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ORIGINAL PAPER

Methylphenidate Increases Glutamate Uptake in Bergmann Glial Cells

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Abstract Glutamate, the main excitatory transmitter in the vertebrate brain, exerts its actions through the activation of specific membrane receptors present in neurons and glial cells. Over-stimulation of glutamate receptors results in neuronal death, phenomena known as excitotoxicity. A family of glutamate uptake systems, mainly expressed in glial cells, removes the amino acid from the synaptic cleft preventing an excessive glutamatergic stimulation and thus neuronal damage. Autism spectrum disorders comprise a group of syndromes characterized by impaired social interactions and anxiety. One or the most common drugs prescribed to treat these disorders is Methylphenidate, known to increase dopamine extracellular levels, although it is not clear if its sedative effects are related to a plausible regulation of the glutamatergic tone via the regulation of the glial glutamate uptake systems. To gain insight into this possibility, we used the well-established model system of cultured chick cerebellum Bergmann glia cells. A time and dose-dependent increase in the activity and protein levels of glutamate transporters was detected upon Methylphenidate exposure. Interestingly, this increase is the result of an augmentation of both the synthesis as well as the insertion of these protein complexes in the plasma membrane. These results favour the notion that glial cells are Methylphenidate targets, and that by these means could regulate dopamine turnover.

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Introduction

Glutamate (Glu) is the main excitatory amino acid neurotransmitter in the Central Nervous System (CNS). As many as 80–90 % of the synapses in the brain are glutamatergic [1], therefore this neurotransmitter is involved in a plethora of functions, from sensory motor information and coordination to emotions and cognition. Glu exerts its actions through the activation of specific membrane receptors that have been traditionally divided into two main categories: ionotropic (iGluRs) and metabotropic receptors (mGluRs).

A family of sodium-dependent Glu transporters, also known as excitatory amino acid transporters (EAATs) are responsible for Glu removal from the synaptic cleft [2]. Thus far, five EAAT subtypes have been described, being EAAT1 and EAAT2 preferentially expressed in glial cells and their activity represents more than 80 % of the total brain Glu uptake activity. Within the cerebellum, most of the Glu uptake takes place in Bergmann glial cells (BGC), which express exclusively EAAT1, a transporter also known as Na^+- glutamate/aspartate transporter (GLAST). In contrast, in other CNS structures, EAAT2 or Glutamate transporter 1 (GLT-1) is the major Glu carrier, in fact, it is known that this transporter represents roughly 2 % of total brain protein [3].

Glu has a critical role in brain physiology and higher brain functions [4, 5]. A tight control of its extracellular levels is critical to prevent its well-characterized neurotoxic effect. In fact, all known neurodegenerative diseases are related to an excess of extracellular Glu, as in amyotrophic lateral sclerosis (ALS) and Alzheimeŕs disease

[6, 7]. Moreover, it has also been suggested that dysregulation of Glu extracellular concentrations is involved in autism spectrum disorders (ASD) [8–10].

Autism comprises a complex neurodevelopment disorders characterized by social interaction impairment, communication and stereotyped behaviours [11, 12]. Multiple factors participate in its pathophysiology, it is known that genetic and environmental cues are involved, although the precise mechanisms underlying these disorders remain to be determined. In this scenario, the selection of an appropriate treatment is difficult.

Methylphenidate (MPH) is currently used for ASD treatment, although its efficacy is highly controversial [13]. Even though its effects over the dopaminergic system have been described, MPH also targets the glutamatergic system [10, 14]. Given the fact that Glu turnover is dependent on the *glutamate/glutamine shuttle,* a biochemical interplay between neurons and glial cells, in this contribution we focused on the effect of MPH on EAAT-1/GLAST-mediated [^{3}H]-D-Aspartate (D-Asp) uptake activity. To this end, we decided to use the model of cultured cerebellar Bergmann glial cells.

A time and dose-dependent increase in [^{3}H]-D-Asp uptake was observed, suggesting a rapid up-regulation of the Glu removal, likely to be relevant for the anti-anxiety effect of MPH. These results support the involvement of glial cells in the pathological features of complex brain disorders such as autism.

Methods

Materials

Tissue culture reagents were obtained from GE Healthcare (Carlsbad, CA, USA). D-Asp and Glu were obtained from Tocris-Cookson (St. Louis, MO, USA). [^{3}H]-D-Asp was from Perkin Elmer (Boston, MA, USA). Actinomycin D, cycloheximide, cytochalasin B, choline chloride, Rp-8-Bromo-β-phenyl-1,N2-ethenoguanosine 3′,5′-cyclic monophosphorothioate (8-Br-cGMP), bisindolylmaleimide II (Bis), 12-O-Tetradecanoylphorbol-13-acetate (TPA) and methylphenidate hydrochloride (MPH) were obtained from Sigma-Aldrich, Mexico. A 1:1000 dilution of GLAST-antibody (produced and characterized in our laboratory [15]) was used. Horseradish peroxidase-linked anti-rabbit antibodies (1:4000 dilution), and the enhanced chemiluminescence reagents were obtained from Amersham Biosciences (Buckinghamshire, UK).

Cell Culture and Stimulation Protocol

Primary cultures of cerebellar Bergmann glial cells were prepared from 14-day-old chick embryos as previously described [16]. Cells were plated in 24-well plastic culture dishes in DMEM containing 10 % foetal bovine serum, 2 mM glutamine, and gentamicin (50 μg/ml) and used on the 4th–7th day after culture. Before any treatment, confluent monolayers were switched to solution A containing 25 mM HEPES-Tris, 130 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 0.8 mM $MgCl_2$, 33.3 mM glucose and 1 mM NaH_2PO_4 at pH 7.4, for experiments that included long-term exposure to MPH, confluent monolayers were switched to non-serum DMEM media containing 0.5 % bovine serum albumin (BSA). Inhibitors were added 30 min before agonists.

[^{3}H]-D-Asp Influx

Confluent Bergmann glial monolayers seeded in 24-well plates were washed three times to remove all non-adhering cells with 0.25 ml of solution A. When indicated, NaCl was replaced by choline chloride. The [^{3}H]-D-Asp influx was initiated at $t = 0$ by the addition of 0.25 ml solution A containing 0.4 μCi/ml of [^{3}H]-D-Asp. The reaction was stopped by aspirating the radioactive medium and washing each well within 15 s with 0.25 ml aliquots of an ice-cold solution A. For the determination of the kinetic parameters, the Asp concentration was modified to a final 10, 25, 50, 100 μM concentrations with unlabelled Asp and the uptake time was 30 min [17]. The uptake was stopped as described above. The cells in the wells were then exposed for 2 h at 37 °C to 0.25 ml NaOH 0.1 M and an aliquot of the radioactivity present determined in a Perkin Elmer scintillation counter in the presence of a scintillation cocktail. Experiments were carried at least three times with a minimum of quadruplicate determinations.

[^{3}H]-D-Asp Binding

Equilibrium-binding experiments were performed in confluent Bergmann glial cells monolayers that were washed three times to remove all non-adhering cells with 0.5 ml of solution A. Cells were incubated for 30 min with or without a fixed 100 μM MPH concentration. Na^+-free solutions were prepared replacing NaCl with choline chloride. In the Ca^{2+}-free solutions, $CaCl_2$ was omitted and 5 mM EDTA was added. The [^{3}H]-D-Asp binding was initiated at $t = 0$ by the addition of 0.5 ml solution A with or without modifications containing 0.4 μCi/ml of [^{3}H]-D-Asp at 4 °C. The assay was stopped after 15 min by aspirating the radioactive medium and washing each well within 15 s with 0.5 ml aliquots of an ice-cold solution A. The cells in the wells were solubilized for 2 h at 37 °C to 0.5 ml NaOH and an aliquot of that solution mixed with scintillation cocktail and the radioactivity present determined counted in a Perkin Elmer scintillation counter.

Experiments were carried out at least three times with a minimum of quadruplicate determinations. Non-specific binding was determined in the presence of 100 μM Asp. [^{3}H]-D-Asp binding to GLAST/EAAT1 was defined as the difference between the specific binding in normal solution A minus the binding in Na^+-free solution A.

SDS-PAGE and Western blots

Cells from confluent monolayers were harvested with phosphate buffer saline (PBS) (10 mM K_2HPO_4/KH_2PO_4, 150 mM NaCl, pH 7.4) containing phosphatase inhibitors (10 mM NaF, 1 mM Na_2MoO_4 and 1 mM Na_3VO_4). The cells were lysed with RIPA buffer (50 mM Tris-HCl, 1 mM EDTA, 150 mM NaCl, 1 mM phenylmethylsufonyl fluoride, 1 mg/ml aprotinin 1 mg/ml leupeptin, 1 % NP-40, 0.25 % sodium deoxycholate, 10 mM NaF, 1 mM Na_2MoO_4 and 1 mM Na_3VO_4, pH7.4). Cell lysates were denatured in Laemmli's sample buffer, resolved through 10 % SDS-PAGE and then electro blotted to nitrocellulose membranes. Blots were stained with Ponceau S stain to confirm that protein content was equal in all lanes. Membranes were soaked in PBS to remove the Ponceau S and incubated in TBS containing 5 % dried skimmed milk and 0.1 % Tween 20 for 60 min to block the excess of non-specific protein binding sites. Membranes were then incubated overnight at 4 °C with the primary antibodies indicated in each figure, followed by the adequate secondary antibodies. Immunoreactive polypeptides were detected by chemiluminescence with a MicroChemi (DNR Bio-Imaging System). Densitometric analyses were performed and data analysed with the Prism 5, GraphPad Software (San Diego, CA, USA).

RNA Extraction and qRT-PCR

Total RNA was isolated from confluent Bergmann glial cell cultures (treated, non-treated) and extracted using the Tri-Reagent (Sigma). Every treatment condition per experiment was analysed as technical duplicates. PCR was performed in a reaction volume of 10 μl. Real-time quantitative reverse transcription-PCR (qRT-PCR) was performed by a one-step method with 20 ng of total RNA and using KAPA SYBR FAST One Step qRT-PCR system (KAPA BIOSYSTEMS). Samples were subjected to quantitative PCR (qPCR) using Step one Plus Real time PCR system (Applied Biosystems). The qPCR profile consisted of an initial cDNA synthesis by M-MuLV Reverse Transcriptase at 42 °C for 5 min, an inactivation of the reverse transcriptase at 95 °C for 5 min, followed by 40 cycles of 95 °C for 3 s, 60 °C 30 s. A melt curve stage was added. To quantify GLAST mRNA levels, we used previously reported oligonucleotides designed by our workgroup as *chGLAST*: GLAST Forward 5′-GGCTGCGGGCATTCCTC-3′ and GLAST Reverse 5′-CGGAGACGATCCAAGAACCA-3′. As an endogenous control we amplified ribosomal protein S17 mRNA with the following primers: S17 Forward 5′-CCGCTGGATGCG CTTCATCAG-3′ and S17 Reverse 5′-TACACCCGTCTG GGCAAC-3′. The relative abundance of GLAST mRNA is expressed as sample versus a control normalized to S17 chick ribosomal mRNA levels and was calculated as $2 - \Delta\Delta C_t$. Data are presented as mean values ± SDs and analysed by one-way ANOVA ($p < 0.05$ was considered statistically significant).

Statistical Analysis

Data are expressed as the mean (average) ± standard error (SEM). A one-way analysis of variance (ANOVA) was performed to determine significant differences between conditions. When this analysis indicated significance (at the 0.05 level), a Dunnett's multiple comparison analysis was used to determine which conditions were significantly different from each other with the Prism 5, GraphPad Software (San Diego, CA, USA).

Results

Glutamate Uptake is Up-Regulated by MPH in Bergmann Glial Cells

The possibility that MPH could have an important effect on the glutamatergic transmission has long been suggested [14, 18, 19]. Despite of these contributions, its effect in glial cells have not been well characterized. Therefore, we decided to investigate whether an established glial cell function such as Glu uptake, could be modified by MPH.

As depicted in panel a of Fig. 1, when the cultured cells were treated with 100 μM MPH, a significant increase in [^{3}H]-D-Asp was detected as early as 2 h after MPH and the effect was still present after 12 h. In order to characterize the effect from a pharmacological perspective, we decided to expose the cultured cells to different MPH concentrations (1, 10, 100, 1000 μM) for 1 or 4 h. The results are shown in panels b and c of Fig. 1. Note that the MPH effect is not detected after 1 h, but after a 4 h pre-incubation a significant increase in uptake is detected at a 100 and 1000 μM MPH doses. As a control of our experiments we pre-treated the cells for 30 min with 1 mM D-Asp, treatment that we have shown, down-regulates GLAST-mediated [^{3}H]-D-Asp uptake [20].

MPH Increases Plasma Membrane GLAST Transporters

The results described in the previous section, demonstrate an increase in GLAST activity after MPH treatment; this

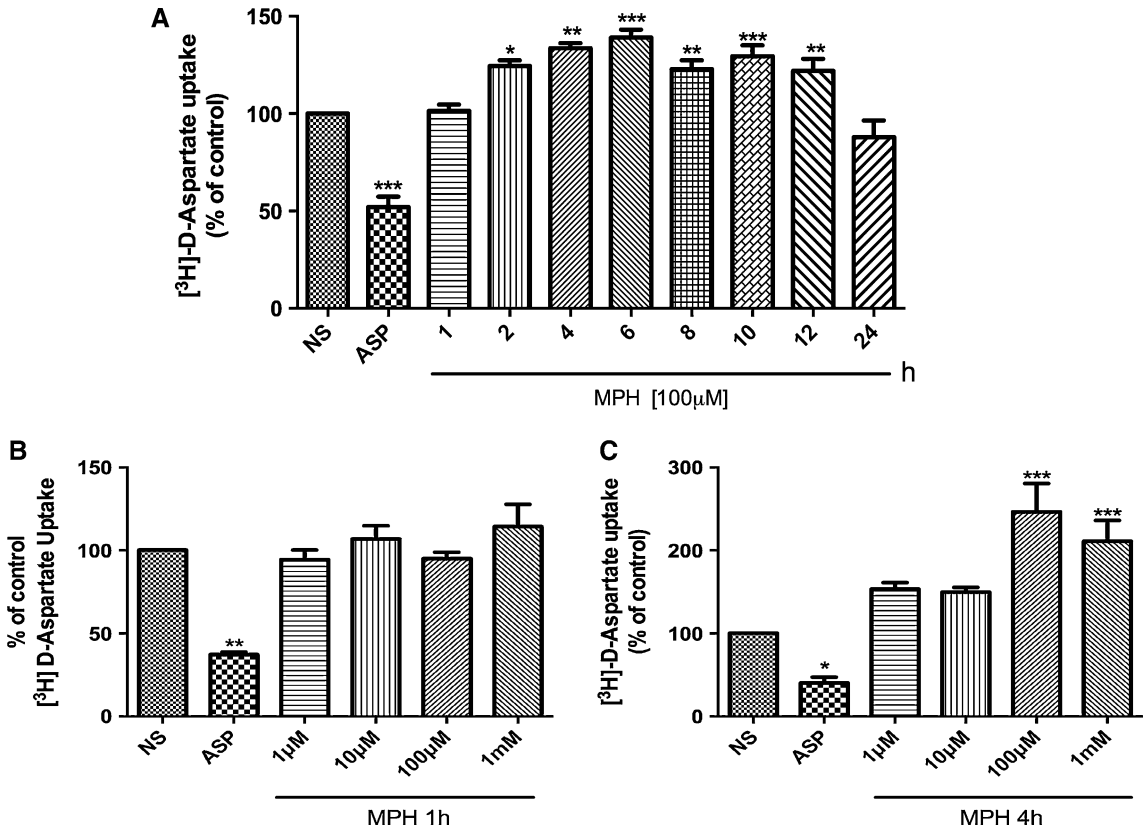

Fig. 1 Methylphenidate (MPH) increases GLAST activity. BGC were pre-incubated with MPH at a 100 μM concentration for indicated time periods (1, 2, 4, 6, 8, 10, 12 or 24 h) (**a**); or pre-incubated for 1 h (**b**) or 4 h (**c**) with increasing MPH concentrations (1, 10, 100 or 1000 μM), the [3H]-D-Asp uptake was performed for 30 min. As control of the experiment, cells were pre-incubated 30 min with 1 mM Glu [20]. *Non-stimulate* (NS), *Glutamate* 1 mM (Glu). Data are expressed as the mean ± SEM of at least three independent experiments, each tested in quadruplicate; a one-way analysis of variance (ANOVA) with a Dunnett's multiple comparison test was performed to analyse the data (***$p < 0.001$, **$p < 0.01$, *$p < 0.05$)

increase could be the result of an increased number plasma membrane transporters or a change in the affinity of the transporter towards its substrate. In order to clarify this issue, we decided to perform a Michaelis–Menten analysis of [^{3}H]-D-Asp uptake in control or MPH treated cells. The results are shown in Fig. 2 panel a. An increase in V_{MAX} (NS = 102.8 ± 13.7 pmol/min*mg, MPH = 206.4 ± 41.7 pmol/mg*min) was detected with a decrease in affinity (K_M = NS: 8.946 ± 5.2 M, MPH: 15.62 ± 10.8 M). These results suggest that MPH increases the number of Glu transporters in the plasma membrane. To confirm this interpretation, we measured the number of plasma membrane transporters via [^{3}H]-D-Asp equilibrium binding experiments. As shown in panel b of Fig. 2, MPH treatment increases plasma membrane [^{3}H]-D-Asp binding sites, an index of GLAST molecules present in that compartment. If indeed, MPH is linked to the regulation of plasma membrane GLAST molecules, manipulation of the cytoskeleton dynamics by means of treatment with cytochalasin B should be sufficient to block the MPH effect. This is the case, a pre-exposure to 50 μM cytochalasin-B for 30 min is sufficient to prevent the documented MPH increase in GLAST at the plasma membrane (Fig. 2c).

Signalling of MPH Increase in [^{3}H]-D-Asp Uptake

Previous findings from our group have demonstrated that signalling through the Ca^{2+}/diacylglicerol dependent protein kinase (PKC) or through the cGMP-dependent protein kinase (PKG) regulates GLAST. In the former case, PKC down regulates GLAST activity and even its gene expression [27, 28]. In contrast, cGMP through PKG, increases GLAST activity in a very similar manner as described in this contribution [21]. With this in mind, we pre-exposed the cultured cells to blockers of PKC, bisindlolylmaleimide I (BisI) and of PKG (Rp-8-Br-PET-cGMPS), and to a PKC activator (12-*O*-Tetradecanoylphorbol-13-acetate, TPA) prior to MPH treatment. A slight decrease in the MPH effect could be noticed in presence of the PKG blocker, although the reduction was not statistically significant. Blockage of PKC did not

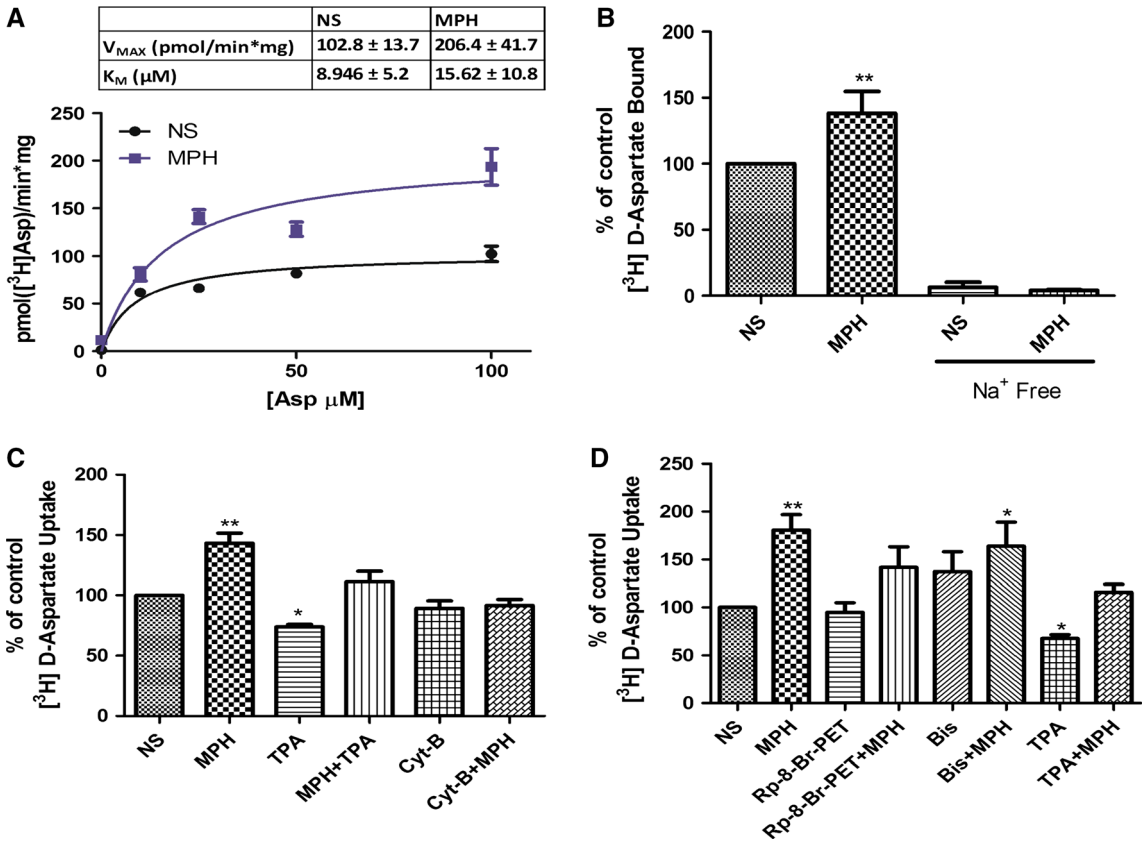

Fig. 2 MPH increases plasma membrane GLAST molecules. **a** BGC were pre-incubated with 100 μM MPH (*filled square*) or vehicle (NS) (*filled circle*) for 4 h and the [^{3}H]-D-Asp uptake assay was performed with increasing non-labelled D-Asp concentrations (0, 10, 25, 50 and 100 μM) for 30 min. Data are expressed as the mean ± SEM of at least three independent experiments, each assayed in quadruplicate. Transport kinetics V_{MAX} (pmol/min*mg) and K_M (μM) were determined by non-linear regression with Prism 5 software (GraphPad). **b** BGC monolayers were stimulated with MPH 100 μM for 4 h in normal or Na^+-free solution A. [^{3}H]-D-Asp binding assay was performed at 4 °C. Data are expressed as the mean ± SEM of at least three independent experiments, each tested in quadruplicate; a one-way analysis of variance (ANOVA) with a Dunnett's multiple comparison test was performed to analyse the data (**$p < 0.01$, *$p < 0.05$). **c** BGC were pre-incubated with 50 μM cytochalasin B (Cyt-B) for 30 min and exposed to 100 μM MPH or vehicle for 4 h; [^{3}H]-D-Asp uptake was performed for 30 min. Statistical analysis was performed as in (**b**). As experimental control, cells were pre-incubated 30 min with 12-O-Tetradecanoylphorbol-13-acetate (TPA). **d** BGC were treated for 4 h with 100 μM h MPH 4 h in the presence or absence of the indicated drugs; PKG inhibitor, Rp-8-Br-PET 10 μM, PKC inhibitor, Bisindolylmaleimide I (Bis) 2 μM, or PKC activator TPA 100 nM; the kinase inhibitors were added 30 min before MPH treatment; [^{3}H]-D-Asp uptake was performed for 30 min. Data are expressed as the mean ± SEM of at least three independent experiments, each performed in quadruplicate; a one-way analysis of variance (ANOVA) with a Dunnett's multiple comparison test was performed to analyse the data (**$p < 0.01$)

modify the MPH response, however TPA slightly decreases MPH effect. At this point, we cannot rule out the involvement of PKC or PKG in MPH signalling (Fig. 3d). A detailed analysis of these signalling cascades in the context of MPH treatment is needed to clarify this issue.

Given the fact that the MPH effect is slow, meaning it is present only after a 2 h treatment, we decided to explore the role of protein synthesis in the MPH effect. To this end, we pre-incubated the cultured cells with a 1 mM concentration of the protein synthesis inhibitor cycloheximide (CHX). As shown in panel a of Fig. 3, CHX treatment prevents the MPH-induced increase in [^{3}H]-D-Asp uptake, suggesting that MPH increases GLAST translation. Moreover, a transcription inhibitor such as actinomycin D, used at a 0.2 g/ml concentration does not modify the MPH effect, again suggesting an augmentation of GLAST mRNA translation (Fig. 3b). Accordingly, GLAST mRNA levels are not changed upon exposure of the cultured cells to 100 M MPH (Fig. 4a), whereas an increase in GLAST protein is present (Fig. 4b). These results are consistent with our interpretation that this psychostimulant regulates the efficacy of GLAST mRNA translation and its plasma membrane insertion.

Discussion

MPH is one of the most widely used CNS stimulants, commonly prescribed for attention-deficit/hyperactivity disorder, depression, obesity and autism spectrum disorders, among other mental illness [22, 23]. Although it has

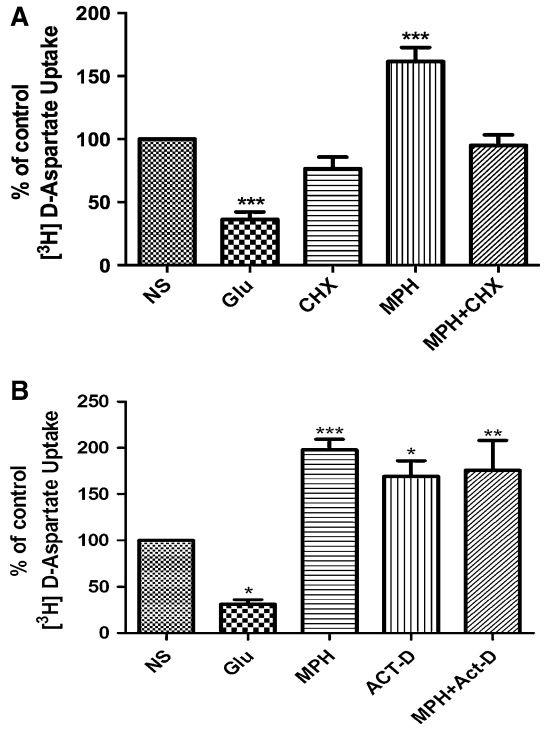

Fig. 3 MPH increases GLAST translation. **a** BGC were pre-incubated with MPH 100 μM for 4 h in the presence or absence of the protein synthesis inhibitor, cycloheximide (CHX) 1 mM, the [^{3}H]-D-Asp uptake was performed for 30 min. Data are expressed as the mean ± SEM of at least three independent experiments, each tested in quadruplicate; a Student *t test* with a Dunnett's multiple comparison test was performed to analyse the data. **b** BGC were pre-incubated with MPH 100 μM for 4 h in the presence or absence of the transcription inhibitor, actinomycin D (ACT-D) 10 μg/ml, the [^{3}H]-D-Asp uptake was performed for 30 min. In (**a**) and (**b**), the cells were pre-incubated with 1 mM Glutamate (Glu) for 30 min as a control of the experiment [20]. Data are expressed as the mean ± SEM of at least three independent experiments, each tested in quadruplicate; a one-way analysis of variance (ANOVA) with a Dunnett's multiple comparison test was performed to analyse the data (***$p < 0.001$, **$p < 0.01$, *$p < 0.05$)

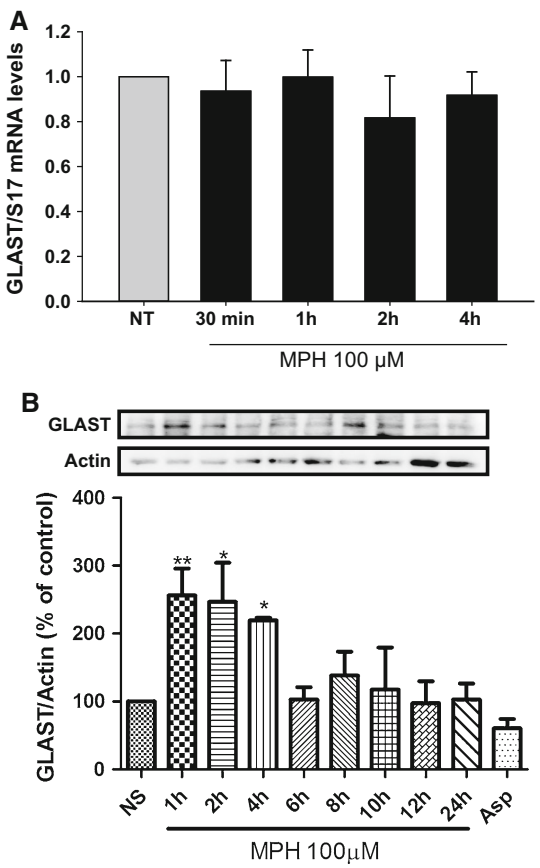

Fig. 4 MPH increases GLAST protein but not mRNA levels. **a** qRT-PCR assays were performed in primary cultures of BGC either non-treated or treated with MPH for the indicated time periods. Cells were harvested and total RNA was collected and analysed for *chglast* mRNA levels; data were normalized against ribosomal protein s17 mRNA levels. Data are expressed as the mean ± SEM of three independent experiments analysed in technical duplicates. A one-way ANOVA detected no significant difference amongst groups. **b** Confluent BGC monolayers were exposed to 100 μM MPH for the indicated time periods; GLAST was detected via Western blots (1:1000 GLAST-antibody was used); actin was used to normalize protein levels. 1 mM Aspartate (Asp) was used as a control. Results are presented as the mean ± SEM of at least three independent experiments, a representative Western blot is shown, a one-way analysis of variance (ANOVA) with a Dunnett's multiple comparison test was performed to analyse the data

been generally accepted that MPH blocks dopamine transporters and by these means increase dopamine intrasynaptic levels [24], it is clear that MPH actions are not restricted to dopamine transporters. In this context, we decided to explore a plausible interaction of MPH with the glutamatergic system, since it has been described that this drug enhances ionotropic Glu receptors response in dopaminergic neurons [25, 26]. Nevertheless, we reasoned that if MPH regulates dopaminergic tone by means of a direct modulation of Glu ionotropic receptors, then it might well be that MPH regulates the availability of this neurotransmitter in the synaptic cleft, availability that is controlled by glial-specific Glu transporters. To challenge our hypothesis, we decided to use the well-established model of glutamatergic neurons-associated glial, namely chick cerebellar Bergmann glial cells [28].

A relevant increase in GLAST activity was found upon exposure to 100 μM MPH, a concentration that has been widely used into evaluate MPH effects [14]. It should be noted that Bergmann glial cells express exclusively GLAST [17]. We were able to characterize a time and dose-dependent increase in [^{3}H]-D-Asp uptake. It is a rather slow phenomenon, since it is detected after a 2 h treatment and persists for up to 12 h. Kinetic analysis points out that the increase in Glu uptake activity is the result of an increase in the number of plasma membrane transporters, reflected in a significant two-fold increase in V_{MAX}. This interpretation was proved to be right, since

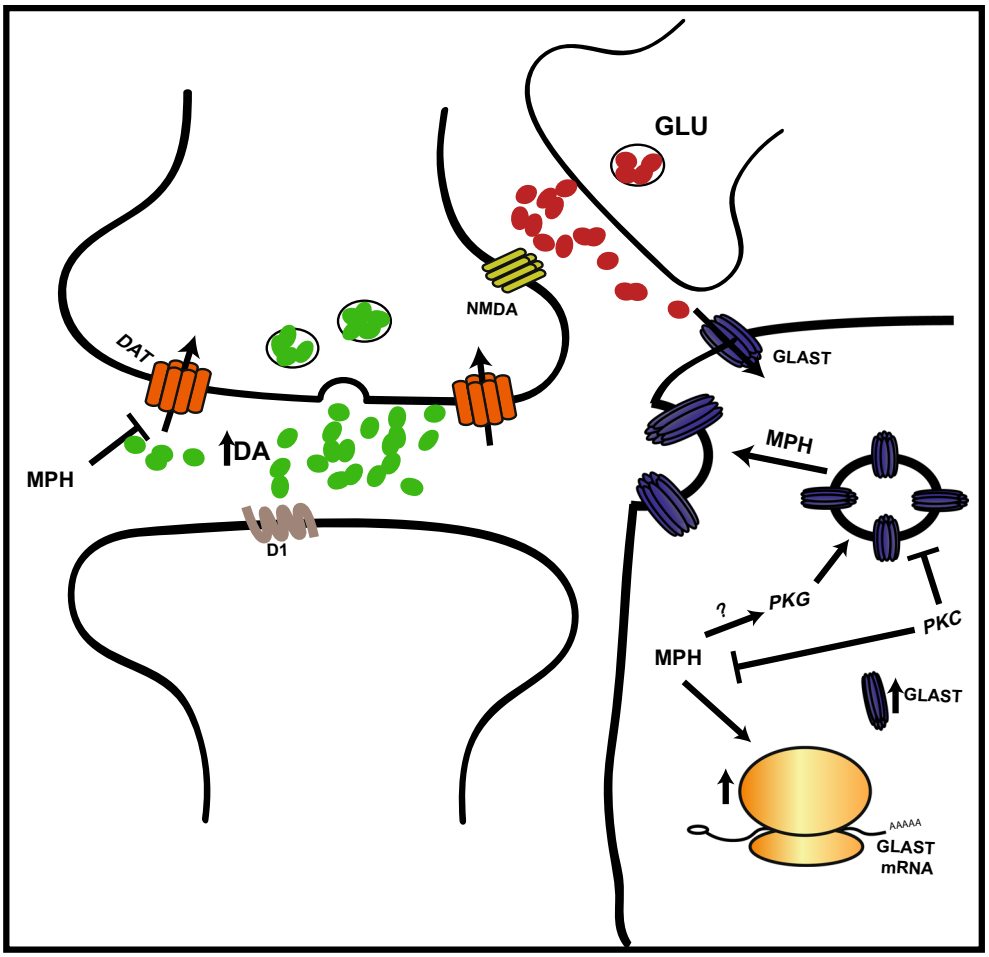

Fig. 5 Current model for MPH effect in GLAST/EAAT1 in cultured Bergmann glial cells. MPH affects GLAST-mediated Glu the uptake two levels: increasing GLAST mRNA translation and GLAST protein translocation to the plasma membrane, with a plausible role of PKG and PKC. This model illustrates that besides dopamine transporters, MPH also targets glutamate transporters in glial cells regulating both dopaminergic and glutamatergic transmission

[^{3}H]-D-Asp equilibrium binding experiments in whole cell monolayers, detected an augmentation in specific D-Asp binding sites in MPH treated cells, reflecting, once again, that the increase in uptake activity is related to an increase in functional transporters at the plasma membrane.

At this stage, an increased GLAST membrane trafficking was evident; therefore we decided to disrupt the cytoskeleton integrity by exposing the cells to cytochalasin B. As expected, MPH was no longer capable to increase GLAST activity. Our interpretation of the data, prompted us to explore the level(s) of regulation elicited by MPH, first we decided to evaluate if two signalling cascades, already known to regulate GLAST, could be the target of this psychostimulant. Although no conclusive evidence could be found, it is likely that the both PKG and PKC are involved in MPH action. Interestingly, while PKG might mediate some of MPH actions, PKC apparently blocks the MPH effect. In any event, it is clear that a detailed study of these signalling cascades in the context of MPH exposure, is mostly needed.

The fact that CHX but not actinomycin D reduces the MPH effect, points out that an increase in GLAST mRNA translation is present (Fig. 4). Note that actinomycin D treatment increases GLAST activity, this is probably due to a decrease in the expression of Ying–Yang 1, a transcription factor known to decrease *chglast* transcription in cultured chick Bergmann glia cells [27, 28]. Evidently, a MPH-dependent increase in plasma membrane GLAST molecules is present. Whether, this augmentation is related to an increase in the rate of plasma membrane transporter insertion or a decrease in its rate of removal is not known at this moment.

A schematic representation of our findings is presented in Fig. 5. Work currently in progress in our lab is aimed to the molecular characterization of the MPH effect in GLAST mRNA translation. In summary, we demonstrate

here that one of the most widely used psychostimulants targets ensheating glial cells associated to glutamatergic neurons.

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Compliance with Ethical Standards

Conflict of interest The authors certify that there is no conflict of interest with any financial organization regarding the material discussed in this manuscript.

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