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Short Communication

GTPase ACTIVITY IN MOUSE HIPPOCAMPUS MEMBRANES FOLLOWING PRENATAL EXPOSURE TO HEROIN AND PHENOBARBITAL

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Abstract—Low K_m high affinity GTPase activity, with and without muscarinic receptor stimulation with 1 mM carbachol, was measured in membrane preparations of mouse hippocampus prenatally exposed to phenobarbital or heroin. Basal and carbachol-stimulated low K_m GTPase activities after prenatal exposure to phenobarbital exhibited a statistically significant ($P < 0.05$) decrease both in K_m and V_{max} values. Basal V_{max} values were reduced from 152 ± 10 in controls to 112 ± 13 (pmol/mg protein/min, mean ± SEM) in exposed mice. The K_m values in the offspring of mice treated with phenobarbital were reduced from 1.55 ± 0.21 to 0.96 ± 0.11 (μM, mean ± SEM); V_{max} and K_m values after carbachol stimulation were similarly affected. Prenatal exposure to heroin did not change the GTPase activities, basal or carbachol-stimulated, with only a non-significant increase in both V_{max} and K_m values. It is postulated that these changes in Gα protein activity may be related to the teratogenic effect of these drugs.

Key words: brain hippocampus; phenobarbital; heroin; prenatal treatment; low K_m GTPase; carbachol-stimulated GTPase

G-proteins carry information from cell surface receptors to intracellular effector proteins by following a controlled cycle of activation and deactivation. The functions of G-proteins in the brain are regulated by association of GTP† with the α-subunit, hydrolysis of GTP to GDP and P_i, and dissociation of GDP. Dissociation of GDP is linked with the rate-limiting step and is receptor controlled [1]. Cholinergic systems, especially those of the hippocampus and forebrain, appear to be critical for the normal operation of memory [2]. The major cholinergic muscarinic receptors in the hippocampus are of the M1-type, which can be induced by carbachol [3]. These receptors mediate the activity of G_α proteins [4, 5].

Previous studies from our laboratory have stressed the importance of the brain hippocampus in the learning behaviour of mice, and have demonstrated the effect of prenatal exposure to phenobarbital and heroin on this behaviour [6]. In these studies inositol phosphate formation in brain tissue was not significantly affected by prenatal exposure to phenobarbital, but it was markedly stimulated by carbachol [7].

We report here studies on the effect of prenatal exposure to phenobarbital and heroin on low K_m intrinsic GTPase activity of the α-subunits and its stimulation by carbachol. This appears to be a convenient measure of G_α protein activity in hippocampus membranes [8].

Materials and Methods

Materials. Chemicals used were obtained from the following sources: carbamylcholine chloride (Aldrich Chemical Co., Milwaukee, WI, U.S.A.); [γ-^{32}P]GTP, 10 Ci/mmol (Amersham, Arlington Heights, IL, U.S.A.), AppNHp, creatine phosphate, creatine phosphokinase, BSA, ATP, GTP, EGTA, $MgCl_2$, and DTT (Sigma Chemical Co., St. Louis, MO, U.S.A.); NaCl, sucrose and activated charcoal (Merck, Darmstadt, Germany); EDTA and phosphoric acid (Analar, BDH Ltd, Poole, U.K.); liquid scintillation, Quick safe A (Zinsser Analytic, Frankfurt, Germany); Tris (International Biotechnologies Inc., New Haven, CT, U.S.A.); Bio-Rad dye reagent for protein assay (Bio-Rad Laboratories GmbH, Munich, Germany).

Drug administration. Mice were exposed to heroin or phenobarbital prenatally, between the ninth and eighteenth days of gestation [6, 9]. Pregnant females received daily subcutaneous injections of 10 mg/kg body weight of either heroin solution in saline, or saline alone (controls). Exposure to phenobarbital was obtained by adding this substance (in acid form) at a concentration of 3 g/kg to milled mouse food. Water was given to all groups *ad libitum*.

Tissue preparation. Treated mice and their respective controls were weighted (average 20 g) and sacrificed by decapitation at the age of 50 days. The brain was rapidly removed and the hippocampus was dissected on an ice-cold tray. The hippocampus membranes from several mice similarly treated and of the same sex were combined and stored at −80°, for 1–3 days. The hippocampus membranes were homogenized in a buffer solution containing 0.31 M sucrose, 0.1 mM EDTA, and Tris-HCl (pH 7.5 at 4°) with a Brinkmann Polytron mechanical homogenizer according to the method described by Ghodsi-Hovsepian *et al.* [3]. These experiments were performed in accordance with governmental guidelines and have been approved by our institutional review committee.

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† Abbreviations: [γ-^{32}P]GTP, [γ-^{32}P]-guanosine 5′-triphosphate; AppNHp, 5′-adenylyl-[β,γ]-imidodiphosphate lithium; GTP, guanosine 5′-triphosphate lithium; carbachol, carbamylcholine chloride, DTT, dithiothreitol.

Preparation of washed hippocampus membranes. The hippocampus crude homogenate (prepared from two different mice) was washed several times by centrifugation in TED buffer (10 mM Tris-HCl, 0.1 mM EDTA, 5 mM DTT, pH 7.5, at 4°) by the method of Hoss *et al.* [10] and Ghodsi-Hovsepian *et al.* [3]. The final pellet was resuspended in TED buffer, homogenized in a Teflon–glass homogenizer, divided into several portions, and stored at −80°. Membrane proteins concentration was determined using the Bio-Rad protein assay with BSA as a standard.

GTPase assay. GTPase activity was measured according to the modification of Hoss *et al.* [10] to the method developed by Cassel and Selinger [8]. In brief, the reaction mixture, in a final assay volume of 100 μL, consisted of 0.25 μM [γ-^{32}P]-GTP (0.05 μCi/tube), 1 mM ATP, 2 mM AppNHp, 10 mM creatine phosphate, 60 units/mL of creatine phosphokinase, 2 mM DTT, 6 mM $MgCl_2$, 0.1 mM EDTA, 0.1 mM EGTA, 16 mM Tris-HCl (pH 7.5), 100 mM NaCl. Unlabelled GTP was added to reach concentrations in the range of 0.15–5 μM. Carbachol, when added, was in the concentration of 1 mM. This concentration was shown to be most effective in stimulating GTPase activity [3, 11]. The reaction was started by adding 30 μL of washed hippocampus membranes containing 20–28 μg protein in TED buffer pH 7.5. The mixtures were incubated at 37° for 10 min. The reaction was terminated by adding 1 mL ice-cold 5% (w/v) activated charcoal in 20 mM phosphoric acid (pH 2.3). The reaction tubes were centrifuged at 2000 *g* for 20 min and 500 μL of the supernatant were transferred into scintillation vials. Four millilitres of scintillation fluid were added and the $^{32}P_i$ released was counted by liquid scintillation spectrometry. All GTPase assays were performed in triplicates.

Correlation with amount of tissue. P_i release from [γ-^{32}P]GTP was measured in four experiments, after adding hippocampus membrane preparations in the range of 13.3–53.3 μg of protein in 30 μL of TED buffer.

Data analysis. Row data were corrected for background and converted to P_i (expressed in picomoles) released by GTPase activity and to percentage stimulation. Each data point represents the average of ten experiments, assayed in triplicate. Data are given as mean ± SEM for the number of separate experiments performed, with significant differences assessed by ANOVA with the criterion of $P < 0.05$. The high-affinity low K_m GTPase activity was calculated by subtracting the P_i (in cpm) liberated from [γ-^{32}P]GTP at high GTP concentration (50 μM) from the P_i (in cpm) liberated at lower (0.15–5 μM) GTP concentrations [8].

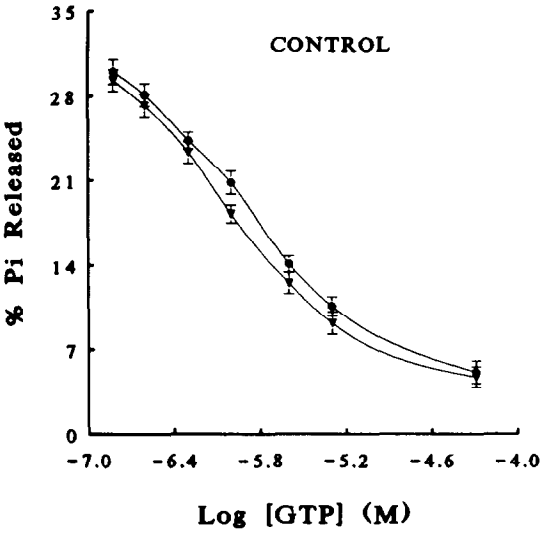

Fig. 1. Hydrolysis of [γ-^{32}P]GTP hydrolysis by mouse hippocampus membranes. In a representative experiment, 20 μg of membrane protein were incubated for 10 min in tubes containing 0.25 μM [γ-^{32}P]GTP with increasing amounts of unlabelled GTP. Release of $^{32}P_i$ from [γ-^{32}P]-GTP in the absence (▼) or presence (●) of 1 mM carbachol was measured. Data are presented as mean percentage release ± SEM in 10 experiments each performed in triplicate.

Results

Low K_m GTPase activity in washed mouse hippocampus membranes was measured as the liberation of ^{32}Pi from [γ-^{32}P]GTP (Fig. 1). While at a concentration of 1.5×10^{-7} M GTP 28% of the P_i was released; a plateau with about 7% release was reached at concentrations above 5×10^{-5} M (either without or with 1 mM carbachol) suggesting the existence of a high-affinity GTPase.

The rate of low K_m GTP hydrolysis mediated by hippocampus membranes was greater in the presence of 1 mM carbachol (Fig. 2). In the Lineweaver–Burk plots (Fig. 2, inset) two sets of points for GTP hydrolysis over the GTP concentration range 0.15–5 μM indicate a V_{max} ± SEM of 152 ± 10 (pmol/mg protein/min) in the absence of carbachol stimulation and 201 ± 16 in the presence of carbachol.

The low K_m high-affinity enzyme has a K_m ± SEM of 1.55 ± 0.21 μM in the absence of carbachol stimulation, and 2.04 ± 0.30 μM in the presence of carbachol.

A linear correlation was found between P_i released from [γ-^{32}P]GTP and the amount of protein derived from hippocampus membrane preparations ($r = 0.98$ for basal values and $r = 0.97$ after carbachol stimulation).

Carbachol (1 mM) increased high affinity GTPase activity in all experiments, but to different degrees (Table 1). Prenatal exposure to phenobarbital induced a statistically significant decrease in basal GTPase activity. The same effect was observed in carbachol stimulated GTPase activity. With prenatal exposure to heroin, however, changes in both basal and carbachol stimulated GTPase activities were not significant. An apparent but not statistically significant difference was observed between carbachol stimulated GTPase activity in hippocampus membrane preparations of phenobarbital controls (which had not received any treatment) and heroin controls (which had received repeated saline injections). No significant difference in GTPase activity in any of the groups was found between male and female hippocampus membrane preparations (data not shown).

Discussion

The teratological effect of prenatal exposure to phenobarbital or heroin was demonstrated in children of addicted mothers [12, 13]. Similarly, when these drugs were administered to pregnant mice, their offspring showed a disturbed behaviour and reduced learning ability [6, 9]. We decided to look for biochemical changes in the brain of these mice exposed prenatally to either phenobarbital or heroin. We focused on the receptor stimulated low K_m GTPase activity in hippocampus membranes, as a convenient measure of G_α protein activity [8]. The different routes of administration for the two drugs, oral for phenobarbital and parenteral for heroin, parallel those usually used by human addicts and have frequently been used in previous studies. Previous reports demonstrate the presence of enzymes exhibiting GTPase activity in the brain hippocampus [3, 10, 14, 15]. We found that

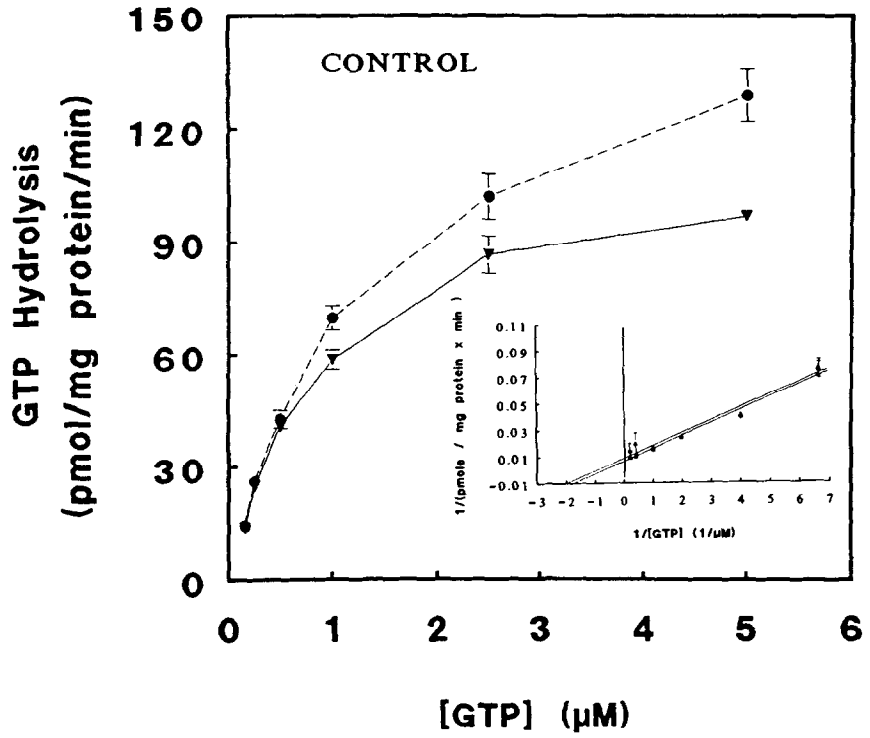

Fig. 2. Kinetics of GTP hydrolysis in control mouse hippocampus membranes. Low K_m GTPase activity was determined over the range 0.15–5 μM GTP concentrations, in the absence (▼) or presence (●) of 1 mM carbachol. Reactions were carried out at 37° and were terminated after 10 min. Data shown are the means ± SEM from 10 experiments each performed in triplicate. Inset: A Lineweaver–Burk plot. Where the error bars are not shown error is < 5%.

Table 1. Carbachol-stimulated low K_m GTPase activity in mice prenatally treated with heroin and phenobarbital and in controls

Prenatal treatment	V_{max} (pmol/mg protein/min)			K_m (μM)		
	Basal	Carbachol	Stimulation (%)	Basal	Carbachol	Stimulation (%)
Control	152 ± 10	201 ± 16	33 ± 8	1.55 ± 0.2[1]	2.04 ± 0.30	31 ± 9
Phenobarbital	112 ± 13*	148 ± 18*	32 ± 6	0.96 ± 0.11**	1.27 ± 0.14*	33 ± 9
Control	160 ± 33	262 ± 67	67 ± 21	1.62 ± 0.27	2.74 ± 0.59	77 ± 28
Heroin	192 ± 29	231 ± 53	16 ± 10	2.13 ± 0.52	2.55 ± 0.71	19 ± 13

Data are means ± SEM in pmol/mg protein/min for V_{max}, and in μM for K_m (N = 10, for each group). Statistical significance was assessed by analysis of variance (ANOVA).
* $P < 0.05$, ** $P < 0.01$, as compared with the respective controls.

phenobarbital and heroin have different effects on GTPase activity, even though both drugs impair normal development after prenatal exposure. While exposure to phenobarbital decreased GTPase activity in the hippocampus membranes, prenatal exposure to heroin did not have this effect. This difference can perhaps be understood if we consider the variability of these enzymes in the brain. Thus, after chronic exposure to opioids the K_m values of the GTPase in the striatum, periaqueductal grey matter and spinal cord were diminished, while those in the hypothalamus and rest of the mesencephalon were increased. This difference was attributed to different ratios of G_o subtypes of the neural structures studied [14, 16]. In our studies, GTPase activity was estimated only in the hippocampus. It is possible that opiates and phenobarbital, which affect brain function and development in distinct ways [17] have a different effect on the intrinsic GTPase activity of the α-subunits. Furthermore, G-proteins are shared by other neurotransmitter systems so that the effect of these drugs on different sites of the receptor–G-protein–effector cascades or the sites of gene expression of G-protein may induce secondary effect changes in GTPase activity. GTPase

activity was stimulated by carbachol in all experimental groups. As in previously reported experiments measuring inositol phosphate formation [7], the increase in stimulated low K_m GTPase activity was higher in mice exposed prenatally to phenobarbital (Table 1). The similar 33% stimulation of the GTPase by carbachol in the control and phenobarbital-treated groups suggest that the hippocampal cholinergic system remains rather unaffected functionally. After prenatal exposure to heroin, carbachol stimulation of low K_m GTPase activity was less marked (16%).

It seems possible that the teratogenic effects of phenobarbital and heroin are accompanied by biochemical changes in the brain. Changes in GTPase activity after prenatal exposure to these drugs, which have been found in this study, could be related to the behavioural changes previously described in our laboratory [7].

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