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Cannabinoids inhibit sodium-dependent, high-affinity excitatory amino acid transport in cultured rat cortical astrocytes

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ABSTRACT

Cannabinoids have been shown to increase the extracellular levels of glutamate in vivo and in vitro, but no studies have evaluated the possible involvement of glial glutamate reuptake system. The present study investigates whether cannabinoids and endocannabinoid, anandamide have an effect on astroglial excitatory amino acid (EAA) transport. The kinetics of glutamate transport was studied in rat cortical astrocytes, using the radiolabeled, non-metabolized amino acid, D-[^{3}H] aspartate in the absence or presence of cannabinoid receptor agonists. The results show that in vehicle controls the uptake of D-aspartate was rapid, sodium-dependent and saturated within the first 5 min, resulting in a K_m 7.365 ± 1.16 μmol/L (n = 5) and the maximum velocity (V_{max}) 1207 ± 51 nmol/mg protein/min. Addition of the synthetic cannabinoid analog R(+)-[2,3-dihydro-5-methyl-3-[(morpholinyl)methyl]pyrrolol][1,2,3de]-1,4-benzoxazinyl]-(1-naphthalenyl)methanone (WIN 55,212-2; 3 μmol/L) increased the K_m (26.25 ± 4.84 μmol/L) without affecting the V_{max} (1122 ± 77 nmol/mg protein/min), suggesting the inhibition was competitive and reversible. Various other cannabinoid agonists also inhibited D-aspartate uptake in a dose-dependent and stereospecific manner. The cannabinoid inhibition of EAA transport was partially blocked by the cannabinoid type-1 (CB1) receptor antagonist N-(piperidin-1-yl-5(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamidehydrochloride (SR141716A; 100 nmol/L). The inhibitory effects of WIN 55,212-2, or its endogenous counterpart anandamide were reversed by 98,059, an inhibitor of mitogen-activated kinase (MAPK) kinase (MEK). These results suggest that cannabinoids and endocannabinoids may constitute a novel class of inhibitors of astroglial glutamate transport system.

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Abbreviations: AN, anandamide; CB1, cannabinoid subtype-1 receptor; CP 55,940, (−)-*cis*-3[2-hydroxy-4-(1,1-dimethylheptyl)phenyl]-*trans*-4-(3-hydroxypropyl) cyclohexanol; DMEM, Dulbecco's modified Eagles medium; DMSO, dimethyl sulfoxide; EAA, excitatory amino acids; EAAT, excitatory amino acid transporter; GFAP, glial fibrillary acidic protein; GLAST, glutamate aspartate transporter; GLT1, glutamate transporter-1; LDH, lactate dehydrogenase; MAPK, mitogen-activated protein kinase; MEK, MAPK kinase; SR141716A, N-(piperidin-1-yl-5(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamidehydrochloride; WIN 55,212-2, R(+)-[2,3-dihydro-5-methyl-3-[(morpholinyl)methyl]pyrrolol][1,2,3de]-1,4-benzoxazinyl]-(1-naphthalenyl)methanone; WIN 55,212-3, S(−)-[2,3-dihydro-5-methyl-3-[(morpholinyl)methyl]pyrrolol]1,2,3de]-1,4-benzoxazinyl]-(1-naphthalenyl)methanone

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1. Introduction

Cannabinoids and their endogenous counterparts, the endocannabinoids, induce various neurobehavioral and neurochemical changes, including changes in memory, locomotion, and cognition [1]. These functions appear to be controlled by the activation of cannabinoid subtype 1 (CB1) receptors, which are distributed mainly in the hippocampus, striatum, and cortex [2]. Previous studies have shown that CB1 receptor activation controls motor function by inhibiting the release of glutamate from presynaptic neuroterminals [3]. Acute in vivo and in vitro exposure to cannabinoids has shown to increase the extracellular levels of endogenous glutamate [4]. Subsequently another study demonstrates that cannabinoids affect synaptic transmission by regulation of both glutamate release and re-uptake [5]. However, the cellular and molecular mechanisms by which cannabinoids increase extracellular levels of glutamate remain largely unknown.

In the brain, extracellular glutamate is normally kept at a low concentration ($<1\ \mu M$) by robust Na^+-dependent high-affinity glutamate transporters [6,7]. At least two of the glutamate transporters, EAAT1 and EAAT2 (corresponding to the rat homolog, glutamate aspartate transporter [GLAST] and to glutamate transporter-1 [GLT1], respectively) are localized in brain astrocytes [8–10]. Inhibition of astroglial glutamate transporters has been shown to increase extracellular levels of glutamate, which by spilling over to the nearest synaptic terminal activates metabotropic glutamate receptors, thereby enhancing the brain motor activity [11]. It is unclear whether cannabinoid-induced changes in motor activity involve inhibition of glutamate transport system. The purpose of the current study was to determine whether cannabinoids affect astroglial glutamate transport in cultured rat cortical astrocytes. The results show that cannabinoids inhibited the excitatory amino acid (EAA) uptake by increasing the K_m without affecting the V_{max}. The cannabinoid inhibition of EAA transport was partially reversed by the cannabinoid CB1 receptor antagonist SR141716A, and completely reversed by a non-specific inhibitor of mitogen-activated kinase (MAPK). These results suggest that cannabinoids and endocannabinoids may be a novel class of competitive inhibitors of astroglial glutamate transport system affecting brain motor activity.

2. Materials and methods

2.1. Materials

Chemicals and reagents used in this study were obtained from the following companies: Dulbecco's modified Eagles medium (DMEM), GIBCO (Grand Island, NY, USA); fetal bovine serum, Hyclone (Atlanta Biologicals, GA, USA); D-[^{3}H] aspartate (sp.activity, 35.0 Ci/mmol), Amersham Life Sciences (Arlington Heights, IL, USA); non-radioactive D-aspartate, Sigma chemical company (St. Louis, MO, USA); anandamide, and its non-hydrolyzable derivative, methanandamide, the synthetic cannabinoid analogs, CP 55,940, (−)-*cis*-3[2-hydroxy-4-(1,1-dimethylheptyl)phenyl]-*trans*-4-(3-hydroxypropyl)cyclohexanol, R(+)-[2,3-dihydro-5-methyl-3-[(morpholinyl)methyl]pyrrolol]1,2,3de]-1,4-benzoxazinyl]-(1-naphthalenyl)methanone (WIN 55,212-2), the inactive stereoisomer S(−)-[2,3-dihydro-5-methyl-3-[(morpholinyl)methyl]pyrrolol]1,2,3de]-1,4-benzoxazinyl]-(1-naphthalenyl)methanone (WIN 55,212-3), were from Tocris, Inc. (Ellisville, MO, USA) and *N*-(piperidin-1-yl-5(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1*H*-pyrazole-3-carboxamidehydrochloride (SR141716A) was supplied through the National Institute on Drug Abuse Chemical Synthesis and Drug Supply Program (Bethesda, MD, USA).

2.2. Drugs

Stock solutions (10 mmol/L) of the cannabinoid agonists CP 55,940, WIN 55,212-2, its inactive stereoisomer, WIN 55,212-3, the CB1receptor antagonist SR141716A, and the MEK inhibitor and PD 98059 were all prepared in DMSO. The respective stock solutions were further diluted, unless otherwise mentioned, in sodium-containing buffer or DMEM to the required concentration just before each experiment (the final concentration of DMSO ranged between 0.0001 and 0.05%). The ethanol stock solutions of anandamide (14 mmol/L), methanandamide (9.7 mmol/L) were evaporated under nitrogen and suspended in DMSO to their original concentration.

2.3. Astrocyte cell culture

Animals were used in accordance to the guidelines stipulated by the National Institute of Health and the protocols approved by the Institutional Animal Care and Use Committee of Texas Southern University. Primary cultures of rat cortical astrocytes were grown from 1-day-old Sprague–Dawley rats (Harlan, Sprague–Dawley, Houston, TX) as previously described [12,13]. The cellular homogeneity of the primary cultures of rat cortical astrocytes was previously established [12] by the criteria of immunocytochemical staining with anti-glial fibrillary acidic protein (GFAP), the known marker for astrocytes, and the cultures were found to be >90% homogeneous. After 12–14 days in culture, the confluent cell monolayer containing glial GFAP-positive cells was washed twice in Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline (PBS) and lifted by trypsinization (0.15% trypsin in PBS containing 0.2 mM EDTA). Cells were sedimented by centrifugation for 10 min at $1000 \times g$ and suspended in DMEM containing 10% fetal bovine serum and penicillin/streptomycin mixture (Sigma–Aldrich, USA). After 12–14 days, the primary cells were seeded into 24-well culture plates (35,000–50,000 cells/well) and maintained for 3–5 days before use.

2.4. Transport assays and kinetic studies

The transport assay procedure used was essentially identical to that previously described [14], with minor modifications. Astrocytes were washed three times with 1 mL of either sodium- or choline-containing buffer (Tris base 5 mM; HEPES, 10 mM; NaCl or choline chloride, 140 mM; KCl, 2.5 mM; $CaCl_2$, 1.2 mM; MgCl2, 1.2 mM; K_2HPO4, 1.2 mM; and dextrose, 10 mM). The cells were preincubated for 10 min in the respective buffers. The incubation was continued for another 10 min after adding various concentrations of D-[^{3}H] aspartate (0.01, 0.1, 1.0, 3.0, 10.0, 30.0, and 100 μmol/L) in atotal

volume of 500 μL. The uptake was terminated by gentle washing of the cells three times each with 1 mL ice-cold choline-containing buffer. Cells were lysed in 500 μL of 1N HCL and a 250 μL aliquot was counted for radioactivity by scintillation spectrometry (Beckman, LS 6000 SC). Sodium-dependent uptake was calculated as the difference between the amounts of radioactivity accumulated in the cultures incubated in sodium- and choline-containing buffers. In some experiments, cells were incubated in sodium-buffer containing WIN 55,212-2 (3 μmol/L) and various concentrations of D-[^{3}H] aspartate to examine the effect of WIN 55,212-2 on the astroglial excitatory amino acid transport kinetics. In the vehicle controls the final concentration of DMSO ranged between 0.0001 and 0.05%. The highest concentration of drug contained 0.05% DMSO and it was not toxic to cells as determined by LDH assay and the uptake was equal to that of untreated controls.

2.5. Concentration–response curves

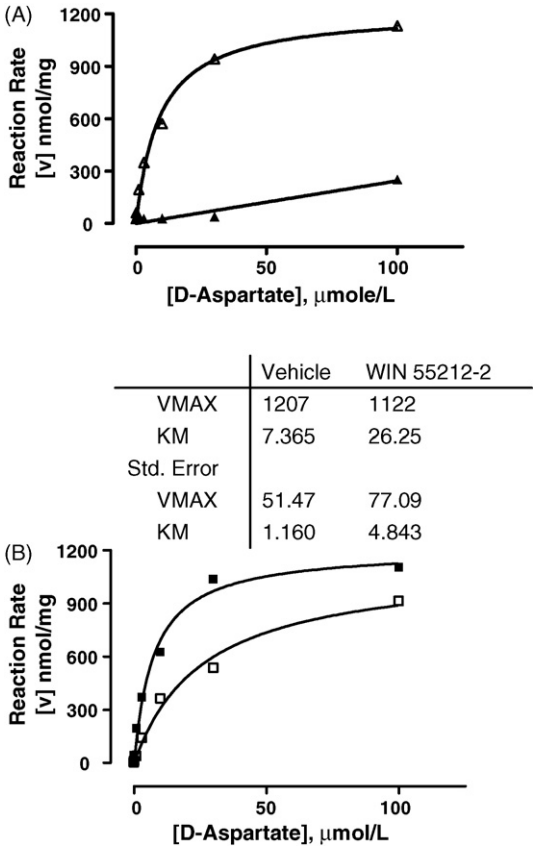
In the next set of experiments, dose response curves were generated with various concentrations of the cannabinoid agonists CP 55,940, WIN 55,212-2, its inactive stereoisomer WIN 55,212-3, and anandamide. The washed monolayer of astrocytes was incubated in duplicate for each concentration of cannabinoid in the uptake solution (10 × concentration) containing D-aspartate (10 μmol/L) and 0.1 μCi D-[3H] aspartate/well. After 10 min the uptake was terminated and the cell-incorporated radioactivity was determined as described above. The effects of various doses of cannabinoid CB1 receptor-selective antagonist SR141716A (0.1–10 μmol/L) were determined and compared with the highest concentrations of vehicle-DMSO (0.05%, v/v) and untreated controls. In some experiments, the effect of WIN 55,212-2 (3 μmol/L) on the D-aspartate uptake was measured in the absence or presence of cannabinoid CB1 receptor-selective antagonist SR141716A (0.1 μmol/L), or a non-specific MAP kinase inhibitor PD 98059 (10 μmol/L).

2.6. LDH release assay

LDH activity was determined as a measure of cell viability. After a 10 min incuabation at 37 °C either with the highest concentration of vehicle (DMSO 0.05%) or in the presence of highest concentration of various cannabinoid agonists (10 μmol/L) in the uptake solution containing D-aspartate (10 μmol/L) (without the radioactive isotope), aliquots of medium were assayed for LDH activity using a diagnostic LDH kit (Sigma Chemical Co., St. Louis, MO) according to the procedure described by the manufacturer, and the enzyme activity was expressed as percent of total activity per milligram of protein.

2.7. Protein estimation

Protein concentrations were determined using BSA as standard by the dye-binding method according to the procedure described by the manufacturer (BioRad, Hercules, CA, USA), and the values were normalized to mg protein/1 × 10^6 cells for data analysis.



	Vehicle	WIN 55212-2
VMAX	1207	1122
KM	7.365	26.25
Std. Error		
VMAX	51.47	77.09
KM	1.160	4.843

Fig. 1 – Saturation plots showing the rate of D-aspartate uptake in neonatal rat cortical astrocytes. (Panel A) Saturation plots showing the kinetics of D-[3H]aspartate uptake measured over a period of 10 min in sodium-containing (open triangle) or choline-containing buffer (solid triangle) at increasing concentrations of D-aspartate. The rate of sodium-dependent and sodium-independent (nonspecific uptake) uptake was measured as described in Section 2. (Panel B) Saturation plots of D-aspartate uptake in the control (absence of WIN 55,212-2; solid square) or in the presence of WIN 55,212-2 (open square; 3 μmol/L). The base-line corrected kinetic curve fitting graph showing the best curve fit after subtracting the non-specific uptake from the total uptake. The table in B shows the values for the kinetic constants at half maximal velocity (K_m) in μmol/L and maximum velocity (V_{max}) in nmol/mg of protein with standard errors. Data shown are mean of triple determinations from three independent cultures.

2.8. Data analysis

Data values are expressed as mean ± S.E.M. for at least three experiments done in duplicates unless otherwise indicated. The transport velocity as a function of substrate concentration was plotted by ''curve fitting'' of the dose response curve. The cell-associated radioactivity (dpm/well) from three independent experiments was used to calculate the concentration of D-asparatate (nmol/L) and was expressed as mean with standard error. The K_m, and V_{max} for D-aspartate transport, EC_{50} values for inhibition of aspartate transport, and linear regression

analyses were calculated with Graph Pad PRISM version 4.0 (Graph Pad Software, Inc., San Diego, CA, USA). The initial velocity (Km) and the maximum velocity (V_{max}) were calculated from the Lineweaver–Burk equation using the GaphPad software. Significance at $^{*}P < 0.05$ or $^{**}P < 0.001$ was calculated by unpaired *t*-test analysis.

3. Results

3.1. Kinetics of D-aspartate transport

Transport kinetics was examined using the non-metabolized amino acid D-[^{3}H] aspartate in the secondary cultures of astrocytes. Secondary cultures of astrocytes were used because these cultures were found to contain very few microglial cells as previously determined [12]. The initial rate of uptake increased almost linearly with increasing concentrations of D-[^{3}H] aspartate and with further increase formed a rectangular hyperbola (Fig. 1). The uptake was sodium-dependent, as evident from the data showing a drastic reduction in the D-aspartate uptake when sodium in the incubation buffer was replaced by choline chloride (Fig. 1A). The uptake was also time-dependent, attaining a maximum within the first 5 min (data not shown). The baseline-corrected substrate velocity graph (Fig. 1B) showed that one-half of maximum velocity (K_m) in the untreated cells was 7.365 ± 1.32 μmol/L ($n = 5$) and the maximum velocity (V_{max}) was 1207 ± 57 nmol/mg protein/min, as determined by double-reciprocal or Lineweaver–Burk plot (Fig. 1B table). Incubation of astrocytes with the synthetic cannabinoid analog WIN 55,212-2 (3 μmol/L) decreased the initial rate of uptake and resulted in a three-fold increase in the K_m for D-aspartate uptake (26.25 ± 5.23 μmol/L) without affecting the V_{max} (1122 ± 93 nmol/mg protein/min) (Fig. 1B), indicating that WIN 55,212-2 inhibited the high affinity, sodium-dependent EAA transport system and that the inhibition was competitive and reversible at higher substrate concentration.

3.2. Comparative effects of cannabinoid analogs

The concentration–response curves for various cannabinoid analogs (Fig. 2A) show that the synthetic analogs CP 55940 and WIN 55,212-2 were the most potent inhibitors of D-aspartate uptake with a $-\log EC_{50}$ of 223 ± 10.6 and 522 ± 12 nM, respectively (Table 1). The less active stereoisomer WIN 55,212-3 showed no inhibition or very weak inhibition only at the highest concentrations (10 μmol/L), indicating a stereospecific effect. Table 1 shows EC_{50} values for all the analogs that were tested in this study. The synthetic cannabinoids CP 55,940 and WIN 55,212-2 inhibited astroglial D-aspartate uptake with EC_{50} values in the nanomolar range, whereas the endocannabinoid anandamide inhibited glutamate uptake with EC_{50} values at micromolar range. The relative potency of various cannabinoids to inhibit glutamate uptake was in the order CP 55,940 > WIN 55,212-2 ≫ AN. Furthermore, the concentration–response effect of SR141716A (0.1–10 μmol/L range) alone resulted in a dose-dependent inhibition of aspartate uptake (Fig. 2A inset).

The LDH assay data (Fig. 2B) showed that the highest doses of cannabinoid analogs used in this study released not more than 10% of the total LDH activity, implying that cannabinoid inhibition of glutamate uptake was not due to failure of the membrane gradient.

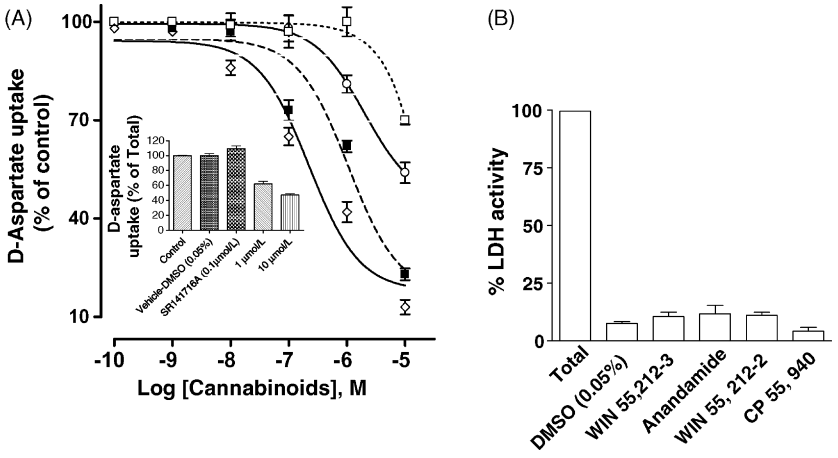


Fig. 2 – Log-concentration–response curves for the inhibition of D-aspartate uptake and cell viability. (Panel A) Cortical astrocytes that were incubated in the presence of WIN 55,212-3 (open squares), anandamide (open circles), WIN 55,212-2 (closed squares), or CP 55,940 (open diamonds) over the concentration range of 10^{-9} to 10^{-5} M ($n = 6$) and D-[^{3}H] aspartate (10 μmol/L) in sodium-containing buffer. Data are plotted as percentage change (inhibition) from control (uptake in the absence of added cannabinoids: 117 ± 17 nmol/mg) and EC_{50} values were estimated by non-linear regression analysis. Data are mean values ± S.E.M. for triple determinations from three independent cultures. The inset shows the effect of various concentrations of cannabinoid CB1 receptor-specific antagonist, SR141716A on D-aspartate uptake in the absence of any exogenously added agonists. Panel B shows the %LDH activity released into the incubation medium by the highest concentrations of each cannabinoid analog as calculated according to the manufacturer (Sigma–Aldrich, USA) suggested experimental procedure.

Table 1 – Relative EC_{50} values[a] for cannabinoid inhibition of D-aspartate uptake into cortical astrocytes

Cannabinoids	EC_{50} (nM)
Anandamide	1998 ± 134
CP 55,940	223 ± 10.6
WIN 55,212-3	UC[b]
WIN 55,212-2	522 ± 12

[a] Values are mean ± S.E.M. calculated from the non-linear regression curves from Fig. 2 using GraphPad Software version 4.0.
[b] Unable to calculate the EC_{50} from the regression curves.

3.3. *Effects of CB1 receptor antagonist and MAPK inhibitor*

The next sets of experiments were performed to determine the possible cellular mechanisms of cannabinoid inhibition of astroglial glutamate uptake. The data in Fig. 3 show that WIN 55,212-2-induced inhibition of D-aspartate uptake was blocked by the CB1 cannabinoid receptor antagonist SR141716A. However, SR141716A (0.1 μmol/L), in the absence of WIN 55,212-2, inhibited about 12% of D-aspartate uptake. In astrocytes, the activation of CB1 receptors has been shown to activate MAP kinases pathway, which consists of three protein kinases: a MAPK kinase kinase (MEKK) that activates a MAPK kinase (MEK) which, in turn, activates the terminal MAPK/ERK or extracellular regulated kinase enzyme activity [15]. Since the MAP kinases have been linked to regulation of glutamate transporters [16], the effect of a non-specific MEK inhibitor PD 98059 (10 μmol/L) on the D-aspartate uptake was tested in the absence or presence of WIN 55,212-2. As shown in Fig. 3, the MEK inhibitor completely reversed the WIN 55,212-2 induced inhibition of glutamate uptake and produced a slight stimulatory response (14%) over the control, implying that cannabinoid inhibition of D-aspartate uptake may involve activation of MAP kinase signaling pathway.

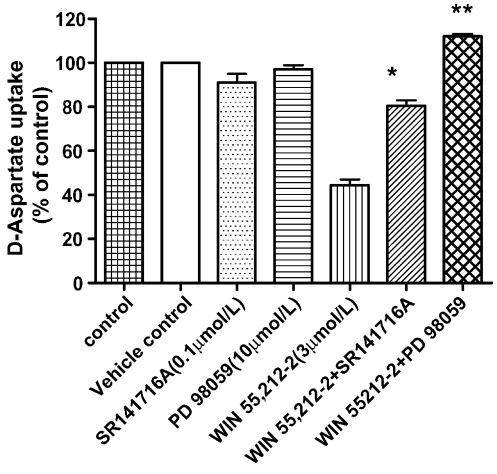


Fig. 3 – Bar diagram showing the effects of SR141716A, or PD 98059, on WIN 55,212-2 inhibition of D-aspartate uptake in cortical astrocytes. D-[3H] Aspartate uptake was measured for 10 min at 37 °C in intact astrocytes as described in Section 2. The control uptake (117 ± 17 nmol/mg; unfilled bar) in the medium or in the presence of DMSO (final < 0.05%) was considered as 100% uptake to calculate the percent of aspartate uptake by SR 141716A(0.1 μmol/L; dotted bar), or PD 98059 (10 μmol/L; horizontal line bar). The effect of WIN55212-2 (3 μmol/L) on the uptake of D-aspartate was measured in the absence (vertical line bar), or in the presence of SR141716A(0.1 μmol/L; angled line bar), or with MEK inhibitor PD 98059 (10 μmol/L; crossed line bar). The data values are mean of three experiments done in duplicate with SM deviation.

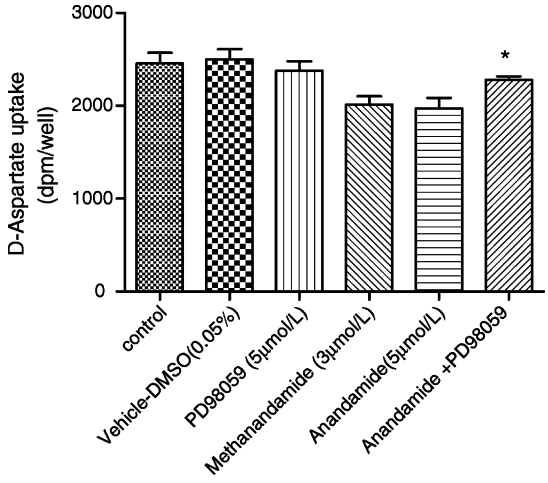


Fig. 4 – Bar diagram showing the effect of MAPK inhibitor, PD 98059 on endocannabinoid anandamide inhibition of -aspartate uptake in cortical astrocytes. D-[3H] Aspartate uptake was measured for 10 min at 37 °C in intact astrocytes in the absence or presence of PD 98059 (5 μmol/L) as described in Section 2. The vehicle controls were incubated with the highest concentration of DMSO present in a particular drug solution (0.05%). The data show that aspartate uptake in 0.05% DMSO vehicle was not different from that observed in untreated control cells. The data values are mean of triplicates with SM deviation of a single experiment.

Next the effect of MAPK pathway on endocannabinoid anandamide inhibition of aspartate uptake was determined. As shown in Fig. 4, aspartate uptake was about 79% of the control in the presence of anandamide (5 μmol/L). Anandamide inhibition of aspartate uptake was reversed by PD 98059 (5 μmol/L), and it was statistically significant ($^{*}P < 0.05$), indicating the possible contribution of MAPK pathway. The inhibition of aspartate uptake by methanandamide (3 μmol/L), a methylated stable derivative of anandamide, was not very different from that of anandamide (70% of control).

4. Discussion

This study demonstrates for the first time that cannabinoids competitively inhibit sodium-dependent, high affinity EAA transport system. This is evident from the kinetic data showing that cannabinoids altered the K_m but not the V_{max} of non-metabolized EAA, D-aspartate transport in astroglial cells. Among the cannabinoid agonists tested, CP 55,940 and WIN 55,212-2 are the most potent inhibitors of astroglial EAA

transport. Cannabinoid inhibition of EAA transport was stereospecific: the less active isomer WIN 55,212-3 failed to show any significant inhibition compared to its active isomer WIN 55,212-2. Furthermore, WIN 55,212-2 inhibition of EAA uptake was partially antagonized by the CB1 receptor antagonist SR141716A, and fully reversed by the MEK inhibitor PD 98059. Furthermore the MEK inhibitor also reversed the aspartate uptake inhibition by the endocannabinoid anandamide. These results suggest that cannabinoids are a novel class of competitive inhibitors of EAA transporters and the inhibition may be regulated by cross-talk between multiple signaling pathways, mainly cannabinoid CB1 receptor-dependent/independent activation of MAP kinases pathway. In the present study, the synthetic cannabinoids CP 55,940 and WIN 55,212-2 inhibit astroglial D-aspartate uptake with EC_{50} values in the nanomolar range. This observation is consistent with the previous in vivo and in vitro studies wherein acute and low doses of WIN 55,212-2 caused an elevation in the extracellular levels of endogenous glutamate [4]. In their subsequent studies, the authors showed that a decrease in the release of glutamate in response to stimulation by potassium in awake rats that were chronically treated in utero with WIN 55,212-2 [17–19]. While these differences in the extracellular levels of glutamate in acute and chronically treated rats are unknown, these findings clearly suggest that cannabinoids play a vital role in brain glutamate neurotransmission. However, it is not clear from these studies whether the increased levels of extracellular glutamate is due to inhibition of glutamate transporters and the decreased levels of glutamate release is due to subsequent depletion of glutamate storage rather than inhibition of release. The decrease in the release may be possible as a result of feed-back inhibition by prolonged activation of glutamate receptors [5]. Similarly, an initial increase in the extracellular levels of glutamate may be due to inhibition of glutamate transport. Since the present study demonstrates that the kinetics of inhibition is competitive and reversible at higher concentrations of substrate, it is conceivable to predict that a steady increase in the extracellular levels of glutamate concentration may overcome the cannabinoid-inhibition, thus making this process reversible and transient. At this point it is unclear whether the glutamate homeostasis is regulated by the brain endocannabinoids, anandamide in particular.

In the present study, the ability of anandamide to inhibit D-aspartate transport is interesting because, it suggests that endocannabinoids may play a role in the modulation of glutamate transport system. However, the concentration of anandamide required to inhibit glutamate transport was in the micromolar range and this may be due to its rapid degradation into arachidonic acid and ethanolamine by fatty-acid amide hydrolase. This may not be the case because there is only a slight increase in the aspartate inhibition by methanandamide, the FAAH resistant, methylated derivative of anandamide. However, the existence of fatty-acid amide hydrolase activity has not been clearly demonstrated in cortical astrocytes [13]. Therefore the observed low potency for anandamide may be due to its own rapid transport into astroglial cells, since astroglial cells are known to take up anandamide through a novel transporter system [20]. Further studies are needed, addressing these issues in more details.

The fact that the CB1 receptor antagonist SR141716A partially reversed WIN 55,212-2 inhibited glutamate uptake, suggests the possible involvement of CB1 receptors in the regulation of EAA transport. However, SR141716A alone inhibited EAA uptake in a dose-dependent manner, suggesting an agonist-like activity, or an activity unrelated to CB1 pathway. This agonist-like activity of SR141716A has previously been shown to be responsible for hyperactivity [21]. Since the brain locomotor activity appears to be mainly regulated by the glutamate neurotransmitter, it remains to be further elucidated whether SR141716A-induced hyperactivity may be due to an increase in extracellular glutamate concentration.

It is interesting to note that cannabinoids represent a novel class of glutamate transport inhibitors with potencies several-fold higher than the known glutamate transport inhibitor drugs, such as dihydrokianate and L-*trans* pyrrolidine-2,4-dicarboxylate (L-*trans* PDC). Of these, L-*trans* PDC, a structural analog of glutamate, inhibits glutamate uptake in cultures of astrocytes and neurons with IC_{50} value around 50 μmol/L, while dihydrokianate is a weak inhibitor requiring mmol/L concentrations [22,23]. As far as the mechanisms of action of glutamate uptake inhibitors is concerned, L-*trans* PDC does not seem to alter ligand binding to the EAA receptors, does not interact with EAA receptors, and does not directly cause inward currents in primary hippocampal neurons [23]. In contrast, dihydrokianate directly activates postsynaptic EAA receptors [23].

In the present study, the complete reversal of WIN 55,212-2 inhibition of D-aspartate uptake by the MEK inhibitor PD 98059 suggest the possible involvement of the ERK1/2 signaling pathway. This is in agreement with a previous study demonstrating regulation of glutamate transporters by the MAPK signaling pathway [16]. The coupling of the MAPK pathway to cannabinoid CB1 receptor activation has been demonstrated in cultured astrocytes [15]. The MAPK kinase pathway activated by cannabinboid receptor agonists may be directly responsible for the ultimate covalent modification of astroglial glutamate transporters, or may act indirectly by phosphorylation of other target enzymes such as cytosolic phospholipase A_2, as previously demonstrated [24,25]. The MAPK-mediated activation of cytosolic phospholipase A_2 may release fatty acids, mainly arachidonic acid. Arachidonic acid is one of the fatty acids, the accumulation of which is known to inhibit glutamate uptake [26–29]. It should also be noted that other chemical agents, such as mellitin, that are known to release arachidonic acid by activating phospholipase A_2, are potent inhibitors of astroglial glutamate uptake [28]. Cannabinoid-stimulation of arachidonic acid release is previously demonstrated in a variety of tissues and cell types [30,31], and in cultured neurons [32]. The present results are consistent with the author's previous study demonstrating that Δ^9-THC and anandamide release endogenous radiolabeled arachidonic acid in cultured astrocytes [13]. The CB1 receptor-mediated activation of MAPK pathway may play a central role in the regulation of glutamate transporters which in turn modulate brain glutamatergic transmission. However, the exact signaling events downstream of MAPK needs to be further elucidated in order to understand the molecular mechanisms of cannabinoid interactions with glutamate transporters that

are localized both in neurons and astrocytes. It is interesting to note that the MAPK inhibitor also reversed anandamide inhibition of aspartate uptake, requiring further evaluation on the effect of cannabinoids on MAPK pathway in the regulation of synaptic glutamate transmission. Thus, interactions between cannabinoids or their endogenous counterparts, endocannabinoids with glutamate transporters may have profound clinical implications, since alteration in glutamate neurotransmission is often associated with neurological and psychiatric diseases, such as Alzheimer's, Huntington's chorea, schizophrenia and amyotropic lateral sclerosis [33–35]. Inhibition of astroglial glutamate transporters will increase extracellular levels of glutamate, which may spill over to the nearest synaptic terminal and activate metabotropic glutamate receptors, thereby enhancing the brain's motor activity [11]. Chronic activation of metabotropic glutamate receptors can inhibit further release of glutamate from the presynaptic terminal [5]. Alternatively, glutamate transporters can increase extracellular glutamate levels by working in the reverse direction transporting glutamate out of the cells. Taken together, it is apparent that in many neurodegenerative diseases, astrocytes may lose their ability to regulate glutamate homeostasis through a malfunction of glutamate transporters, EAAT-2 in particular [36]. Therefore, future studies should be directed towards understanding the molecular mechanisms of cannabinoid interaction with different glutamate transporters, which may play a role in the development of various neurodegenerative diseases affecting memory, cognition and motor functions of the brain.

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