 10.8	8	0.266
tetrahydrolipstatin (10 µM)	102.6 ₅ ± 9.9	10	0.795
WIN55212-2 (500 nM)	106.4 ± 10.3	11	0.550
WWL70 (1 µM)	94.1 ± 3.2	5	0.137

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5