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Brain Research 680 (1995) 9–15

BRAIN RESEARCH

Research report

Activation of glycogen phosphorylase by serotonin and 3,4-methylenedioxymethamphetamine in astroglial-rich primary cultures: involvement of the 5-HT_{2A} receptor

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Accepted 31 January 1995

Abstract

Neurotransmitters, neuropeptides, and ions regulate glycogen levels in the brain by modulating the activity of glycogen synthase (GSase) and glycogen phosphorylase (GPase). GPase is co-localized with glial fibrillary acidic protein (GFAP), an astroglia-specific marker, suggesting that glycogen is localized in astroglial cells. Additionally, functional serotonin (5-HT) receptors are found in both neurons and glia, and 5-HT is known to stimulate glycogenolysis. It is reported that 3,4-methylenedioxymethamphetamine (MDMA), a drug of abuse, stimulates the release and inhibits the reuptake of 5-HT, and selectively inhibits the activity of MAO-A. These biochemical consequences of MDMA lead to increased extra-cellular 5-HT levels. This study investigates the effects of MDMA(+) and serotonin (5-HT) on glycogen metabolism in the rat brain. A histochemical method was designed to visualize active glycogen phosphorylase (GPase) in an astroglial-rich primary culture.

Serotonin activated GPase in a concentration-dependent manner (100 nM–100 μM). Maximal activation by 5-HT was achieved by 50 μM and resulted in a 167% increase in the number of reactive sites ($P < 0.001$). MDMA(+) (500 nM–50 μM) directly stimulated GPase activity with maximal activation induced by 5 μM, which caused a 70% increase in the number of reactive sites ($P < 0.001$). The 5-HT_2 receptor agonist, 1-(2,5-dimethoxy-4-bromophenyl)-2-aminopropane (DOB), also displayed a concentration-dependent increase in the number of GPase reactive sites. Maximal stimulation by DOB occurred at 100 nM which increased the number of reactive sites by 166% ($P < 0.001$). These effects of 5-HT and MDMA(+) were significantly attenuated by mianserin (200 nM), a 5-HT_2 receptor antagonist.

An astrocyte-neuron metabolic link may be vital for synaptic homeostasis. By increasing 5-HT levels in the synapse, MDMA(+) may increase GPase activity and promote glycogenolysis via activation of the 5-HT_2 receptor. Prolonged GPase activity may lead to depletion of synaptic energy stores, thereby compromising the energy state of the synapse. The resulting deficiency in synaptic energy may contribute to terminal degeneration induced by substituted amphetamines.

Keywords: Glycogen phosphorylase; Serotonin; Astrocyte; 3,4-Methylenedioxymethamphetamine; Glycogen; Metabolism

1. Introduction

The single largest energy reserve in the brain is glycogen. In the mammalian central nervous system (CNS), glycogen is mainly localized to astrocytes. Glycogen phosphorylase (E.C. 2.4.1.1, GPase) is the rate-limiting enzyme in glycogenolysis and is likewise localized within astrocytes [15,30]. It is suggested that changes in glycogen levels within the CNS parallel changes in neuronal activity [22] and respond to several neurotransmitters, neuropeptides, and ions [6,13,23,32]. Specifically, micromolar amounts of serotonin (5-HT) stimulate the breakdown of [^{3}H]glycogen in mouse cerebral cortex [31].

Cellular responses to 5-HT are mediated by several families of serotonin receptors. Among the different subtypes in the 5-HT_1 receptor family, characterized by its high affinity for 5-HT, are the 5-HT_{1A}, 5-HT_{1B}, 5-HT_{1D} and 5-HT_{1E}. The 5-HT_2 family is characterized by its low affinity for 5-HT and a high affinity for serotonergic antagonists and includes the 5-HT_{2A}, 5-HT_{2B}, and the 5-HT_{2C} [7,29]. The 5-HT_3 family is characterized by its moderate affinity for 5-HT and is a

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SSDI 0006-8993(95)00201-4

ligand-gated ion channel [9,19]. Given the affinities of the different receptor families for the endogenous ligand, 5-HT, it therefore appears that glycogenolysis involves the low-affinity 5-HT_2 receptor.

3,4-methylenedioxymethamphetamine (MDMA) is a specific 5-HT neurotoxin that produces degeneration by an unknown mechanism. MDMA is reported to have a high affinity for 5-HT transporter ($K_I = 610$ nM) and for the 5-HT_2 receptor ($K_I = 5.1$ μM) in the rat brain [2]. In culture, MDMA produces neuropathology of primary serotonergic neurons at a micromolar concentration (5.0 μM). In addition, the toxic effects in culture can be prevented by the presence of a 5-HT_2 receptor antagonist, ketanserin [1]. In vivo, chronic MDMA(+) exposure produce degeneration of the serotonergic system in several species [3,28,33], while ketanserin pretreatment was similarly protective against MDMA(+) toxicity [38]. Thus, it can be hypothesized that the toxic effects of MDMA may be mediated by the 5-HT_2 receptor.

In this study, we examined the activation of GPase by serotonin and MDMA(+) in astroglia-rich primary cultures. Furthermore, we investigated the role of 5-HT receptors in the activation of GPase. The results of the current study indicate (a) that 5-HT and MDMA(+) activate GPase, (b) that DOB, a selective 5-HT_2 receptor agonist, produces an activation of comparable magnitude, and (c) that mianserin, a selective 5-HT_2 receptor antagonist, significantly attenuates the activation of GPase by 5-HT and MDMA(+).

2. Materials and methods

Primary cultures of astrocytes were derived from neonatal Sprague–Dawley rats which were anesthetized on ice and rapidly decapitated (postnatal days 1–3). The cerebral hemispheres were dissected and cell suspension was plated at an initial density of 3×10^4 cells/cm^2 onto 24-well plates (Nunc, Denmark) previously coated with poly-D-lysine (0.02 mg/ml) as previously described [39]. Media was supplemented with 10% horse serum (dialyzed with 1,000 kDa MW cutoff, Sigma), 5% fetal bovine serum (dialyzed with 1,000 kDa mol.wt. cut-off, Sigma), MEM vitamins (GIBCO), and MEM non-essential amino acids (Gibco). Dialyzed sera were used in the study to eliminate the effect of free 5-HT normally found in sera. Cells were incubated in a humidified atmosphere of 95% air and 5% CO_2 at 37° C. After 1 day, cultures were gently agitated and the media was changed to remove neurons, as well as dead and unattached cells. Media was changed every four days until the cells reached confluence (14–18 days) and were subsequently used for the studies.

Media was changed 24 h prior to drug incubations and cultures were pre-incubated with different concentrations of 5-HT (100 nM–100 μM, Sigma), MDMA(+) (5 nM–50 μM, National Institute on Drug Abuse), and 1-(2,5-dimethoxy-4-bromophenyl)-2-aminopropane (DOB or 2,5-dimethoxyamphetamine hydrobromide, 100 pM–100 nM, RBI, Natick, MA) for 60 min. For the mianserin experiments, cultures were incubated with mianserin (200 nM, RBI) 20 min prior to the addition of 50 μM 5-HT and 50 μM MDMA(+). Cells were then assayed for glycogen phosphorylase activity by a modification of the method of Harley et al. [10]. Briefly, cells were incubated for 30 min at 37° C in the GPase media (200 mg Na · EDTA · $2H_2O$, 160 mg NaF, 4 g Dextran, 800 mg α-D-glucose-1-phosphate, 100 ml. 0.1 M acetic acid/acetate buffer, pH 6.0). This condition promotes the reversal of the activated GPase reaction and allows for the deposition of α-D-glucose-1-phosphate into glycogen. Following incubation in GPase media, the cells were fixed with 40% ethanol for 3–5 min. The glycogen deposited in the cells were stained with Lugol's iodine/11% sucrose for 1.5–2 min, rinsed with deionized water to wash off excess Lugol's iodine solution and thoroughly air dried prior to inspection under an Olympus IMT-2 inverted microscope. The number of GPase reactive sites for each condition were determined using a computer-aided densitometric software (OPTIMAS; Bioscan, Inc.). Control cultures showed variability in the number of reactive sites in plates generated at different days (coeff. of variability: 60%). The reason for this variability is unknown. However, control and experimental groups were compared from the same plates in all cases, and experiments were done in multiples of four per group. In addition, all experimental findings were replicated a minimum of three times on different days. Statistical significance was determined using one-way ANOVA and the post-hoc Tukey Test with experimental conditions compared to corresponding control conditions. In brief, the increase in staining for glycogen correlates with the increase in number of activated glycogen phosphorylase (α-GPase) reactive sites [11]. Even though α-GPase hydrolyzes glycogen, the reaction condition of the GPase media promotes the reversal of the α-GPase reaction [26]. Thus, glycogen is deposited from α-D-glucose-1-phosphate using active glycogen phosphorylase (Fig. 1).

3. Results

In order to visualize GPase activity, astroglial-rich primary cultures were histochemically stained for glycogen. Levels of Lugol's iodine staining directly correlate to the amount of glycogen-positive reactive sites found in the cultures. Deposited glycogen sites were visible within the cytoplasm of stained cells and, in some cases, staining appeared granular (Fig. 2). No

staining was detected within the nuclei of stained cells. Not all cells in these astroglial cultures showed activated GPase in response to drug treatment. Consequently, only a subpopulation of astrocytes were glycogen-positive which formed clusters or patches reactive sites.

Serotonin produced a significant and concentration-dependent activation of GPase (Fig. 3A). Maximal stimulation of GPase was achieved by 50 μM 5-HT (151.8 ± 16.1; 167% change from control, $P < 0.001$) which resulted in an increase in the number of reactive sites from control (56.8 ± 4.7). The EC_{50} for the activation of GPase by 5-HT was approximately 5 μM. The staining pattern of GPase activity in control and in the 10 μM 5-HT conditions are shown in Figs. 2A and 2B, respectively. The activation of GPase by 10 μM 5-HT was maintained up to 24 h (data not shown).

MDMA(+) also produced a significant concentration-dependent increase in the number of GPase reactive sites (Fig. 3B). Maximal activation of GPase was achieved by 5 μM MDMA(+) (146.8 ± 5.0; 70% change from of control, $P < 0.001$), which caused a significant increase from control (86.3 ± 9.8). The EC_{50} for the activation of GPase by MDMA(+) was approximately 500 nM. The staining pattern of GPase activity in control and in the 5 μM MDMA(+) conditions are shown in Fig. 2C and 2D, respectively, and are similar to those produced by 5-HT. The activation of GPase by 5 μM MDMA(+) was maintained up to 24 h (data not shown).

The role of the 5-HT_2 and 5-HT_{1A} receptors in GPase activation was investigated using specific agonists and antagonists. DOB (100 pM–100 nM), a 5-HT_2 receptor agonist, produced a significant concentration-dependent increase in the number of GPase reactive sites in the astroglial-rich primary cultures (Fig. 4). Maximal stimulation of GPase was achieved by 100 nM (83.8 ± 4.6; 166% change from control, $P < 0.001$), which produced a significant increase from control (31.5 ± 5.6). The EC_{50} was approximately 1 nM. To further characterize the functional role of the 5-HT_2 receptor in GPase activation, mianserin (200 nM), a 5-HT_2 receptor antagonist, was incubated 20 min prior to the addition of either 50 μM 5-HT or 50 μM MDMA(+). Mianserin attenuated the stimulation of GPase induced by either 5-HT or MDMA(+) (Fig. 5). The high-affinity 5-HT_{1A} receptor does not seem to be involved in the regulation of GPase in astroglial-rich primary cultures since ipsapirone (40 nM), a selective 5-HT_{1A} agonist, and nanomolar concentrations of 5-HT did not produce significant changes in GPase activity (data not shown).

Glycogen ($glucose_n$) + P_i

α -d-glucose-1-phosphate

+

Glycogen ($glucose_{n-1}$)

Fig. 1. Glycogen phosphorylase (GPase) reaction. GPase is the rate-limiting enzyme in the breakdown of glycogen to glucose-1-phosphate and glycogen with one less glucose unit. This reaction is in equilibrium at pH 6.8, but proceeds in the direction of glycogen breakdown because the orthophosphate (P_i): glucose-1-phosphate ratio is usually greater than 100 in vivo. In our assay system, lowering the pH to 6.0 and adding glucose-1-phosphate in excess facilitates the artificial reversal of the active glycogen phosphorylase reaction. Thus, allowing the deposition of glucose into glycogen using dextran as a glucosyl acceptor. The increase in glycogen deposition can be correlated with the increase in active glycogen phosphorylase.

4. Discussion

Glycogen phosphorylase is localized in astrocytes in the CNS [15,30]. In this study, we utilized primary astroglial-rich cultures to study the effects of 5-HT, MDMA(+), and DOB on glycogen phosphorylase activity and, consequently, glycogenolysis. We report that all three compounds stimulate GPase activity in astroglial-rich primary cultures in a concentration-dependent manner (Figs. 3 and 4). The effect of 5-HT and MDMA(+) on astroglial GPase activation is attenuated by mianserin, a 5-HT_2 receptor antagonist (Fig. 5). These data indicate that 5-HT activates GPase by the 5-HT_2 receptor. This observation is consistent with previous results which show that micromolar concentrations of serotonin ($K_{act} = 20$ μM) stimulate the breakdown of [^{3}H]-glycogen [31,32] in mouse cortical slices, suggesting the involvement of the low-affinity 5-HT_2 receptor rather than the high-affinity 5-HT_1 receptors. The K_d of 5-HT for the 5-HT_2 receptor is 2.7 μM [29] which corresponds to the activating concentrations of 5-HT in this study. In addition, it is reported that 5-HT_2 receptor mRNA is expressed in rat primary astrocyte cultures, however not all astrocytes express this receptor subtype [8]. This may account for the activation of a subpopulation of astrocytes in these cultures. Therefore, we conclude that glycogenolysis in the brain is regulated by 5-HT via the 5-HT_2 receptor in astrocytes.

Recently, the 5-HT_2 receptor family was characterized using pharmacological and molecular biological techniques [21]. The 5-HT_{2A} receptor has a low affinity for 5-HT and a high affinity for antagonists [25]. In comparison, the 5-HT_{2C} receptor subtype has a relatively high affinity for 5-HT and a low affinity for

antagonists. Therefore, the 5-HT_{2A} receptor subtype appears to be involved in the activation of GPase in astrocytes, because 5-HT was effective in the micromolar range, and this effect was attenuated by mianserin, a 5-HT_2 receptor antagonist. In addition, DOB, a selective 5-HT_{2A} receptor agonist [14], also activated GPase in these astroglial-rich cultures. Finally, 5-HT_{2A} receptor subtype mRNA and functional receptors are present in primary astroglial cells [8,12].

It is interesting to note that the maximal responses elicited by 5-HT (50 μM; 167% change from CTL) and by DOB (100 nM; 166% change from CTL) were similar, suggesting that there exists a maximal stimulation of GPase within primary astroglial-rich cultures in response to 5-HT_2 receptor agonists. In addition, 100 μM 5-HT did not produce a significant increase in the number of reactive sites when compared to control. We believe the 5-HT_2 receptor is desensitized as a result of its activation by high concentrations of 5-HT [18,24]. Furthermore, the activation of GPase by MDMA(+) suggests that it has an agonist-like action on the 5-HT_2 receptor.

The highest glycogen content in the rat brain is seen during the perinatal period [5]. Levels of glycogen begin to decrease between postnatal day (PND) 1–3 and reach adult levels from PND 7-21. In addition, MDMA does not induce any long-term neurochemical deficits in rats when administered either prenatally or up to PND 10 [4,34]. However, MDMA was able to partially deplete 5-HT and 5-HIAA when administered on PND 15 or PND 20, and induce even further depletion when given to adult rats (PND 150) [4]. Hence, we propose that the potential of MDMA to induce 5-HT neurotoxicity is decreased when adminis-

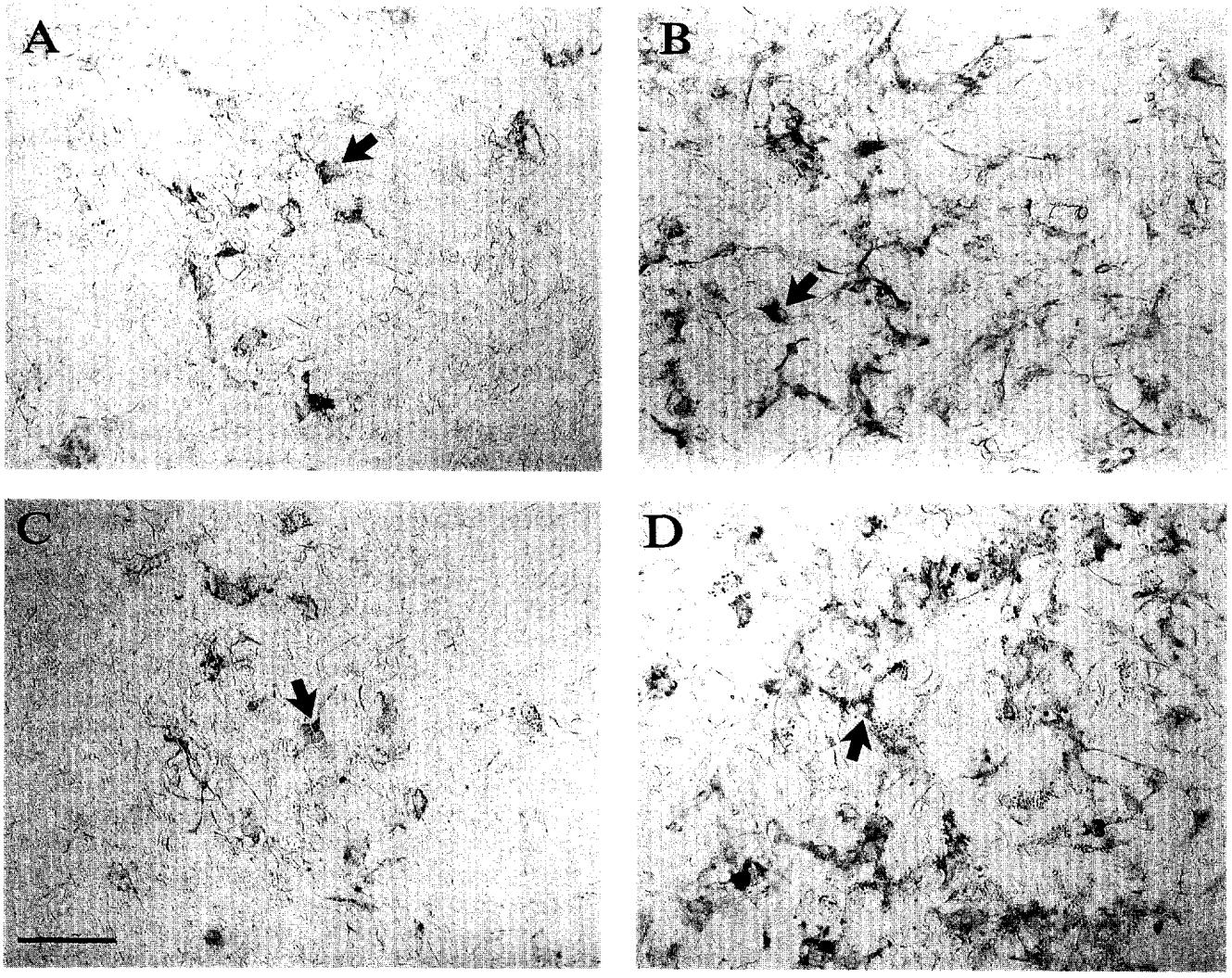

Fig. 2. Effect of 10 μM 5-HT and 5 μM MDMA(+) on glycogen reactive sites in primary astroglial-rich cultures as visualized using Lugol's Iodine staining. Test compounds were incubated for 60 minutes prior to glycogen phosphorylase assay. (A) Control condition for 5-HT; (B) 10 μM 5-HT; (C) Control condition for MDMA(+); and, (D) 5 μM MDMA(+). Stained areas indicated by arrows show a typical glycogen-positive reactive site. Bar = 300 μm.

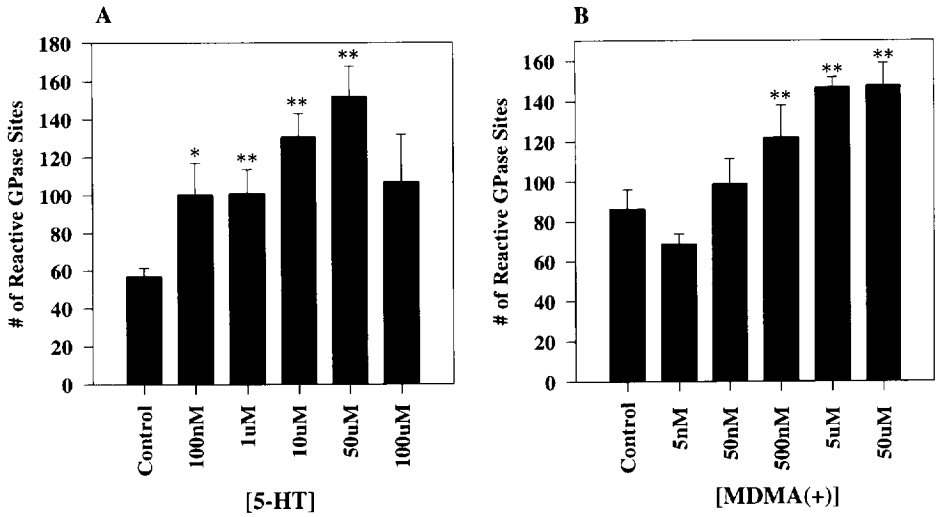

Fig. 3. Activation of glycogen phosphorylase (GPase) by 5-HT and MDMA(+). (A) Response of GPase to increasing concentrations of 5-HT. One-way ANOVA (P-value = 5.93×10^{-3}; $F = 4.88$, $F_{crit} = 2.81$) and post-hoc Tukey statistical test showed: * $P < 0.005$, ** $P < 0.001$ relative to corresponding control. B) Response of GPase to increasing concentrations of MDMA(+). One-way ANOVA (P-value = 1.77×10^{-4}; $F = 9.19$, $F_{crit} = 2.77$) and post-hoc Tukey statistical test showed: * $P < 0.005$, ** $P < 0.001$ relative to corresponding control. The number of reactive sites were determined using a computer-aided densitometric software (Optimas: Bioscan, Inc.). All data points represent the average of four experiments (sampling area = 421,828 μm^2). Error bars represent the standard error of the means.

tered during the perinatal period, possibly due to the presence of larger energy reserves in the form of glycogen.

In support of this idea, MDMA(+) is reported to have a micromolar affinity for the 5-HT_2 receptor ($K_I = 5.1$ μM) [2], and 5-HT_2 antagonists attenuate MDMA(+)-induced neuropathology in culture [1] and in vivo [36,38]. Furthermore, MDMA(+) binds to the 5-HT high affinity uptake transporter ($K_I = 610$ nM) [2], and acts on this protein by stimulating the release of 5-HT and by inhibiting the reuptake of 5-HT [16,17,27,37]. In addition, MDMA(+) preferentially inhibits monoamine oxidase-A (MAO-A; $K_I = 22$ μM) activity which is the catabolic isozyme that has a higher affinity for 5-HT than MAO-B [20]. As a result of the

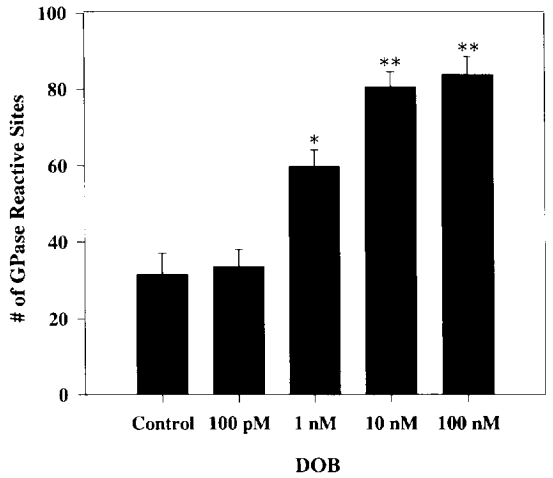

Fig. 4. Effect of DOB on glycogen phosphorylase (GPase) activation in primary astroglial-rich cultures. The number of reactive sites were determined using a computer-aided densitometric software (Optimas: Bioscan, Inc.). All data points represent the average of four experiments (sampling area = 122,261 μm^2). Error bars represent the standard error of the mean. One-way ANOVA (P-value = 6.35×10^{-7}; $F = 29.22$, $F_{crit} = 3.05$) and post-hoc Tukey statistical test showed: * $P < 0.005$, ** $P < 0.001$ relative to control.

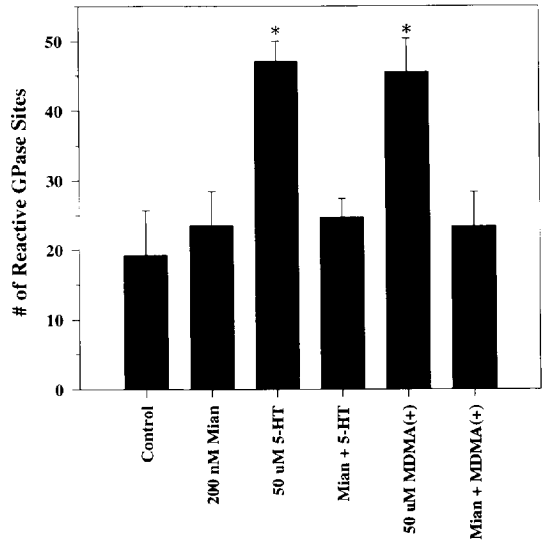

Fig. 5. Effect of 200 nM mianserin on 50 μM 5-HT- and 50 μM MDMA(+)-induced activation of glycogen phosphorylase (GPase). The number of reactive sites were determined using a computer-aided densitometric software (Optimas: Bioscan, Inc.). All data points represent the average of at least four experiments (sampling area = 74,108 μm^2). Error bars represent the standard error of the mean. One-way ANOVA (P-value = 4.74×10^{-4}; $F = 6.51$, $F_{crit} = 2.59$) and post-hoc Tukey statistical test showed: * $P < 0.025$ relative to control.

binding of MDMA(+) to the transporter and the inhibition of MAO-A, levels of extracellular 5-HT is increased. Fluoxetine, a selective 5-HT uptake inhibitor with no affinity for the 5-HT_2 receptor, is also capable of preventing the MDMA-induced nerve terminal degeneration in vivo [35] and in cultured 5-HT neurons [1]. Thus, although MDMA directly activates the 5-HT_2 receptor, stimulation of this receptor would be augmented and prolonged by the build up of extracellular 5-HT induced by MDMA. Fluoxetine, by preventing extracellular build up of 5-HT, and mianserin, by antagonizing the 5-HT_2 receptor, are both effective at reducing MDMA induced nerve terminal degeneration.

Our results indicate that 5-HT and MDMA(+) activate GPase in astroglial-rich primary cultures via the 5-HT_2 receptor. This mechanism is established by the attenuation of this effect by mianserin. The activation of GPase by DOB further specifies the role of the 5-HT_{2A} receptor subtype in the breakdown of glycogen in astrocytes. We are suggesting that the increase in extracellular 5-HT activity induced by MDMA(+) leads to an increase in the breakdown of glycogen which may contribute to nerve terminal degeneration by depleting synaptic energy reserve in the CNS.

Acknowledgements

The authors would like to acknowledge the help of Dr. Monroe Yoder, H. Kenneth Kramer, Efthimia Kokotos Leonardi, Nancy Kheck, Pamela Hou and Christine M. Poblete in the preparation of this work. Help in establishing astroglial cultures was provided by Dr. Patricia Whitaker-Azmitia. This work was supported by the National Institute on Drug Abuse (NIDA; Contract No. 271-90-7403 to Efrain C. Azmitia, Ph.D.)

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