Regulation of PI3K/Akt/GSK-3 pathway by cannabinoids in the brain

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Abstract
Delta9-tetrahydrocannabinol (THC), the main psychoactive component in Cannabis sativa preparations, exerts its central effects mainly through the G-protein coupled receptor CB1, a component of the endocannabinoid system. Several in vitro and in vivo studies have reported neuroprotective effects of cannabinoids in excitotoxicity and neurodegeneration models. However, the intraneuronal signaling pathways activated in vivo by THC underlying its central effects remain poorly understood. We report that THC acute administration (10 mg/kg, i.p.) increases the phosphorylation of Akt in mouse hippocampus, striatum, and cerebellum. This phosphorylation was mediated by CB1 receptors as it was blocked by the selective CB1 antagonist rimonabant. Moreover, PI3K inhibition by wortmannin abrogated THC-induced phosphorylation of Akt, but blockade of extracellular signal-regulated protein kinases by SL327 did not modify this activation/phosphorylation of Akt. Moreover, administration of the dopaminergic D1 (SCH 23390) and D2 (raclopride) receptor antagonists did not block the activation of PI3K/Akt pathway induced in the striatum by cannabinoid receptor stimulation, suggesting that this effect is independent of the dopaminergic system. In addition, THC increased the phosphorylation of glycogen synthase kinase 3 beta. Therefore, activation of the PI3K/Akt/GSK-3 signaling pathway may be related to the in vivo neuroprotective properties attributed to cannabinoids.

Keywords: Akt, cannabinoid receptor, CB1, delta9-tetrahydrocannabinol, dopamine, GSK-3, rimonabant.


Introduction
Cannabinoids are the constituents of marijuana plant (Cannabis sativa) of which the principal psychoactive compound is delta9-tetrahydrocannabinol (THC). Cannabinoid compounds exert a wide spectrum of actions through the activation of two cannabinoid receptors, CB1 and CB2 (Matsuda et al. 1990; Munro et al. 1993), and recent evidence supports the existence of additional receptors (Begg et al. 2005). Cannabinoids might play a physiological and pharmacological role as neuroprotective agents (reviewed in van der Stelt and Di Marzo 2005). Several studies have shown that cannabinoids can protect neural cells from different insults, such as glutamatergic excitotoxicity, oxidative damage, traumatic injury, and ischemia (for review, see Guzman 2005). Some of these effects are linked to the activation of the PI3K/Akt pathway, which is closely involved in the survival signaling in many cell types including neurons. Cannabinoids can activate PI3K/Akt pathway by acting on both CB1 and CB2 receptors (Sanchez et al. 2003), although the protective effects on primary astrocytes (Molina-Holgado et al. 2002) have been reported to involve CB1 receptor. The stimulation of the PI3K/Akt pathway is also required for the neuroprotective effects of the synthetic cannabinoid HU-210 in primary cortical neurons (Molina-Holgado et al. 2005).

The signaling mechanisms activated in response to cannabinoid agonist stimulation have been described mainly in in vitro systems. In these models, cannabinoid receptor activation triggers the inhibition of adenylyl cyclase activity through its coupling to Gi/Go-proteins (Howlett and Fleming 2000).
1984), closure of Ca\(^{2+}\) channels (Mackie and Hille 1992), opening of K\(^{+}\) channels (Deadwyler et al. 1995), and activation of mitogen-activated protein kinases (MAPKs) (Bouaboula et al. 1995; Wartmann et al. 1995; Rueda et al. 2000) and PI3K/Akt signaling pathways (Gomez Del Pulgar et al. 2002; Molina-Holgado et al. 2002, 2005). Activated Akt, also known as protein kinase B, phosphorylates intracellular substrates, promoting cell survival by inhibiting apoptosis through several targets, including Bad, caspase 9 (Cardone et al. 1998) and Forkhead transcription factors (Brunet et al. 1999). One of the essential functions of Akt is the phosphorylation of glycogen synthase kinase 3 (GSK-3) at Ser21 in GSK-3\(\alpha\) and Ser9 in GSK-3\(\beta\) (Cross et al. 1995) causing its inactivation. GSK-3 activation has been reported to induce apoptosis (Pap and Cooper 1998; King et al. 2001; Watcharasit et al. 2002; Jope and Johnson 2004), and this enzyme can be pharmacologically inhibited by lithium (Klein and Melton 1996). Therefore, much effort is currently directed towards understanding the functions of GSK-3 and identifying methods capable of diminishing the deleterious impact of GSK-3 in pathological conditions.

The aim of the present study was to investigate the signaling cascades activated by THC in vivo by analyzing the activity of the PI3K/Akt/GSK-3 pathway. We found a close regulation of Akt and GSK-3 phosphorylation by THC in brain, acting on CB1 receptors, that could be related to the neuroprotective effects induced by cannabinoids in insults such as ischemia, glutamatergic excitotoxicity, mechanical trauma, and oxidative damage through the modulation of these crucial components of the cell survival pathway.

Materials and methods

Animals

Male CD-1 mice (Charles River, Lyon, France) weighing 30–34 g were used. After arrival animals were housed in cages of five and maintained at a controlled temperature (21 ± 1°C) and humidity (55 ± 10%). The mice were given access to food and water ad libitum. Lighting was maintained at 12-h cycles (on at 8 AM and off at 8 PM). All the experiments were performed during the light phase of the dark/light cycle. The animals were habituated to the experimental room and handled for 1 week before the start of the experiments. All animal procedures were conducted in accordance with the standard ethical guidelines (European Communities Directive 86/609 EEC) and approved by the local ethical committee (CEEA-IMAS-UPF).

Drugs and treatments

Delta(9)-tetrahydrocannabinol was provided by THC Pharm GmbH (Frankfurt, Germany), SL327, wortmannin, and Cremophor EL were provided by Sigma (Madrid, Spain). Rimonabant was kindly provided by Sanofi-Aventis (Sanofi-Aventis Recherche, Montpellier, France). \(R^+\)-(SCH 23390 and \(S^+\)-raclopride were provided by RBI (Madrid, Spain). THC was diluted in vehicle preparation (5% ethanol : 5% cremophor EL : 90% saline). Rimonabant was diluted in vehicle preparation (10% ethanol : 10% cremophor EL : 80% saline). SL327 (50 mg/kg) was dissolved in 50% dimethylsulfoxide (DMSO). All other compounds were dissolved in 0.9% bacteriostatic sodium chloride (saline). All the compounds, except wortmannin (see cannulation and wortmannin perfusion), were administered intraperitoneally in a volume of 10 mL/kg, except SL327 that was administered in a volume of 4 mL/kg.

Rimonabant (3 mg/kg or vehicle) and SL327 (50 mg/kg in 50% DMSO or vehicle) were administered 30 min and 1 h respectively before THC injection (10 mg/kg or vehicle). Mice were killed 30 min after receiving THC or vehicle and their brains were rapidly removed. Brain areas were dissected on ice, frozen on dry ice and stored at −80°C until used.

Sample preparation

Samples from all animal groups, in each experiment, were processed in parallel, to minimize inter-assay variations.

Total solubilized fraction

Frozen brain areas were dounce-homogenized in 30 volumes of lysis buffer (50 mmol/L Tris-HCl pH 7.4, 150 mmol/L NaCl, 10% glycerol, 1 mmol/L EDTA, 1 µg/mL aprotinin, 1 µg/mL leupeptine, 1 µg/mL pepstatin, 1 mmol/L phenylmethylsulfonyl fluoride, 1 mmol/L sodium orthovanadate, 100 mmol/L sodium fluoride, 5 mmol/L sodium pyrophosphate, and 40 mmol/L beta-glycerophosphate) plus 1% Triton X-100. After 10 min incubation at 4°C, samples were centrifuged at 16 000 g for 30 min to remove insoluble debris. Protein contents in the supernatants were determined by DC-micro plate assay (Bio-Rad, Madrid, Spain), following manufacturer’s instructions.

Membrane and cytosolic fractions

Frozen brain areas were dounce-homogenized in 30 volumes of lysis buffer and immediately centrifuged at low speed (1000 g) for 10 min at 4°C. The supernatant was recovered and spun at 16 000 g for 30 min at 4°C. The supernatant was recovered and used as cytosolic fraction, while the pellet was resuspended in lysis buffer plus 1% Triton X-100 and used as crude membrane fraction. Protein content in both the cytosolic and the membrane fraction were determined by DC-micro plate assay (Bio-Rad, Spain), following manufacturer’s instructions.

Western blot analysis

Equal amounts of brain lysates were mixed with denaturing 5x Laemmli loading buffer and boiled for 5 min. Samples with equal amounts of total protein (20 µg per lane) were separated in 10% sodium dodecyl sulfate-polyacrylamide gel before electrophoretic transfer onto nitrocellulose membrane (Bio-Rad, Spain). Membranes were blocked for 1 h at 21 ± 1°C in Tris-buffered saline (TBS) (100 mmol/L NaCl, 10 mmol/L Tris, pH 7.4) with 0.1% Tween-20 (TBS-T) and 5% non-fat milk. Afterwards, membranes were incubated for 2 h with the primary antibodies. Bound antibodies were detected with horseradish peroxidase-conjugated anti-rabbit or anti-mouse antibodies (Pierce; diluted 1 : 5000) and visualized by enhanced chemiluminescence detection (SuperSignal, Pierce, Spain). When necessary, nitrocellulose membranes were stripped.
THC modulates Akt/GSK-3 signaling

Cannulation and wortmannin perfusion
Mice were anesthetized with an intraperitoneal injection (0.2 mL/10 g body weight) of a mixture of ketamine (100 mg/kg) and xylazine (20 mg/kg), and mounted on a stereotaxic apparatus with a flat skull (Franklin and Paxinos 1997). A small hole was drilled randomly on the left or on the right side of the skull, and the cannula was implanted vertically in the left/right lateral ventricle (antero-posterior, −0.22 mm; mediolateral, +1.00/−1.00 mm; and dorso-ventral, −2.25 mm from bregma) and then fixed to the skull with dental cement. Mice were housed individually and allowed 4 days of post-operative recovery before handling. The irreversible inhibitor of PI3K, wortmannin (stocked at 1 mmol/L in DMSO) was used as a marker for membrane subcellular fractionation. Statistical analyses were performed by Student’s t-test using Microsoft Excel software.

Results
THC phosphorylates and activates Akt in the mouse brain
As mentioned above, cannabinoids have been shown to modulate Akt activity in vitro (Gomez Del Pulgar et al. 2002; Molina-Holgado et al. 2002, 2005). To examine if cannabinoids could phosphorylate Akt in vivo, mice received THC (10 mg/kg, i.p.) or its vehicle and the phosphorylation of Akt was examined after 30 min (Fig. 1a). This dose of THC produces behavioral effects such as anti-nociception, catalepsy and decreased locomotor activity (Hutcheson et al. 1998; Valverde et al. 2000). We focused our study on four brain areas involved in the central action of cannabinoids: hippocampus, cerebellum, striatum, and frontal cortex. Protein extracts from these brain areas were analyzed by western blot. We used a specific p-Akt antibody because phosphorylation of this residue is required for the kinase activity of Akt (Chan et al. 1999). A significant increase of p-Akt in response to THC treatment occurred in three of the four areas analyzed (Fig. 1a). After normalizing the values of the active p-Akt with the amount of total Akt (Akt) in each sample and comparing them with the control treatment (animals receiving vehicle), we observed a twofold increase in the hippocampus (p < 0.01), 2.9-fold in the cerebellum (p < 0.01), 2.6-fold in the striatum (p < 0.01), and a slight but non-significant increase in the cortex (n = 6 per treatment).

We indirectly studied the activation of Akt by measuring the phosphorylation of its downstream target GSK-3β in the same brain areas (Fig. 1b), using a specific antibody that recognizes p-GSK-3β (Fig. 1b). The values of p-GSK-3β were normalized to the levels of the ubiquitous glycolytic enzyme GAPDH – used as gel loading control. Comparing THC-treated mice with control mice, the statistical analysis showed a 2.5-fold increase in the hippocampus (p < 0.01), 1.8-fold in the cerebellum (p < 0.01), 2.3-fold in the striatum (p < 0.01), and 1.7-fold in the frontal cortex (p < 0.05) (n = 6 per treatment), concordantly with a similar pattern of the activated Akt.

Akt/GSK-3β pathway modulation by THC is dose dependent
We analyzed the dose dependency of the effect of THC on the phosphorylation of Akt and GSK-3β in the hippocampus. Different groups of mice (n = 4 per group) were treated with the following doses of THC: 0.3, 1, 10, and 20 mg/kg. Animals were killed 30 min later and the phosphorylation levels of Akt and GSK-3β were examined by western blot (Fig. 2) as described before. Akt phosphorylation increased dose dependently from 0.3 to 10 mg/kg (2.1-fold, p < 0.05), but did not show a further increase at 20 mg/kg (twofold, p < 0.01) (Fig. 2a). GSK-3β phosphorylation (Fig. 2b) followed a similar dose dependency as Akt, showing no change at 0.3 mg/kg of THC, but a significant increase at one, that reached the maximum values at 10 mg/kg (1.7-fold, p < 0.05) (Fig. 2b).

Membrane-bound Akt is involved in the signaling through CB1 receptors
Akt activation occurs during its translocation from the cytosol to the plasma membrane where it is phosphorylated.
on two critical residues, Thr308 and Ser473. Then, Akt can detach from the membrane and targets both cytosolic and nuclear substrates (for review, see Brunet et al. 2001). To better characterize the molecular events regulated by THC in brain, we performed a crude subcellular fractionation on hippocampi of mice treated either with vehicle or with THC (10 mg/kg, i.p.). Western blot analysis of the subcellular fractions (Fig. 3) revealed that the increase in p-Akt because of THC administration was exclusively located in the membrane fraction (Fig. 3(a and c)), with no changes in the level of activation associated to the cytosolic fraction (Fig. 3(b and c)). Meanwhile, the increase in p-GSK-3β associated to THC administration was observed in both membrane and cytosolic compartments to a similar extend (Fig. 3(a, b, and d)). N-cadherin and GAPDH immunoreactivity were used as markers for membrane and cytosolic fractions, respectively.

THC induces Akt phosphorylation through CB1 receptors

To assess the contribution of CB1 receptors to the activation of the serine/threonine Akt kinase, mice were pre-treated with rimonabant (3 mg/kg, i.p.) or its vehicle 30 min before THC (10 mg/kg, i.p.). The levels of p-Akt and p-GSK-3β were analyzed in the hippocampus by western blot. The increase in phosphorylation of both Akt and GSK-3β kinases induced by THC was totally abolished by the pre-administration of the cannabinoid antagonist, demonstrating the involvement of the CB1 receptor in the activation of this pathway (Fig. 4). On the other hand, rimonabant administration alone did not modulate the phosphorylation of Akt or GSK-3β. This critical role of CB1 receptors in the acute activation of the Akt/GSK-3 signaling cascade by THC was also observed in cerebellum, striatum, and frontal cortex (data not shown).

THC-mediated Akt phosphorylation is PI3K dependent, but independent of MAPK/ERK signaling pathway in the hippocampus

It is well established that Akt is a downstream target of PI3K (reviewed in Downward 1998). We therefore examined the role of PI3K on cannabinoid-mediated Akt phosphorylation by pre-treating mice 1 h before THC administration with the PI3K inhibitor wortmannin (1 nmol, i.c.v.). Mice were killed 30 min after THC injection (10 mg/kg, i.p.). Wortmannin reduced Akt phosphorylation induced by CB1 receptors in the hippocampus (Fig. 5a) suggesting that this phosphorylation is mediated by PI3K. Interestingly, GSK-3β phosphorylation induced by THC was non-significantly diminished, suggesting that such a GSK-3β phosphorylation might be modulated by other kinases. We therefore turned to the mitogen-activated protein kinase/extracellular signal-regulated protein kinase (ERK) pathway that has been shown to be modulated by cannabinoids by immunohistochemical
techniques in the hippocampus (Derkinderen et al. 2003) and is abundantly expressed in this area (Fiore et al. 1993), as a candidate in the phosphorylation of GSK-3β.

To assess whether MAPK/ERK signaling might be involved in the regulation of Akt or GSK-3β by THC, mice received an injection of SL327 (50 mg/kg, i.p.), a selective inhibitor of the mitogen-activated protein kinase or vehicle (DMSO), 1 h before THC (10 mg/kg, i.p.) administration. Then, 30 min after THC injection hippocampi were processed for immunoblot. THC administration did not modify the phosphorylation levels of ERK1/2 detected by immunoblot, in agreement with previous results from Rubino et al. 2004; Administration of SL327 in mice resulted in a marked reduction of phospho-ERK1/2 levels (Fig. 5b), demonstrating that drug treatment had worked appropriately. Nevertheless, inhibition of ERK phosphorylation did not result in a decrease of p-Akt nor p-GSK-3β levels (Fig. 5b), indicating that ERK signaling is not contributing to the phosphorylation of Akt or GSK-3β induced by THC in the mouse hippocampus.

THC-induced Akt activation is independent of dopaminergic system in the striatum

A common neurochemical effect of all drugs of abuse, including THC, is the induction of dopamine (DA) released

Fig. 2 Delta9-tetrahydrocannabinol (THC) produces a dose-dependent phosphorylation of Akt and glycogen synthase kinase 3 beta (GSK-3β) in the hippocampus. Western blot analysis of hippocampal whole cell lysates at different THC doses (0.3, 1, 10, and 20 mg/kg, i.p.) was performed 30 min after drug administration (n = 4 mice/group). (a) Densitometric values were analyzed for the density of p-Ser473-Akt related to that of Akt in THC-treated mice compared with vehicle-treated mice. (b) Densitometric values for the density of p-Ser9-GSK-3β related to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) values in THC-treated mice compared with vehicle-treated mice. Results represent mean ± SEM. *p < 0.05; **p < 0.01; and ***p < 0.001 as compared with vehicle-treated mice.

Fig. 3 Subcellular localization of phosphorylated Akt and glycogen synthase kinase 3 beta (GSK-3β) in hippocampus. Hippocampal samples from vehicle-treated and delta9-tetrahydrocannabinol (THC)-treated mice were subcellularly fractionated into crude membrane fraction (a and filled bars) and cytosolic fraction (b and open bars), and the phosphorylation of Akt and GSK-3β promoted by THC was measured in both fractions and compared with that observed in vehicle-treated mice (n = 6 mice/group). Activated Akt was observed mainly in the membrane fraction (a and filled bars on c) of THC-treated mice, but not in cytosolic fraction (b and open bars on c). GSK-3β phosphorylation was observed in both subcellular compartments (a, b, and d). N-Cadherin was used as a marker for membrane fraction and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as a marker for cytosolic fraction. Results represent mean ± SEM. *p < 0.05; **p < 0.01 as compared with vehicle-treated mice.
in the nucleus accumbens (NA) (Chen et al. 1990; Tanda et al. 1997; Malone and Taylor 1999). DA receptor stimulation is also involved in THC-induced ERK phosphorylation, as the D1-receptor antagonist SCH 23390 totally blocked THC-induced Akt phosphorylation in the dorsal striatum and NA, and the D2-receptor antagonist raclopride significantly reduced the levels of phosphorylated ERK in these areas (Valjent et al. 2001). Therefore, we evaluated the involvement of the dopaminergic system in THC-induced Akt phosphorylation in the striatum. For this purpose, mice were pre-treated with D1 (SCH 23390, 0.3 mg/kg, i.p.) or D2 (raclopride, 0.3 mg/kg, i.p.) receptor antagonists, followed by the acute injection of THC (10 mg/kg, i.p.) 30 min later, as described previously (Valjent et al. 2001). SCH 23390 induced a significant decrease on Akt phosphorylation by itself, while raclopride had no effect on Akt phosphorylation (Fig. 6). Mice were killed 30 min after THC administration. Western blot analysis of the striatal area showed no reduction in THC-induced Akt phosphorylation after the administration of the D1-receptor antagonist SCH 23390 (Fig. 6a), nor in the phosphorylation of its main downstream target GSK-3β. On the other hand, the D2 receptor antagonist raclopride (Fig. 6b) did not modify the activation of Akt produced by THC. These results suggests that the activation of Akt observed in the striatum is independent of the dopaminergic stimulation produced by THC.

**Discussion**

This study demonstrates for the first time the activation of Akt in several brain areas *in vivo* after acute administration of THC, that is associated to the phosphorylation/inactivation of GSK-3β, which has been reported to play a protective effect against apoptosis (Jope and Johnson 2004). The modulation of both kinases by THC was mediated by CB1 receptors and PI3K, in a dose-dependent manner, and was independent of the activation of MAPKs in the hippocampus. Moreover, in the striatum, the modulation of Akt/GSK-3β by THC did not depend on dopaminergic transmission.

Considerable evidence exists demonstrating that cannabinoids play a role as neuroprotective agents by both receptor-dependent (reducing Ca²⁺ conductances and excitability) and receptor-independent mechanisms (anti-oxidative properties of cannabinoid compounds) (reviewed in Sarne and Mechoulam 2005). The signaling events involved in this beneficial action produced *in vivo* are largely unknown. PI3K/Akt pathway promotes cell survival by both enhancing the expression of anti-apoptotic proteins and inhibiting the activity of pro-apoptotic ones. Direct intracellular targets of PI3K/Akt involved in the control of apoptosis include Bad, caspase 9, transcription factors of the Forkhead family, and GSK-3β (reviewed in Brunet et al. 2001). The ability of cannabinoids to activate the pro-survival PI3K/Akt pathway has been reported in some *in vitro* studies and may account for their protective role (Gomez Del Pulgar et al. 2002; Molina-Holgado et al. 2002, 2005). Nevertheless, the signaling events mediated by CB1-receptor stimulation *in vivo* remains poorly understood.

The results presented herein show that *in vivo* acute THC administration in mice activated Akt by enhancing Ser473 Akt phosphorylation in both the hippocampus, cerebellum, striatum and, to a minor extend, in the frontal cortex. This effect was common to all the brain areas tested, supporting the idea that this signaling mechanism is closely related to the activation of CB1 receptors in the brain. The activation of Akt was dose dependent with a modest effect at 0.3 mg/kg of THC, reaching the maximum peak at 10 mg/kg. Therefore, the

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**Fig. 4** Pharmacological blockade of CB1 receptors prevents the delta9-tetrahydrocannabinol (THC)-induced phosphorylation of both Akt and glycogen synthase kinase 3 beta (GSK-3β) in the hippocampus. Mice were pre-treated with rimonabant (RIM, 3 mg/kg, i.p.), a CB1-receptor antagonist, 30 min before THC (10 mg/kg, i.p.) administration and killed 30 min later (n = 6 mice/group). Western blot analysis of hippocampal samples showed a complete blockade of THC-induced Akt and GSK-3β phosphorylation by the cannabinoid antagonist rimonabant. Densitometric values for p-Ser473-Akt were normalized by those of Akt detection and p-Ser9-GSK-3β were normalized by those of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) detection. Results represent mean ± SEM. **p < 0.01 as compared with vehicle–vehicle treated mice; *p < 0.05 as compared with vehicle–THC treated mice.**
A dose of 10 mg/kg was used to characterize this signaling pathway in vivo.

Experiments of subcellular localization addressing the mechanism of activation of Akt by THC in the hippocampus showed that the pool of Akt phosphorylated at Ser473 corresponded to that associated to the cellular membrane. On the other hand, the pool of cytosolic Akt did not increase its phosphorylation level as a consequence of THC administration. This is in agreement with the mechanism that has been proposed for the activation of Akt, i.e. binding through the pleckstrin homology domain of Akt to the plasma membrane and activation through phosphorylation at this location (Scheid et al. 2002). However, phosphorylated GSK-3β pools as a consequence of THC administration distributed equally between the cytosolic fraction and the membrane fraction, indicating that the inactivation of GSK-3β by THC is independent of its subcellular location (membrane vs. cytosol).

The effect of THC on the phosphorylation of Akt was prevented by the selective CB1-receptor antagonist rimonabant, discarding the involvement of CB2 receptors or other non-specific mechanisms in the acute effects of THC modulating the Akt pathway in the brain areas studied. The blockade of CB1 receptors by rimonabant also abolished the phosphorylation of GSK-3β by THC, strengthening the idea that the inactivation of GSK-3β is closely modulated by the stimulation of CB1 receptors in the brain.

CB1 receptors are coupled to a variety of downstream signal transduction pathways after the interaction with heterotrimeric G proteins (especially Gi/o) (Howlett 1998). This results in inhibition of adenylyl cyclase and decreased activation of protein kinase A (Childers and Deadwyler 1996), as well as diminished Ca²⁺ influx through voltage-gated Ca²⁺ channels (Mackie and Hille 1992). CB1-receptor stimulation can also activate signaling pathways involving MAPK-ERK (Bouaboula et al. 1995; Galve-Roperh et al. 2002; Derkinderen et al. 2003), p38 (Derkinderen et al. 2001) and c-Jun N-terminal kinase (Rueda et al. 2000), as well as PI3K (Gomez del Pulgar et al. 2000) and focal adhesion kinase (Derkinderen et al. 1996). To further characterize the phosphorylation of Akt/GSK-3β, downstream kinases of PI3K, induced by THC, wortmannin, an irreversible inhibitor of PI3K was infused, into the cerebral ventricle. Wortmannin perfusion inhibited THC-induced Akt phosphorylation in the hippocampus, corroborating the major role of PI3K in the effects of THC on Akt. However, GSK-3β

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**Fig. 5** Characterization of the signaling cascades implicated in the delta9-tetrahydrocannabinol (THC)-induced activation of Akt in the hippocampus. (a) Administration of the PI3K inhibitor wortmannin (1 nmol, i.c.v.) 1 h before THC administration prevented the phosphorylation of Akt, and diminished the phosphorylation of glycogen synthase kinase 3 beta (GSK-3β) (n = 6 mice/group). Results represent mean ± SEM. *p < 0.05; **p < 0.01 as compared with vehicle–vehicle treated mice; #p < 0.05 as compared with vehicle–THC treated mice. (b) Administration of the mitogen-activated protein kinase cascade inhibitor SL327 (50 mg/kg, i.p.) 1 h before THC injection did not modify the phosphorylation of Akt or GSK-3β detected 30 min later. Instead, SL327 decreased the phosphorylation level of p42 and p44 ERKs (n = 5 mice/group). Results represent mean ± SEM. *p < 0.05; **p < 0.01 as compared with vehicle–vehicle treated mice; #p < 0.05 as compared with vehicle–THC treated mice.
phosphorylation was reduced but not abolished by wortmannin, suggesting the existence of an alternative pathway independent of Akt activation in the phosphorylation of GSK-3β induced by THC. The wortmannin-insensitive phosphorylation of GSK-3β could involve the activity of other intracellular signaling systems, such as MAPK–ERK, protein kinase A, protein kinase C or p90Rsk (Jope and Johnson 2004). The effect of the blockade of MAPK–ERK was also investigated through the systemic injection of the mitogen-activated protein kinase inhibitor SL327. This treatment inhibited the phosphorylation of MAPK–ERK in the hippocampus, but did not modify the effect of THC on Akt/GSK-3β, discarding the possibility that GSK-3β phosphorylation is mediated by MAPK–ERK pathway. Further investigation will be needed to better characterize the modulation of GSK-3β exerted by cannabinoids through the activation of CB1 receptors.

Glycogen synthase kinase 3 beta activity is inhibited when phosphorylated by Akt at Ser9 (Cross et al. 1995). Active GSK-3β phosphorylates and thereby regulates the function of multiple substrates, including the induction of apoptosis by inhibiting survival-promoting transcription factors, such as cAMP-response element binding protein and heat shock factor-1 (Grimes and Jope 2001) and facilitating pro-apoptotic transcription factors, such as p53 (Watcharasit et al. 2002). Moreover, GSK-3β plays a crucial role in neurodegenerative disorders such as Alzheimer’s disease, contributing to the formation of neurofibrillary tangles by phosphorylating Tau, and amyloid plaques by promoting β-amyloid peptide production (Bhat et al. 2004; Jope and Johnson 2004). Furthermore, GSK-3 activity inhibition by lithium seems to underlie its mood-stabilizing properties in bipolar disorders (Gould and Manji 2005). Therefore, the relevant role of GSK-3β in several neuronal disorders underlies the current interest in identifying selective inhibitors of GSK-3β that might be therapeutically useful (Meijer et al. 2004). Therefore, the inhibition of GSK-3β here reported in several brain areas after CB1-receptor activation represents an important finding to identify a new possible therapeutic target to modulate the different neuronal functions triggered by this kinase.

Previous microdialysis studies have reported that THC, as other drugs of abuse, activates DA transmission in the dorsal striatum and NA (Chen et al. 1990; Tanda et al. 1997; Malone and Taylor 1999). On the other hand, DA, D1 and D2 agonists rapidly increase phosphorylation of Akt on Thr308 and activate the ERK pathway in primary striatal neurons in culture (Brami-Cherrier et al. 2002), and blockade of D1 receptors in vivo by SCH 23390 decreases the phosphorylation of Akt in the striatum. In our study, phosphorylation of
Akt on Ser473 in response to THC was independent of the activation of the dopaminergic system, as D1 and D2 receptor antagonists did not reduce p-Akt levels in the striatum after THC administration. These results support the idea that Akt activation by THC in the striatum is not mediated by the dopaminergic system, in contrast to its involvement on the activation of MAPK–ERK by acute THC (Valjent et al. 2001, 2004). The activation of both Akt/GSK-3 (described here) and MAPK–ERK (described elsewhere, Valjent et al. 2001, 2004) signaling pathways in the striatum by acute THC could happen simultaneously in response to CB1 activation. Activation of PI3K/Akt/GSK-3 pathway could modulate the expression and activity of genes involved in cell survival, whereas the MAPK–ERK pathway, through the activation of the dopaminergic system, would affect the motivational aspects related with THC, including the rewarding effects (Valjent et al. 2000). In this sense, a causal relationship between the activation of the ERK pathway in the striatum and immediate early genes (c-fos, Zif-268, and Egr-3) induction has been reported in response to THC (Valjent et al. 2001). On the contrary, the inhibition of the MAPK–ERK pathway by pre-treatment with SL327 did not affect the modulation of Akt/GSK-3 by THC in the striatum (data not shown).

In conclusion, the present results demonstrate for the first time in vivo that an exogenous cannabinoid, such as THC, activates the neural-protective PI3K/Akt pathway and negatively regulates GSK-3β activity in the mouse brain. These findings highlight the existence of cannabinoid-induced activation of survival signaling pathways in the brain, as previously reported in in vitro models. These molecular events provide new insights for better understand the specific mechanisms involved in the neuroprotective effects that have been reported after the activation of CB1 receptors by cannabinoid agonists.

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